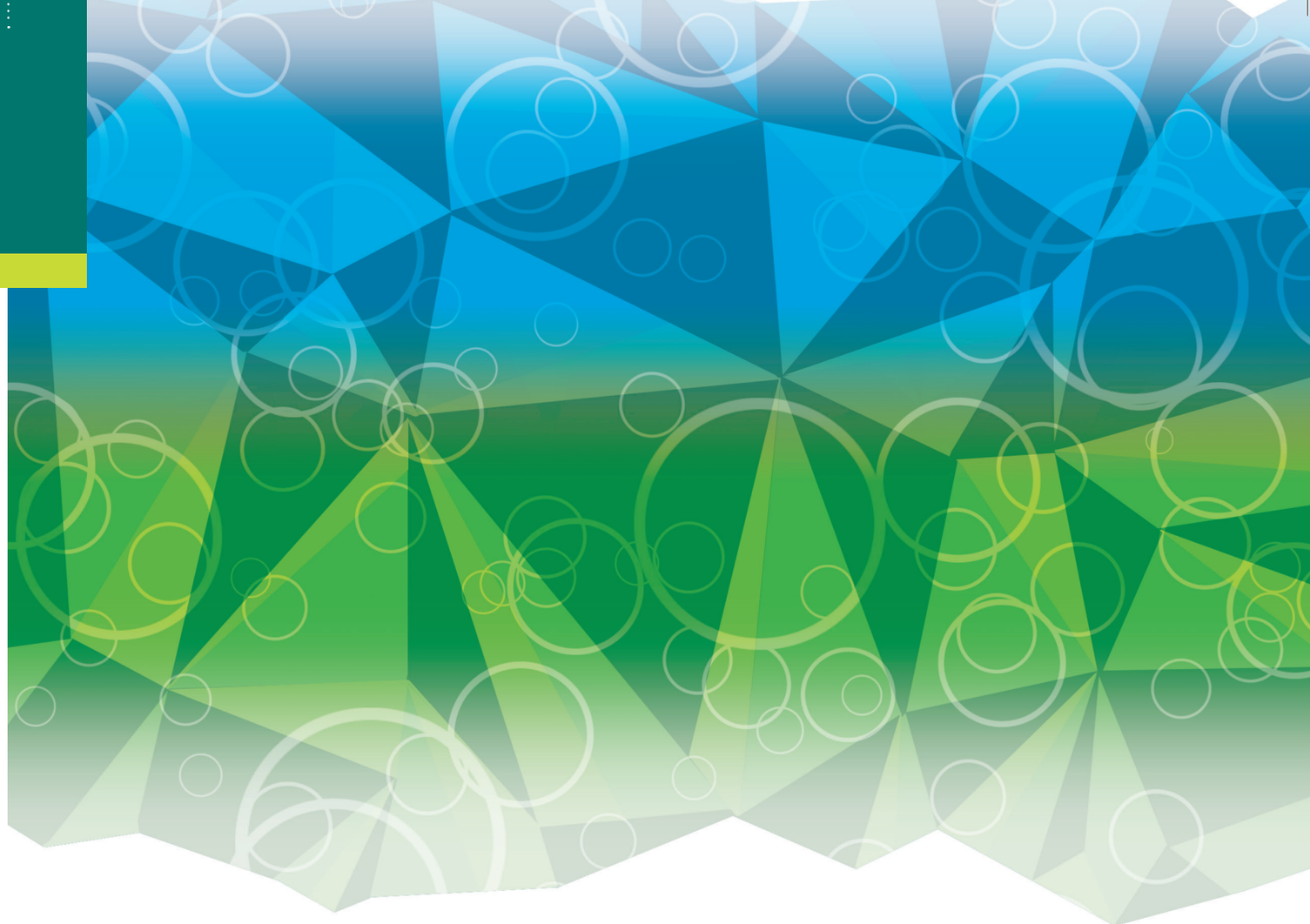
AUTOIMMUNITY From Bench to Bedside

Juan-Manuel Anaya | Yehuda Shoenfeld
Adriana Rojas-Villarraga | Roger A. Levy
Ricard Cervera
-Editors-



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UNIVERSIDAD DEL ROSARIO

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Centro de Estudio
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UNIVERSIDAD DEL ROSARIO
EDITORIAL

AUTOIMMUNITY

From Bench to Bedside

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CONTENT

Icons used in this book X

Preface XIII
Francesco M. Marincola

SECTION I

INTRODUCTION TO IMMUNOLOGY AND MOLECULAR BIOLOGY

1. Gene expression and regulation 3
Sandra Ramírez-Clavijo, Gladis Montoya-Ortíz

2. Innate immune system 31
Beatriz Aristizábal, Ángel González

3. Toll-like receptors and autoimmunity 47
Gloria Vásquez, Soraya Zorro

4. The complement system 57
Rafael Parra-Medina, Paula Quintero-Ronderos, Édgar Garavito Rodríguez

5. Introduction to T and B lymphocytes 77
Luz Elena Cano R, Damaris E. Lopera H

6. B lymphocytes in autoimmunity 97
Jorge H. Izquierdo, Carlos A. Cañas, Gabriel J. Tobón

7. T lymphocytes in autoimmunity 113
Alejandro Ruiz-Argüelles

8. Are autoimmune disease autoimmune epithelitis? 123
Édgar Garavito Rodríguez, Grégory Alfonso García Morán

9. Cytokines, chemokines and growth factors 133
Grégory Alfonso García Morán, Rafael Parra-Medina, Ananías García Cardona, Paula Quintero-Ronderos, Édgar Garavito Rodríguez

10. Major histocompatibility complex: Antigen processing and presentation 169
Paola Cruz-Tapias, John Castiblanco, Juan-Manuel Anaya

11. Immunosenescence 185
Gladis Montoya-Ortíz

12. The endocrine system and autoimmunity 201
Luis J. Jara, Gabriela Medina, Stephanie Voorduin, Griselda Romero-Sánchez, Miguel A. Saavedra, Olga Vera-Lastra

13. Cell Death 219
Luisa Marina Matheus Merino, Rafael Parra-Medina, Mónica Ararat Sarria, Jaime Alberto Díaz Galvis

SECTION II

FROM THE MOSAIC OF AUTOIMMUNITY TO THE AUTOIMMUNE TAUTOLOGY

14. From the mosaic of autoimmunity to the autoimmune tautology 237
Juan-Manuel Anaya, Adriana Rojas-Villarraga, Yehuda Shoenfeld

15. Evolution and autoimmunity 247
Carlos A. Cañas, Fabio Bonilla-Abadía, Gabriel J. Tobón

16. Introduction to genetics of autoimmune diseases 257
John Castiblanco, Mauricio Arcos-Burgos, Juan-Manuel Anaya

17. HLA association with autoimmune diseases 271
Paola Cruz-Tapias, John Castiblanco, Juan-Manuel Anaya

18. Non-HLA genes and autoimmune diseases 285
Juan-Manuel Anaya, John Castiblanco, Christopher J. Lessard

19. Infection and autoimmune diseases 303
María-Teresa Arango, Yehuda Shoenfeld, Ricard Cervera, Juan-Manuel Anaya

20. Autoimmune ecology 321
Adriana Rojas-Villarraga, Juan Castellanos-de la Hoz, Oscar M. Pérez-Fernández, Jenny Amaya-Amaya, Juan-Sebastián Franco, Juan-Manuel Anaya

21. Molecular mimicry in autoimmunity and vaccinations 343
Miri Blank, Eitan Israeli, Smadar Gertel, Howard Amital, Yehuda Shoenfeld

22. Epigenetics and autoimmune diseases 351
Paula Quintero-Ronderos, Gladis Montoya-Ortíz

23. Immunological computation 373
Gerardo Quintana López, Luis Antonio Morales, Luis Fernando Niño

SECTION III

AUTOIMMUNE DISEASES

24. Rheumatoid arthritis 381
Jenny Amaya-Amaya, Adriana Rojas-Villarraga, Rubén D. Mantilla, Juan-Manuel Anaya

25. Systemic lupus erythematosus 427
Mario García-Carrasco, Claudia Mendoza Pinto, Juan Carlos Solís Poblano, Ivet Etchegaray Morales, Ricard Cervera, Juan-Manuel Anaya

26. Antiphospholipid syndrome 443
Mario García-Carrasco, Claudia Mendoza Pinto, César Jiménez Hernández, Juan Carlos Solís Poblano, Ivet Etchegaray Morales, Socorro Méndez Martínez

27. Systemic autoimmune diseases and pregnancy 455
Roger A. Levy, Claudia Mendoza Pinto, Vinicius Domingues, Guilherme de Jesús, Mario García-Carrasco, Nilson Ramírez de Jesús, Ricard Cervera

28. Sjögren's syndrome 475
Juan Camilo Sarmiento-Monroy, Rubén D. Mantilla, Adriana Rojas-Villarraga, Juan-Manuel Anaya

29. Autoimmune diabetes mellitus (Type 1A) 519
Andrés F. Echeverri, Gabriel J. Tobón

30. Thyroid disease and autoimmune diseases 537
Juan-Sebastián Franco, Jenny Amaya-Amaya, Juan-Manuel Anaya

31. Autoimmune hepatitis 563
Nazarena Ferreyra Solari, Alejandra Claudia Cherñavsky

32. Primary biliary cirrhosis 573
Dahyana Cadavid, Andrés F. Echeverri, Carlos A. Cañas

33. Celiac disease ... 583
Rafael Parra-Medina, Alejandra Claudia Cherñavsky

34. Dermatological autoimmune diseases ... 597
Esperanza Ávalos-Díaz, Rafael Herrera Esparza

35. Systemic sclerosis ... 611
Paola Ximena Coral-Alvarado, Paul Alejandro Méndez Patarroyo, Gerardo Quintana López

36. Systemic vasculitis ... 621
Ana Beatriz Santos Bacchiega, Manuella Lima Gomes Ochtrop, Alexandre W S de Souza

37. Autoimmune uveitis ... 643
Manuel J. Amador-Patarroyo, Alba Cristina Peñaranda, María Teresa Bernal

38. Cardiovascular involvement in autoimmune diseases ... 653
Jenny Amaya-Amaya, Juan Camilo Sarmiento-Monroy, Adriana Rojas-Villarraga

39. Autoimmune diseases and cancer ... 683
Michael Ehrenfeld

40. The early autoimmune disease clinic. The case of rheumatoid arthritis ... 691
Carlos E. Toro-Gutiérrez, Lorena Bejarano-Pineda, Juan Manuel Pino, Yiset Adriana Fernández, Alejandro López, León Felipe Jaramillo, Álvaro Arbeláez-Cortés

SECTION IV

INNOVATIVE THERAPIES

41. Intravenous immunoglobulin-customized therapy ... 699
Gisele Zandman-Goddard, Yehuda Shoenfeld

42. Autologous peripheral hematopoietic stem cell transplantation ... 705
Álvaro Arbeláez-Cortés, Lorena Bejarano-Pineda, Carlos E. Toro-Gutiérrez

43. RNAi and autoimmunity ... 719
Felipe Cañas-Orduz, Gabriel J. Tobón, Carlos A. Cañas

44. Nanotechnology and autoimmunity ... 727
Arley Gómez López

SECTION V

BASIC PRINCIPLES OF LABORATORY

45. Cell culture and cell analysis ... 741
María-Teresa Arango, Paula Quintero-Ronderos, John Castiblanco, Gladis Montoya-Ortíz

46. Analysis of nucleic acids ... 755
Paola Cruz-Tapias, John Castiblanco, Nidia E. Correa, Gladis Montoya-Ortíz

47. A primer on current and common sequencing technologies ... 777
John Castiblanco

48. Analysis of proteins and antibodies ... 793
Paula Quintero-Ronderos, María-Teresa Arango, John Castiblanco, Nidia E. Correa, Gladis Montoya-Ortíz

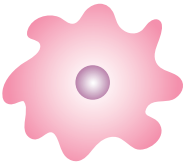
49. Epigenetic tools in autoimmune diseases ... 813
Paula Quintero-Ronderos, Marcela Burgos, Gladis Montoya-Ortíz

Contributors ... 835
Index ... 839

Icons used in this book


Macrophage


Dendritic cell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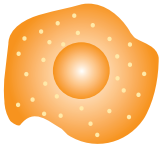
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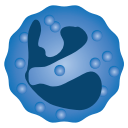
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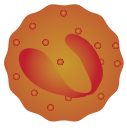
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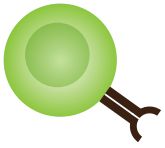
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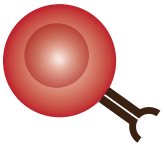
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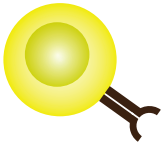
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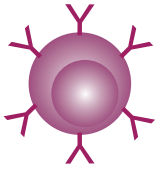
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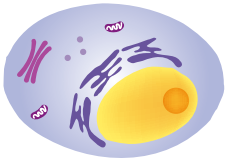
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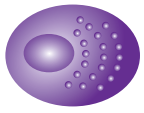
NK cell


Erythrocyte


Apoptotic cell


Endothelial cell


Epithelial cell

Foam cell


Microorganisms


C3


TCR


BCR


MHC I


MHC II

"In the end, we only have what we have given"
Isabel Allende
In *"Ines of my Soul"*

"Everything is autoimmune until proven otherwise"
Yehuda Shoenfeld

PREFACE

Complex biomedical problems remain such till profound insights are obtained that can segregate irrelevant epiphenomena and can set aside confounders to identify commonalities and principles that govern their occurrence. This book attempts to organize the knowledge collected by those who have extensively studied autoimmune diseases (ADs) into a comprehensive overview of how the immune system, governed by its own complex yet limited pathways, turns against self targeting distinct tissues in different conditions according to a balance determined by the genetic predisposition of the host, its gender, environmental circumstances and other co-factors that modify the outcomes of a similar pathological process. As well described in chapter 14, the tautology of autoimmunity is reflected by several aspects of ADs that span at least 10 converging observations (see Table 1). The hypothesis that ADs stem from common and overlapping mechanisms is clinically and scientifically relevant because, if correct, it suggests that the identification of commonalities among distinct aspects of AD may provide insights about salient mechanisms that are necessary and perhaps sufficient for autoimmunity to occur. Consequently, this information may suggest treatments endowed with broader efficacy.

It should be emphasized that autoimmunity is not alone among immune-mediated disorders and evidence suggests that other diseases may follow similar principles; cancer rejection during immunotherapy may represent a facet of autoimmune "tumoritis". In addition, allograft rejection in its chronic and acute form, graft versus host disease and some aspects of acute and chronic infection may follow similar principles although the triggering mechanism may differ. We refer to such converging mechanisms leading to immune-mediated tissue specific destruction as the "immunologic constant of rejection". This book clearly attempts to present a balanced overview of the causality and phenomenology associated with ADs providing a comprehensive view of basic immunologic principles in the first 13 chapters.

The novelty intensifies in the following section II, where the attempt is made to present general principles regulating the physiopathology of ADs describing cutting edge technologies to investigate them in the realm of direct human observation. Indeed, ADs exemplify the need for observational studies including epidemiologic investigations, broad genome-wide discovery and assessment of environmental exposure to complement mechanistic studies performed in the comfort of experimental conditions. ADs are multifaceted because of the diversity of the human genome, its epigenetic adaptations and its response to environmental stimuli. This is something that cannot be reproduced experimentally and needs to be tackled only through direct and skilled human observation. In this respect, immunologists who study autoimmunity have been at the forefront of this type of investigations and have produced in the years evidence of the genetic basis of several ADs, their dependence on common congenital and post-partum adaptations, the influence of accidentally acquired infections and other environmental conditions that modulate the switch even between identical twins from a dormant predisposition to full blown pathology.

The third section, following a more canonical pattern present individual pathology for those who need specific information since, in spite of the commonalities included in tautology of autoimmunity, peculiarities remain that affect the diagnosis, prognosis and treatment of individual diseases till more basic principles will be uncovered. The final two sections dwell with innovative therapies and laboratory and diagnostic tools that remain the ultimate goal for any clinical investigation and consequently are of interest to any responsible basic scientist attentive to the welfare of the recipient of his efforts: the patient.

This book strikes, therefore, a great balance at the experimental and translational intersection in presenting cutting edge views of ADs in the broader context of autoimmunology.

Francesco M. Marincola, MD, FACS.
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Section I

Introduction to immunology and molecular biology

1 GENE EXPRESSION AND REGULATION

Sandra Ramírez-Clavijo
Gladis Montoya-Ortíz

BASIC ELEMENTS OF GENE EXPRESSION

Life exists due to the emergence of molecules that store and transmit genetic information. Most of them are common to all living beings as are several of the mechanisms that intervene to allow the flow of information. Replication is one example of these mechanisms and, through it, cells obtain a semi-conserved copy of all the genetic material during the S phase of the cell cycle. This process only occurs in cells involved in cell division events. Other mechanisms are transcription, which consists of reading the genetic material fragments to produce different types of RNAs, and translation, in which RNA and ribosomes synthesize proteins. Living beings store genetic information in the form of single- or double-strand RNA or DNA nucleic acids. Therefore, the flow of genetic information between one cell and its progeny is guaranteed through replication while the flow of genetic information inside the cell is transmitted by reading the information contained in genes through transcription and translation mechanisms. These three mechanisms are strongly regulated and are activated or inactivated by signals detected and interpreted by cells, thus leading to the generation of a response with the participation of thousands of molecules that the cell signaling pathways consist of. This chapter focuses on gene expression and its regulation including transcription and translation processes. Once the concept of the gene and its structure are defined, the sequential stages of transcription, translation, and the molecules involved will be described. Moreover, the fact that gene expression is regulated during the entire process and is carried out through diverse mechanisms must be highlighted. Some examples of these mechanisms are: remodeling of molecules which serve as a template such as in the case of DNA in transcription and messenger RNA (mRNA) in translation; activation of protein factors which participate in different stages and can also be targets on signaling pathways; maturation and transportation of end products so they can acquire their functional structures and reach the sites where they carry out their function (inside or outside the cells) through the recognition of signals inscribed in the products of these mechanisms. Finally, some epigenetic properties of the genome that are important for gene expression will be discussed. These include the position of the gene in the chromosome and nucleus, the degree of chromatin condensation, and the multifractal characteristics of the genome. The latter property has recently been associated with gene regulation and genomic stability.

GENE STRUCTURE

The human genome has three billion pairs of bases of which only 1.5% is coding: the DNA used to synthesize proteins or different types of RNA such as ribosomal RNA (rRNA), transference RNA (tRNA), and messenger RNA (mRNA). These pairs of bases are DNA fragments that form structural reading units of genetic information called genes. Moreover, the human genome contains 20,000 genes and even though the functions of every one of them are not known, they are recognized as DNA fragments with promoter and coding regions which can be transcribed. The promoter region is indispensable for transcription because it guides the positioning of the transcriptional machinery. The region transcribed contains sequences of coding nucleotides called exons, non-coding regions known as introns, and 5'UTR and 3'UTR regions with regulatory functions. The initiator element (Inr) –site where transcription begins– is found between the promoter and the transcribed region. The non-encoding 5'UTR region is found

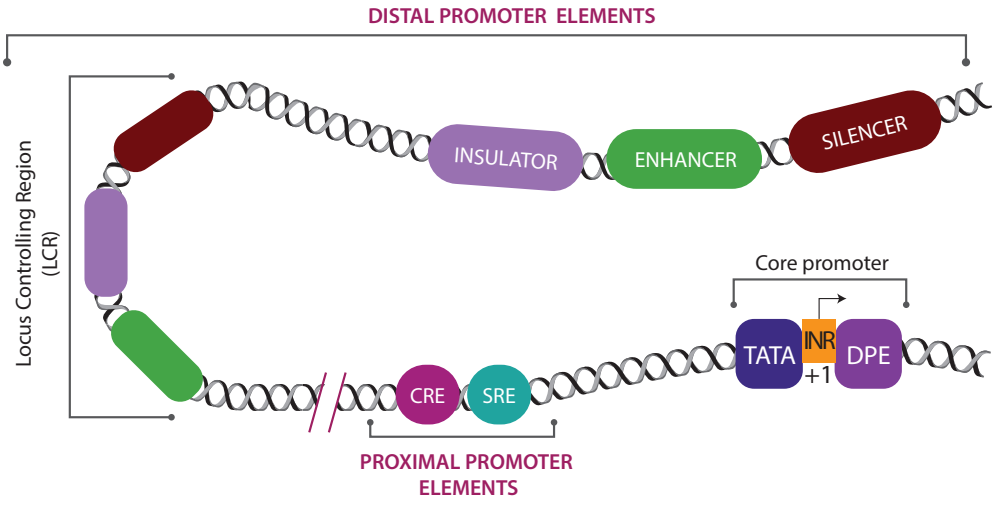

Figure 1. Eukaryotic promoter gene structure. There are two regions in the promoter. The first one is the core or basal promoter which consists of the TATA box located upstream of the transcriptional start site +1, the initiator element (INR) and the downstream promoter element (DPE). The second region consists of the upstream promoter elements, which are proximal and distal DNA sequences and provide a control point for gene transcription regulation. Modified from (17).

between the initiator and the first exon, which is then followed by the first intron. Thereafter, exons and introns are interspersed and after the last exon, there is a non-coding 3'UTR sequence containing the signal sequence to add the polynucleotide A tail (poly A) to the 3'end mRNA (Figure 1).

Promoter. This regulatory region has variable lengths and contains both specific and consensus sequences common to several promoters. Consensus sequences can keep genes in permanent activity and specific sequences regulate gene expression in response to different signals. These sequences are denominated proximal elements when they are close to the transcription initiation site and distal elements when they are located at distances that are greater than 1 Kb. Examples of proximal elements are TATA, CAT, SRE, CRE, AP1, and Sp1 boxes. The basic elements necessary for a promoter to be functional are the TATA box located -25bp upstream from the transcription start site, the Initiator (Inr) that includes the start site located at +1bp, and the promoter element (DPE) located +30bp downstream from the Inr. Distal elements also regulate gene expression and are located at variable distances. Some of them are: the locus controlling region (LCR) that regulates gene families expressed only during embryonic development such as homeotic genes; the isolator element, which is a sequence acting as a border between regions of transcriptionally active euchromatin or inactive heterochromatin. For instance, one isolator element that has been described in β-globin genes separates orodate receptor genes on one side from the folate receptor genes on the other. The last distal elements to mention are the enhancer and the silencer that promote or block transcription respectively. These act at long distances and in different positions and directions in relation to the gene.

The sequences mentioned above are part of the DNA elements acting *in cis* and are generally recognized by their association with transcription factors acting *in trans*. These factors locally modify the chromatin structure to assemble the transcriptional complex if they are activators, or to block the approach of this complex to the transcription initiation site if they are repressors.

At this point, it is important to define the transcription factors (TF) which are protein or lipid molecules with regions in their structure that constitute functional domains for the DNA interaction and/or other proteins and also for transcription activation or repression. Furthermore, TF recognize and bind to specific nucleotide sequences in the DNA. However, genes can have different nucleotide sequences in their promoters: a large percentage have some consensus sequences such as TATA and CAT boxes, but different elements which provide specificity to genes are found in proximal and distal regions. Therefore, alterations in the structure or function of TFs modify the gene expression which is under their regulation; even variations of a single nucleotide (SNP) in gene sequences codifying for TF and promoter elements have been associated with the development of diseases.

GENE EXPRESSION

Gene expression is determined by reading gene information during the transcription and translation processes. The product of transcription is mRNA and the product of translation is a protein. Not all genes encode proteins which means that they can be transcribed but their mRNA is not translated. Both processes are carried out in consecutive stages making it difficult to establish the boundary be-

BOX 1. Some aspects of promoter gene regions and Autoimmune Diseases

Promoter regions of genes associated with Autoimmune Diseases (ADs) have been the target of recent investigation due to their important role in gene regulation. In these regions, any variation could represent differences in gene expression patterns between healthy and sick individuals. Several studies have focused on the potential role of Single Nucleotide Polymorphisms (SNPs) in the development of ADs. Even though the results from most studies have not been evaluated at a functional level, the location of SNPs can provide important clues about their potential roles. Thus, further research on SNPs located in the promoter region of genes associated with autoimmunity should be done because they can affect differential binding of TFs on target sequences (Table 1). Regarding this matter, Maurano *et al*. did a study of DNase I sensitive sites in 349 cells and tissue samples through whole genome mapping (GWAS). This study identified 3,899,693 positions that were hypersensitive to DNase I. Many of these were located and concentrated in gene regulatory regions associated with Reumathoid Arthritis (RA), Multiple Sclerosis (MS), Systemic Lupus Erythematosus (SLE), and Type 1 Diabetes (T1D). Interestingly, sequence variants common to those diseases were systematically and specifically enriched in the recognition sequences of TF involved in the regulation of physiological processes.

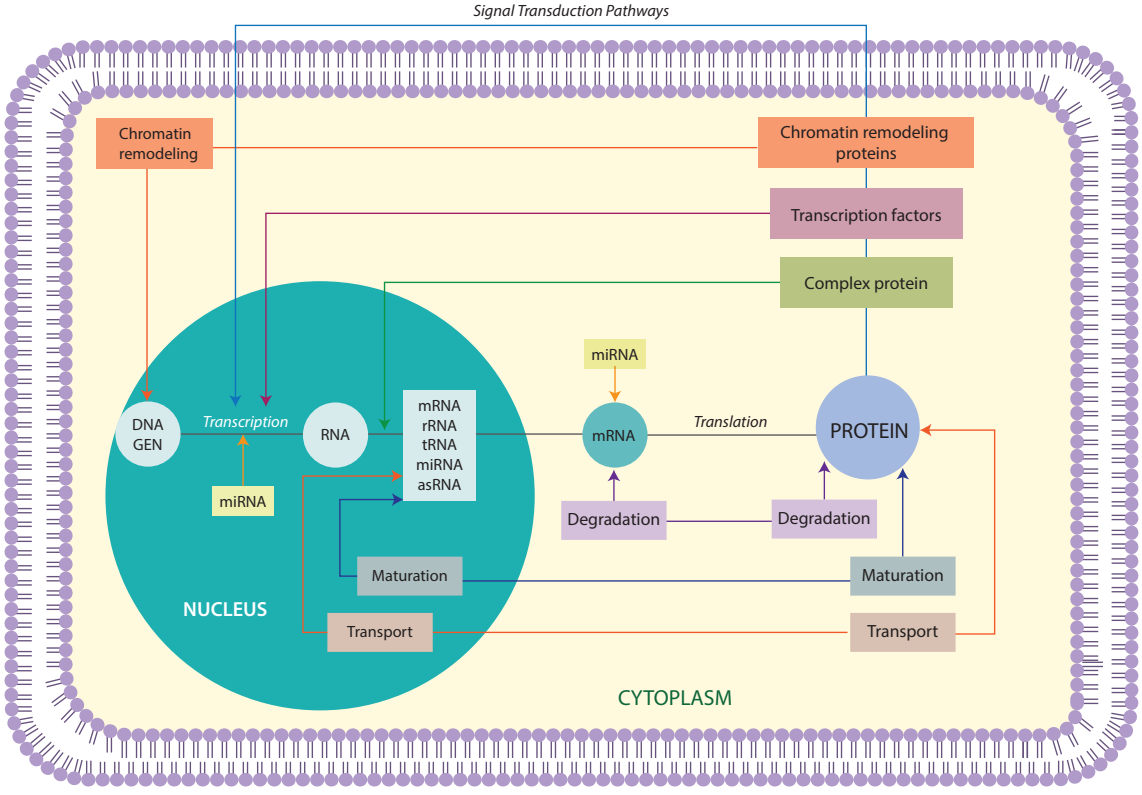

Figure 2: Pathways for gene expression regulation. There are many events associated with control of gene expression which are integrated into the following groups: events associated with RNA and protein molecules, epigenetic mechanisms, and signal transduction pathways. All of them can act at different times and sites during transcriptional or translational processes. Before transcription starts, the promoter is recognized by transcription factors targeted by signal transduction pathways. The transcription factors recruit chromatin remodeling complexes into the promoter which relaxes (open state allowing transcriptional activity) or condenses (closed state repressing the transcriptional process) the chromatin structure. Also, small RNAs such as miRNA can prevent the transcription. The transcription product is RNA, and it is processed by protein complexes in order to obtain mature RNA. Those RNAs remain there. However, those playing a role in the cytoplasm are transported through a nuclear envelope. Before translation starts, the RNA may be targeted by small RNAs or protein complexes in order to promote or block protein synthesis. Also, RNA stability is regulated: it is degraded or kept within the cytoplasm. Proteins are the translation products and the target for structural modifications by other protein complexes. Those changes are required in order to achieve their functional structure, transport them to the locations where they interact, or degrade them. All these steps are regulated by mechanisms responding to signals produced outside or inside of the cells. These constitute large networks of cellular communication regulating gene expression.

tween them. Moreover, each one includes multiple protein complexes in which subunits are recruited as the process advances or, instead, are released and replaced by others once they fulfill their function. Transcription, translation, and their regulatory mechanisms require a permanent recognition of molecular structures, interactions and associations between compounds, and the identification of signals that constitute true communication codes (Figure 2).

TRANSCRIPTION

Transcription is a process in which the fragment of genetic information with a structure or DNA template is read in order to synthesize RNA molecules. The phases of transcription are: pre-initiation, initiation, promoter clearance, elongation, and termination. All of them include the participation of protein complexes the components of which are assembled and disassembled as the process advances. Most of the mechanisms which regulate the flow of genetic information act in the period prior to the reading process, where signals and molecules must come together to assemble the pre-initiation complexes that will be modified to form the initiation complexes. Furthermore, different factors facilitate, review the process, and keep the machinery active during elongation and, during termination, spatial structures or *cis* sequences are recognized to stop the reading machinery. There are three types of genes which codify RNAs or proteins. Type-I genes encode rRNA (28S, 18S, and 5,8S) and are transcribed by RNAP I holoenzyme. Type-II genes encode mRNA, hnRNA, snRNA, miRNA, and telRNA and are transcribed by RNAP II. Finally, Type-III genes encode tRNA, snRNA, and 5S rRNA and are transcribed by RNAP III holoenzyme. The three RNAP complexes consist of different subunits and are accompanied by general transcription factors specific to each complex. In all of these types of genes, the promoter is the axis where protein complexes arrive, assemble, and also accompany RNAP during the beginning of transcription. RNAP provides the site of assembly for the complexes which are in charge of the continuity of the process and of modifying the growing RNA molecule. Nevertheless, RNA modification is different for each type of RNA. For example, to obtain mature mRNA (the template used in protein synthesis), co-transcriptional modifications such as capping, splicing, cleavage, and poly (A) addition must be made.

Transcription details of type II genes transcribed by RNAP II are the ones that are best understood so far. Even though some miRNA can also be transcribed by RNAP III, they have promoters with structures that are different from those described above. Note that RNAP II is a holo-

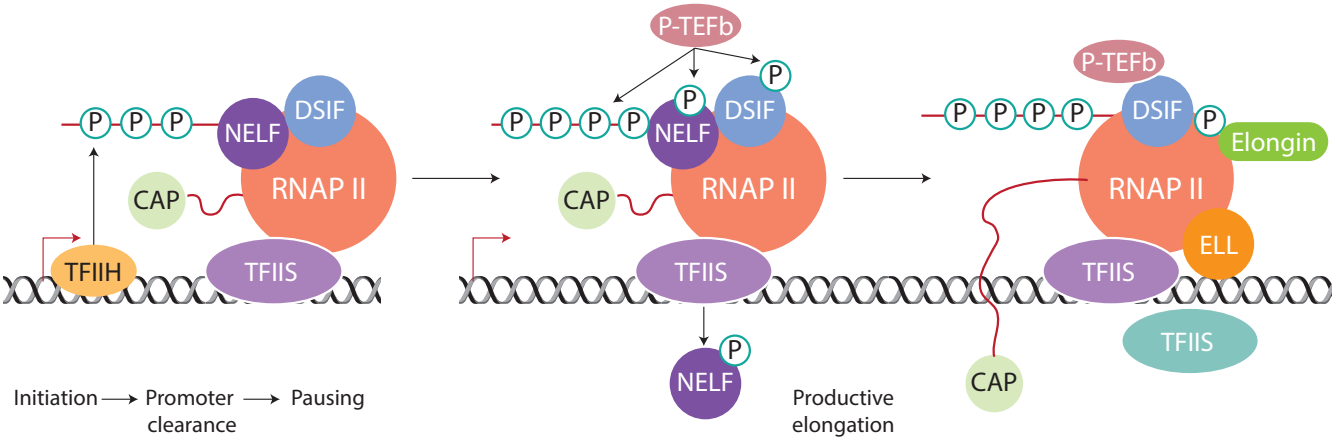

Figure 3. The C-terminal repeat domain (CTD) from the holoenzyme RNAP II. The RNAP II enzyme is in charge of reading gene sequences and synthesizes RNA molecules. This holoenzyme is composed of 60 polypeptides. The largest RNAP II subunit - Rpb1 - has a C-terminal repeat domain (CTD), which is an unusual peptide extension. The CTD consists of multiple heptad repeats (consensus Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7) varying in number from 26 in yeast to 52 in vertebrates. The CTD serves as a flexible binding scaffold for numerous nuclear factors at different steps during the transcription process. These interactions depend on the phosphorylation patterns within the CTD. Thus, CTD is phosphorylated on Ser5 and Ser7 by CDK7, which stimulates RNAP II polymerase activity and thus, allows transcription to begin. Afterwards, CDK9 and p-TEFb phosphorylate Ser2 which leads to promoter clearance and the recruitment of capping enzyme into the vicinity of nascent mRNA. Subsequently, NELF binds to CTD and stops the transcription while splicing factors are recruited into the primary transcript. DSIF immediately interacts with CTD as an anti-terminator factor to continue the transcription. Next, phosphate groups on Ser5 and Ser7 are gradually removed by phosphatases such as Rtr1and Ssu72. At this point, Elongin and ELL complexes bind to RNAP II to promote the elongation. Also, phosphorylated Ser2 serves to recruit 3' processing complexes involved in polyadenylation and cleavage mechanisms. As RNAP II is finishing the process, Fcp1 dephosphorylates Ser2 to regenerate unphosphorylated RNAP II which can be recycled for another transcription round. Modified from (25).

enzyme made up of 60 polypeptides with an approximate weight of 3-million daltons (MDa). It is organized in 12 subunits denominated Rpb1-Rpb12 and a core that consists of Rpb1, Rpb2, Rpb3, Rpb6, and Rpb11. Moreover, the carboxyl terminal end (CTD) of the Rpb1 subunit has a domain of 7 amino acids (Tyr1, Ser2, Pro3, Thr4, Ser5, Pro6, Ser7) YSPTSPS which is repeated 22 times in yeasts, 44 in mice, and 55 in human cells (Figure 3). However, the sequence can have subtle differences between repeated segments and ends in a 10 amino acid sequence. Therefore, CTD is an interaction site for multiple factors including factors that promote chromatin remodeling.

Other functions of CTD are: guarantee the processing of the RNAP complex, avoid early termination, modify the new RNA co-transcriptionally, and promote elongation. The CTD's binding site to different complexes is called a "docking" domain and the large combination of complexes that binds to this domain constitutes the CTD code, which determines the recruitment and release of proteins at the appropriate moment during transcription. Furthermore, during a transcription cycle, the CTD structure is modified by phosphorylation and dephosphorylation on both Ser2 and Ser5. However, it must remain hypophosphorylated during initiation and hyperphosphorylated during promoter clearance and elongation, but it must be dephosphorylated in order to be recycled.

The CTD domain can be phosphorylated at different transcription stages by three main kinases: Cdk7, Cdk8, and Cdk9 and is dephosphorylated by phosphatases. The Cdk7/Kin28 complex is a TFIIH subunit that phosphorylates the CTD on Ser5, which is essential to recruit Set1 and enzymes participating in cap formation and promoter clearance regulation. Cdk8 is associated with the Srb/mediator, which is a complex that connects enhancers and silencers with RNAP II before elongation. Additionally, Cdk9 and cyclin T are subunits of the positive transcription elongation factor b (P-TEFb). Cyclin T recognizes a sequence rich in histidine on CTD. On one hand, phosphorylation on Ser2 by Cdk7, Cdk8, and Cdk9 favors the recruitment of factors facilitating continuity of transcription and splicing. On the other, dephosphorylation of the CTD domain is mediated by Ser2 specific phosphatase (FCP1) and Ser5-selective phosphatase (SCP1) with the participation of TFIIH. FCP1 is a factor that is also involved in the modulation of transcriptional pauses and RNA processing. It interacts with the DRB-sensitivity-inducing factor (DSIF) and RNA polymerase-associated factor 1 (PAF1) and is inhibited by proteins from the capping complex (Figure 3).

PRE-INITIATION

The first phase of transcription is pre-initiation, which consists of formation of the pre-initiation complex (PIC) on the promoter of the gene. PIC is composed of general and specific transcription factors, coactivators, and the RNAP complex. Its assembly is stimulated when an activator binds to one of the promoter sequences thus generating a spatial structure recognizable to coactivators located in the same region. This region also recruits ATP-dependent complexes to produce chromatin relaxation.

Therefore, these changes provide the space necessary for the arrival of basal factors including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and the RNAP II complex. PIC formation is nucleated by the TATA binding protein (TBP), which is a TFIID subunit. In the absence of the TATA box, another TFIID subunit can bind to another element of the promoter and support PIC formation. Afterwards, TFIIB binds to and surrounds the DNA-TBP complex, and its N-terminal domain extends to the transcription start site and provides support for the binding of RNAP II. Moreover, TFIIF and RNAP II bind to the promoter guided by TFIIB and, at the same time, TFIIE joins the complex and acts as a signal to recruit TFIIH. At this point, PIC is assembled on the promoter core (Figure 4).

TFIID is composed of TBP and 14 TBP associated factors (TAF). Some TAF subunits have acetyltransferase activity or

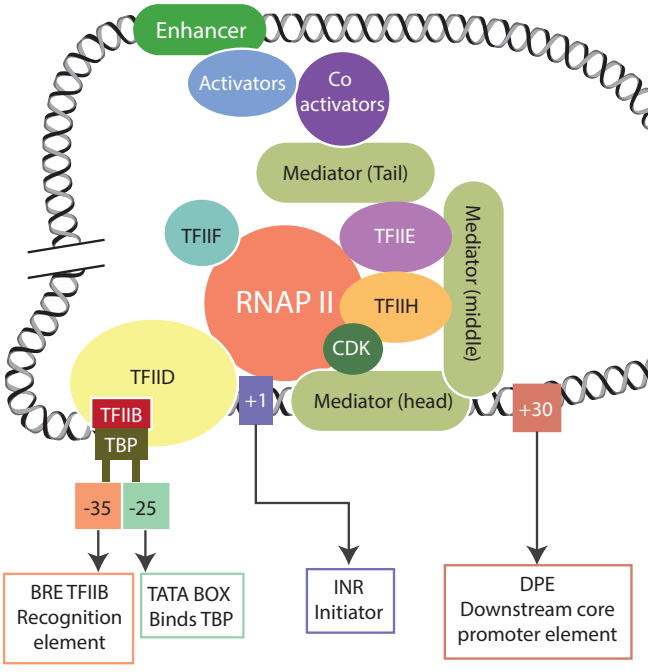

Figure 4. RNAP II transcription preinitiation complex (PIC). PIC is made up of general and specific transcription factors, coactivators, and the RNAP II complex. PIC assembly is stimulated when an activator binds to one of the promoter´s sequences. Then, TBP binds to TATA box and the assembly of RNAP II begins by the stepwise binding of general transcription factors, TFIIB, TFIIF, TFIIE, and TFIIH. Afterwards, coactivator complexes are recruited including chromatin modifiers and mediators, which interact directly with RNAP II and general transcription factors. Next, TFIIH Cdk7 subunit phosphorylates Ser5 within the CTD and induces the activation of XPB helicase function. This activation produces the single DNA strand which stimulates RNAP II polymerase activity. Modified from (13).

are associated with chromatin remodeling complexes such as **S**pt-**A**da-**G**cn5-**A**cetyltransferase (SAGA). TAF1 and TAF2 bind to the Inr element, while TAF6 and TAF9 bind to DPE. Additionally, TFIIH consists of 10 subunits and has helicase activity on the XPB (Xeroderma pigmentosum factor D, also known as protein ERCC2) subunit. Furthermore, XPB is a 3'-5' DNA-dependent helicase required for transcription and DNA repair through nucleotide excision (NER) and belongs to Superfamily II which consists of ATP-dependent helicases containing iron-sulphur cluster domains. Therefore, TFIIH facilitates promoter melting during initiation and regulates the transition from initiation to elongation by Cdk7-catalysed phosphorylation of RNAP II CTD in its Ser5 residue. The presence of mediator complexes during PIC formation has been described in some promoters. The mediator complex, which is composed of at least 24 subunits and has a mass greater than 1 MDa, acts as a general transcription factor. It integrates regulating signals from enhancers and silencers, associates with the hypophosphorylated RNAP II complex, and stimulates the TFIIH kinase activity.

INITIATION

Initiation occurs after PIC formation when the reading machinery is assembled and two initiating nucleoside triphosphates (NTPs) are linked according to the DNA sequence and, thus, lead to the formation of the first phosphodiester bond. When RNAP II is assembled on the promoter, the TFIIH Cdk7 subunit phosphorylates Ser5 on CTD and induces the activation of the XPB helicase function. Activation of XPB produces the single DNA strand that stimulates RNAP II polymerase activity which marks the beginning of transcription. Afterwards, CTD is phosphorylated in Ser2 by the CDK9 subunit of P-TEFb which is associated with cyclin T1, T2a, and T2b or cyclin K. Phosphorilation of CTD by P-TEFb leads to promoter clearance.

PROMOTER CLEARANCE

After the bonding of 20 to 40 ribonucleotides, CTD is dephosphorylated on Ser5 by Rtr1 and Ssu72 phosphatases. In contrast, it is phosphorylated on Ser2 by CDK9. These changes produce the release of general transcription factors from the RNAP II complex which will remain on the promoter thus serving as a scaffold for the formation of the other RNAP II Initiation Complex (IC) (figure 5).

It has been noted that some Ser5 must remain phosphorylated along with Ser2 to allow promoter clearance, which consists of the movement of the transcriptional machinery away from the promoter. The stability of the complex increases when TFIIB is released and TFIIF remains in it thus producing a pause or termination of the process.

ELONGATION

Elongation is a very important step for the integration and coordination of multiple events such as RNA synthesis which guarantees the process of the reading, inhibition of premature transcription termination, and prevention of errors during the process. Other important events are related to RNA maturation. The binding of other proteins to the RNAP II complex generates the transcription elongation complex (TEC) (figure 6), which is composed of two protein families classified as active (TFIIF, Elongins, ELL, DSIF, NELF, CSB, FCP1, TFIIS, Spt6,

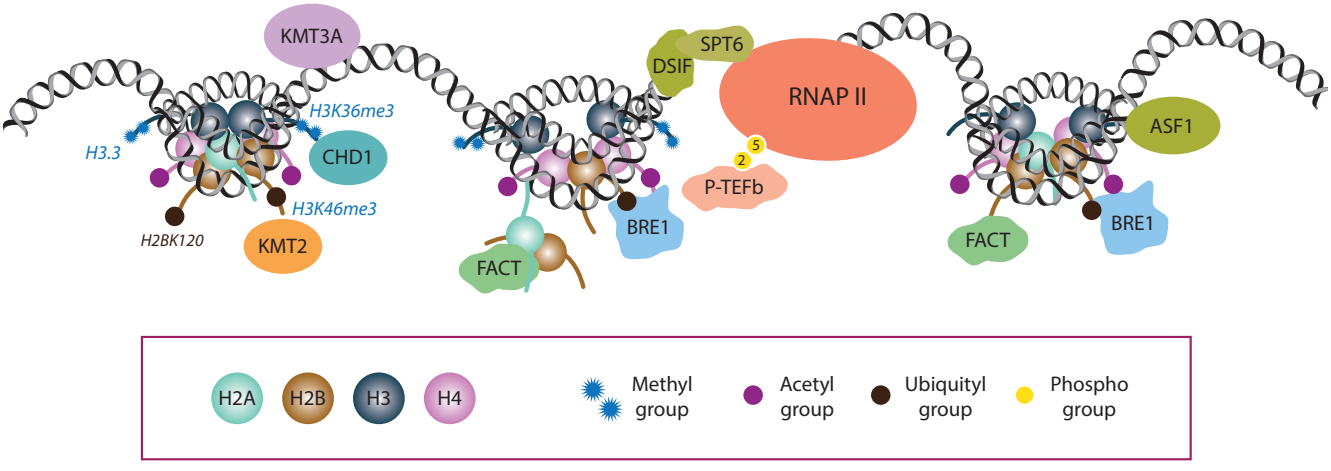

Figure 5. RNAP II transcription initiation complex (IC). IC complex is organized after transcription preinitiation complex (PIC) is assembled, and 20 to 40 ribonucleotides are linked according to the DNA sequence. CTD is phosphorylated on Ser5 and Ser2 producing the release of general transcription factors to allow promoter clearance. Finally, TFIIB is released while TFIIF remains into the promoter, IC is assembly, and the pause or termination of the process takes place. Modified from (4).

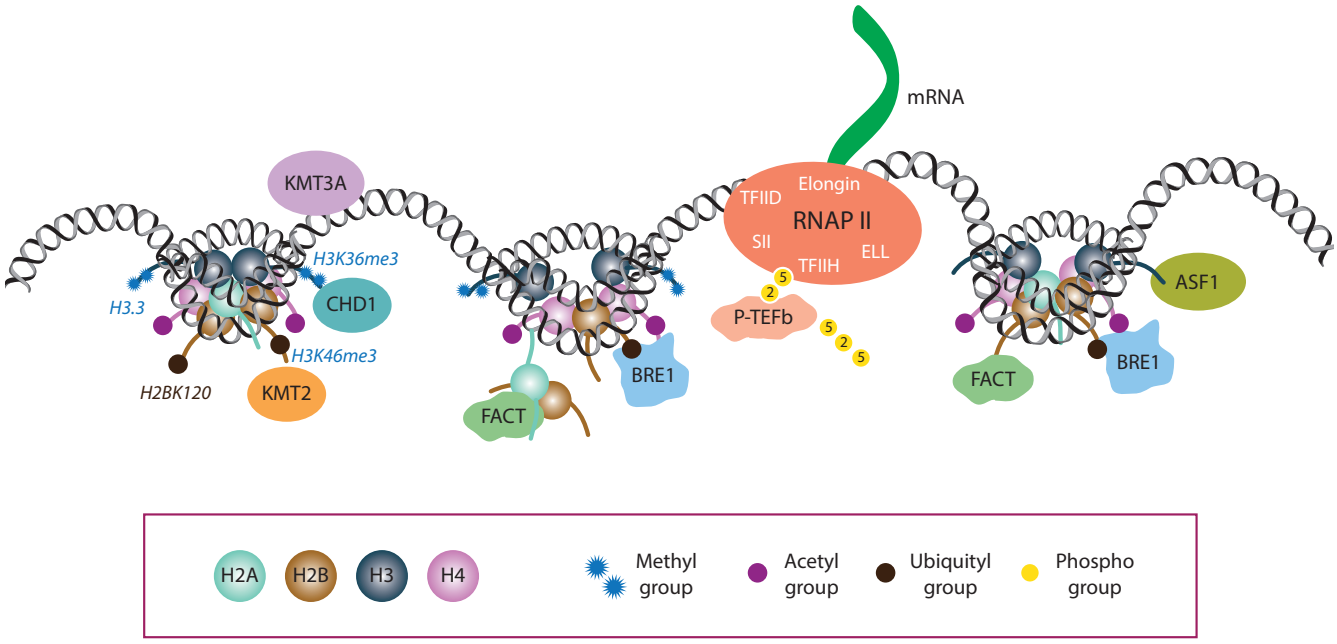

Figure 6. RNAP II transcription elongation complex (TEC). The TEC assembly is a very important step for multiple such as processivity of the reading, premature transcription termination inhibition, and error prevention during the process. TEC consists of approximately 23 elongation complexes including: TFIIF, TFIID and TFIIH, Elongin SIII, NELF and the eleven-nineteen lysine-rich leukemia (ELL) complex. RNA Capping, splicing and polyadenylation processes occur during the elongation step. Modified from (4).

19S Proteasome) or passive proteins (P-TEFb, Ssu72, SWI/SNF, Isw1p, CHD1, FACT, Set1, Set 2, Paf, tho, TREX, and Iws1/Spn1) on the basis of their action on RNAP II catalytic activity. Over 23 elongation complexes are in charge of preventing RNAPII from pausing or stopping after the transcription process has started. The activity of TFIIF, an active protein, is regulated through phosphorylation by TFIID and TFIIH subunits. TFIIF lessens pausing and stimulates the rate of RNAP II. At the same time, TFIIH contributes to stabilizing the RNAP II complex, releasing TEC from the promoter, and keeping TEC close to the promoter. Other active proteins belong to the Elongin SIII complex, which consist of subunits A, B, and C, prevents RNAP II from being paused or stopped, and promotes its advance along the DNA. Subunits B and C also participate in the ubiquitination-dependent mechanism and are able to stop the transcription process when DNA mutations are detected. The eleven-nineteen lysine-rich leukemia (ELL) complex belongs to a three-member family composed of ELL1, ELL2, and ELL3 and acts as an antitermination factor during the elongation. Another active protein is DSIF, a heterodimeric complex with Spt1 and Spt5 subunits that binds directly to RNAP II and pauses transcription in the presence of 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole. It also acts along with NELF to prevent a premature termination and interacts with several factors such as TFIIF, TFIIS, CSB, chromatin modeling factors including Spt6, FACT, CHD1, and the PAF complex. Subunit Spt5 also interacts with mRNA stability and maturity factors. It also favors continuation of elongation through processes of methylation in Arginine residues and in vitro phosphorylation by PRMT1 and PRMT5. As a result, DSIF stimulates elongation, suppresses early termination of the transcription process, and stimulates capping. As was mentioned before, NELF acts as an active protein complex which consists of NELF-A, NELF-B, NELF-C, NELF-D, and NELF-E. It binds to the DSIF/RNAP II complex and halts transcription to allow the assembly of factors participating in 5'- capping, maturation and transport of mRNA. P-TEFb, in turn, accompanies RNAP II during its displacement along the gene, lessens the pause mediated by NELF through phosphorylation of Ser2-CDT and DSIF (Spt5) thus allowing dissociation of the NELF complex and enabling the elongation process to continue.

The protein CSB is a DNA dependent ATPase which binds directly to the RNAP II complex and modulates TFIIS activity. It also participates in transcription-coupled repair (TCR) and nucleotide excision repair (NER) systems. Furthermore, it has been found that the NER system is defective in patients with premature aging syndrome or Cockayne Syndrome (CS) in whom CSB mutations have been identified. Finally, the TFIIS complex restores the transcription process after a pause, improves the efficiency of the process, and increases the rate of synthesis after it has been reduced during the pause. It is physically associated with Spt5 and contributes to the "proofreading" activity of the RNAP II complex.

BOX 2. Gene transcription elongation step regarding autoimmune phenotype

Regarding participation of molecules in the elongation step during the gene transcription process, a recent study found evidence of the involvement of the transcription factor (Aire) in this specific step. Although Aire has been reported to interact with a large number of proteins involved in transcription, its mechanism of action has remained elusive. Recently, an isoform variant of this factor which is transcriptionally inactive was found in a patient with autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). This variant is characterized by the loss of its extreme C-terminus and it is noteworthy that in the absence of this region, Aire is unable to interact with positive transcription elongation factor b (P-TEFb) through a transcription activation domain accessory (TAD). This interaction is important because it allows Aire to increase the levels of RNAP II, particularly on the gene body and the 3'-end, but not in promoter regions. Additionally, it also allows enhanced mRNA splicing of genes regulated by this transcription factor. This is possible due to the Ser2 phosphorylation of CTD. Dissection of this mechanism mediated by P-TEFb and Aire is very interesting in the context of central tolerance and the prevention of responses against itself.

CO-TRANSCRIPTIONAL EVENTS

Protein complexes approach the primary transcript for maturing process, achieve the functional structure and regulate its permanence inside the cell and its export outside the nucleus as elongation progresses in a co-transcriptional manner. Modifications vary among types of RNA (e.g., modifications of mRNA include capping, addition of polynucleotides of Adenine to form the poly A tail, 3' cleavage, and splicing) and always follow this order. CTD is the platform on which most of these events are coordinated and its phosphorylation status determines the structural conformations promoting association or dissociation of complexes as needed.

In yeasts, a model with 26 repetitions of the CTD heptapeptide revealed the formation of a left-handed beta spiral structure which is stabilized by phosphorylation on Ser2 and is not compatible with phosphorylation on Ser5. This structure favors the assembly of pre-mRNA modifying complexes.

Capping

Pre-mRNA is the raw material for the maturation process where it is transformed into a functional mRNA for translation. The capping reaction occurs after the newly synthesized pre-mRNA has a length of approximately 20–30 bases. At that point, RNAP II pauses, and three proteins approach the 5' end of the pre-mRNA. The first protein is the 5 –triphosphatase that converts the 5' triphosphate nucleotide into diphosphate. This is followed by the fusion of one GMP catalyzed by guanylyltransferase. Afterwards, a methyltransferase adds a methyl group to the N7 of GMP while the first and sometimes second nucleotides of the primary transcript are methylated in their 2'-hydroxyl groups to complete the capping structure. The capping process depends on the presence of Ser5 phosphorylated CDT and on the interaction between capping enzymes and DSIF. There is a checkpoint that verifies the capping mechanism's fidelity and contributes to RNAP II stability. The capped end of the mRNA is protected from exonucleases and is recognized by specific proteins belonging to the splicing, transport, and translational machineries. Once the capping structure has been synthesized, the FCP1 enzyme helps to remove the enzymes participating in its assembly.

3'cleavage and poly (A) addition

Cleavage occurs on dinucleotide GU at the 3' end of pre-mRNA. Pre-mRNA is released from TEC and followed by the addition of a variable length fragment called poly A tail. Meanwhile, RNAP II continues transcribing several kilo bases ahead of the GU, thus forming an RNA strand that will be degraded. Poly A polymerase builds the poly A tail (about 200–300 nucleotides long) and the poly A binding protein (PAB) catalyzes its union with the 3' end of mRNA, which is guided by the recognition of a AAUAAA signal present in the primary transcript (approximately 10-30 bp away from DNA dinucleotide CA) where the cleavage takes place. Cleavage factors CFI and CFII cut the 3' end of pre-mRNA, which requires the localization of the cleavage poly adenylation stimulating factor (CPSF) in the AAUAAA pre-mRNA sequence and the binding of cleavage stimulation factor (CstF) to a pre-mRNA sequence rich in GU. Note that CPSF is a TFIID subunit recruited during PIC formation which shows a connection between transcription initiation and termination processes.

Splicing

Splicing consists of the selective elimination of fragments (primarily introns) from pre-mRNA, which is an essential step in the expression of multiple genes. The cell machinery that splices pre-mRNAs uses information at the splice junctions to determine where to cut and where to rejoin the mRNA. The elimination of exons, through which different mRNAs

are obtained and are translated into protein isoforms, is less common and is called alternative splicing. Exons are relatively short sequences (typically, 50–250 base pairs in length) separated by larger sequences called introns (typically, hundreds to thousands or more base pairs in length), which account for approximately >90% of the primary transcript. The complex responsible for this process is called spliceosome and it consists of more than 100 core proteins and five small nuclear RNAs (snRNA) (U1, U2, U4, U5, and U6). The spliceosome is recruited in the presence of hyperphosphorylated CTD and requires additional regulatory proteins to perform its functions. Some of these regulatory proteins have ATP- dependent helicase activity and others belong to the serine/arginine-rich (SR) family of proteins which bind to the ESE element next to the 3′ end exon. Moreover, core splicing signals are three sites in every intron: the 5′ splice site (5′ss: 5′A/C AG - GU A/C AGU 3′), the 3′ splice site with the polypyrimidine (Y) sequence (3′ss: 5′YYYYYYYYYYYYN C/U AG-G G/U3′), and the branch point sequence (BPS).

The process starts when U1 binds to the 5′ss (U1 bears a complementary sequence to 5′ss) and is followed by the binding of U6. At the same time, U2AF joins the 3′ss sequence and SF1/mBBP is recruited in BPS. Later, U2 is associated with BPS- SF1/mBBP complex (Figure 7). After this, U2AF recruits U2 to the intron's central region, ESE, and it, in turn, associates with SR and constitutes the U4, U6, and U5 binding platform. At this point, U1 and U4 abandon the complex, followed by the formation of a loop and activation of U6 ribozyme function which cuts the 5′ and 3′ ends of the intron, thus producing the release of the intron in order to bind the exons. During the intron's cutting process, U5 keeps the exons together. Finally, the exons are ligated and the spliceosome is disassembled. Splicing is regulated by cis-elements (ESE, ESS, ISS, and ISE) and trans-acting factors (SR proteins, hnRNP, and unknown factors), and occurs with very high accuracy.

TERMINATION

Termination leads to RNA release and disassembly of the reading machinery. RNAP I and RNAP II have a common termination factor: TTF2 which is independent from CTD and has a mechanism which is not well understood. TTF2 is also associated with RNAP II surveillance activity and nuclear export.

Once the mRNA is synthesized and checked, the exosome complex with 3′-5′ exorribonuclease activity elimi-

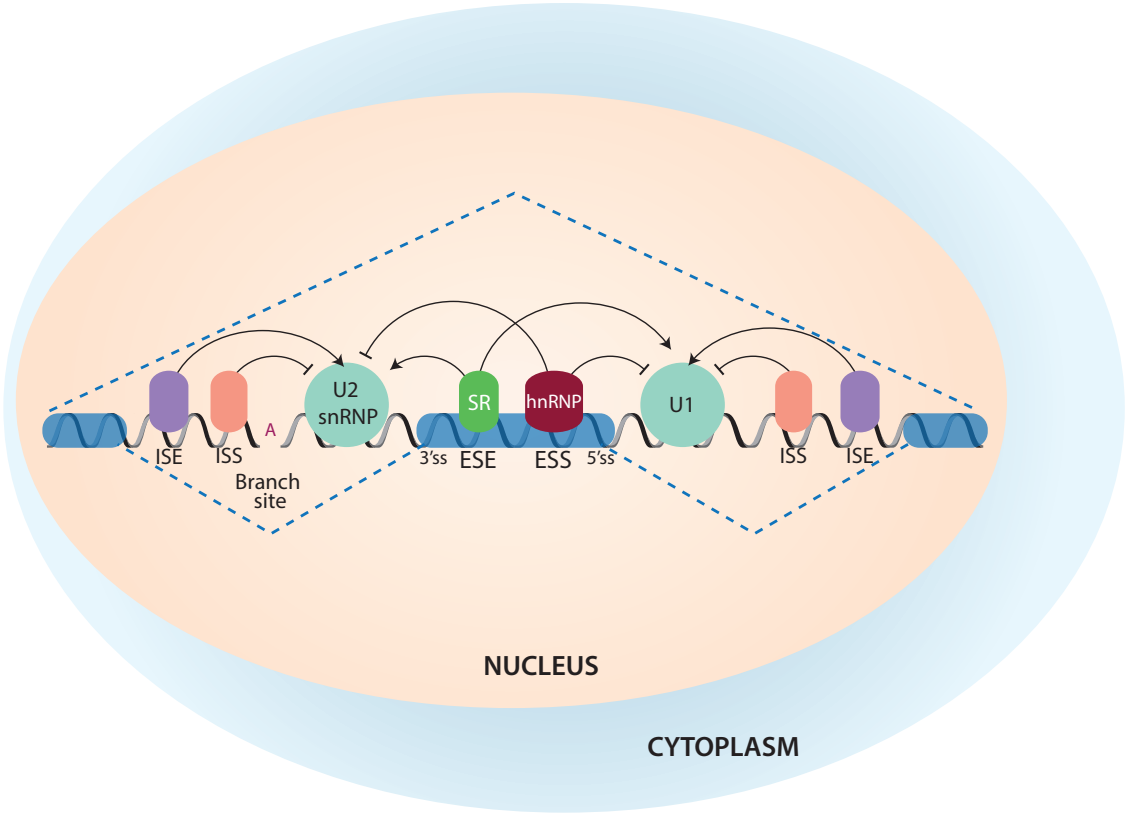

Figure 7. Alternative splicing mechanism. Two alternative splicing pathways where the middle exon is either included or excluded are shown. The consensus motifs of 3′ and 5′ ss are indicated as the branch point adenosine. The regulator cis-elements (ESE, ESS, ISS, and ISE) and trans-acting splicing factors (SR proteins and hnRNP) are marked. Modified from (30).

BOX 3. Alternative splicing and its relationship with Autoimmune Disease

The alternative-splicing mechanism has been extensively studied in the context of AD and involves multiple genes that are important in the development of these diseases. Alternative splicing may result from mutations in the regulatory elements (cis and trans) which may directly cause the development of the disease, influence its severit,y or modify its susceptibility. One specific example is the CTLA4 gene transcripts (molecule critical for the negative regulation of T cell proliferation). There are two variants of the gene produced by splicing, a trans-membrane (exon 1-4) form and a soluble (exon 3 is lost) form. The former has been involved in protection mechanisms for T1D. In this context, the allele associated with susceptibility (+49 A/G) produces decreased levels of the soluble form, which increases susceptibility to T1D. Furthermore, interleukin receptor 7 (IL7R) transcripts are another important case where alternative splicing is related to ADs. This gene has a non-synonymous SNP (rs6897932, allele T or C) located in exon 6 which is associated with multiple sclerosis (MS). The presence of allele C in patients with MS increases the loss of exon 6 and this loss has been related to decreased expression of the trans-membrane form and an increase in the soluble form. Moreover, this condition appears to compromise the signal transduction downstream, thus affecting the survival and proliferation of T cells mediated by this receptor and the appropriate function of these cells in patients with MS. Other molecules in autoimmunity that suffer significant alternative splicing are summarized in Table II.

nates the mRNA carrying alterations. An exportin complex of the mRNA called transcription/export (TREX) participates in mRNA transport to the cytoplasm and it is also associated with elongation.

TRANSLATION

Translation is the process in which the information contained in the genes is transformed into proteins. The information expressed in the language of nucleotides (nucleic acids) is translated into the language of amino acids (proteins). In eukaryote cells, translation takes place in the cytoplasm, hence implying an additional regulation point in comparison to prokaryote cells because, in the case of eukaryote cells, it is necessary to export the molecules which will be used in protein synthesis such as mRNA, rRNA, and tRNA from the nucleus to the cytoplasm. The central organelle of translation is the ribosome, which has enzymatic properties that guarantee a balance between precision and rate of protein synthesis. Ribosomes have two main functions: facilitate the correct interpretation of the genetic code and form the peptide bonds. The ribosome is the site where information contained in the mRNA is read and interpreted according to the rule established by the genetic code in which three ribonucleotides are equivalent to one amino acid. Inside the ribosomes, each amino acid provided by tRNAs is assembled and the peptide bond is formed. All the elements required during translation must be present in the cytoplasm including: tRNAs carrying amino acids, ribosomal subunits, the mRNA template, and translation factors. Prior to ribosome assembly, the formation of the pre-initiation complex (PIC) upstream from the start codon on the mRNA's 5UTR region (figure 8) and the complex in charge of finding the AUG initiation codon which determines the open reading frame (ORF) is necessary. Afterwards, the larger 60S ribosomal subunit is added to the structure, thus forming the 80S ribosome and marking the beginning of translation. During translation and the ribosome moves along the mRNA while the protein is synthesized until it identifies the stop codon which produces the disassembly of the ribosomal units and the release of the protein.

The ribosome has three interaction sites: the first one, which is called A site, is where the aminoacyl tRNA enters and the codon-anticodon is recognized, the next is the peptidyl site (P site) where the peptide bond is catalyzed, and the last one is where the deacetyl tRNA without amino acid is before it leaves the ribosome (E site).

PRE-INITIATION

Prokaryote cells have a specific sequence that is 3 to 9 nucleotides in length (UAAGGAGG) in 5'UTR mRNA called Shine–Dalgarno, which is complementary to the 3' end of the ribosome's minor 16S rRNA subunit. Their interaction results in the formation of a duplex and the bond between the ribosome's minor subunit and the mRNA. Thereafter, the duplex is destabilized and the subunit is displaced to where it finds the start codon. A similar sequence has not been described in eukaryotes. Rather, eukaryotic initiation factors (eIFs) have been found to facilitate the bond of the minor ribosomal subunit (40S) and the pre-initiation complex slides to the start codon. However, it has been reported that some mRNAs carry internal sequences called internal ribosome entry sites (IRESs) which are in charge of recruiting PIC to the start codon, thus starting a capping-independent translation. Some of them correspond to viral mRNA. In eukaryotes, PIC weighs 48S. It is initially located on the mRNA's 5'- UTR capped region and

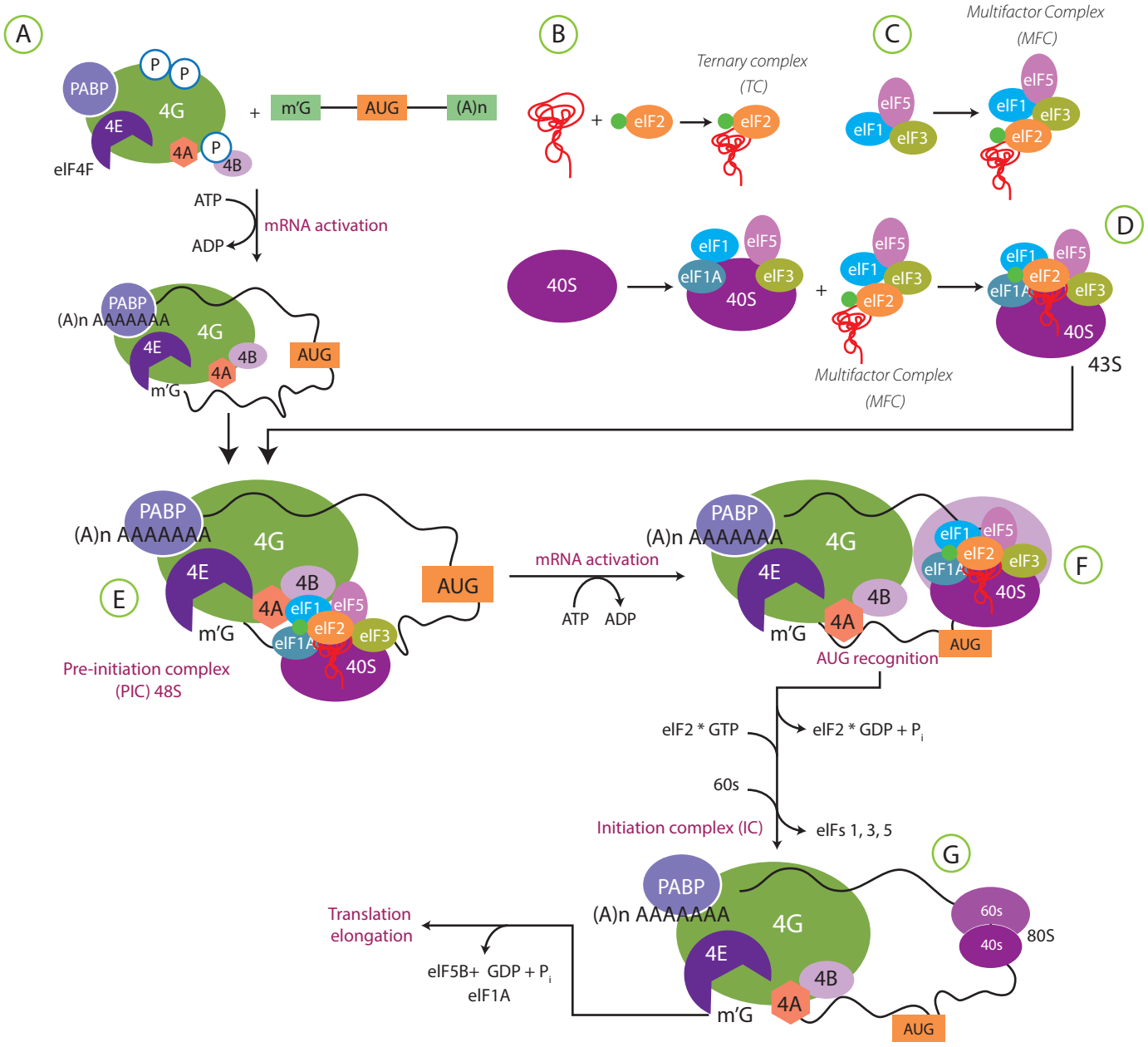

Figure 8. Eukaryotic Translation pathways. Many complexes are assembled in order to initiate the translational process. First of all, (A) mRNA is activated by eIF4F complex binding (eIF4E-eIF4G-eIF4A) to the cap and PABP to the poly (A) tail thus encircling the mRNA. Second, the ternary complex (TC) (B) and the multifactor complexes (MFC) (C) are assembled with the ribosomal subunit 40S to organize the 43S complex (D). Then, the active mRNA is associated with the 43S complex and they form the initiation translational complex PIC (48S) (E). Next, PIC moves along the mRNA in the 5' to 3' direction to where it finds the start codon AUG (F). When the PIC reaches this point, there is a perfect match between the start codon and its Met-tRNAi anticodon, which then produces the GTP hydrolysis of the eIF2-GTPMet-tRNAi. At the same time, eIF5B mediates the binding of the 60S ribosomal subunit to the PIC to allow ribosome 80S assembly. Therefore, the initiation translation complex (IC) consists of 80S ribosome, mRNA, aminoacyl tRNAs, and initiation translation factors (G). Modified from (28).

moves along the mRNA through a scanning mechanism until it reaches the start codon (AUG). The assembly of this complex is guided by the eIF4F, eIF4A, and eIF4B factors present in the capping complex while the protein binding poly A (PABP) guides the approach of the 3'end to the same capping complex. This leads to the formation of a loop and the closure of the 5'and 3' ends. PABP interacts with eIF4G and promotes mRNA circling to form a "closed loop" which facilitates the reinitiation of translation by the ribosomal units released during the termination of a cycle (Figure 8a). Moreover, the eIF4F factor is comprised of eIF4A (a helicase, the activity of which is enhanced by eIF4B), eIF4E (cap-binding protein), and eIF4G (a scaffold for eIF4E and eIF4A that also binds eIF3). The factors, eIFs 4A, 4B, and 4F, relax the

region proximal to the capping and promote the bonding of a 43S complex in order to form PIC 48S. Prior to this, in order to form 43S complexes, the multifactor complexes (MFCs) and ternary complexes (TCs) are organized. Unlike capping, the formation of TCi (TC containing the metionil-tRNA, the first residue in all eukariotic proteins) occurs through the association between eIF2 and GTP-Met-tRNAi, which is always the first to be located within the ribosome and to carry the initiator methionine (Figure 8b). Other GTP-aminoacyl-tRNAs are bound to the elongation factor in eukaryotes (eEF1A) to form other TCs. Subsequently, eIF1, eIF3 and eIF5 bind to TC in order to form the MFC (figure 8c). Meanwhile, eIF5 binds to the 40S ribosomal subunit and recruits it to the multifactor complex (MFC). As a result, the 43S complex is formed (figure 8d). Finally, the 43S complex is recruited into 5'UTR next to the capping, thus completing PIC 48S (figure 8e). Afterwards, PIC 48S moves along the mRNA in direction 5' to 3' to where it finds the start codon flanked by the GCC(A/G) CC**AUG**G sequence in eukaryotes (Figure 8f).

INITIATION

A perfect match between the start codon and its Met-tRNAi anticodon produces hydrolysis of the GTP in the eIF2-GTPMet-tRNAi and, at the same time, eIF5B mediates the bond of the 60S ribosomal subunit. This process leads to the assembly of the functional 80S ribosome or initiation complex (IC) and the release of eIF2-GDP+Pi, eIF1, eIF3, and eIF5 factors (Figure 8g). Some mRNAs have a secondary structure on the 5'UTR region and require ATP helicase activity to increase the bond of the 43S complex. During the assembly of ribosomes, the Met-tRNAi remains on the P site and the A site is available to receive the aminoacyl-tRNA which carries the next amino acid. In addition, the eIF1A factor binds to the minor subunit at the beginning and participates in the recruitment of TC. It also accompanies the 48S complex during scanning and temporarily occupies the A site in the ribosome.

ELONGATION

Once the ribosome is assembled, a second GTP-aminoacyl-tRNA reaches the A site and the peptide bond is formed in the P site between the first two amino acids, which are catalyzed by peptidyl transferase. The first tRNA without the amino acid remains located on the E site in order to exit the ribosome during the next movement. Furthermore, elongation consists of synchronized movements of the ribosome and tRNAs, through which tRNAs are shuttled through A, P, and E sites until they exit the ribosome. The ribosome selects the amino acid that must enter from a pool of 40 different aminoacyl-tRNAs and the correct codon-anticodon pairing induces changes in the ribosomal structure which contribute to its subsequent displacement on the mRNA. TCs are accompanied by eEF1A and their entry into the ribosome is facilitated by the ribosome GTPase activity. GTP hydrolysis causes a dramatic change in eEF1A structure which decreases its affinity for aminoacyl-tRNA and leads to its dissociation from aminoacyl-tRNA, thus allowing the accommodation of the aminoacyl tRNA on the A site and the synthesis of the peptide bond. The eEF2 catalyzes a rapid and coordinated movement which leads to the translocation of the tRNA from the A site to the P site. Several antibiotics such as amino glycosides, macrolides, and tetracyclines target the prokaryotic ribosome and alter the elongation rate and accuracy.

BOX 4 Post-transcriptional regulation in Autoimmune Diseases

An important proinflammatory cytokine implicated in autoimmunity is tumor necrosis factor alpha (TNF-α). Its transcript contains an AU-rich element (ARE) at the 3 'UTR region which interacts with several binding proteins. These regulate mRNA transportation, rate of decay and the translation efficiency of the TNF-α transcript. In addition to the mRNA stability given by capping and polyadenilation processes, the protein regulation synthesis of TNF-α has also been associated with the presence of ARE sequences by the binding the T Cell intracellular antigen (TIA)-1 protein to these elements. TIA-1 has a negative regulatory effect since some studies have shown that mutants lacking this molecule have increased protein synthesis with no change in the mRNA level. Another silencer of TNF-α is the TIA-1-related protein (TIAR protein). TIA-1 and TIAR are structurally and functionally homologues and they are also regulators of translational TNF-α expression. Furthermore, translational silencing is also done through a mechanism that involves the dependent signaling pathway of Mitogen Activated Protein Kinase (MAPK2). Recently, no report of possible associations between TNF-α protein synthesis and autoimmune diseases has been done. However the mRNA of TNF-α, like other proteins, is actively regulated through micro RNA (miRNA) molecules which control multiple transcripts in cytoplasm. Regarding this, there is a recent study showing that in patients with RA, miRNA 146a was positively correlated with TNF-α production (1)

TERMINATION

This takes place when the stop codon is recognized by the ribosome, thus generating a modification of the ribosome structure which halts translation, induces peptide release, and disassembly of the ribosome's components and elongation factors to start another translation cycle. There are eRF1 and eRF3 factors which participate in the termination process and it is unknown if GTP hydrolysis will be required at this point. In addition, evidence suggests that the interaction between PABP, eRF1 and eRF3 factors stimulates the activity of IC and the bond with the 60S subunit.

GENE EXPRESSION REGULATION

Genes can have two states depending on whether their information is being read or not. Moreover, a gene is said to be active when it is transcribed and inactive when it is not; hence, the product of the transcription of some genes can be translated until the protein sequence is complete. Furthermore, it was observed that some genes are continually expressed while there are others with spatiotemporally regulated expression. This leads us to think about the presence of gene expression regulation systems. The first studies related to gene expression regulation were focused on the mechanisms of transcriptional activation and positive control, whereas transcriptional repression and negative control was less studied. Thus, the promoter was the center of several studies that concentrated on *cis* proximal and distal sequences and also on transcription factors which recognize promoters and have a trans-acting action. However, in recent years, studies have shown that transcriptional repression mediated by chromatin structure and repressor proteins is a common regulatory mechanism in mammals. This mechanism has generated new approaches to understanding gene activation as a relief of repression by the nucleosomal structure of the chromatin.

These mechanisms are at a different level from the gene itself. They are called epigenetic regulation and include chromatin remodeling through changes in DNA and histones as well as regulation by miRNAs and asRNA. Furthermore, it was observed that the amount of protein far exceeds the number of genes described. All these facts suggest that there is a very efficient regulation system in gene expression within cells and also strategies to generate a diversity of proteins. Regarding the variety of proteins, alternative splicing has been postulated as the mechanism generating the majority of mRNA diversity from one gene. However, other mechanisms include the beginning of an alternative transcription. For example, the microphtalmia factor gene (MITF) has several transcription initiation sites located between the TATA box and the first exon, thus generating different mRNA isoforms which are translated. Other mechanisms are alternative polyadenylation, gene fusion, and *trans*-splicing. These processes are not usually in superior eukaryotes. Hence, gene expression regulation contemplates all the previously mentioned events and these events are integrated into the following topics: events associated with RNA and protein molecules, epigenetic mechanisms, and signal transduction pathways. They can act at different moments during transcription or translation depending on how the process is analyzed.

EVENTS ASSOCIATED WITH RNA AND PROTEINS

The abundance, location, and activity of RNA and proteins must be regulated to make the cell function well. The abundance is regulated through a balance between synthesis and degradation rates and the mean lifetime of molecules. The location depends on the efficiency and reliability of the transportation systems which work mainly by recognizing signals present in the molecules which need to be transported. This activity is regulated by the acquisition of the functional structure through protein folding, modifications after the synthesis process, and the interaction and association with other compounds.

RATE OF SYNTHESIS

The RNA synthesis rate depends on what the cell needs. The highest rate belongs to rRNA. Synthesis of this takes place in the nucleolus which is a region in the nucleus enriched with proteins which allow the transcriptional machinery to be assembled. This rate of synthesis is regulated by the signals present in the promoter, the availability of transcription factors, the epigenetic program, the chromatin structure, and the position of the gene in the nucleus and the chromosome. In addition, the presence of the proximal (TATA, SRE, CRE, etc.) or distal (enhancer, isolator, silencer, RCL) *cis* elements is specific for each gene. These elements are susceptible to being recognized by transcription factors. Transcription factors are proteins which bind to the promoter prior to the RNAP II. They bind to the promoter by recognizing specific elements and are classified into general and specific factors. General transcription factors are those present in almost all types of cells while the expression of the specific factors is restricted in both time and cell type.

Transcription factors have at least three functional domains in their structure: for DNA binding, for protein-protein interaction, and for transcription activation (TAD) or repression (TRD). All of them can be the activation target of signal transduction pathways which modify their structure through post-translational processes such as phosphorylation and dephosphorylation. The DNA-binding domains have tridimensional structures such as the zinc finger, the leucine zipper, the helix-turn-helix (HTH), and the helix-loop-helix (HLH) structures. Their association is weak and occurs through 20 contact points by the recognition of short and specific sequences with high DNA-Protein structural complementarity, which provides stability and specificity. The protein-protein interaction domains, in turn, are

BOX 5 Transcription Factors related to Autoimmune phenotype

Gene expression is one of the main processes involved in the development of ADs because it can be regulated by different mechanisms and strategies. One important element in this regulatory process is transcription factors (TFs) and their binding to corresponding target sequences. In fact, there are genes coding for TFs such as Aire, FoxP3, NF-κB, STAT4, and even steroid hormone receptors within genes associated with ADs. Table I lists some TFs involved with ADs. For example, NF-κB is known to be involved in the regulation of innate and adaptive immune response. Alterations in its function have been associated with rheumatoid arthritis (RA), psoriasis, inflammatory bowel disease, autoimmune thyroiditis (AITD), multiple sclerosis (MS), and Type 1 Diabetes (T1D). For a more extensive information on this topic, see (14)

important for the dimers and complex formation. An example of this is the SRF-SRF complex. This dimer binds to the serum response element (SRE) and recruits other proteins in order to form complexes of higher molecular weight that consist of subunits (PIC, IC and RNAP II). Activation domains have rich regions in glutamines and prolines, whereas repression domains have lysines. The transcription factors regulate the initiation transcription rate by facilitating the entry of RNAP complexes, which may stay in order to keep the promoters permanently open and have a higher transcription rate. This is the case for rRNA genes.

During translation, microRNAs can bind to the 3'UTR and block the process when the "closed loop" is formed via PABP. However, this structure also facilitates PIC and IC assembly and the reading of the same mRNA strand by several ribosomes at the same time (polyribosomes). Additionally, the poly A tail blocks the RNA helicase activity of eIF5B, which stimulates the binding of the tail to the ribosome's major subunit and the release of the IC ribosome. The presence of IRES sequences on the mRNA of certain viruses such as Hepatitis C virus (HCV) promotes the assembly of ribosomes and the beginning of 5'cap-independent translation as well as increasing the efficiency of the process.

MATURATION

Maturation processes of primary transcripts are different for each type of RNA. Even though some of the mRNA modification has been mentioned, we will emphasize splicing regulation and the modifications of other RNA types. Alternative splicing of pre-mRNA is a major contributor to proteomic diversity and regulation of gene expression. Diversity is acquired by generating mRNA isoforms containing different combinations of exons which will be translated into multiple protein isoforms. Splicing is tightly regulated in different tissues and developmental stages, and its disruption can lead to human diseases. Several studies are focused on determining a set of rules or "code" to predict the splicing pattern for any primary transcript, and they have already shown a larger network of pathways to regulate the splicing process. For example, a trans-splicing event between distal genes has been described, and it results from the fusion between primary transcripts leading to the combination of exons from different genes.

Nitrogenated bases on tRNA, in turn, undergo covalent modifications which lead to the formation of loops and double-chain fragments, the acquisition of a trefoil shape structure, and the addition of an amino acid to its 3' end. In contrast, the rRNA undergoes several cleavages in its primary transcript to obtain the 5S, 5.8S, and 28S rRNAs. This is followed by the addition of 49 ribonucleoproteins to form the 60S subunit, and the binding of 33 ribonucleoproteins with the 18S rRNA to form the 40S subunit. Finally, proteins, which are the final product of translation, are covalently modified by transferring phosphate, methyl, acetyl, glycosyl, and ubiquitin groups, etc., to its structure. Several enzymes participate in this dynamic and reversible process. For example, kinases transfer phosphate groups, while phosphatases eliminate them. The combination of these modifications regulates protein activity, stability, and degradation. Moreover, most of these modifications are also regulated by components of the signaling pathways.

TRANSPORTATION

RNAs, in eukaryotes, must be transported to the cytoplasm where they carry out their function in protein synthesis. Regions of the RNA are exposed and recognized by adapter proteins such as Nab2, which interacts with Mex67 to form a complex recognized by the Mex67-Mtr2 receptor (TAP-p15 or NXF1-Nxt1 in higher eukaryotes) (Figure 9). Then, it is guided toward the nuclear pore complex (NPC), where it will be transported to the cytoplasm through the FG-nucleoporins lining the central channel. Hence, protein transportation is regulated by the exposure of peptide signals present in the structure. For example, proteins from the nucleus have an NLS signal (Pro, Pro, Lys, Lys Lys, Arg, Lys, Val) recognized by nuclear importins, which mediate

transportation through the nuclear pore. In contrast, proteins from the peroxisome have the Ser, Lys, Leu peptides. Additionally, proteins synthesized in free ribosomes are transported to the mitochondria, nucleus, peroxisome or stay in the cytoplasm. This depends on the peptide signals in their structure recognized by proteins named chaperons belonging to transportation systems which ensure the correct location of the proteins. Ribosomes that adhere to the rough

BOX 6 Post-translational modifications of autoantigens in autoimmunity

Post-translational modifications in autoantigens are closely related to autoantibody production. A recent example of this issue is the discovery of 20 different protein isoforms from U1 small nuclear ribonucleoprotein 68K (U1-68K), which is the main autoantigen target for autoantibodies. This protein is very diversified in its molecular weight, which seems to be a reflection of different phosphorylation states. One particular isoform, 66.5 KDa, is higher in patients with RA and SLE which suggests the importance of differential phosphorylation states in the generation of specific AD autoantibodies. Another important modification is the citrullination of autoantigens, which consists of deimidation of arginine residue, to convert them into citrulline. This reaction is catalyzed by the peptidylarginine deiminase (PAD) enzyme. This mechanism has been extensively involved in RA and MS and in citrullinated antibody generation. Moreover, four proteins found in joints are recognized as autoantigens and are also targets of anti-citrulline: fibrinogen / fibrin, vimentin, collagen type II, and α-enolase. Thus, environmental factors such as exposure to cigarette smoke have been associated with the development of citrullinated proteins and anti-citrulline antibody production in RA patients. In Table III, the main post-translational modifications of autoantigens which have been associated with ADs are listed.

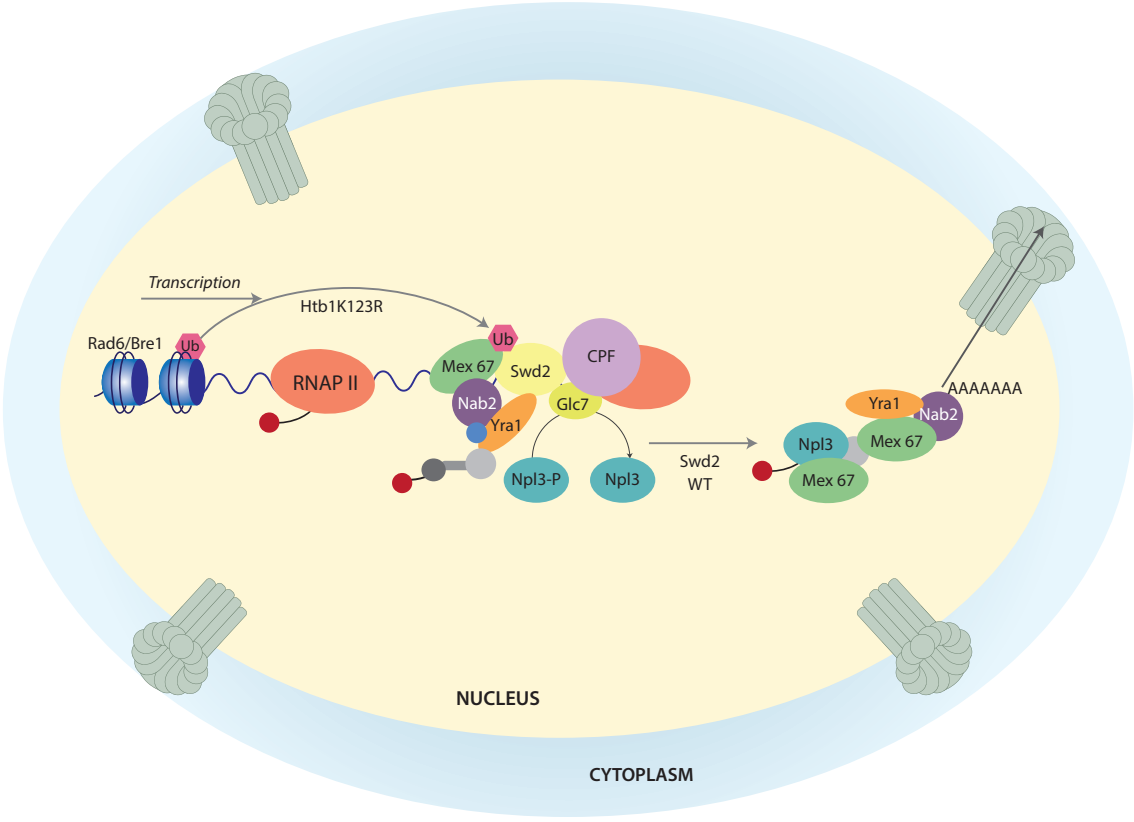

Figure 9. The mRNA transport mechanism. The mRNA transportation to the cytoplasm is guaranteed by adapter proteins (Nab2-Mex67–Yra1), which bind to mRNA through recognized regions on RNA. In eukaryotes, a receptor made up of the Mex67-Mtr2 proteins is located in the nuclear pore complex which recognizes the Nab2-Mex67–Yra1- mRNA complex and then transports it to the cytoplasm. Modified from (29).

BOX 7 Cell traffic of autoimmune mediators

Newly made proteins are sorted within several cell compartments and this mechanism is the major regulatory process in terms of their functionality. In order to have a biological function, proteins have to reach their correct place in the cell. Many proteins may go through alterations regarding their mobilization capability within the cell and these alterations could eventually be associated with ADs. For instance, there is evidence of alterations on the mechanism of TNF-α secretion in macrophages that has been very well dissected. In addition, two other proteins related to this topic are toll-like receptors 7 and 9 (TLR7 and TLR9). These are intracellular receptors involved in innate immune response to nucleotides derived from both host and pathogen cells; therefore, they are relevant in ADs. The mechanism that allows the regulation of these molecules is their retention within the endoplasmic reticulum when cells are in a resting state. Such sequestration is done through the action of three proteins: glucose-regulated protein 94 (GRP94), protein A associated with the TLR4 (PRAT4A), and UNC93B1. GRP94 is a stress response protein from the endoplasmic reticulum. Recent studies have shown that this protein is induced in inflammatory lesions, during inflammation and in autoimmunity. Interestingly, GRP94 antibodies have been detected in patients with SLE, RA and myasthenia gravis (MG). Another striking example in this context are the molecules of the major histocompatibility complex class I (MHC I). The density of these molecules on the cell surface determines some immune responses. Their passage through the trans Golgi network (TGN) is accompanied by CD99 protein. During this passage, there is a physical association between the two molecules which then go to the transport step (through vesicles). Note that CD99 binds to p230, an important protein for vesicular trafficking from TGN to cell membrane. Recently, patients with SS were found to have an isoform of this protein which is produced by alternative splicing of its extreme amino-terminus. This isoform was also detected in the TGN. However, the implications of these results in autoimmune pathology and vesicular trafficking are unknown. Taken all together, these results suggest a possible compromise of intracellular trafficking in patients with ADs.

endoplasmic reticulum (RER) synthesize proteins which remain in the membrane or in the RER matrix. These proteins are then located in the plasma membranes, in lysosomes, or in transportation vesicles for storage or secretion. Note that chaperons mediate transportation systems to guarantee correct localization of the proteins.

DEGRADATION

Some mRNAs and proteins have high cellular permanence and others are rapidly degraded. The mRNAs with a long poly A tail have greater permanence and, generally, are degraded by exoribonucleases. Studies in Xenopus oocytes have found that the length of the poly A tail depends on the balance between the cytoplasmic synthesis mediated by GLD2 polymerase and the degradation carried out by PRNA ribonuclease. Note that, in addition to other ways, proteins can be degraded in the lysosome or by ubiquitination signals recognized by the proteasome or by autophagy. Almost all require the recognition of degradation signals which act as markers on the proteins. For example, histone degradation is mainly regulated by the ubiquitination system through ubiquitin peptides that can be attached in their PEST domain for recognition and degradation by proteasomes. The proteasome is made up of a 19S regulatory subunit and a 20S subunit with chymotryptic, tryptic, and caspase type proteolytic activity. The 19S subunit also participates in transcription regulation by providing energy for the complex formation composed of SAGA acetyltransferase and the transcription initiation factor. The energy is supplied by subunits containing six AAA-type (Rpt1-6) ATPases and two non-ATPases (Rpn1-2). Subunit 19S also interacts with a complex that is named facilitates chromatin transcription complex (FACT) to reorganize histones during elongation in the transcription process.

EPIGENETIC MECHANISMS

Epigenetic regulation of gene expression is mediated by mechanisms which modify access to the region containing the promoter or the start codon which is not related to cis signal sequences. This regulation is associated with DNA methylation, histone modification, chromatin remodeling, formation of double-strand structures between ncRNA complementary regions, and genome organization. The DNA is packed around nucleosomal units which are made up of octamers of histone proteins named H2A, H2B, H3, and H4. These are separated by an H1 molecule. For transcription, the DNA must be accessible, which means that the chromatin must be in a relaxed state. Modulation of the condensation state is done through protein complex-

BOX 8 mRNA TNF-α regulation and stability

mRNA stability in the cytoplasm is a critical point during protein production. It is not surprising that several regulatory sequences could be located on all the transcripts in order to protect them from possible degradation. A clear example of this regulatory mechanism has been described for TNF-α. In addition to the mRNA translation efficiency of TNF-α transcripts mediated by ARE sequences, several studies have shown the relationship between mRNA TNF-α stability and these kinds of motifs. Studies in CD3-stimulated lymphocytes showed that the mRNA of TNF-α is labile, while co-stimulation with CD3 and CD28 makes it more stable. Moreover, this ARE sequence is also important for mRNA TNF-α stability and may be dependent on or independent of MAP kinases but strictly dependent on the Tristetraprolin molecule (TTP). Recently, MAPK phosphatase-1 (MKP-1) has been shown to be a negative regulator inflammatory response that controls the half-lives of several cytokines including TNF-α, IL-10, and IL-6 mRNAs through association with AUF1. Other molecules involved in TNF-mRNA stabilization and related to ARE sequences are HeL-N1, HuR and AUF1. The decay constitutive element (CDE) is another element in the 3 'UTR, which could be repressing the expression of TNF-α by reducing its half-life. Other cytokines and growth factors are also regulated through associations with ARE sequences. In an autoimmune condition, all these elements and mechanisms related to cytokine mRNA stability could be extremely important during inflammatory processes.

es which modify the amino terminus end of the histones, thus making them release DNA fragments locally. Nucleosomes are not completely removed during transcription. As a result, the relaxation of the structure allows the transcriptional complex to circle the nucleosome as it reads the DNA template. Although epigenetic mechanisms will be discussed thoroughly in the chapter on epigenetics and autoimmune diseases, we will describe the main epigenetic mechanisms in the context of this section.

DNA METHYLATION

DNA methylation occurs on CpG dinucleotides located on the promoters to prevent their recognition by transcriptional factors and inhibit the transcription process. Methylation of miRNA gene promoters has been frequently observed in tumor cells, e.g., the miR-124a promoter is hypermethylated in colon cancer cell lines but not in normal tissue.

BOX 9 Chromatin remodeling and autoimmunity

The mosaic of autoimmunity refers to the multifactorial origin and diversity of expression of autoimmune diseases. The term implies that many different combinations of factors involved in autoimmunity produce variations representing a broad spectrum of diseases. Among the factors described to date, one, the epigenetic component, has been highlighted and the interest in it has increased in recent years. Particularly, the process of chromatin remodeling during gene transcription is important in the development of the autoimmune phenotype. An example of the chromatin structure and ADs will be discussed later with reference to the MHCII.

There is evidence that the autoimmune regulator (Aire), regulates gene expression in this context. This molecule is responsible for ectopic induction of peripheral tissue antigens (PTAs) in the thymus (tissue-restricted antigens (TRAs) in medullary thymic epithelial cells) and thus plays an essential role by fostering immune tolerance. The mechanism by which this molecule may regulate thousands of genes is through specific recognition of the amino terminal tail of histone 3 (H3) by its PHD1 domain. Therefore, Aire directly interacts with nucleosomes and preferentially with chromatin regions depleted of H3K4me3, and this recognition of H3 tails contributes significantly to interaction stability. However, Aire's H3 tail binding activity is necessary but not sufficient for Aire gene regulation encoding PTAs.

BOX 10 DNA methylation and Autoimmune Diseases

Epigenetic mechanisms play an important role in the understanding of the molecular mechanisms involved in the autoimmune phenotype. Moreover, DNA methylation actively participates in the development of autoimmune processes, particularly in the case of Systemic Lupus Erythematosus (SLE). This is a B cell disease which produces a variety of autoantibodies directed against surface, cytoplasmic, and nuclear antigens. One of the abnormalities in these cells is the reduced expression of the CD5 molecule on the B cell surface. Complete CD5 form (membrane, CD5-E1A) is supposed to be involved in modulation of the signaling from the B cell receptor (BCR) and the autoreactivity control. When the cytoplasmic form (CD5-E1B) is present, there is no translocation of complete CD5 form to cell membrane, thus producing alterations in the negative regulation of this molecule in BCR. Note that the balance between cytoplasmic and membrane forms is regulated by an epigenetic process. SLE patients have a demethylated promoter region of cytoplasmic isoform unlike control individuals. Therefore, as a result of this demethylation, CD5-E1B mRNA is transcribed at the expense of the CD5-E1A mRNA transcription which explains the altered signaling on B cells. This could, in turn, promote the activation and expansion of autoreactive B cells in SLE patients.

MODIFICATION OF HISTONES

Histones can be the object of covalent modifications such as methylation, acetylation, ubiquitination, phosphorylation, isomerization in proline, ADP ribosilation, and citrulination. They can also be degraded due to the action of proteolytic pathways. Altogether, these changes constitute the so-called "histone- code," which acts as a signal for recognition and interaction with regulatory proteins and epigenetic modifiers. These modifiers may have specific interaction domains, for example, a bromo-domain recognizes acetylated lysine. Moreover, acetylated histones in lysine residues destabilize internucleosomal interactions and the bond between histones and DNA. Note that these modifications are reversible. For example, lysine acetylation mediated by acetyltransferases (HATs) is associated with gene activation and its action can be reversed by histone deacetylase (HDACs) proteins, which eliminate lysine acetyl groups. Histone hypoacetylation is associated with gene repression.

CHROMATIN REMODELING

Chromatin remodeling consists of the modification of chromatin condensation states affecting DNA accessibility, especially in the promoter regions. This remodeling is the product of interaction between remodeling complexes such as SWI/ PAF and the combination of DNA methylation with histone methylation and acetylation. Furthermore, two chromatin regions can be distinguished: a more condensed heterochromatin that is transcriptionally inactive and a less condensed euchromatin with transcriptional activity. The HP1 protein has a chromo-domain which binds to H3K9me. Both are heterochromatin markers in most eukaryotes and act as a dynamic platform to recruit factors in processes such as cell-type switching, sister chromatid cohesion, RNAi, and transcriptional gene silencing.

ATP-dependent chromatin Remodeling

Homothallic switching deficient/sucrose nonfermenting (SWI/SNF) complex is an ATP-dependent complex recruited by activator molecules on gene promoters and it also binds to TFIIS. It acts mainly in the elongation phase of transcription. In addition, the ATPase chromo-ATPase/helicase-DNA binding domain (CHD1) remodels nucleosomes during elongation and termination, interacts with the Set-2, SWI/SNF, Paf, DSIF, and FACT complexes that follow it, and has a chromo-domain which binds to methylated histones. Another ATP-dependent chromatin remodeling complex is the ISWI protein family (imitation switch). However, their components can vary as well as their actions during transcription. The Isw1a complex composed of Isw1p and Ioc3p (Isw one complex) negatively affects nucleosome positioning during transcription initiation. The Isw1b complex, in turn, which consists of Isw1p, Ioc2p, and Ioc4p, regulates and links the transcription elongation and termination phases with mRNA maturity. Isw1p preferentially recognizes di and trimethylated H3-K4 which contribute to its recruitment on RNAP II.

Chromatin remodeling by histone chaperone proteins

A general remodeling complex, FACT, is an hSpt16 heterodimer and also a specific structure recognition protein-1 (HMG-box protein SSRP1). Several of its subunits have been identified as part of the components of DNA polymerase alpha which interacts with DSIF, Spt6, PAF, Chd1, and histones. Recruitment of FACT to promoters is achieved through the intervention of heat shock chaperone proteins of the Hsp family which destabilize the nucleosome through the selective removal of an H2A/H2B dimer. Hence, this allows RNAP II to surround the nucleosome to transcribe the DNA rolled around it; this activity is mediated by direct H2A/H2B nucleosome-FACT-dimer interactions. Moreover, Spt6 interacts with

RNAP II, DSIF, TFIIS, PAF, FACT, and H3 histone to promote assembly of the nucleosome when transcription takes place.

ORGANIZATION OF THE GENOME

Genomic architecture is not collinear; instead, it is cross-linked and modular. Moreover, several sequences are multifunctional because they are used for multiple transcripts and also as regulatory regions. Inside the nuclei, the genome is spatially organized within chromosomes, and there are genes occupying preferential positions relative to each other and to various nuclear and nucleolar landmarks. There are intra- and inter-chromosomal communications between different genomic regions which appear to play important roles in genome expression, function, and regulation. Some evidence of gene expression regulation mediated by genome organization is described below.

Transcriptional factories: these are regions of the genome with high transcription rates and high concentration of transcription factors and RNAP subunits. These subunits have a tissue-specific tridimensional self-organization for regulating gene expression. Actin can collaborate in the organization of factories, and it is implicated in several nuclear processes such as chromatin remodeling, transcription, and mRNA export. It is also required for the action of all three RNA polymerases during transcription in vivo and in vitro. Transcription factories may be attached to a type of nuclear actin scaffold. In genes transcribed by RNAP II, regulating regions such as LCR and enhancers, which are located close together in transcriptional factory regions, can act on genes far from their chromosomal location.

Position in the genome: the gene position in the chromosome and nucleus is also important for determining gene expression. For example, genes located in the centromeric region or close to it are inactive, and telomeric genes may be lost due to telomere shortening. In addition, centromeres are specialized regions at the center of chromosomes that are compounded of heterochromatin, which plays a critical role in the accurate segregation of duplicated chromosomes during cell division. In contrast, telomeres are regions located at the termini of linear chromosomes and consist of repeated DNA sequences (TTAGGG) and different telomere binding proteins. Telomere shortening refers to incomplete replication of chromosomal ends during cell division (S phase) which results in the loss of a small fraction of telomeric DNA. This may be used as a marker to determine the biological age of cells, tissues, organs, and probably humans. Moreover, telomerase enzymes synthesize telomere DNA and preserve telomere length and function, but they are absent in most cells. Telomerase can be found in cell types such as stem cells, embryonic cells, germline cells, lymphocytes, and tumor cells. On average, telomeres are longer in naïve cells than in memory T cells, and are longer in $CD28^+$ than in more differentiated $CD28^-$ $CD8^+$ T cells. In contrast, the leukocyte and PBMC telomeres are short. Telomere shortening has also been described in association with a number of immune related diseases and as a risk factor for some type of tumors. It has been reported that patients with rheumatoid arthritis and diabetes mellitus (type 1 and type 2) display significantly shortened telomeres in leukocytes or PBMC compared to age-matched healthy controls. This topic will be addressed in depth in the chapter on immunosenescence.

Multifractal analysis genomic: The human genome is one of the most complex molecular structures because it presents mosaicisms between coding and non-coding sequences. The human genome has highly regionalized structures

BOX 11 Long-range interaction in MHC Class II Complex

With respect to deregulation of gene expression associated with ADs, transcriptional factories, which are involved in long-distance transcription regulation, are closely linked to chromatin organization. An interesting example of this is related to the major histocompatibility complex region (MHC) on chromosome 6. The gene transcription located in this region is finely regulated by simultaneous regulation of several genes (Figure 10). In vitro studies have shown that MRC5 cells (human fibroblasts) are stimulated with interferon gamma (INFγ) and form large chromatin loops on the surface of chromosome territories, which correspond to specific regions of simultaneous multi-gene transcription. This promotes a functional and coordinated compartmentalized control of gene expression by the interaction between transcriptional regulatory elements. Furthermore, the transcriptional cofactor CIITA (class II transactivator) functions as an adapter molecule for several chromatin-remodeling factors and for the general transcriptional machinery. This factor is the master regulator of gene expression in the MHC class II (MHCII) region. Transcription of DRB1 and DQA1 genes can be favored by interchromosomal interaction between transcriptional machinery from each gene and the intergenic region, XL2. Such interaction may be effective only in cells expressing MHCII. At the same time, this is dependent on the presence of the CIITA-RXF complex, which forms a loop that would favor simultaneous transcription of both genes.

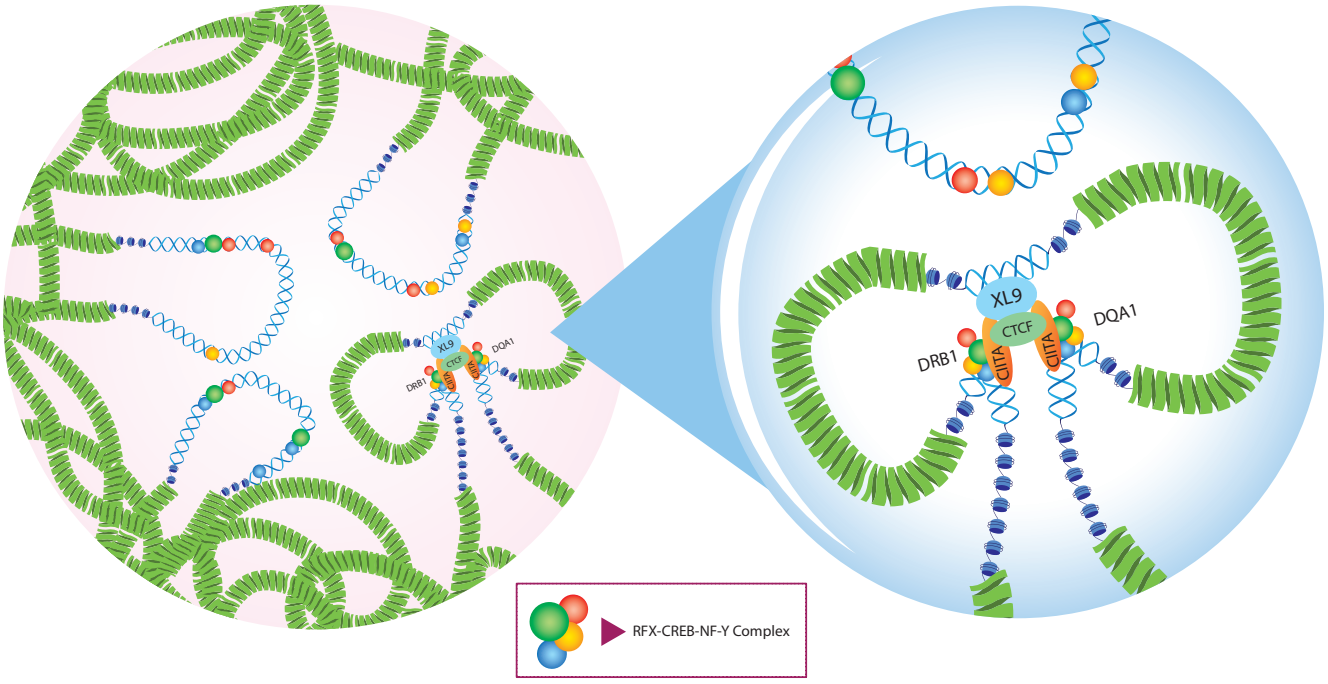

Figure 10. Schematic representation of HLA-DRB1 and HLA-DQA1 and the XL9 region interchromosomal interaction. In an active state, the CCCTC binding factor (CTCF) and class II transactivator (CIITA) are positioned near proximal promoter regions of HLA-DRB1 and HLA-DQA1 genes which are interacting with the XL9 region. One or both genes can interact with the XL9 region and thus be transcribed alone or at the same time respectively. Modified from (5).

which consist of a complex pattern for gene structure and expression regulation. In order to analyze the fractal characteristics of the human genome, fractal methodologies were used. These methodologies are derived from an approach focused on the degree of fragmentation (or irregularity) existing in nature. Therefore, the fractal nature of the human genome was studied using several sets. Each set produces a fractal dimension translated into a continuous spectrum of exponents, called a singularity spectrum. Then, the degree of multifractality (MD) obtained from this continuous spectrum allows the genetic information content to be measured. Hence, multifractal analysis of chromosomal fragments, chromosomes, and chromosomal regions reveals the presence of repetition units such as the Alu sequences, which confer fractal characteristics on the genome. This is the reason chromosomes can be classified as having low (4, X, 13, 5,18,3, 6, Y, 8, 2, 11), medium (21, 14, 9, 10, 7, 12, 1, 20, 15), or high (16, 22, 17) fractality. Regions with low fractality are related to low genome stability while those with high fractality are associated with high genetic stability, e.g., chromosome 19 presents the highest fractality (Figure 11).

RNA systems: RNA mapping techniques show that, in the nucleus, there are more non-polyadenylated and non-coding snRNA than mRNAs. These snRNAs are identified as 'transcripts of unknown function (TUF) and are denoted by the ENCODE consortium. Several transcriptional units overlap and are denominated as sense and antisense, thus many mRNAs have antisense transcripts. With the discovery of several non-coding RNA (ncRNA) species, regulation systems of gene expression based on RNA began to be elucidated. The control provided by non-coding RNA regulates the compound's stability, modifies its structure, contributes to transcription initiation and translation temporal-spatially and tissue specifically, modulates chromatin organization, regulates alternative splicing, controls sub cellular localization of proteins, and regulates heat shock sensing. Furthermore, functional ncRNAs vary significantly in size from ~22 bp for miRNAs to ~18 kb for XIST (X-inactive-specific transcript) and to ~108 kb for AIR (antisense IGF2R RNA) ncRNAs. Size variation and diversity of events in which ncRNAs participate could exceed the protein regulation system in quantity. The best-known RNA-dependent regulation systems are those mediated by snRNAs: non-coding RNA (ncRNAs), small nucleosomal RNA (snoRNA), and microRNAs (miRNAs).

miRNAs are a family of small ribo-oligonucleotides which constitute the main group of post-transcriptional gene regulators, but they do not translate into proteins. miRNAs regulate cell proliferation, apoptosis, and differentiation and intervene in cell processes such as signal transduction, metabolism, and mRNA useful life. Moreover, genes encoding for miRNA have been located in cancerous cells in regions with fragilities or with a high rate of chromosomal aberrations and often cause a loss of function. A cluster of miRNA genes was described on human chromosome 19 (C19MC) among repeated Alu sequences. Some were

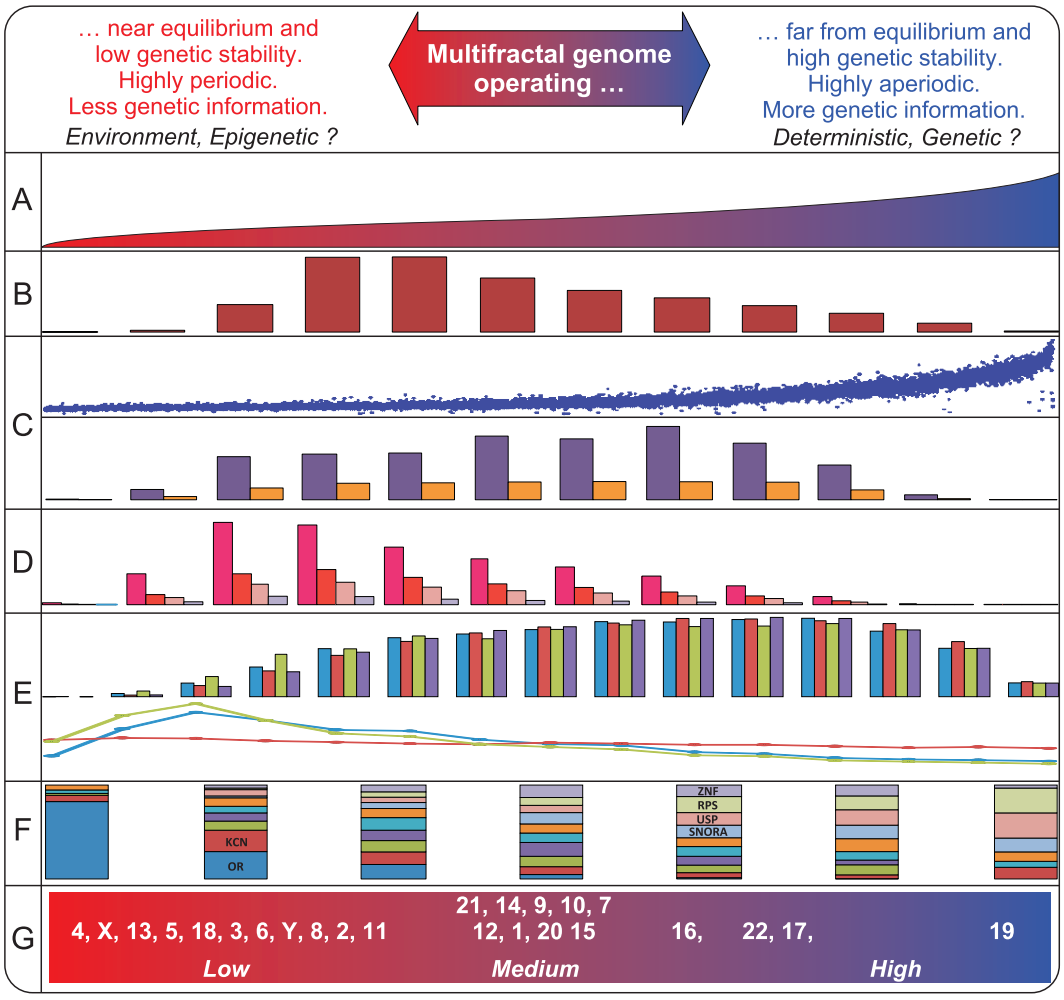

Figure 11. Summary diagram: a conceptual non linear model for the human genome. From left to right multifractality increases. A. Multifractality profile from 9,379 chromosome fragments (from 0.79 to 1.56). B. Number of chromosome fragments per Range of multifractality (RM). Upper C. Alu profile of the content from 9,379 chromosome fragments. Lower C. Analyses of molecular parameters: Alu content per range of multifractality degree (ΔDq). D. Analyses of molecular parameters: Long interspersed element (LINE), Mammalian wild- interspersed repeat (MIR), Medium reiterated element (MER) and Long terminal repeat (LTR) contents per RM. E. Distributions by gene function, gene family, and gene length: Gene functional distributions per RM. These distributions are strongly significant up to 80% of the ranges. F. Distributions by gene function, gene family, and gene length: Percentage of gene families per RM Gene families. G. Multifractal classification for the human chromosomes: Discrimination method based on multifractal formalism in a distribution of two-dimensional points, R2 ~0.967, p <0.05. On top: hierarchical clustering for the averaged multifractal parameters by chromosome between -20 and 20 Dq (color scale bar is indicated). Minimum similarities are indicated near nodes and the asterisks show the only two exceptions found. Abbreviations: CA: Carbonic anhydrase, CD: differentiation cluster, GPR: G protein-coupled receptors, KCN: potassium channels, OR: olfactory receptor, RPS: ribosomal proteins, SLC: solute carrier, SNORA: small nucleolar RNA, USP: ubiquitin-specific peptidases, ZNF: Zinc fingers, C2H2-type. Reproduced with permission from (19).

located in intronic regions and others, in exonic regions. Thirty percent of the genes are believed to be regulated by miRNAs. In the immune system, several processes regulated by miRNAs have been described: ablation of the miRNA machinery as well as the loss of function of some molecules contribute to the formation of certain components and are associated with the development of autoimmunity and cancer. For example, miR-223 controls generation and activation of granulocytes in the inflammatory response. Its loss in mice results in increased granulocyte progenitor cells, hyper mature granulocytes that are hypersensitive to activation stimuli, and increased antifungal activity. In psoriasis, a chronic inflammatory cutaneous disease, over-expression of miRNA-203 and a reduction of its target gene – the suppressor of cytokine signaling 3 (SOCS-3) – was found in psoriatic plaques. SOCS-3 participates in the inflammatory response and in some functions of keratinocytes. A good example of RNA system regulation of gene

expression is the tumor-suppressor gene called phosphatase and tensin homolog (PTEN). PTEN is a negative regulator of the PI3K-Akt pathway and is epigenetically silenced in several cancers. Also, PTEN is an indispensable regulator of B cell homeostasis. PTEN expression is post-transcriptionally regulated by the action of a PTEN pseudogene (PTENpg1). This is a long, noncoding RNA (lncRNA) that sequesters numerous PTEN-targeting miRNAs by acting as a miRNA sponge (Figure 12).

SIGNAL TRANSDUCTION PATHWAYS

Cells are in constant communication with the extracellular medium. Cells are able to detect and interpret extracellular signals that regulate the expression of target genes through mechanisms which vary from posttranslational modification of proteins leading to their activation, inhibition, or degradation to the activation or repression of genetic programs. There is permanent cell signaling among nearby and distant cells belonging to the same individual in whom signals coming from different sources can induce cell responses. These responses include activation or inactivation of genetic programs which lead to proliferation, differentiation, cell death, senescence, or accomplishment of cell functions within a tissue.

The signals can have different molecular structures or sizes, especially those that are hydrophilic or hydrophobic in nature, and have specific mechanisms to transmit information within the cell. Likewise, signals can also be physical, magnetic, electric, etc. Hydrophobic signal molecules such as vitamin D, thyroid and steroid hormones, retinoids, and eicosanoids can cross the plasma membrane and bind to receptor molecules at the cytoplasm or inside the nucleus.

These signal molecules are associated with long-lasting responses due to their high blood permanence. Receptors for these compounds have interaction domains with the ligand, the DNA, and transcription activation or repression processes, thus conferring the characteristics of transcription factors on these receptors.

Hydrophilic signal molecules such as neurotransmitters, protein hormones, glycoprotein hormones, and local chemical mediators, require the presence of a receptor molecule in the plasma membrane. These signal molecules mediate short-duration responses and have low blood permanence. Their ligand-receptor complex may be endocytosed or not, the ligand may be destroyed, and the receptor can be recy-

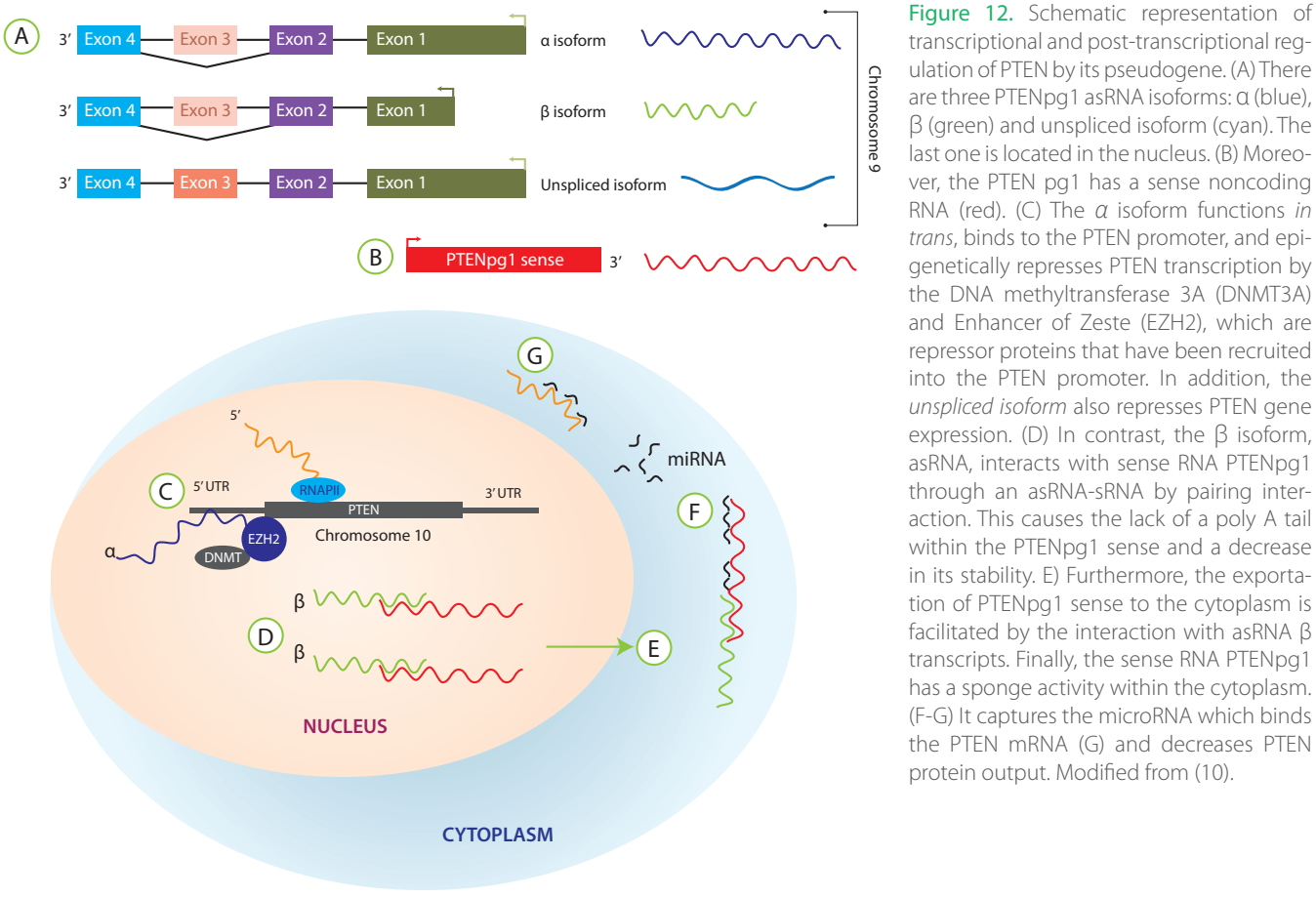

Figure 12. Schematic representation of transcriptional and post-transcriptional regulation of PTEN by its pseudogene. (A) There are three PTENpg1 asRNA isoforms: α (blue), β (green) and unspliced isoform (cyan). The last one is located in the nucleus. (B) Moreover, the PTEN pg1 has a sense noncoding RNA (red). (C) The *α* isoform functions *in trans*, binds to the PTEN promoter, and epigenetically represses PTEN transcription by the DNA methyltransferase 3A (DNMT3A) and Enhancer of Zeste (EZH2), which are repressor proteins that have been recruited into the PTEN promoter. In addition, the *unspliced isoform* also represses PTEN gene expression. (D) In contrast, the β isoform, asRNA, interacts with sense RNA PTENpg1 through an asRNA-sRNA by pairing interaction. This causes the lack of a poly A tail within the PTENpg1 sense and a decrease in its stability. E) Furthermore, the exportation of PTENpg1 sense to the cytoplasm is facilitated by the interaction with asRNA β transcripts. Finally, the sense RNA PTENpg1 has a sponge activity within the cytoplasm. (F-G) It captures the microRNA which binds the PTEN mRNA (G) and decreases PTEN protein output. Modified from (10).

cled or destroyed. Note that hydrophilic molecules act on G-coupled receptors, ion channels, or tyrosine kinase receptors. When the ligand-receptor bond occurs, it can undergo modifications in its structure which lead to cascading molecular changes until a cell response is generated. In the case of receptors coupled to G trimeric (α, β and γ subunits) proteins, ligand binding induces their dissociation (β and γ units run) and mobilization within the membrane until they interact with effector proteins. These proteins in turn activate molecules downstream and the effect falls on second messengers such as AMPc, GMPc, Ca2+, and DAG. These, in turn, act and modulate the state of activation of target molecules such as PKA, PKG, Calmodulin, PKC, and Serine/Threonine kinases respectively. These molecules also act on different targets including transcription factors, cytoskeleton structures, and mitochondria proteins. Expression of genetic programs may be modulated in the cascade, thus leading to proliferation, differentiation, cell death, senescence, or functional activity on a tissue. On the same pathway, cell characteristics such as adhesion, migration, and survival can also be modulated. Receptors associated with ion channels, in turn, modulate the transportation of substances between the extracellular medium and the interior of the cell by opening or closing the channels, thus altering the membrane's permeability and excitability. Finally, receptors with autocatalytic activity located in the intracytoplasmic region have a tyrosine kinase domain which is activated when the ligand binds to the receptor, which generates a structural change making the receptor accessible to adapter proteins. Afterwards, proteins are activated in cascade and act upon different target molecules. The MAPK pathway is the classical example for these types of signal transduction pathways.

Recently, a plethora of signaling pathway members as well as molecules involved in the interaction between pathways have been identified. This reveals a communication network system that is difficult to interpret and understand but provides clues to the complex regulation system of gene expression.

TF	RA	SLE	MS	T1D	IBD	Psoriasis	SS	SSc
NF-kappaB	**	**		**	**	+		
RELA (p65)	**	+		+	**			
c-REL	**		+	**	**			
NF-κB1 (p105/p50)	**	+	**	**	**			
Aire				**				
STAT1	+	+	**	+	**			
STAT3	+	**	**		+	+		
STAT4	**	**	**	**	**			
STAT6	+	**			+			
NFATc1	+							
NFATc2	+	+						
Foxp3	+	+	+	+	+	+		
Foxd1		+						
Foxj1		+						
Foxo3a	**	+						
AP-1	**	+	+	+	+	+		
Ifi202		+						
IRF-1	**	**	**	**				
IRF-2						**		

Table 1. Transcription factors involved in autoimmune diseases. Adapted from (22). Supported strongly (**); supported by limited, controversial and/or conflicting studies (+); empty spaces indicate insufficient knowledge. Abbreviations: *AIRE*, Autoimmune Regulator; *AP-1*, Activating Protein-1; *c-REL*, c- v-rel Avian Reticuloendotheliosis viral; *ERa*, Estrogen Receptor 1; *ERb*, Estrogen Receptor 2; *Foxd1*, Forkhead Box D1; *Foxj1*, Forkhead Box J1; *Foxo3a*, Forkhead Box O3a; *Foxp3*, Forkhead Box P3; *GATA-3*, GATA-binding factor 3; *IBD*, Inflammatory Bowel Disease; *Ifi202*, IFN-inducible 202; *IRF*, Interferon Regulatory Factor; *MS*, Multiple Sclerosis; *NFAT*, Nuclear Factor of Activated T cells; *NF-kappaB*, Nuclear Factor kappaB; *PPAR*, Peroxisome Proliferator-Activated Receptor; *RA*, Rheumatoid Arthritis; *RELA*, v-rel Avian Reticuloendotheliosis Viral Homolog A (nuclear factor of kappa light polypeptide gene enhancer in B Cells 3 (p65); *RELB*, Avian Reticuloendotheliosis Viral (v-rel); *RAR*, Retinoic Acid Receptor; *RORa*, RAR-related Orphan Receptor alpha; *RORg*, RAR-related Orphan Receptor gamma; *RXR*, Retinoid X Receptor; *SS*, Sjögren Symdrome; *SSc*, Systemic Sclerosis; *STAT*, Signal Transducer and Activator of Transcription; *SLE*, Systemic Lupus Erythematosus; *T-bet*, T-box expressed in T cells; *T1D*, Type 1 Diabetes; *VDR*, Vitamin D (1.25- dihydroxyvitamin D3) Receptor.

TF	RA	SLE	MS	T1D	IBD	Psoriasis	SS	SSc
IRF-2						**		
IRF-3	+							
IRF-4	+	+	+	+				
IRF-5	**	**	**		**		**	**
IRF-7		**				+		**
IRF-8		**	**					**
T-bet	+	**	**	**	**			
GATA-3	+							
PPAR				+				
VDR	**		**	**	**	**		
RORα			+					
RORγ	+	+	+		+	+		
RXR			+	+				
RAR				+				
ERα	+	+	+					
ERβ		+						

Table 1 continuation. Transcription factors involved in autoimmune diseases. Adapted from (22). Supported strongly (**); supported by limited, controversial and/or conflicting studies (+); empty spaces indicate insufficient knowledge. Abbreviations: *AIRE*, Autoimmune Regulator; *AP-1*, Activating Protein-1; *c-REL*, c- v-rel Avian Reticuloendotheliosis viral; *ERa*, Estrogen Receptor 1; *ERb*, Estrogen Receptor 2; *Foxd1*, Forkhead Box D1; *Foxj1*, Forkhead Box J1; *Foxo3a*, Forkhead Box O3a; *Foxp3*, Forkhead Box P3; *GATA-3*, GATA-binding factor 3; *IBD*, Inflammatory Bowel Disease; *Ifi202*, IFN-inducible 202; *IRF*, Interferon Regulatory Factor; *MS*, Multiple Sclerosis; *NFAT*, Nuclear Factor of Activated T cells; *NF-kappaB*, Nuclear Factor kappaB; *PPAR*, Peroxisome Proliferator-Activated Receptor; *RA*, Rheumatoid Arthritis; *RELA*, v-rel Avian Reticuloendotheliosis Viral Homolog A (nuclear factor of kappa light polypeptide gene enhancer in B Cells 3 (p65); *RELB*, Avian Reticuloendotheliosis Viral (v-rel); *RAR*, Retinoic Acid Receptor; *RORa*, RAR-related Orphan Receptor alpha; *RORg*, RAR-related Orphan Receptor gamma; *RXR*, Retinoid X Receptor; *SS*, Sjögren Symdrome; *SSc*, Systemic Sclerosis; *STAT*, Signal Transducer and Activator of Transcription; *SLE*, Systemic Lupus Erythematosus; *T-bet*, T-box expressed in T cells; *T1D*, Type 1 Diabetes; *VDR*, Vitamin D (1.25- dihydroxyvitamin D3) Receptor.

AUTOIMMUNE DISEASE	ALTERNATIVELY SPLICED PRE-mRNA	CONSEQUENCE
Rheumatoid arthritis	IL6R	Soluble receptor
	IL7R	Soluble receptor induced by pro-inflammatory cytokines
	Foxp3	increased expression of FoxP3 splice forms in synovial CD4+ T cells
	PTPN22	Lacks nearly the entire phosphatase domain and can function as a dominant negative isoform of the full length PTPN22

Table 2. Alternative spliced genes in autoimmune diseases. Adapted from (7). Abbreviations: AChE, acetylcholinesterase; Aire, Autoimmune regulator; ASS, Argininosuccinate synthase; BANK1, B Cell scaffold protein with ankyrin repeats; CR2, complement receptor 2; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; CXCL12, chemokine (C-X-C motif) ligand 12; FGFR1, Fibroblast growth factor receptor 1; FoxP3, forkhead box P3; HLA-G, Major histocompatibility complex, class I, G; IA-2, insulinoma-associated tyrosine phosphatase-like protein; Ig-like, immunoglobulin-like; IGRP, Islet-specific G6CP-related protein; IL4, interleukin 4; IL7, interleukin 7; IL1R2, interleukin-1 receptor type II; IL6R, interleukin 6 receptor; IL7Rα, interleukin 7 receptor α chain; IL20R, interleukin 20 receptor; iNOS, inducible Nitric Oxide Synthase; IL-32, Interleukin 32; IRF5, interferon regulatory factor 5; ITPKC, inositol 1,4,5-triphosphate 3-kinase C; KLC1, kinesin light-chain 1; LILRA2, leukocyte immunoglobulin-like receptor A2; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; NFKB2, Nuclear factor of kappa light polypeptide gene enhancer in B Cells 2 (p49/p100); OAS1, 2′,5′-oligoadenylate synthetase 1; PLP, proteolipid protein; PTPN22, protein tyrosine phosphatase, non-receptor type 22; PTPσ, protein-tyrosine phosphatase sigma; RasGRP1, Ras guanyl-nucleotide releasing protein 1; SNP, Single nucleotide polymorphism; TCRζ, T Cell receptor ζ chain; TAP2, transporter 2, ATP-binding cassette, subfamily B; TNC, Tenascin-C; TNFR2, receptor for tumor necrosis factor α2; 3′-UTR, 3′-untranslated region; VEGF, Vascular endothelial growth factor.

AUTOIMMUNE DISEASE	ALTERNATIVELY SPLICED PRE-mRNA	CONSEQUENCE
Rheumatoid arthritis	CXCL12	High affinity y for heparin sulfate (HS)
	TNFR2	(skipping of exons 7, 8) novel soluble receptor
	TNC	Increase in large isoform
	IL-32	Different splice variants. Increased IL-32γ
	CD1d	Soluble isoforms
	CD44	Multiple splice variants
	Fibronectin	Isoform containing EDA region
	CD137	Soluble receptor isoforms
	VEGF receptor type 1 and 2	
	Soluble isoforms	
	Tie1receptor	Multiple isoforms
	FGFR1	Two isoforms
Systemic lupus erythematosus	BANK1	(SNP at branch point) isoform lacking exon, enhanced and increased potential for multimerization
	LILRA2	Activation of cryptic 3' splice site, novel isoform lacking 3 amino acids
	CD5	A truncated variant produced by hipomethylation of E1B-cd5 gene
	TCRζ	(skipping of exon 7; alternative splicing of 3'-UTR) different isoforms
	IRF5	(alternative 5' splice sites) specific exon 1 used
	RasGRP1	Aberrant splice variants
	CD72	(13-nucleotide repeats and 4 bp deletion in intron 8) isoform lacking exon 8
	IL20R	(alternatively spliced in the mouse model of SLE) soluble receptor
	CD45	C77G polymorphism that produces hyperactive version of CD45.
	CR2	(SNPs) decreased splicing efficiency of exon 11
Multiple sclerosis	IL7R	(skipping of exon 6) more sIL7R produced
	IL7	Multiple isoforms. IL-7δ345 expression is raised
	OAS1	(new 3' splice site) new isoforms
	CD45	C77G polymorphism that produces hyperactive version of CD45.
	KLC1	(SNP in intron 13)
	MBP	Multiple isoforms
	MOG	Multiple isoforms
	PLP	(alternative 5' splice sites) two isoforms
	HLA-G	Multiple isoforms. sHLA-G levels elevated in CSF from patients with relapsing-remitting condition

AUTOIMMUNE DISEASE	ALTERNATIVELY SPLICED PRE-mRNA	CONSEQUENCE
Type 1 diabetes	TAP2	Two isoforms
	IA2	Differential isoform expression
	iNOS	Isoform lacking exon 8
	ASS	Isoform lacking exon 1
	CTLA-4	Two isoforms. Soluble CTLA-4 is lowered
	NFKB2	Isoform lacking exon 22
	IGRP	Multiple isoforms
	IA-2	Isoform lacking exon 13
Myasthenia gravis	AChE	Two isoforms
	CTLA-4	Different isoforms
Kawasaki disease	ITPKC	(SNP in intron 1 disrupts a poly-G run) Decreased splicing efficiency
Scleroderma	IL4	Truncated isoform
Systemic sclerosis	CD45	C77G polymorphism that produces hyperactive version of CD45.
Ulcerative colitis	PTPσ	(skipping of exons 8, 9) isoform lacking Ig-like domain
Autoimmune inner ear disease	IL1R2	Two isoforms
Autoimmune polyendocrine syndrome type 1	Aire	Functional missense AIRE mutation c.463G>A (p.Gly155Ser) which generates a truncated protein

Table 2. Alternative spliced genes in autoimmune diseases. Adapted from (7). Abbreviations: AChE, acetylcholinesterase; Aire, Autoimmune regulator; ASS, Argininosuccinate synthase; BANK1, B Cell scaffold protein with ankyrin repeats; CR2, complement receptor 2; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; CXCL12, chemokine (C-X-C motif) ligand 12; FGFR1, Fibroblast growth factor receptor 1; FoxP3, forkhead box P3; HLA-G, Major histocompatibility complex, class I, G; IA-2, insulinoma-associated tyrosine phosphatase-like protein; Ig-like, immunoglobulin-like; IGRP, Islet-specific G6CP-related protein; IL4, interleukin 4; IL7, interleukin 7; IL1R2, interleukin-1 receptor type II; IL6R, interleukin 6 receptor; IL7Rα, interleukin 7 receptor α chain; IL20R, interleukin 20 receptor; iNOS, inducible Nitric Oxide Synthase; IL-32, Interleukin 32; IRF5, interferon regulatory factor 5; ITPKC, inositol 1,4,5-triphosphate 3-kinase C; KLC1, kinesin light-chain 1; LILRA2, leukocyte immunoglobulin-like receptor A2; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; NFKB2, Nuclear factor of kappa light polypeptide gene enhancer in B Cells 2 (p49/p100); OAS1, 2',5'-oligoadenylate synthetase 1; PLP, proteolipid protein; PTPN22, protein tyrosine phosphatase, non-receptor type 22; PTPσ, protein-tyrosine phosphatase sigma; RasGRP1, Ras guanyl-nucleotide releasing protein 1; SNP, Single nucleotide polymorphism; TCRζ, T Cell receptor ζ chain; TAP2, transporter 2, ATP-binding cassette, subfamily B; TNC, Tenascin-C; TNFR2, receptor for tumor necrosis factor α2; 3'-UTR, 3'-untranslated region; VEGF, Vascular endothelial growth factor.

MODIFICATION	AUTOANTIGEN	AUTOIMMUNE DISEASE
Citrullination	Fillagrin	RA
	Fibrin	RA
	Vimentin	RA
	Collagen	RA
	α-Enolase	RA
	Aporin	RA
	CapZα-1	RA
	MBP	MS

MODIFICATION	AUTOANTIGEN	AUTOIMMUNE DISEASE
Phosphorylation	αB-crystallin	MS
	Multiple	SLE
	La/SSB	SLE
	U1-68k	RA, SLE
	SmD1, SmD2	SLE
Acetylation	αB-crystallin	MS
	DEK	JIA
Oxidation	Insulin chain A	T1D
	β2 glycoprotein 1	APS
	HMGB1	SLE
Methylation	SmD1, SmD2	SLE
Deamidation	Transglutaminase	CD
	snRNP D, H2B	SLE
Endoprotease cleavage	Pso27	PSO
Malondialdehyde	MOG	MS
Carbamylation	Fillagrin	RA
Caspase cleavage	U1-70K	SLE
Lipid peroxidation	Self-proteins	SLE
Hydroxylation and glycosylation	Collagen type II	RA
N- glcyosylation	MBP	MS

Table 3. Posttranslational autoantigen modifications in autoimmune diseases. Adapted from (6). Abbreviations: APS, Antiphospholipid Syndrome; CapZα-1, Capping protein (actin filament) muscle Z-line, alpha 1; H2B, H2B histone family, member M; JIA, Juvenile Idiopathic Arthritis; MBP, Myelin Basic Protein; HMGB1, High Mobility Group Box 1; La/SSB, Sjogren Syndrome Antigen B (autoantigen La); MOG, Myelin Oligodendrocyte Glycoprotein; MS, Multiple Sclerosis; PSO, psoriasis; Pso27, Psoriasis-associated antigen pso p27; RA, Rheumatoid Arthritis; SLE, Systemic Lupus Erythematosus; SmD1, Small Nuclear Ribonucleoprotein D1 polypeptide 16kDa; SmD2, Small Nuclear Ribonucleoprotein D2 polypeptide 16.5kDa; snRNP D, Small Nuclear Ribonucleoprotein D; U1-68k, Small Nuclear Ribonucleoprotein 68k; U1-70k, Small nuclear Ribonucleoprotein 70k; T1D, Type 1 Diabetes.

Recommended Lectures

1. Abou-Zeid A, Saad M, Soliman E. MicroRNA 146a expression in rheumatoid arthritis: association with tumor necrosis factor-alpha and disease activity. Genet Test Mol Biomarkers. 2011;15:807-12.
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2 INNATE IMMUNE SYSTEM

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INTRODUCTION

The innate immune response is the first mechanism for host defense found in all multicellular organisms. The innate immune system is more ancient than the acquired or adaptive immune response, and it has developed and evolved to protect the host from the surrounding environment in which a variety of toxins and infectious agents including bacteria, fungi, viruses and parasites are found (1).

The immune system is complex and is divided in two categories: i) the innate or nonspecific immunity, which consists of the activation and participation of preexistent mechanisms including the natural barriers (skin and mucosa) and secretions; and ii) the adaptive or specific immunity, which is targeted against a previously recognized specific microorganism or antigen. Thus, when a given pathogen is new to the host, it is initially recognized by the innate immune system and then the adaptive immune response is activated (2). Innate immunity is the host's first line of defense and is intended to prevent infection and attack the invading pathogens.This nonspecific mechanism is fast (minutes to hours) while the adaptive response takes longer (days to weeks).

Innate immunity is comprised of different components including physical barriers (tight junctions in the skin, epithelial and mucous membrane surfaces, mucus itself); anatomical barriers; epithelial and phagocytic cell enzymes (i.e., lysozyme), phagocytes (i.e., neutrophils, monocytes, macrophages), inflammation-related serum proteins (e.g., complement, C-reactive protein, lectins such as mannose-binding lectin, and ficolins); surface and phagocyte granule antimicrobial peptides (e.g., defensins, cathelicidin, etc.); cell receptors that sense microorganisms and signal a defensive response (e.g., Toll-like receptors); and cells that release cytokines and inflammatory mediators (i.e., macrophages, mast cells, natural-killer cells). Once the interaction host-invader pathogen enters, a signaling cascade is initiated which enhances the immune response and activates specific mechanisms (3-5). This natural immune response is designed to: a) prevent infection, b) eliminate invader pathogens, and c) stimulate the acquired immune response.

COMPONENTS OF THE INNATE IMMUNE SYSTEM

The innate immune system includes physical and anatomical barriers as well as effector cells, antimicrobial peptides, soluble mediators, and cell receptors (Table 1). Skin and mucosa provide an effective immune barrier between the internal and external environment. Skin acts as not only a physical barrier but also a chemical shield. The most external layer of epidermis mainly consists of keratinocytes, which are tightly linked by desmosomes and embedded in a layer of extracellular matrix proteins. Keratinocytes not only act as a physical barrier but also express pattern recognition receptors (PRRs) and are capable of producing cytokines and antimicrobial peptides that, in turn, induce an inflammatory cascade and microbial destruction respectively (6,7). Furthermore, sebaceous glands associated with hair follicles produce large amounts of fatty acids which create an acidic environment that is hostile to microorganisms. Mucous membranes in the digestive, respiratory, and genitourinary tracts have a continuous epithelium that prevents microorganisms from entering the host. In addition, these epithelial cells produce antimicrobial peptides such as defensins. The production of defensins is also enhanced by the action of inflammatory cytokines including interleukin (IL)-1 and tumor necrosis factor alpha (TNF-α) which are produced by macrophages and other immune cells in response to invading pathogens (2,8-11).

COMPONENT	FUNCTION
Barriers	
Skin	Prevents microbial entrance
Mucosa	Prevents microbial entrance, secretes proteins and enzymes, absorbs metabolic substrates
Effector cells	
Granulocytes	Phagocytosis, cytokine production, protein and enzyme secretion, destruction of pathogens
Monocytes/macrophages	Phagocytosis, cytokine production, protein and enzyme secretion, destruction of pathogens
Dendritic cells	Phagocytosis, cytokine production, protein and enzyme secretion, destruction of pathogens
Natural killer (NK) cells	Lysis of infected and tumoral cells, activation of macrophages through cytokine production
Innate lymphoid cells	Mediate immune response and regulate tissue homeostasis and inflammation
Endothelial/epithelial cells	Microbial recognition, cytokine production
Antimicrobial peptides	
	Destruction of invading pathogens
Soluble mediators	
Cytokines	
TNF-α, IL-1, chemokines	Mediate immune response and inflammation
IFN-α	Involved in resistance to viral infection
IFN-γ	Involved in resistance to intracellular pathogen infection and activation of macrophages
IL-12	Stimulates IFN-γ production by NK cells and T lymphocytes
IL-15	Stimulates NK cell proliferation
IL-10	Regulates and controls the inflammation process
TGF-β	Regulates and controls the inflammation process
Seric proteins	
Complement system	Opsonization, destruction of pathogens, and T lymphocyte activation
Collectins	Opsonization of pathogens and complement activation
C reactive protein	Opsonization of pathogens and complement activation
Coagulation system	Localization of damage or infected tissue
Cellular receptors	
TLRs	Recognize a variety of microbial components
NLRs	Sense bacterial components present in the cytoplasm
CLRs	Recognize sugar moieties of bacteria and fungi
RLRs	Sense viral RNA

Table 1. Components of the innate immune system. *TNF*: Tumor necrosis factor; *IFN*: interferon; *IL*: interleukin; *TGF*: transforming growth factor; *TLR*: Toll-like receptor; *NLR*: Nod-like receptor; *CLR*: C-type lectin receptor; *RLR*: RIG-I-like receptor.

A malfunction in the epidermis can lead to an inadequate host response to a pathogen or a persistent inflammatory state. Atopic dermatitis is the most common inflammatory skin disorder. It is characterized by abnormalities in the skin barrier structures (i.e., stratum corneum and tight junctions), a robust TH2 response to environmental antigens, defects in innate immunity, and an altered microbiome. Many of these abnormalities may occur as a consequence of epidermal dysfunction through pattern recognition receptors.

Epithelial cells in the gastrointestinal and respiratory mucosa have cilia, an extension of the cell surface which has the ability to move back and forth and thus keep the mucosa clear of mucus, dust, and possible invading microorganisms. In addition, intraepithelial lymphocytes are located in the skin and mucosal epithelium.These lymphocytes are

predominantly gamma/delta T lymphocytes (LT-γδ), which are involved in host defense through cytokine production, phagocyte activation and, destruction of infected and tumoral cells. There is a subpopulation of B lymphocytes (LB-1) in this compartment that secretes immunoglobulin M (IgM), which are also known as natural antibodies.These natural antibodies protect against microbial pathogens through recognition of highly conserved epitopes and also exert homeostatic functions (2,10,11).

STRUCTURE OF AND IMMUNOLOGICAL THREAT TO THE AIRWAYS

The human respiratory apparatus consists of nose, oropharynx, larynx, conducting airways, and the respiratory surface. Despite containing a volume of approximately 5 L, the total respiratory surface of the lung exceeds 120 m^2 (12), which is more than 60 times the body surface. This is due to the presence of millions of small alveoli, spheroid sacs at the terminal end of the conducting airways that provide an extremely thin epithelium. This is optimized for the diffusion of respiratory gases. A recent re-estimation of the total number of alveoli in the human lung represent 480 million units with a remarkably narrow size distribution that is around 4.2*106 /ml. This is equal to an alveolar radius of approximately 100 ml (13). There is continuous intense confrontation between the extensive surface of the respiratory tract and noxious airborne threats and potentially pathogenic microorganisms. As a result, the mucosal tissue in the nasal passages and oropharynx is always colonized by a multitude of bacteria.

An effective system of surveillance and cleaning has evolved in order to constantly monitor and maintain the sterility of the lung. This system is characterized by a unique design for the conducting airways and alveoli. Starting in the nose, a coarse filter consisting of hair and mucus will obstruct the entry of material exceeding a certain size limit. Combined with a rapid sneezing reflex, potentially hazardous or allergenic material will immediately be removed from the airways or trapped in the mucus. Despite being very sticky and viscous, the mucus also contains many antibiotic factors such as antimicrobial peptides or oxidizing enzymes (14). Thus, mucus not only constitutes a physical trap but also has considerable antibiotic properties. Specialized epithelial cells containing a ciliated surface line the airways. The design of an alveolus directly reflects its main function in the respiratory surface. It is covered by two types of alveolar epithelial cells (AECs), type I and type II. Type I AECs provide the thin respiratory surface of an alveolus. Type II AECs are almost round in appearance and contain the so-called lamellar bodies. They are storage sites for surfactant, a thin liquid film that is constantly produced by type II AECs (15). This film covers the entire surface of the alveolus and has important functions for the biology of the lung. AECs II are considered precursors for type I AECs and can replace them at sites of alveolar damage (16). Individual alveoli are separated from each other by thin septae, within which the capillaries of the pulmonary blood vessels flow. Alveoli are connected to each other by multiple holes within the septae, the so-called pores of Kohn. Immune cells recruited to the surface of alveoli can migrate through these pores (17).

IMMUNOLOGICAL CONTROL OF ALVEOLAR INTEGRITY: SURFACTANT

Surfactant is a compound mixture of phospholipids (90%) and proteins (10%). An important physical effect of the thin surfactant layer is that it compresses cells lying under its surface very flat. In the conducting airways, surfactant surface forces transport particulate matter from the rigid surface (gel phase) of the surfactant layer into the more liquid underlying sol phase, which is in direct contact with the mucocilliary border of the epithelium. The presence of particles in the sol phase facilitates their mucocilliary transportation (18).

Surfactant contains four types of proteins (SP-A to SP-D) of which three have important immunological functions. Among them are the binding of bacterial lipopolysaccharide to or the direct absorption of surfactant proteins into the surface of pathogens. Surface binding of surfactant proteins can lead to pathogen aggregation and direct killing or the increase of the phagocytosis and killing activity of attached immune cells. In addition, surfactant proteins can also interfere with dendritic cell (DC) maturation or inhibit T cell proliferation and thus have an immunoregulatory function. Absence of surfactant proteins leads to the deviation of a protective T-helper 1 (Th1) towards a non-protective Th2 response during pulmonary hypersensitive reactions against *Aspergillus* antigens (19).

EFFECTOR MOLECULES AND MICROBICIDAL MECHANISMS OF INNATE IMMUNITY

There are several chemical and enzymatic compounds capable of inhibiting and destroying microbial pathogens. These include: lysozyme, which is present in the saliva, tears, and nasal secretions and is able to affect microbial growth; hydrochloric acid and digestive proteins such as pancreatin and peptidase in the gastrointestinal tract, which destroy microbial pathogens; and fatty and bile acids, transferrin, lactoferrin and fibronectin that can control the growth of the host's normal microbiota as well as the entrance of microbial pathogens through the mucosa (4,20).

Plasma proteins include the secreted PRRs: MBL and CRP. These molecules recognize carbohydrates which are acting as opsonins. In addition, these PRRs may bind and activate complement factors such as C1q thus enhancing the inflammatory response (21,22).

The coagulation system, in addition to its role in controlling bleeding and clotting formation during a tissue injury, is also involved in the innate immune response by preventing microbial dissemination. Fibrinogen, one of the coagulation system components, can sense microorganisms and act as an opsonin (21).

Complement is considered one of the most important enzymatic systems involved in the innate immune response (See chapter 4). This enzymatic system is activated three different ways. Some of the components of this system act as opsonins or anaphylatoxins that enhance the immune response (23).

Activation of the innate immune system is initiated by soluble pattern recognition molecules, which may be expressed on innate immune cells, bound to the extracellular matrix, or circulate in the blood as soluble molecules. One such soluble pattern recognition molecule is MBL, which is primarily synthesized in the liver and secreted to circulation (24,25). Small amounts of MBL are also synthesized in the kidney, thymus, tonsils, small intestine, and vagina, where mRNA has been detected (25-27).

MBL protein has also been found in other organs such as the skin, brain, and lung although its mRNA has not been detected in those areas (24-29). In the lung, MBL is found in the bronchial alveolar lavage of healthy individuals and also on the smooth muscle in airways following infection (28,30). In the skin and the brain, MBL is observed only following burn and trauma injury respectively (28,29,31).

MBL functions as an opsonin and activates the complement through the lectin complement pathway. The lectin pathway is also activated by ficolins, which are structurally similar to MBL and circulate in the blood. The lectin pathway requires activation of MBL-associated serine proteases (MASPs) (24,28,31-34).

There are two genes and five MASP gene products. MASP-1, MASP-3, and MAp44 (or MAP-1) are the alternative splice products of the MASP-1/3 gene while MASP-2 and MAp19 (or sMAP) are the alternative splice products of the MASP-2 gene (35). MASPs form complexes with MBL (35, 36), and MBL binding to carbohydrate ligands is thought to induce conformational changes that enhance proteolytic activities in the associated MASP. MASP-1 and MASP-2 have been shown to activate the alternative pathway and the lectin complement pathway (37–40).

MBL deficiency increases susceptibility to infection by reduced opsono-phagocytic activity and a reduced activation of the lectin complement pathway. The MBL deficiency may manifest as disseminated intravascular coagulation and organ failure with infection.

Successful innate immune protection is achieved through two steps. First, identifying targets, such as pathogens and abnormal tissues and cells. Second, by orchestrating humoral and cell effectors to neutralize and eliminate the identified targets. In this sense, MBL contributes to both immunity from pathogens and maintenance of tissue integrity and homeostasis.

MBL DEFICIENCY

MBL deficiency can be caused by inherited gene defects, which have been identified in 5%–30% of the population. MBL deficiency is a common primary immunodeficiency (41-43). There are three coding region single nucleotide polymorphisms (SNPs) at codons 52, 54 and 57, termed the C, B, and D alleles respectively (44). All of these SNPs are located in the collagen-like domain (all are located close to the N-terminus side of the kink and produce aberrant proteins) [44]. The frequency of these alleles varies depending on ethnicities. While all three alleles are observed in Caucasians, alleles C and D are very rare in Asians (45,46).

Most MBL deficiency is due to the heterozygosity of these SNPs and results in a wide range of MBL blood concentrations from undetectable to as high as 10 μg/ml (45, 47). Some aberrant MBLs were found to be dysfunctional in activating the lectin complement pathway. Mechanisms for this lack of complement pathway activation are related to reduced ligand binding due to decreased oligomerization and decreased activation of MASPs due to impaired association with mutant MBL (48,49).

MBL-deficient hosts may present with systemic infection involving multiple organs, including blood (bacteremia), and uncontrolled inflammation due to cytokine storm. Such infection and subsequent cytokine release may establish an autocrine loop with further escalating complications.

OTHER EFFECTOR MICROBICIDAL MECHANISMS

OXYGEN-DEPENDENT MECHANISMS: REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species (ROS) and reactive oxygen intermediates (ROI) are produced by mammalian cells, particularly phagocytes, as a reaction against several microbial pathogens (50). These molecules are generated by activation of the enzymatic complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) and include superoxide anion (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), peroxynitrite (ONOO-),hypochlorous acid (-OCl), etc. (50). Both ROS and ROI are known to play diverse roles in inflammation, host defense, and homeostasis. Chronic granulomatous disease (CGD) is an inherited condition in which a deficiency of NOX2 results in the inability of phagocytes to generate microbicidal superoxide anion and its metabolites. Thus, patients with CGD have recurrent, life-threatening bacterial and fungal infections as well as chronic inflammatory diseases due to dysregulated inflammatory pathways (51).

OXYGEN-INDEPENDENT MECHANISMS

Innate cells, mainly phagocytes, are equipped with an enzymatic arsenal capable of destroying several microorganisms. These enzymes include proteases, cationic proteins, lysozyme, elastases, capthesin G, defensins, etc., all of which exhibit microbicidal activities (52-55). In addition, antimicrobial peptides and other mechanisms involving reactive nitrogen intermediates (RNI) and DNA extracellular traps have been described and will be discussed below.

ANTIMICROBIAL PEPTIDES (AMPS)

AMPs are host defense peptides secreted mainly by innate and epithelial cells including keratinocytes. Their antimicrobial activity is broadly based especially against fungi, bacteria, and viruses (20). About 1700 AMPs have been described so far.They are found constitutively or can be induced after activation of the host cells through several PRRs during an infection or injury (6). Additionally, these AMPs are involved in other cell processes including cell migration, proliferation, differentiation, cytokine production, angiogenesis, and wound healing, along with other functions (6). Several families of AMPs have been described.

Cathelicidin or LL-37 is released by neutrophils and epithelial cells. This AMP has the ability to kill Gram-negative and Gram-positive bacteria, fungi, and viruses. It induces an immune response which triggers inflammatory cell recruitment and cytokine release by host cells (6). Of note, LL-37 is induced by vitamin D3, and the absence of this vitamin is associated with the development of certain infectious diseases (20,56-59).

Defensins include α- and β-defensins. α-defensins (hαD-1, -2, -3, -4) are stored in the azurophil granules of neutrophils, and HαD-5 and -6 are synthesized by the Paneth cells in the gastrointestinal tract (60). β-defensins (hβD-1, -2, -3) are produced mainly by keratinocytes. Defensins also exhibit antimicrobial activity, and like cethelicidins, they are chemotactic and induce cytokine and chemokine synthesis (6,61,62).

Other AMPs include dermicidin and psoriasin, which also show antimicrobial activities. Alterations in the AMP expression are related to atopic dermatitis and psoriasis (6).

NITRIC OXIDE (NO)

Nitric oxide (NO) is considered to be one of the most important RNI and is produced by an oxidative mechanism involving the catabolism of L-arginine (63). NO production by the enzymatic action of inducible nitric oxide synthase (iNOS) represents one of the major microbicidal mechanisms that phagocytic cells use against several pathogens (64). In turn, iNOS can be induced by several stimuli, including IFN-γ, TNF-α, and LPS, and is expressed by immune cells such as macrophages, neutrophils, dendritic cells, and NK cells (63). Like ROS, NO may be involved in inflammation and its regulation process.

EXTRACELLULAR TRAPS

Extracellular DNA traps are part of innate immunity and are associated with infectious processes and allergic and autoimmune diseases. These structures are generated by different leukocytes including neutrophils, eosinophils, monocytes, and mast cells.They are called NETs, EETs, METs, and MCETs respectively. Extracellular traps are composed of DNA, histones, and the content of the intracellular granules such as elastase, myeloperoxidase (MPO), cathelicidins, tryptase, cationic proteins, and major basic protein, etc. These traps are induced by the action of the granulocyte/macrophage-colony stimulating factor (GM-CSF), interferons, IL-8, C5a, and LPS. Once formed, extracellular traps are capable of binding to and killing microbial pathogens. As was mentioned, these DNA traps may be involved in the development of autoimmune and chronic inflammatory diseases (65) (See chapter 13).

EFFECTOR CELLS OF INNATE IMMUNITY

Cell components encompass phagocytic cells, epithelial and endothelial cells, natural killer cells, innate lymphoid cells, and platelets (Figure 1). Phagocytic cells consist of granulocytes (i.e., neutrophils, eosinophils, basophils, and mast cells), monocytes/macrophages, and dendritic cells.These cells participate in not only the phagocytosis but also the inflammatory process. "The majority of cell components expresses PRRs on the cell surface, and they are able to secrete cytokines: thus exhibiting microbicidal mechanisms". These cells with effector mechanisms of innate immunity are modulated by both the innate and acquired immune systems (66,67).

GRANULOCYTES

Granulocytes are effector cells that predominate during the early or acute phase of the innate immune response. The main function of these cells is to identify, ingest, and destroy microbial pathogens through receptors, oxidative mechanisms, and enzymes including lysozyme, collagenase, and elastase, etc. This group of cells is composed of neutrophils, eosinophils, basophils and mast cells (53,68).

NEUTROPHILS

These cells are most abundant and effective during the inflammation and phagocytosis processes. Neutrophils (PMN) are characterized as being the first cell line that is recruited at the inflammation site after chemotactic stimuli. These stimuli include the complement factors such as the C5a factor, chemokines such as IL-8, and leukotrienes (L) including the L-B4, which exerts a paracrine and autocrine function on other neutrophils. All these substances that allow migration to the injury site are recognized by specific receptors or PRRs.These phagocytes possess Fc or complement receptors (RFc or CR) that recognize the immunoglobulin Fc fractions or complement factors respectively. This allows the phagocytosis of tagged (opsonized) microorganisms by antibodies (mainly IgG) or complement (mainly C3b or iC3b). Moreover, neutrophils have stored an enzymatic arsenal capable of exerting a lytic effect on microbial pathogens or inducing microbicidal systems through oxygen-dependent and -independent mechanisms (53,55,68,69) in their granules. In addition to proinflammatory cytokines, the hematopoietic growth factors, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are critical for recruitment and activation of PMNs (70). Three different PMN subsets have been described in mice based on their cytokine and chemokine production as well as on the toll-like receptor (TLR)

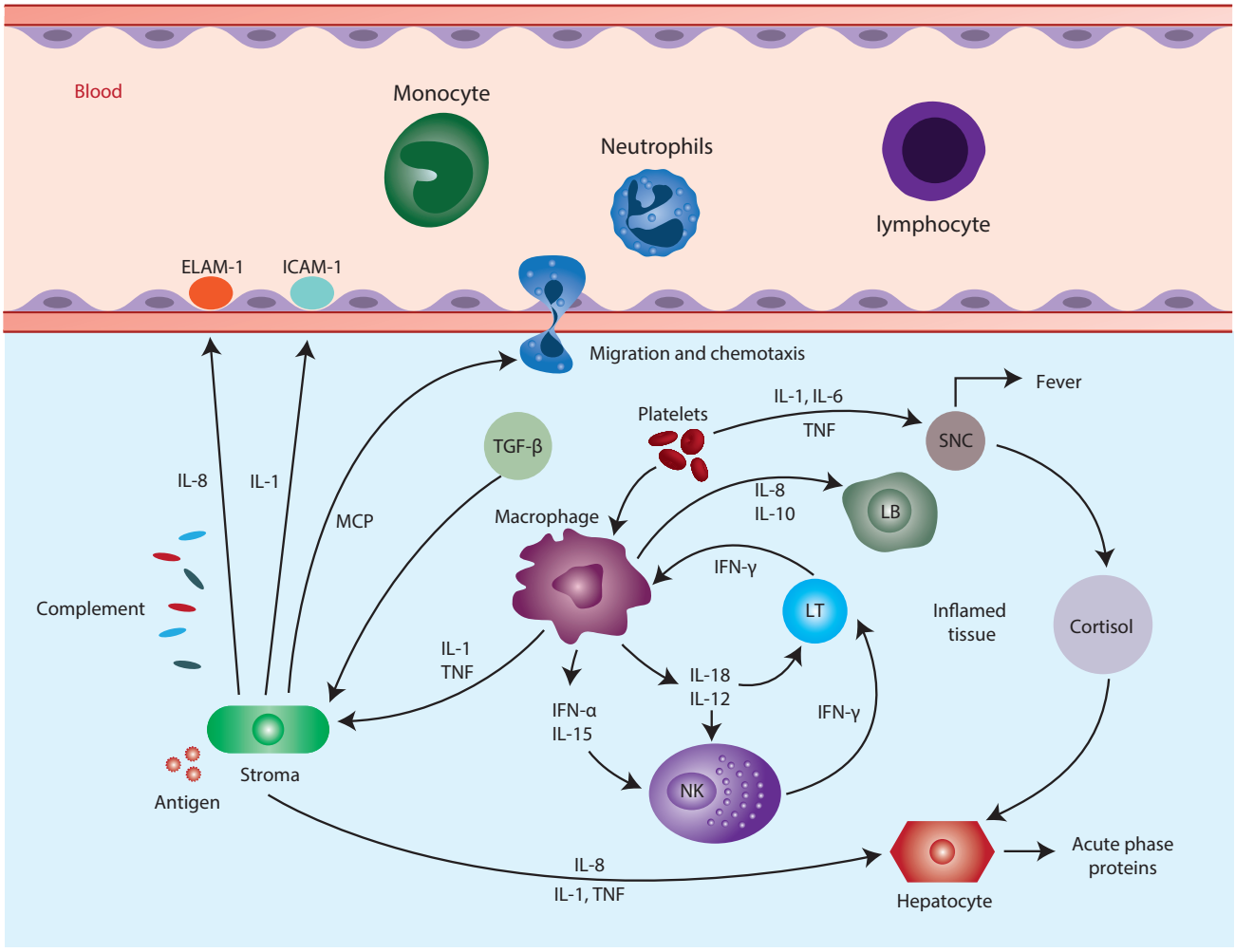

Figure 1. Effector mechanisms of the innate immune response: The innate immune response involves a set of cells that produce cytokines/chemokines that participate in phagocytosis, inflammation, and the synthesis of acute phase proteins.

and surface antigen expression and macrophage activation. Normal PMNs (PMN-N) which are CD49d- and CD11b- express TLR2, TLR4, TLR9, and have no cytokine/chemokine production or effect on macrophage activation. PMN-I subsets which are CD49+ and CD11b- and express TLR2, TLR4, TLR5, TLR8, produce IL-12 and CCL3 and activate type-1 macrophages or classically activated macrophages. PMN-II subsets, which are CD49- and CD11b+, express TLR2, TLR4, TLR7, and TLR9, produce IL-10 and CCL2 and activate macrophages alternatively (71). Two additional populations have been described based on the expression of the surface marker Gr-1 (Gr-1 high and Gr-1 medium) (72). More recently, a subset of mature neutrophils which express the surface markers CD11c brigth, CD62L dim, CD11b bright, and CD16 bright have been identified in humans. Apparently, this circulating population of myeloid cells is capable of suppressing T cell proliferation (73). Once PMNs have completed their tasks, they die by apoptosis, netosis or necrosis (see Chapter 13). The latter process can cause tissue damage through the release of their granule contents, thus prolonging the inflammatory reactions (72).

EOSINOPHILS

These granulocytes are present in the respiratory, gastrointestinal, and urinary tract, and they are less abundant than neutrophils. Their effector function is mediated by degranulation and release of histamine, cationic proteins, major basic protein, sulfatases, and chemotactic factors such as leukotrienes and prostaglandins. The degranulation process is mediated by the IgE or other chemotactic factors, including the IL-5. The main function of these cells is to destroy microbial pathogens, mainly parasites, but they also play an important role in the allergic processes together with mast cells (74).

BASOPHILS AND MAST CELLS

These cells are not phagocytic in nature and have several receptors including IgE receptors. The proportion of basophils in circulation is lower than the proportion of other granulocytes. Mast cells are located in tissues, mainly in mucosa, and their granules contain heparin, serotonin, and histamine. They may also release a variety of cytokines that

enhance the inflammatory process, especially during the early events. These cells are involved in allergic and viral processes. Mast cells are present mainly in the connective tissue. They expressTLR-1, -2, -4, and -6, complement receptors (CR), mannose receptor (MR) in their cell membrane and release TNF-α, IL-8, platelet activator factor, proteases, antimicrobial peptides (catelicidin LL-37 and defensins), and other inflammatory mediators (75-79).

MONOCYTES/MACROPHAGES

Monocyte/macrophages together with DC are considered important actors in both innate and adaptive immunity. Monocytes circulate in peripheral blood and have the ability to not only migrate to the inflammatory site but also exhibit the plasticity to transform themselves into tissue macrophages (80). Once in the tissue, these cells are named macrophages and have different functions: i) they are phagocytic and exhibit a microbicidal mechanism through oxygen -dependent and-independent mechanism; ii) they are able to present antigens and activate lymphocytes; iii) once activated, they release and stimulate cytokine secretion; iv) they modulate the immune response; v) they participate in tissue reorganization after the inflammation process has ceased through production of extracellular matrix proteins (i.e., collagen and elastase) and matrix metalloproteinases; and vi) they produce cytotoxic factors involved in the immunity against tumors (80). Based on the biological function, there are three populations of macrophages: i) classically activated macrophages or type 1-activated macrophages; ii) alternatively activated macrophages; and iii) type 2-activated macrophages (81). Type 1-activated macrophages are usually stimulated by IFN-γ or TNF-α in combination with microbial products such as LPS and are considered effector cells in the Th1 immune response. Once activated, type 1 macrophages up-regulate expression and production of pro-inflammatory cytokines and chemokines [TNF-α, IL-23/IL-12, IL-6, IL-1, IP-10, macrophage inflammatory protein 1 alpha (MIP-1α), and monocyte chemoattractant protein 1 (MCP-1)], major histocompatibility complex (MHC) class II, and co-stimulatory molecules and enhance their ability to kill microbial pathogens through NO and ROS production (81,82). The alternatively activated macrophages are stimulated mainly by IL-4 and glucocorticoids, and once activated, they synthetize IL-10, IL-8, MIP1β, MCP-1, and RANTES.They also produce high levels of fibronectin and other extracellular matrix, as well as arginase. These are involved in polyamine and proline synthesis which, in turn, induces cell growth and collagen formation and thus participates in tissue repair. These alternative macrophages do not produce NO and subsequently fail to kill intracellular microorganisms (81,82). The type 2-activated macrophages are stimulated after recognition of IgG complex and TLR ligands. Once FcγRs recognize their ligands IgG complex), macrophages become activated and produce IL-10, TNF-α, and IL-6. These cells do not produce arginase but induce T cells to produce IL-4 (81,82).

DENDRITIC CELLS (DCS)

DCs are considered to be professional antigen-presenting cells (APC). They reside in and patrol the skin and mucosal surfaces, thus playing an important role in the innate immune system with subsequent activation of T cell responses to provide a cell-mediated immunity against microbial pathogens. Antigen uptake occurs through different mechanisms including phagocytosis, endocytosis, picnocystosis, and macropicnocytosis. DCs have the ability to transport and carry the antigens from peripheral to primary lymphatic nodes where the antigen presentation takes place. These APC lead to the processing and presentation of antigens via major histocompatibility complex (MHC) class II molecules, thus bridging the innate and acquired immune response (83). Additionally, DCs participate in the induction of peripheral immunological tolerance, regulate the types of T cell immune responses, and function as effector cells in innate immunity against several microbial pathogens. These diverse functions depend on the diversity of DC subsets (83). In fact, there are various subsets of DCs including immature DCs (imDCs) and precursors (pre-DCs).

imDCs display different phenotypes and functions and are produced from hematopoietic stem cells (HSC) within the bone marrow. Thus, CD34+ HSC differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CD34+ CMP differentiate into CD34+CLA+ and CD34+CLA-, which, in turn, differentiate into CD11c+CD1a+ and CD11c+CD1a-imDCs respectively (84). CD11c+CD1a+imDCs migrate to the skin epidermis and become Langerhans cells while CD11c+CD1a-imDCs migrate to the skin dermis and other tissues and become interstitial imDCs (85).

There are two types of pre-DCs: monocytes (pre-DC1) and plasmacytoid cells (pre-DC2). Pre-DC1 expresses the myeloid antigens (CD11b, CD11c, CD13, CD14, and CD33), CD1a-d, and mannose receptor. it also produces IL-12 and induces a Th1 pattern and cytotoxic T lymphocyte responses. Pre-DC2 expresses specific markers for lymphocyte lineage. It also produces IL-10 and induces a Th2 profile and CD8+T suppressor cells (83). Functionally, imDCs are involved in the antigen presentation while pre-DCs participate directly as effector cells in innate immunity to microbial pathogens.

INNATE LYMPHOID CELLS (ILC)

ILCs have been identified as new members of the lymphoid linage that are involved in regulating tissue homeostasis and inflammation. These cells do not express a T cell receptor and, consequently, do not respond antigen-specifically. Moreover, these cells do not express cell-surface markers associated with other immune cell lineages (86). These cells are divided in three subsets: i) Group I, ILCs, which is made up of ILC1 and NK cells. Both of these produce proinflammatory and type 1 cytokines and induce cytotoxicity through the expression of perforin and granzymes. ii) Group II, which consists of ILCs2, is characterized by the

production of type-2 cytokines and is present in the mesenteric fat-associated lymph clusters, mesenteric lymph nodes, spleen, liver, intestines, and Peyers' patches. Group II plays a role in the antihelminthic response and allergic lung inflammation. iii) Group III, is composed of ILCs3 and lymphoid tissue-inducer (LTi) cells. ILCs3 express the NK cell activating receptor NKp46 but lack cytotoxic effects and do not produce type-1 cytokines. These ILC3 cells reside in mucosal tissue and appear to play a crucial role in mediating the delicate balance between symbiotic microbiota and the intestinal immune system. LTI cells produce IL-17 and IL-22 and express molecules required for the development of lymphoid tissue. Subsequently they appear to be involved in the generation of lymph nodes and Peyers' patches (86). Note that disruption of the intestinal homeostasis maintained by these ILC cells is associated with the development of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (86).

NATURAL KILLER (NK) CELLS

NK cells exhibit an immunomodulatory role in the cell-mediated immune responses due their cytotoxic activity. They are also involved in antimicrobial defense and in the immunological surveillance by controlling tumoral growth and maintaining the immunological homeostasis (Figure 2). These cells employ a strategy known as "negative recognition." While a T or B cell is activated after recognition of an antigen via MHC, NK cells are activated when the antigen cannot be recognized the same way (87,88). NK cell receptors are "inhibitory receptors" in nature due to the fact that they keep the lytic activity of these cells suppressed since they detect the presence of MHC antigens. These cells detect infected cells (mainly infected by viruses) or malignant cells in which expression of MHC molecules has decreased, is altered or abolished. NK cells have the ability to distinguish the normal host cells through the killer cell immunoglobulin-like receptor (KIR) and CD94-NKG2A inhibitory receptors which recognize the MHC class I expressed on the surface of these normal cells (88,87). The binding of these receptors inhibits lysis and cytokine secretion by NK cells (89). In addition, NK cells have granules with perforins and granzymes that act on target cells inducing lysis or apoptosis and also express PRRs including TLR-2, -3, -4, -5, -7, and -8 (90,91). Once activated, NK cells secrete IFN-γ, TNF-α growth factors, IL-5, IL-10, IL-13, and chemokines (92-94).

EPITHELIAL AND ENDOTHELIAL CELLS

In addition to acting as a physical barrier, epithelial and endothelial cells express PRRs on their surface that recognize pathogen-associated molecular patterns (PAMPs) from microorganisms; secrete proinflammatory cytokines including IL-1, IL-6, and IL-8; and release antimicrobial peptides (8). Epithelial cells, mainly alveolar epithelial cells, are the most studied innate immunity component so far. In addition to providing an anatomic barrier that separates the organism from the external environment, alveolar epithelium serves as a defense mechanism against potential inhaled pathogens (58). This alveolar epithelium consists of two cell types: alveolar type I and alveolar type II cells.The former is ~95% of the alveolar epithelium and expresses TLR-4, a receptor for lipopolysaccharides (LPS). It produces pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β in response to LPS stimulation (57). Type II alveolar cells are~5% of the alveolar epithelium and produce cytokines and chemokines including

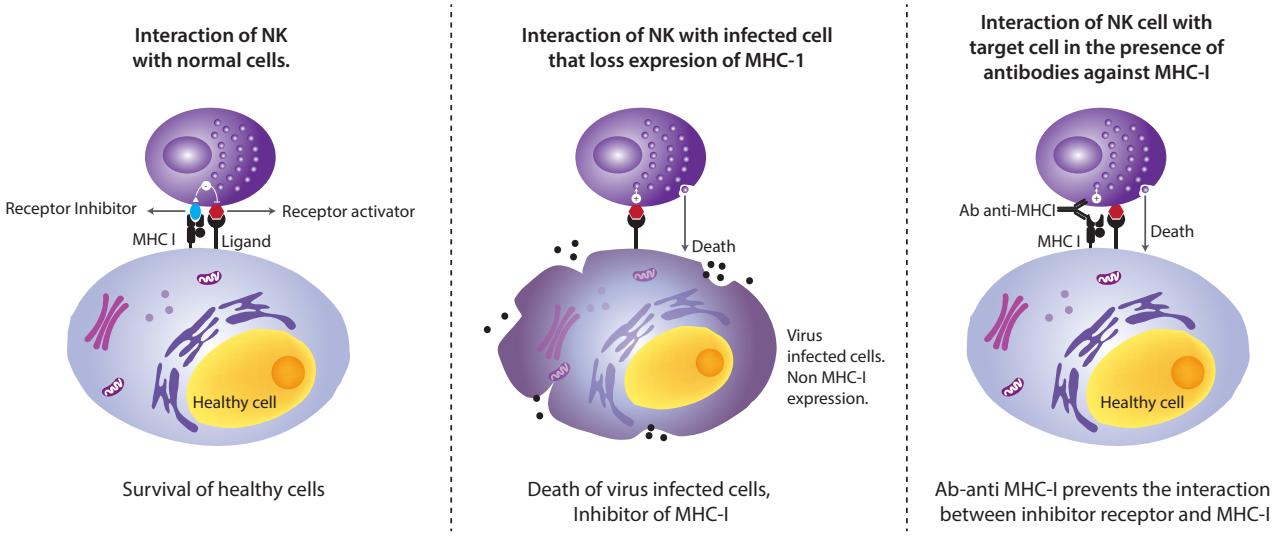

Figure 2. Recognition mechanisms and cellular innate immune response: functional characteristics of NK cells. NK cells trigger their activation once virus-infected or tumor cells suppress the expression of MHC molecules through the interaction of inhibitor or activator receptors with their respective ligands.

TNF-α, IL-6, IL-1β, MCP-1, growth related oncogene alpha (GRO-α), and GM-CSF, etc.,in response to various stimuli such as bacteria and viruses. Moreover, these cells also produce surfactant proteins which enhance chemotaxis and phagocystosis (58). Both type I and type II alveolar epithelial cells are important players in the innate immune response.

PLATELETS

Platelets are recognized by their participation in the coagulation process, control of bleeding, and defense against infectious agents (95,96). These cells express PRRs on their surface and produce cytokines and chemotactic molecules to recruit leukocytes at the inflammatory site. Platelets interact with leukocytes and endothelial cells through the expression of the adhesion molecule, P-selectin, which mediates proinflammatory events (95).

CHARACTERISTICS OF THE INNATE IMMUNE SYSTEM AND ITS RECOGNITION MECHANISMS

Innate immune response is characterized by its ability to distinguish structural components from microbial pathogens, which are present only in these microorganisms and are absent in the normal host cells. This recognition process is mediated by a variety of proteins present in the host cells such as the PRRs, which have already been mentioned (3,96). PRRs are germ-line encoded and do not show variability in comparison to the receptors involved in adaptive immunity. This characteristic indicates that innate immunity may identify clusters of microorganisms while adaptive immunity may distinguish between different antigens from one microorganism, which indicates that innate immunity is not specific. Another characteristic is that the innate immune response does not generate immunological memory after the recognition of the pathogen while adaptive immunity does (3). PRRs are evolutionarily conserved receptors that detect relatively invariant molecular patterns found in most microbial agents, the PAMPs. PRRs not only recognize PAMPs from invading pathogens but also have the ability to sense inflammatory components, also called damage-associated molecular patterns (DAMPs), released from damaged cells. PRRs include TLRs, NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs) (97). Nonetheless, in spite of the microbial recognition by innate cells, several microorganisms have developed evasion mechanisms to avoid recognition by these receptors.

PATTERN RECOGNITION RECEPTORS

PRRs have been divided into 4 subclasses: TLRs, NOD-like receptors (NLRs), retinoic acid–inducible gene (RIG)–like receptors (RLRs), and C-type lectin receptors (CLRs) (98). Although peptidoglycan recognition proteins (PGLYRPs) are not included in this classification, they are also recognized as PRRs and are thought to be important for bacterial infections.

PRRS AND THE EPIDERMAL CHEMICAL BARRIER

In addition to establishing a formidable physical barrier, keratinocytes are the major producers of antimicrobial peptides (AMPs). AMPs serve as a chemical defense against cutaneous pathogens and are increasingly recognized for their effects on wound repair. The classical human AMPs are LL-37 (a cathelicidin) and the β-defensin family. A number of other proteins produced by keratinocytes–including ribonucleases (RNases), S100 family proteins (e.g., S100A7, S100A8, and S100A9), dermcidin, and regenerating islet-derived protein 3a (REG3a)– are also recognized for their antimicrobial properties (99,100). Human keratinocytes constitutively express human β-defensin (HBD) 1, whereas HBD2, HBD3, and LL-37 are produced in response to inflammatory cytokines or PRR signaling (18,101,102). RNase7 has extensive antimicrobial properties and is constitutively expressed by human keratinocytes, but this is further enhanced by inflammation or bacterial exposure (103). Psoriasin (S100A7) is produced by differentiated keratinocytes and is most highly expressed around hair follicles and sebaceous units. Its expression is enhanced by IL-1, TNF-α, IL-17A, and IL-22 and repressed by IL-4 and histamine (104,105). Keratinocytes also express S100A8 and S100A9, which can exist as monomers or heterodimers (calpro- tectin) (106). Dermcidin is constitutively produced by eccrine sweat glands with extensive antimicrobial activity (107). Its proinflammatory actions induce the epidermal production of cytokines and chemokines (62). REG3a is produced in response to wounding or IL-17A exposure and, not surprisingly, is highly expressed in psoriatic skin lesions (100). Lastly, filaggrin is proteolytically cleaved into the hygroscopic amino acids, urocanic acid, and pyrrolidone carboxylic acid, which are referred to as natural moisturizing factors. This is an example of an alteration in the physical barrier that directly affects the chemical barrier.

In addition to their antimicrobial activities, AMPs have been found to play a role in physical barrier repair. The novel AMP REG3a enhances wound repair, at least in part, by inducing keratinocyte proliferation and differentiation (100). Not surprisingly, a number of PRRs are induced in response to wounding. For example, the expression of CD14 and TLR2 rise along the edge of the wound after an injury to the skin (108). This expression is dependent on the CYP27B1 enzyme, which converts 25-hydroxyvitamin D to the active 1.25 dihydroxy vitamin D form. This highlights a role for vitamin D in innate immune responses observed at sites of wounding and suggests that therapeutic approaches that increase vitamin D levels might enhance the host's innate immune response and help repair wounds.

PRRs are located on the surface and/or in the cytoplasm of virtually all nucleated cells. Nonetheless, there is a small

PRR	PAMP RECOGNITION	FUNCTION
TLRs		
Expressed on cell surface TLR-1, -2, -4, -5, -6	Lipopetides, LPS, flagellin, LTA, OxLDL, β-amyloid	Induce synthesis and secretion of cytokines
Expressed on cytoplasm TLR-3, -7, -8, -9, -10	dsRNA, ssRNA, CpG DNA	Induce synthesis and secretion of cytokines
CLRs		
Expressed on cell surface		
Dectin-1 Dectin-2 MR DC-SIGN Gal-3	β-glucan Mannose, α-mannan N-linked mannan Mannan β-mannosides	Phagocytosis and cell activation Phagocytosis and cell activation Phagocytosis and cell activation Phagocytosis and cell activation Complement activation, lysis, and opsonization
Secreted		
Collectins MBL Ficollins-1, -2, -3 SP-A, -D	Mannan Microbial carbohydrates Oligosaccharides	Complement activation, lysis, and opsonization Complement activation, lysis, and opsonization Opsonization
NLRs		
NODs **NLRPs**	Peptidoglycans and DAMPs	Induce synthesis and secretion of cytokines
RLRs		
RLRs	dsRNA	Induce synthesis and secretion of IFN and viral clearance

Table 2. **Pattern recognition receptors, their ligands and functions.** TLR: Toll-like receptor; NLR: Nod-like receptor; CLR: C-type lectin receptor; RLR: RIG-I-like receptor; MR: Mannose receptor; DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; Gal: Galectin; MBL: Mannose-binding lectin; SP: Surfactant protein; CRP: C-reactive protein; PTX3: Pentraxin 3; LPS: Lipopolysaccharide; LTA: lipoteicoic acid; OxLDL: oxidized low density lipoprotein; dsRNA: double-stranded RNA; ssRNA: single-stranded RNA; DAMPs: Damage-associated molecular patterns.

group of PRR molecules that can be secreted and act as a bridge between the microbial or cell target and host cells. PRRs include the TLRs, CLRs, NLRs, and RLRs (Table 2).

TOLL-LIKE RECEPTORS

To date, 10 TLRs have been identified in humans (TLR1-10) and 12 in mice (TLR1-9 and TLR11-13) (109,110) (see Chapter 3). TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface, while TLRs 3, 7, 8, 9, and 10 are found at the cytoplasm level. The main interactions of TLRs and their ligands are the following: TLR1/TLR2 recognize triacylated lipopetides, TLR3 binds double-strand (dsRNA), TLR4 recognizes LPS, TLR5 binds flagellin, TLR2/TLR6 bind diacylated lipopetides and lipoteichoic acid (LTA), TLR4/TLR6 recognize oxidized lipids (OxLDL) and β-amyloid, TLR7 and TLR8 sense single-strand (ssRNA), and TLR9 recognizes unmethylated CpG DNA and hemozoin (110) (Table 2).

TLRs are composed of 1) a leucin-rich repeat (LRR) domain that is usually involved in ligand binding and microbial sensing and 2) a cytoplasmic domain known as the Toll/interleukin-1 receptor (TIR). Thus, after a TLR binds to its ligand, an activation process is initiated through a signaling pathway via TIR domain-containing adaptor proteins. Several adaptor proteins that participate in TLR-mediated mechanisms have been described.These molecules include the myeloid differentiation primary-response protein 88 (MyD88), Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein (TIRAP), MyD88-adaptor-like protein (Mal), TIR domain-containing adaptor-including interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM). These adaptors mediate the activation of transcription factors such as the nuclear factor- κB (NF- κB) and the interferon regulatory factor (IRF), which, in turn, induce the expression of inflammatory and anti-inflammatory cytokine and chemokine genes (111,112). Note that TLR polymorphisms have been said to be associated with an increased risk of developing viral and fungal infections (113,114).

C-TYPE LECTIN RECEPTORS (CLRS)

CLRs are considered the other major PRR family. These PRRs recognize not only sugar moieties from bacteria and fungi but also molecules associated with dead or dying cells (97). This family consists of two groups, those present on the cell membrane and the soluble forms, which are secreted

mainly by immune cells. Membrane CLRs include Dectin-1, which recognizes β-glucans present in the fungal cells; Dectin-2, which recognizes both high-mannose structures and α-mannan; mannose receptors (MR) that recognize N-linked mannan; DC-SIGN (a receptor on the dendritic cells), which also recognizes mannan; and galectin-3, which recognizes β-mannosides (115). It is noteworthy that several of these membrane CLRs, including Dectin-1, DC-SIGN and galectin-3, have been identified as TLR2 co-receptors (115).

The soluble CLRs are divided into two groups as follows:

i) Collectins. These PRRs are proteins that are structurally similar to collagen. They bind carbohydrates present on the microbial cell wall. These soluble molecules act as opsonins, and in addition, they induce lysis of target cells and act as chemoattracting molecules for leukocytes through complement activation. Collectins include MBL, which recognizes mannan, surfactant proteins (SP) that sense oligosaccharides, and ficollins 1, 2 and 3 that bind microbial carbohydrates (3).

ii) Pentraxins. These proteins are highly conserved through evolution and are characterized as having a domain with five subunits of 200 aminoacids searching the C-terminal region. This group of CLRs includes C-reactive protein (CRP), serum amyloid P component, and pentraxin 3 (PTX3). These soluble molecules are secreted by macrophages and DC after activation via TLRs and proinflammatory cytokines (116).

NOD-LIKE RECEPTORS (NLRS)

The nucleotide-binding oligomerization domain (NOD) receptors (NLRs) are intracellular PRRs that sense bacterial components including peptidoglycans, which are directly introduced into the cytoplasm (3,4,96). NLRs include several family members such as NODs (NOD 1-4), NLRPs (NLRP 1-14), and IPAF. These molecules are regulators of immunity in response to a variety of pathogens (117). NLRs in concur with the AIM2 protein, the adaptor protein ASC, and caspase-1 constitute the inflammasome (97). NOD expression is regulated by IFN-γ and TNF-α, and polymorphisms in NOD2 gene influence the risk of acquiring Crohn's disease (79).

RIG-LIKE RECEPTORS (RLRS)

Retinoic acid inducible gen-I (RIG)-like receptor (RLRs) is an intracellular protein able to sense viral dsRNA during viral replication. RIG-I consists of two N-terminal caspase recruitment domains (CARD) and a RNA helicase domain. After interaction with its ligand, this receptor induces the production of antiviral cytokines such as IFNs and thus modulates the anti-viral immune response (3,4,117,96).

PATHOGEN–ASSOCIATED MOLECULAR PATTERNS (PAMPS)

PAMPs are polysaccharides and polynucleotides in nature and they are shared by several groups of pathogens. These molecules are conserved at the molecular level within a class of pathogens. PAMPs include a variety of molecules recognized mainly by PRRs. The most characteristic PAMP molecules are: LPS, an endotoxin found in the Gram negative bacterial membranes, lipoteichoic acid from Gram positive bacteria, bacterial flagellin, peptidoglycan, ssRNA and dsRNA from viruses, unmethylated DNA (CpG motifs), mannose present on yeast surfaces, and β-glucans present on the fungal cell wall, etc. (3) (Table 2).

DAMAGE-ASSOCIATED MOLECULAR PATTERNS (DAMPS)

Besides recognition of microbial PAMPs, the immune system has the ability to sense other signals associated with infection or tissue damage, including host components released from infected, damaged, or necrotic cells, which, in turn, are able to activate and amplify the immune response. These components are called damage-associated molecular patterns (DAMPS) or alarmins. These inflammatory components liberated from damaged cells include nucleic acids, intracellular proteins, extracellular matrix components, oxidized lipids, crystals such as uric acids, silica, β-amyloid, and cholesterol (97,117). One of the differences between PMAPs and DAMPs is that the former stimulate the synthesis of pro-IL-1β, but not its secretion while the latter stimulates the assembly of inflammasome with subsequent activation of caspase-1. This, in turn, cleaves pro-IL-1β into IL-1β thus allowing its secretion (117). Sensing these endogenous ligands by the corresponding PRRs induce persistent inflammation, a phenomenon associated with the development of chronic inflammatory and autoimmune diseases (97).

INFLAMMATION AND INFLAMMASOME

INFLAMMATION

Inflammation is a nonspecific mechanism generated by the host in response to an infectious, physical, or chemical injury with recruitment of peripheral blood leukocytes and plasma proteins to the site of injury or tissue damage. In this process, there is an increase in both blood flow and vascular permeability, mainly in the vascular endothelial at the local level. Vascular permeability is a consequence of the endothelial cell retraction to allow the transmigration of leukocytes and the ingress of plasmatic proteins such as complement, coagulation factors, and antibodies, etc. (118).

After an injury, there is tissue damage with the release of components by epithelial or endothelial cells as well as by cells present in that tissue such as mast cells or ILCs. These substances include histamine, leukotrienes, extracellular matrix components, and pro-inflammatory cytokines and chemokines, all of which have the ability to induce chemotaxis and cell adhesion molecule (CAM) expression in both endothelium and leucocytes. These CAMs include selectins, integrins, immunoglobuline-like superfamily molecules and

cadherins. Expression of these CAMs allows interaction between leukocytes and endothelium and the subsequent leukocyte transmigration at the site of the injury. In the latter process, cells are guided by chemoattractant stimuli (Figure 3). The cell migration process is complex and depends on cell type as well as on the differentiation and activation state of the cells (118). As was mentioned, the first cells recruited at the site of the injury are neutrophils. They are also the most abundant during the first hours or days of the inflammation process followed by mononuclear cells. If the inflammatory reaction cannot be resolved, this process may become chronic with other implications for the host.

During the inflammation process, there is another important event known as phagocytosis. Phagocytosis is considered one of the most important processes during the innate immune response. Once phagocytes arrive at the infectious site, they ingest microbial pathogens in vacuoles called phagosomes. Here, after activation, these microorganisms are destroyed and then presented to lymphocytes via MHC. The microbicidal mechanisms included are, therefore, oxygen-dependent and -independent as described previously (5,119).

The phagocytic process is mediated by the cytoskeleton of the phagocytic cells as well as by endocytic and signaling receptors (96). These receptors, mainly PRRs present on cell surfaces, bind microbial PAMPs, and this interaction usually generates an intracellular signal which, in turn, allows the synthesis and release of proinflammatory cytokines and other effector molecules (3).

Proinflammatory cytokines play an important role during the inflammation process, and they participate in the interactions of the cells involved in not only the innate immune response but also the establishment of acquired immunity. Proinflammatory cytokines participate during the activation and effector phases of the innate immune response. These cytokines include TNF-α, IL-1, and type I IFNs. Nonetheless, other cytokines are also important during the establishment of the innate immune response (Table 3). Functions and characteristics of these cytokines are extensively described in Chapter 9.

INFLAMMASOME

Inflammasome is a complex of proteins consisting of caspase-1, ASC (a CARD-containing adaptor), and NLRs. Once these are activated, they cleave the pro-IL-1β and pro-IL-18 with subsequent maturation and secretion of these cytokines. Inflammasome activation is required for many inflammatory processes. In addition to the initial recognition of PAMPs or DAMPS by TLRs or CLRs, recognition by intracytoplasmic NLRs is necessary. Inflammasome may be also activated by ROS, lysosomal damage, and cytosolic K+ efflux at the intracellular level (110,117). Several members of the NLR family are involved in the assembly of inflammasome. These molecules include NLRP3, NLRP1, NLRP6, and IPAF (NLRC4). Moreover, the AIM2 protein, a non-NLR that is identified as a PYHIN (pyrin and HIN domain-containing protein) family member, is also involved in the inflammasome activation (117).

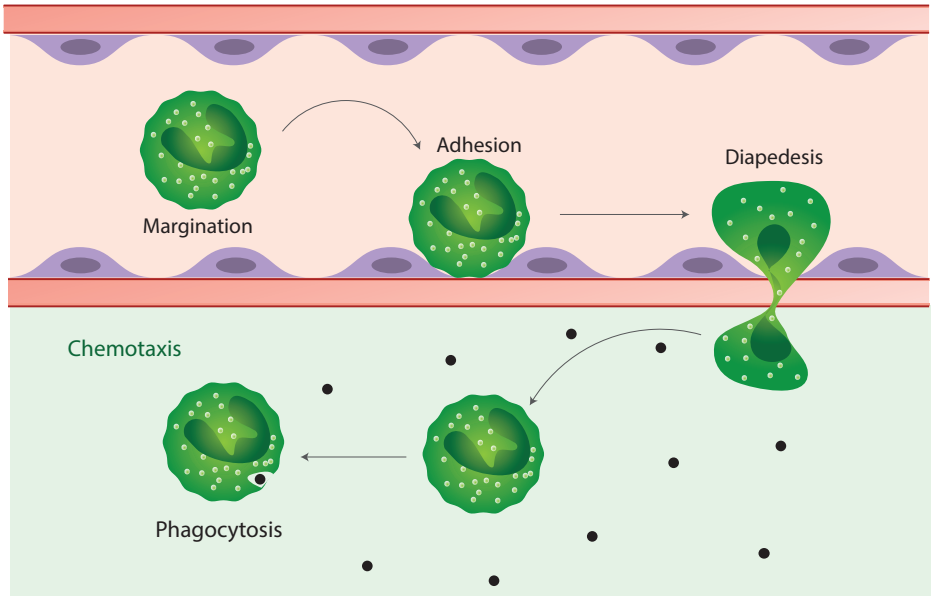

Figure 3. Recruiting phagocytes into the inflammation site and phagocytosis. Phagocyte recruitment involves several phases including: i) marginalization, which decrease leukocyte traffic with a subsequent endothelium approach; ii)adhesion, a process that depends on adhesion expression in both endothelium and phagocyte cells and thus facilitates adhesion of the leukocytes to the endothelium; iii) diapedesis, this process involves the extravasations or passage of leukocytes through the endothelium to the infected t issue or inflammation site; iv) chemotaxis, gradients of chemotactic stimuli (mainly chemokines and anaphylatoxins) allow leukocytes to reach the site of infection; and v) once at the site of infection, phagocytes can sense microbial pathogens or immune complexes through patter recognition receptors (PRRs) present on their own surface and start the phagocytosis process.

CYTOKINE	CELLULAR SOURCE	FUNCTION
TNF-α	Macrophages and T cells	Activates endothelial cells and neutrophils Induces fever and synthesis of acute phase proteins
Type I Interferons (IFN-α, IFN-β)	Macrophages and fibroblasts	Activate NK cells Induce expression of MHC-I
IL-1	Macrophages and endothelial cells	Activates endothelial cells and neutrophils Induces fever and synthesis of acute phase proteins
IL-6	Macrophages, endothelial and T cells	Induces synthesis of acute phase proteins Induces proliferation of B cells and antibody production
IL-10	Macrophages and Th2 cells	Induce proliferation of B cells Inhibits proinflammatory cytokine production and MHC-II expression
IL-12	Macrophages and dendritic cells	Activates NK and T cells Induces synthesis of IFN-γ Increases cytolytic activity Induces differentiation of T cells toward Th1 cells
IL-15	Macrophages	Induces NK and T cell proliferation
IL-18	Macrophages	Activates NK and T cells Induces IFN-g synthesis
Chemokines	Macrophages, endothelial and T cells	Induce chemotaxis and cell activation

Table 3. Cytokines of innate immunity. NK: natural killer; MHC: major histocompatibility complex.

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3 TOLL-LIKE RECEPTORS AND AUTOIMMUNITY

Gloria Vásquez
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INTRODUCTION

The immune response in vertebrates can be divided into innate immune response and adaptive immune response. The adaptive immune response is mediated by B and T lymphocyte clones, which are characterized by their specificity and memory. In contrast, the innate immune response is mediated by macrophages, dendritic cells and, in general, by all cells with the ability to present antigens. These are known as Antigen Presenting Cells - (APCs). At the same time, the innate immunity is characterized by its rapid response, which is very important in the early phase of an infection.

For several decades, the recognition of pathogens by macrophages was considered to be nonspecific as they did not possess receptors that underwent rearrangement. However, there is current evidence which indicates that the innate immune system can recognize non-self pathogens as well as structural alterations or the absence of self molecules.

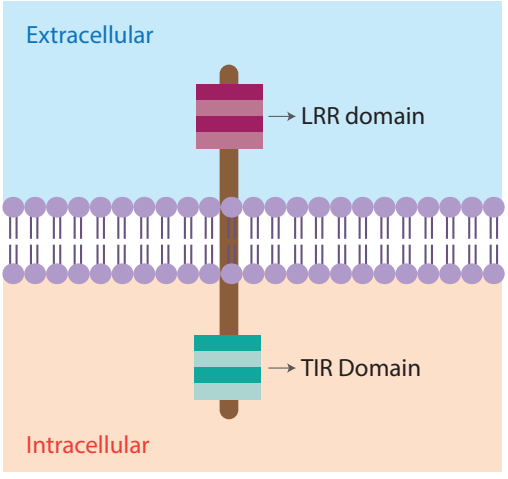

Figure 1. TLR Structure.

The innate immune system cells have pattern recognition receptors (PRRs) to carry out such recognition. These receptors recognize structural components known as pathogen-associated molecular patterns (PAMPs) in microorganisms (1).

Additionally, the innate immune system shares some of these PRRs for the recognition of cell debris with the immune cells and thus facilitates the clearance of products from the constant cell turnover. The molecules recognized by these PRRS are called Danger-Associated Molecular Patterns (DAMPS) and they include all endogenous ligands such as DNA, RNA, Heat Shock Proteins (HSP), etc.

There are several groups of PRRs expressed on the cell surface or residing within cell compartments (1,2). One of the most important PRR families is the Toll-like receptors (TLR) which were initially identified as essential molecules for the Drosophila embryonic development pattern and then, as key molecules in the antifungal immune response (3) (Figure 1).

TLR MOLECULAR STRUCTURE

It was thought that the mammalian TLR counter part was the family of IL-1 receptors. However, this hypothesis was not confirmed until the first member of the mammalian TLR family was found to have structural homology with the intra- and extra-cytoplasmic regions in the molecule previously described in Drosophila. The TLR family receptors belong to the type I membrane glycoproteins. These molecules have common molecular structures characterized by leucine-rich regions (LRR) that comprise 19-25 aminoacids in tandem in their extracellular portion and intracytoplasmic regions which are homologous to the IL-1 receptor. These domains are known as TIR (Toll receptors and Interleukin 1 domains) (TIR) due to these similarities (Figure 2). The crystal structure of TLR3 shows a horseshoe-shaped ligand binding core constituted by the LRR. The sequence homologies suggest that the other TLRs adopt a similar tertiary structure (4).

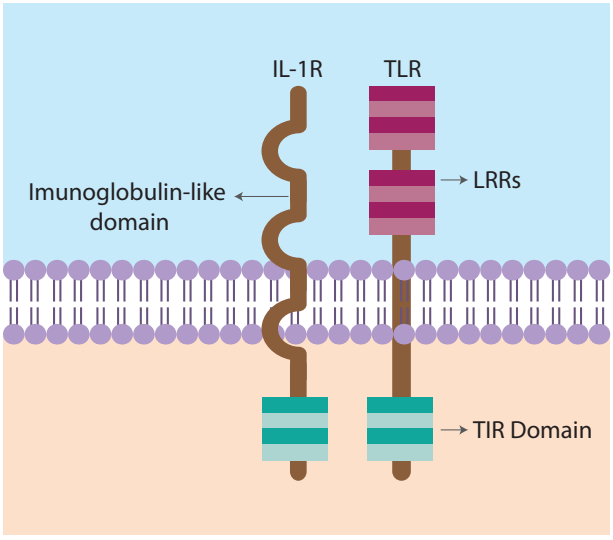

Figure 2. Structural homology between TLR and the IL-1 receptor (IL-1R).

Today, at least 13 TLRs have been described in mammals and nine of these have been shown to be functional receptors.(5-12) (Table 1).

The TLRs have differential expression not only at the cell level but also in the cell compartments. TLR1, 2, 4, 5, 6, and 11 are expressed primarily on the extracellular surface while TLR 3, 7, 8, and 9 are primarily expressed in the endosomal compartment (13). These receptors can be expressed in monocytes, phagocytic cells, dendritic cells, and B cell subsets. TLR8 is not functional in mice, and TLRs 11, 12, and 13 are expressed in mice but not in humans.

TLRs behave like PRRs and have the ability to bind different PAMPs and DAMPs. TLR4 recognizes lipopolysaccharides (LPS) (14); TLR2, lipoproteins and glycolipids (15); TLR3, double-stranded RNA; TLR7, chemical compounds such as imidazoquinolines; TLR 5, flagellin (16); TLR9, CpG sequences (8, 17); and TLR11 recognizes uropathogenic bacteria (12). The TLR 10, 12, and 13 ligands remain unidentified (13). (Figure 3)

There are also other potential TLR ligands. Cells subjected to stress or harm and undergoing apoptosis cannot generate inflammation, but they can induce signals generated

RECEPTOR	EXOGENOUS LIGAND	ENDOGENOUS LIGAND
TLR1	Triacyl lipopeptides	NI
TLR2	Lipoproteins and lipopeptides Peptidoglycans Lipoteichoic acid Lipoarabinomannan Phenol-soluble modulin Glycosyl phosphoinositide phospholipids Glycolipids Porins Atypical lipopolysaccharides Zymosan	HSP70
TLR3	Double-stranded RNA	NI
TLR4	Lipopolysaccharides	HSP 60
	Taxol	HSP 70
	Fusion proteins	Some portions of fibronectin
	Envelope proteins	Hyaluronic acid oligosaccharides Heparan sulfate fragments
TLR5	Flagellin	NI
TLR6	Diacyl lipopeptides	
	Lipoteichoic acid	NI
TLR7	Imidazoquinoline Loxoribine Bropirimine Double-stranded RNA	NI
TLR8	Imidazoquinoline	NI
	Double-stranded RNA	
TLR9	CpG DNA	DNA from apoptotic bodies
TLR10	NI	NI
TLR11	Uropathogenic bacteria	NI

Table 1. TLR receptors and their ligands. HSP: Heat shock protein; NI: not yet identified; TLR: Toll-like receptor.

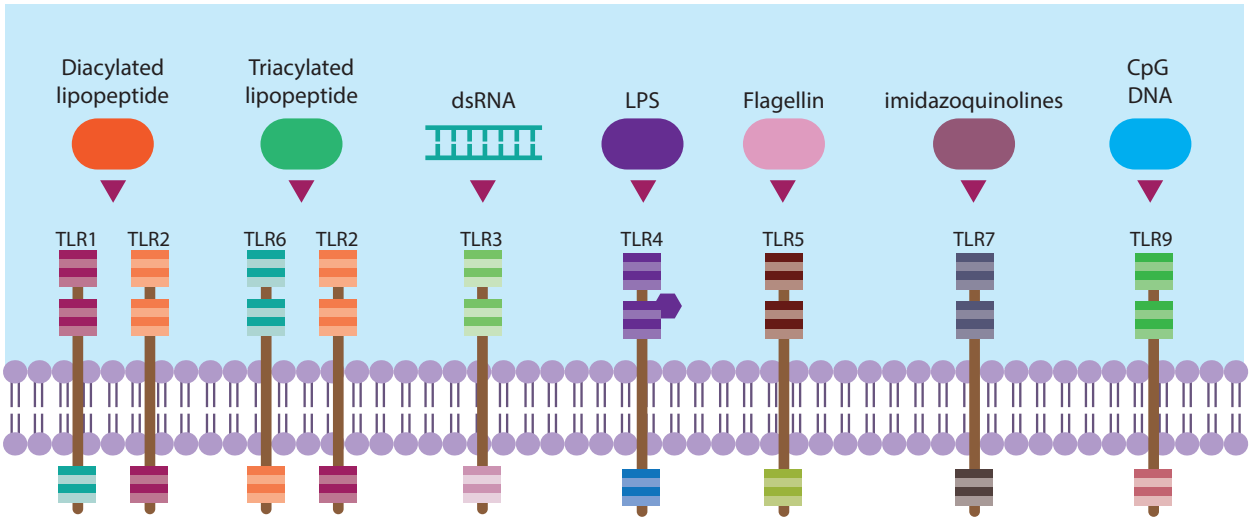

Figure 3. TLR proteins family and their ligands.

by the changes in lipids or carbohydrates found on the cell surface or by the expression of proteins which are not normally found on the external surface of the cell. One group of these molecules is heat shock proteins (HSP), which increase their levels considerably in stressful situations (18). The C3H/HeJ mouse strain, which has a mutation on the TLR4 gene, is resistant to the induction of macrophage activation by HSP-60. This suggests that human HSP-60 transmits signals through TLR4 (17). It has also been stated that fibronectin activates TLR4 and hydrogen peroxide activates NFkB and AP1 through TLR2 due to cell events that lead to the release, during cell death, of other possible endogenous TLR2 ligands.

TLR-MEDIATED RECOGNITION MECHANISMS

The interaction between TLRs and their ligands is low affinity. However, some molecules assist this interaction or recognition. For example, CD14 positions itself close to TLR4 on the cell surface and retains LPS for recognition by TLR4 in order to enable interaction. MD-2 is another associated protein which aids TLR4 interaction. TLRs tend to generate noncovalent dimers in the absence of ligands. TLR2 preferentially forms heterodimers with either TLR1 or TLR6, whereas the other TLRs appear to generate homodimers. Heterodimerization between TLR receptors is another tool which facilitates TLR recognition by their ligands (4,21).

The need for adaptor proteins and the formation of heterodimers that allow the TLRs to establish an adequate interaction with their ligands has suggested that TLRs should be considered signal integrators rather than receptors. The interaction between agonists and TLRs takes place in a focal area of active signaling, where multimeric protein complexes found in lipid rafts may interact. Several PRRs may be included in these complexes. As a result, it has been suggested that direct contact between TLR and their ligands is not necessary but rather fundamental to the stabilization of the interaction with these signaling complexes (22) .

TLR SIGNALING

After ligand recognition, TLRs activate the intracellular signaling pathways which lead to cell proliferation, the production of cytokines, and expression of co-stimulatory molecules. The intracytoplasmic domains of TLRs (TIR) are associated with adaptor molecules like TRIF (TICAM1), TRAM (TICAM2), TIRAP, and MyD88. With the exception of TLR3, all TLRs utilize MyD88 as an adaptor protein (13). This activates a series of signal transduction molecules including interleukin (IL)-1R-associated kinase (IRAK), tumor necrosis factor receptor (TNFR)-associated factor (TRAF-6), transforming growth factor (TGF)-β-activated kinase (TAKI), and the inhibitor of nuclear factor κB (IκB)-kinase complex. These events ultimately lead to activation of mitogen-activated protein (MAP) kinase and nuclear translocation of the transcription factor NF-κB. The TLR3 MyD88 independent pathway leads to activation of IFN regulatory factors (IRF), particularly IRF7, thus inducing high levels of type I IFN production (4).

Observations in MyD88-deficient mice demonstrated that a response is obtained even in the absence of MyD88. It is measured by the activation of NF-kB and MAPKs but with delayed kinetics. These findings have helped to clarify the fact that, so far, there are two TLR activation pathways – one is MyD88-dependent and the other one is independent (13,18). This latter pathway is dependent on the adaptor protein which contains TIR domains (TIRAP) or MyD88-adaptor-like (MAL) protein, which is associated with TLR4 and is involved in the transmission of MyD88-independent signals (Figure 4) (18,23,24).

TLR3 activation uses the MyD88-independent pathway. TLR3 has been shown to be associated with another TIR

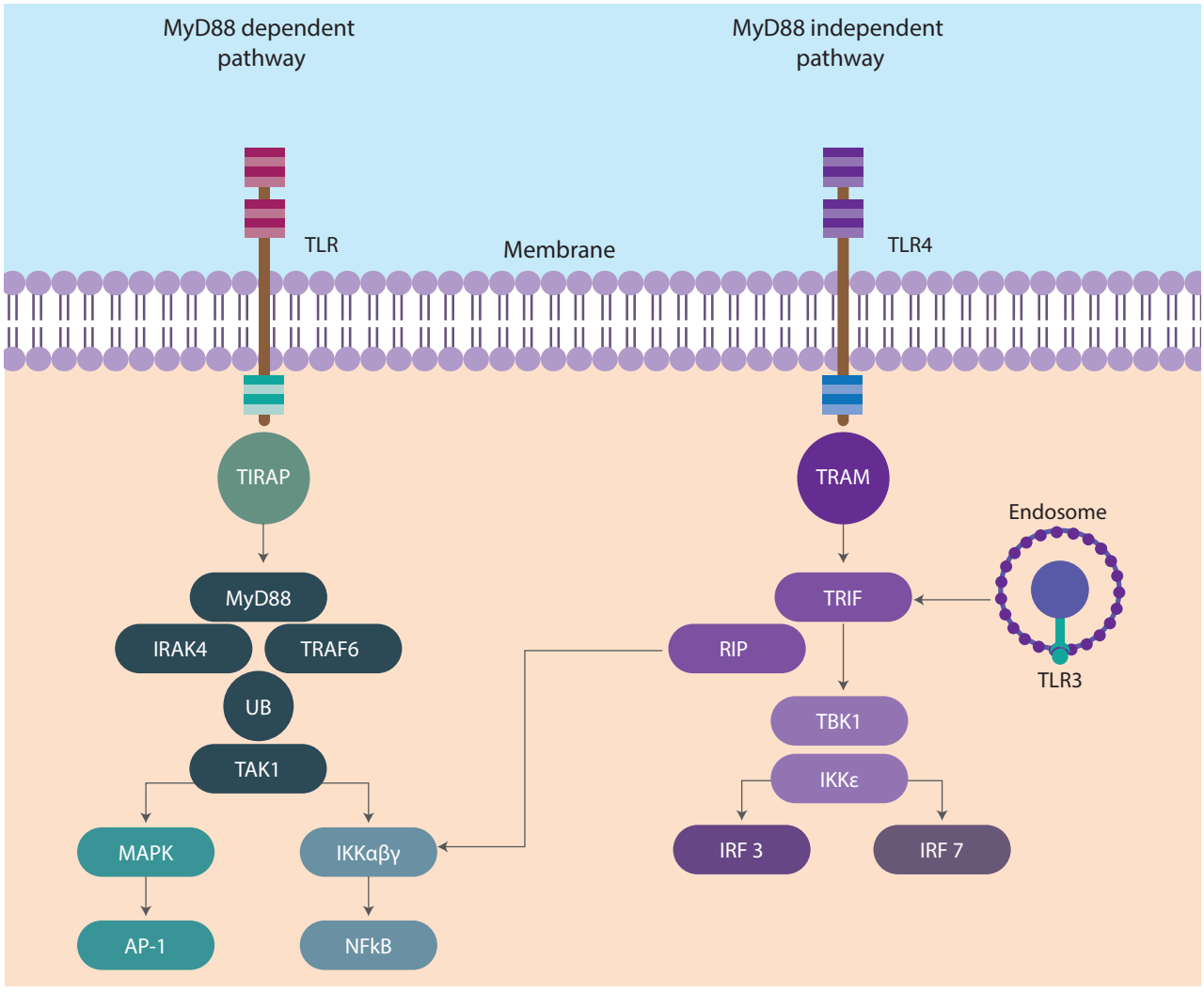

Figure 4. TLR activation pathways.

domain-containing adaptor protein called TRIF/TICAM-1. TRIF is a large protein consisting of 712 amino acids in humans. Over-expression of TRIF as well as MyD88 and TIRAP causes activation of the NF-κB dependent promoter. Furthermore, over-expression of TRIF, but not MyD88 or TIRAP, induces activation of the IFN-β promoter. Dominant negative TRIF inhibits the TLR3 ligand-induced activation of the IFN-β promoter, and RNAi-mediated knockdown of TRIF causes impairment in the TLR3 ligand-induced IFN-β expression. Thus, these in vitro studies indicate that TRIF is involved in the TLR3-mediated MyD88-independent pathway. These adaptors propagate signaling by recruiting kinases which mediate activation of the transcription factors AP-1, NF-κB, IRF3, and IRF7 which are all required for IFN-α/β production (25). The membrane and endosome receptors use the same pathways as MyD88 and TIRAP adaptor proteins. Meanwhile, TLR3, which is located in the endosome, signals using TRIF.

TLR signals differ among family members. For example, the TLR9 signal induces effects similar to those transmitted by TLR4 such as the positive regulation of the expression of co-stimulatory molecules and cytokines. However, unlike TLR4, all effects induced by TLR9, as far as is known to date, are MyD88-dependent (26). These results indicate that TLR4 and TLR9 can activate different signaling mechanisms that lead to similar biological effects. The fact that TIRAP/MAL has been found associated with TLR4, but not with TLR9, may explain the inability of TLR9 to induce MyD88-independent signals (24).

TLRS AND AUTOIMMUNITY

Autoimmunity is strongly inheritable but also dependent upon a variety of stochastic environmental factors. However, the disease expression depends on the integrity of the immune system and many of its components are determinants. The growing understanding of innate immune signaling pathways has provided new paradigms to explain autoimmunity, particularly the systemic autoimmunity associated with lupus (13,27).

The knowledge that endogenous materials, particularly self-nucleic acids and related immune complex, can stimulate the innate immune system by TLR-dependent pathways has added a new dimension to the way self-tolerance is bypassed.

TLRs stimulated by self-constituents are TLRs 3, 7, 8, and 9, and all of them are endosomal TLRs. In these compartments, microbial, but not mammalian, nucleic acids are concentrated by phagocytosis. Moreover, modifications and degradation of DNA and RNA are barriers for the interaction between TLRs and self-molecules.

These barriers are not infallible and can be breached which results in direct or indirect stimulation of autoreactive T and B cells. The first way to break this barrier involves human FcγRIIa (CD32) that mediates Plasmocytoid Dendritic Cells uptake of apoptotic/necrotic materials with chromatin fragments, SnRNPs or the B cells immune complexes. These materials are transported into the endosomal compartments where DNA interacts with TLR9 (28) and RNA, with TLR 7/8. The simultaneous engagement of a BCR and a TLR results in efficient B cell activation (13). (Figure 5)

THE TLR9 AND THE CPG LIGAND

CPGS

A strain of *Mycobacterium bovis*, Bacillus Calmette-Guerin (BCG), is widely known to be an adjuvant in the induction of cell immunity. In the mid-1980's, BCG-derived DNA was found to contribute to immunostimulatory activity. It was also demonstrated that this DNA inhibited tumor growth, increased NK cell activity, and induced the production of interferons by lymphocytes (29). By using synthetic oligonucleotides, the non-methylated cytosine and guanine (CpG) motifs were found to be responsible for this immunological activity. It was also observed that these motifs were capable of activating B cells. CpG motifs are scarce in vertebrates and most of these are methylated which makes them lose their immunostimulatory activity. Hence, the DNA containing these non-methylated CpG motifs is considered a PAMP.

CpG sequences with immunostimulatory activity vary from one species to another; therefore, the CpG immunostimulatory sequences in mice differ from those in humans. The optimal sequence in mice consists of two purines in the

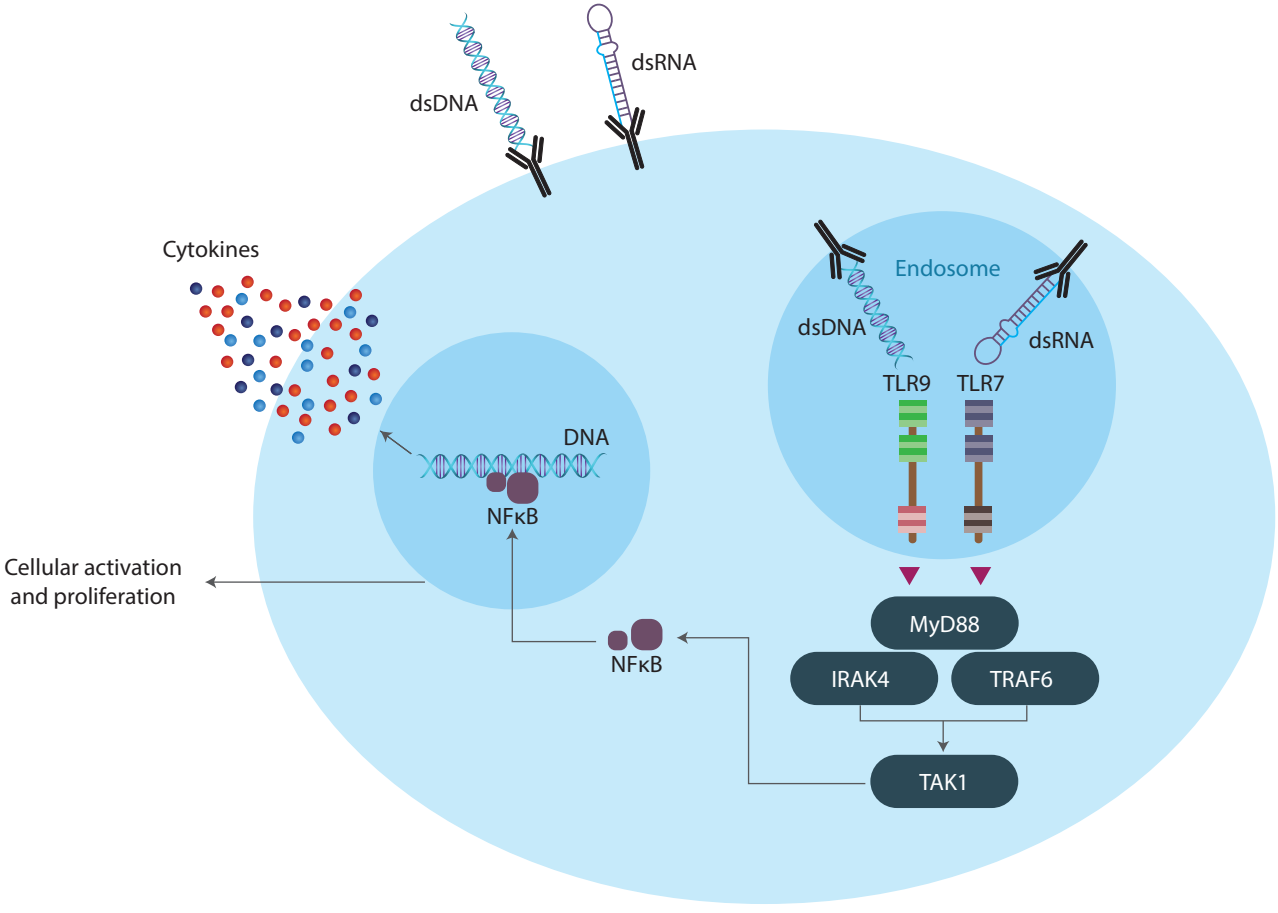

Figure 5. Model of TLRs pathways involved in SLE pathogenesis.

5′ direction and two pyrimidines in the 3′ direction. In contrast, the optimal sequence in humans and in certain other species is TCGTT and/or TCGTA (30) .

The exposure of various APCs to CpG sequences generally induces greater antigen presentation ability. For example, in dendritic cells, sequences with CpG-oligodeoxynucleotides (ODN) induce maturation and a transient increase in antigen processing and in the average lifespan of MHC class II-peptide complexes with the resulting sustained antigen presentation. The presentation mechanisms that require newly-synthesized MHC class II molecules are lost, but the antigen presentation function persists since the mature dendritic cell recycles its class II molecules (30). In human primary dendritic cells isolated from peripheral blood, induction of survival and maturation (measured as CD83, MHC class II, CD40, CD54, and CD86 expression) was higher with CpG stimulation than with stimulation by the granulocyte-monocyte colony stimulating factor (GM-CSF). Compared to stimulation by GM-CSF, dendritic cells treated with CpG showed an increase in functional activity in the mixed lymphocyte culture and induced T cells to increase the secretion of Th1 cytokines. These findings demonstrate the ability of CpG sequences to activate dendritic cells and encourage a Th1-type response (31). In an experimental murine model, CpG DNA injection led to the development of a local lymphadenopathy characterized by the maintenance of cell composition with predominantly dendritic cells. It was also observed that these cells and T cells presented a sustained local production of IL-12 and IFNγ. CpG injection created a local predisposition to an intense cell lymphotoxicity (CLT) response – a Th1-type response. CpG ODNs increased the expression of co-stimulatory molecules (CD40, CD80, CD86, CD54) and the expression of MHC class I and II molecules (32) in tumoral B cells but not in controls (non-CpG ODN).

In addition, macrophages treated with CpG ODN and evaluated by flow cytometry demonstrated a decrease in the surface expression of MHC class II. Analysis with Northern-Blot also showed that treatment with CpG ODN diminished mRNA for I-Ak. At no point in time was an increase in antigen processing by the macrophage observed after treatment with CpG ODN, a result which contrasts with the dendritic cell studies. Therefore, macrophage exposure to CpG results in a decrease in antigen processing and presentation which is largely due to a reduction in the synthesis of MHC class II (33).

A model in which CpG DNA activates cells differentially and does not induce positive regulation of antigen presentation by macrophages but does up-regulate other antigen-presenting cells such as B cells and dendritic cells could explain these results.

TLR9

TLR9 is recognized as the specific receptor of CpG sequences (34). Assessment of TLR9-defficient mice has shown that they are unable to respond to CpG because their macrophages do not induce cytokine production, their B lymphocytes do not proliferate, and their dendritic cell maturation is not induced, which demonstrates the essential function of TLR9 in the response induced by CpG sequences (8).

TLR9 activation by oligonucleotides containing CpG motifs requires the internalization of CpGs into endosomes. At the same time, the internalization process requires maturation and acidification of the endosome in order to enable the TLR9 signals to start. This, in turn, suggests the specificity of the interaction between TLR9 and its ligand and establishes TLR9 expression as endosomal (28,35).

Not all CpG sequences induce similar activation patterns in different cell types. This may be due to the formation of heterodimers with other TLRs or due to interaction with other PRRs (36).

TLR9 expression differs from one species to another. In mice, immune system cells of myeloid lineage (including monocytes, macrophages, and dendritic cells) express TLR9 and respond to stimulation. Existing evidence in humans is controversial. Studies evaluating TLR9 expression by RT-PCR showed TLR9 mRNA in all cell groups that had lymphoid and myeloid lineage, but a differential expression was also seen between cell groups (37). We have observed TLR9 expression in monocytes (CD14+), B-lymphocytes (CD19+), and myeloid dendritic cells (BDCA3+). These findings, though similar to previous studies, differ in the observation of TLR9 expression in myeloid dendritic cells such as BDCA3+ (38).

TLR9, CPG INTERACTION AND AUTOIMMUNITY

The first evidence with respect to the role of TLR in autoimmunity was obtained in a collagen-induced arthritis (CIA) murine model in which the CpG exposure generates a worse arthritis evolution and an increase in IFNγ-induced TH1 response (39). Moreover, evidence presented by Lovgren showed that apoptotic cells obtained by freezing and thawing or UV-light exposure induced IFNα production. But the production was conditional to the presence of lupus patient serum (40).

Using the AM14 cell line derived from transgenic mice for the B cell receptor with rheumatoid factor (RF) specificity, a greater activation of these cells was shown in the presence of antibodies directed against the nucleosome (IgG2a a/j antinucleosome). As mentioned earlier, DNA hypomethylated sequences are common in bacterial DNA. Hypomethylated CpG sequences are also present in mammalian DNA in low quantities. Since the chromatin bound to antibodies is part of the complex that stimulates AM14 cells, TLR9 could be a good candidate for a second receptor. An unique characteristic of the TLR9 signal is its dependence on processing via endolysosoma, which is reflected by the sensitivity to the TLR9 signal to chloroquine and other endosomal acidification inhibitors (41).

The activation of AM14 cells (RF+B cell) by the immune complexes containing anti-chromatin antibodies was blocked by chloroquine which indicated that TLR9 is the TLR responsible for amplifying the B cell receptor signal in this

cell line (41,42). Stimulation of both CpG and anti-chromatin immune complexes can be explained by multiple and sequential coupling (Figure 5).

The stimulation of lupus patients' B cells by CpG induces increased expression of CD86 and the production of IL-10 and IL-6 but only in patients with SLEDAI <6. The cells of another group of patients (SLEDAI >6) did not respond to the CpGs. These findings suggested that the TLR ligands-induced pathways can be exhausted by the previous abundance of CpG ligands (38).

TLR7 AND ITS LIGAND

Christensen et al reported that, in TLR9-/- mice, the antinuclear antibodies changed from a homogeneous to a speckled pattern, and they were associated with high levels of anti-Sm antibodies. The TLR7 KO mice lost the speckled pattern. The glomerular compromise was also evaluated in these mice. The TLR9 KO mice had reduced survival and a worse evolution in contrast to TLR7 KO mice in which the evolution of renal disease was mild (43.

These findings have suggested a role for both TLRs in different forms of SLE. TLR9 is associated with anti-DNA antibodies and TLR7, with anti-RNP (43,44) (Figure 5).

More evidence highlighted the role of TLR7. The Yaa gene determines TLR7 expression and is associated with the presence of autoantibodies. The duplication of this gene induces the presence of multiple antibodies and renal comprise resembling SLE (45).

UNC93B1

UNC93B1 is a protein that is involved in the movement of TLR7 and TLR9 from the endoplasmic reticulum to the endoplasmic vesicles. A murine model KO for UNC93B1 showed a reduction in levels of autoantibodies. These findings emphasize the importance of the TLR pathways in autoimmunity induction(46,47).

TLR AND RHEUMATOID ARTHRITIS

Numerous studies have been done using PAMPs to induce arthritis in animal models. Microbe-derived TLR ligands such as peptidoglycan for TLR2, dsRNA for TLR3, LPS for TLR4, and CpG for TLR9 have frequently been used to provoke or accelerate experimental arthritis. A study was done to investigate the involvement of TLR-2 and TLR-4 in the development of chronic destructive streptococcal cell wall (SCW)-induced arthritis. Chronic SCW arthritis was induced by 4 repeated intra-articular injections of SCW fragments in wild-type, TLR-2 (-/-), and TLR-4 (-/-) mice. The clinical, histopathological, and immunological parameters for arthritis were evaluated. During the acute phase, the swelling was dependent on TLR2, but in the chronic phase, this dependence shifted to TLR-4. Persistent joint inflammation in the latter phase of the model was significantly suppressed in TLR-4 (-/-) mice. TLR-4 (-/-) mice expressed less IL-1beta, tumor necrosis factor alpha, IL-6, and IL-23 that are implicated in IL-17 production. Accordingly, SCW-specific IL-17 production was found to be dependent on TLR-4 activation, since T cells from arthritic TLR-4 (-/-) mice produced markedly less IL-17 upon SCW stimulation, whereas interferon-gamma production remained unaffected. These data indicate the involvement of TLR-4 in the chronicity and erosive character of arthritis coincident with the antigen-specific IL-17 response (48,49).

To clarify the role of TLRs in rheumatoid arthritis, synovial fibroblasts (SF) were stimulated with interleukin-1β and tumor necrosis factor-α (cytokines present in RA synovium) to demonstrate how these cytokines influenced in the TLR gene expression. TLR2 expression in RA SFs was increased after treatment with interleukin-1β, tumor necrosis factor-α, lipopolysaccharide, and synthetic lipopeptide. Synovial tissues from RA joints expressed TLR2 predominantly at sites of attachment and invasion into cartilage and bone. The authors conclude that TLR-associated signaling pathways may contribute to the pathogenesis of RA either by initiating or perpetuating activation of SFs (50). Furthermore, stimulation of cultured synovial fibroblasts by the bacterial peptidoglycan (which is well known as a TLR-2 ligand) demonstrated 74 genes that were up-regulated >2.5-fold. Fourteen CC and CXC chemokine genes were among the genes with the highest up-regulation. Chemokine secretion induced by stimulation of rheumatoid arthritis synovial fibroblasts via TLR-2 was functionally relevant as demonstrated by chemotaxis assays. GCP-2 and MCP-2 expression, which has not been reported previously in rheumatoid arthritis, was demonstrated in synovial tissue sections of patients diagnosed with rheumatoid arthritis but not in those with osteoarthritis. Correspondingly, synovial fluid levels were significantly higher in patients diagnosed with rheumatoid arthritis as compared to patients with osteoarthritis (51).

The expression of TLR 3 and 7 in synovium was also evaluated. TLR-3 and TLR-7 were highly expressed in RA synovium. TLR-2- and TLR-4-mediated stimulation of DCs from RA patients resulted in markedly higher production of inflammatory mediators (TNF-α and IL-6) than stimulation of DCs from healthy controls did. In contrast, upon stimulation of TLR-3 and TLR-7/8, the level of cytokine production between DCs from RA patients and those from healthy controls was equal. Remarkably, both TLR-3 and TLR-7/8 stimulation resulted in a skewed balance toward IL-12. The combined stimulation of TLR-4 and TLR-3-7/8 resulted in a marked synergy with respect to the production of inflammatory mediators.(51) Most of the evidence showed the importance of TLR4 and TLR-4 ligands in the inflammatory response and the development of chronic lesion present in rheumatoid arthritis.

TLR PATHWAY AS A THERAPEUTIC TARGET

Vaccine adjuvants are perhaps the most extensively explored applications for TLR agonists. TLR agonists also feature prominently in efforts to develop therapeutic vaccines against cancer and chronic viral disease (4,52).

A specific inhibitor of TLR7 and TLR9 known as immunoregulatory sequence 954 (IRS 954) inhibits the induction of IFN-alpha by human plasmacytoid dendritic cells in response to DNA and RNA viruses and isolated immune complexes from lupus patients. This inhibitor can prevent progression of disease when injected into lupus prone (NZBxNZW) F1 mice. Following treatment, a significant reduction of serum levels in nucleic acid-specific autoantibodies as well as decreased proteinuria, reduced glomerulonephritis, end-organ damage, and increased survival were described (52,53)

Myeloid differentiation factor 88 (MyD88) is an endogenous adaptor protein which coordinates the inflammatory response to agonists of the Toll-like receptor and interleukin-1 receptor families. The MyD88 pharmacological inhibitor (ST2825 25 mg/kg) is a synthetic peptido-mimetic compound (ST2825) modeled after the structure of a heptapeptide in the BB-loop of the MyD88-TIR domain. This domain, which interferes with MyD88 signaling, has been evaluated for different TLR pathways (54). In vitro, TLR9 triggers memory B cell differentiation into antibody-producing cells, and causes the MyD88-inhibitor - ST2825 - to block TLR9-induced plasma cell (PC) generation. Peripheral blood mononuclear cells from 10 SLE patients in clinical remission and 2 with active SLE were cultured in the presence of CpG with or without ST2825. CpG-induced TLR9 stimulation caused autoantibody secretion in patients with active disease and in the majority of patients in clinical remission. Inhibition of MyD88 completely blocked the de novo generation of PCs and the secretion of auto-antibodies. Targeting the TLR/MyD88 pathway may be a novel therapeutic tool for treating acute disease and preventing relapses in SLE patients (55,56).

CPG SEQUENCES AS ADJUVANT AND AUTOIMMUNITY

ODNs contain CpG sequences which are similar to those found in bacterial DNA and have similar immunostimulatory actions (37,57). These ODNs have various potential therapeutic uses. The objective is to induce an appropriate innate immune response, hence protecting the host from infectious diseases, acting as an adjuvant, and improving the function of APCs to encourage a specific adaptive immune response induction. In addition, it can contribute to allergy prevention by inducing a Th1- response rather than Th2. CpG ODNs are also being evaluated in cancer immunotherapy for hypernephroma, skin tumors, breast cancer, uterine cancer, and immune system cancer (58).

There are some concerns surrounding safety in the clinical use of CpG ODNs. Among these concerns is the possibility that ODNs may increase immunogenicity to self-proteins and, possibly launch the development of organ-specific or systemic autoimmune disease.

The ability of CpGs to induce autoimmune diseases is based on murine studies where it can be observed that high doses of bacterial DNA induce the production of antibodies against double-stranded DNA and thereby accelerate the production of autoantibodies in lupus-prone mice. Furthermore, bacterial DNA stimulates IL-6 production and blocks apoptosis in activated lymphocytes. This predisposes to the development of autoimmune autoimmune diseases by fomenting of autoreactive lymphocyte persistence.

In order to clarify the risk of CpGs inducing autoimmunity, several in vivo experiments were done by injecting mice with repeated, immunostimulatory doses of CpG DNA. Although the number of B cells producing anti-DNA antibodies increased 2-3 times and the anti-DNA antibody titer grew 60%, the magnitude of the effect was insufficient to induce the deterioration of systemic autoimmunity (58).

In the case of organ-specific autoimmunity, the situation becomes more complex as a Th1 response, which is typically induced by CpG DNA, is observed in these entities. In a murine model of IL-12-dependent experimental allergic encephalomyelitis (similar to multiple sclerosis), animals treated with CpG DNA, and later challenged with an antigen, developed effector Th1 cells which caused the disease. However, the mice which were only challenged with the antigen (without CpG sequences) did not develop the disease.

In a “molecular similarity” model, it was demonstrated that the co-administration of antigens derived from Chlamydia and the DNA CpG can induce autoimmune myocarditis. Likewise, it was shown that the presence of CpG increases the susceptibility to arthritis in mice. This evidence suggests that the CpG sequences could favor the development of autoimmunity under certain circumstances (58).

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4 THE COMPLEMENT SYSTEM

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INTRODUCTION

The complement system (CS) is one of the first lines of innate immune defense and plays an important role in the homeostasis of adaptive immunity response. In humans, it was identified as the heat-labile component of serum that assists, or *complements*, the action of antibodies which are in charge of killing bacteria. In addition, the CS comprises more than 60 plasma and surface proteins. These are covered by nine central components of the cascade (C1 to C9), multiple activation products (such as C3a and C3b), regulators and inhibitors (e.g. Factor H and C4BP), proteases and newly assembled enzymes (e.g. C4b2a and Factor B), or effector molecule receptors (such as C3aR and C5aR) (Tables 1-3). However, this system works with inactive components that are activated sequentially as a cascade. Thus, the inactive zymogens together with additional components become effector compounds or active enzymes when they encounter a biological surface with the ability to activate the complement system. All of this process will actives new substrates (1,2).

The complement proteins in plasma are more than 3 gr. per liter, which means that they constitute 5% of total plasmatic proteins (10% of the total proteins) and more than 15% of the globular fraction of plasma. Moreover, the nomenclature of complement proteins is given by their historical discovery. The fragments generated during the activation process are designated by a letter. Generally, if the fragment is large, it is labeled "b" and if it is small, it is labeled "a". However, C2 is an exception: in this case C2a is the biggest one (1). The components of the CS are involved in immune surveillance and in the maintenance of homeostasis in physiological and in stress conditions (3). Thus alterations in complement proteins may trigger diseases states (Figure 1). These proteins interact with non-immune cells (epithelial cells, osteoclast, etc) (4), innate immune cells (macrophages, dendritic cells (DCs), neutrophils, mast cells, eosinophils and basophils) (5) and adaptive immune cells (B and T cells) (6).

The majority of complement proteins are synthesized in the liver, but other cells can also produce them. The main source of C1q is DCs, macrophages, and epithelial cells from the spleen, bowel, thymus, lung and heart. It can also be produced by osteoclasts (7). Specifically, the kidney cells produce C3 and C4, spleen cells C6 and C8, fibroblasts C2, C3, C5 and C9, adipocyte factor D, and pneumocyte C3 and C9. In addition, immune cells such as polymorphonuclear (PMN) can also produce complement factors like C7, C3 and C6 while DCs produce C7, C8 and C4BP regulatory proteins (8). Another protein such as properdin is synthesized by neutrophils, monocytes/macrophages and T cells (9).

The CS has multiple functions. It triggers an immune response to foreign pathogens (1), has pro-inflammatory activity (10), regulates cytokine production (11), helps to remove immune complex and dead or modified cells (following injury, hypoxia, after virus-infection or tumor-caused modification) (12). CS can also regulate tolerance to self-antigens (13), and it plays an important role in immunoregulation of adaptive immunity (14). Other physiological functions attributed to CS are angiogenesis, mobilization of hematopoietic stem/progenitor cells, lipid metabolism, coagulation pathway, calcium metabolism, organ regeneration and neuroprotection (including in migration of neurons and synapse elimination) (2,3).Because of their many functions and the implications in the pathogenesis of several diseases, complement proteins are used as biomarkers of disease (15,16). Recently, complement proteins within tissues have been observed in the murine model through radiologic imaging (17).

THE COMPLEMENT CASCADE

Complement activation can be done through three pathways: classical, lectin and alternative (Figure 2).This mechanism is

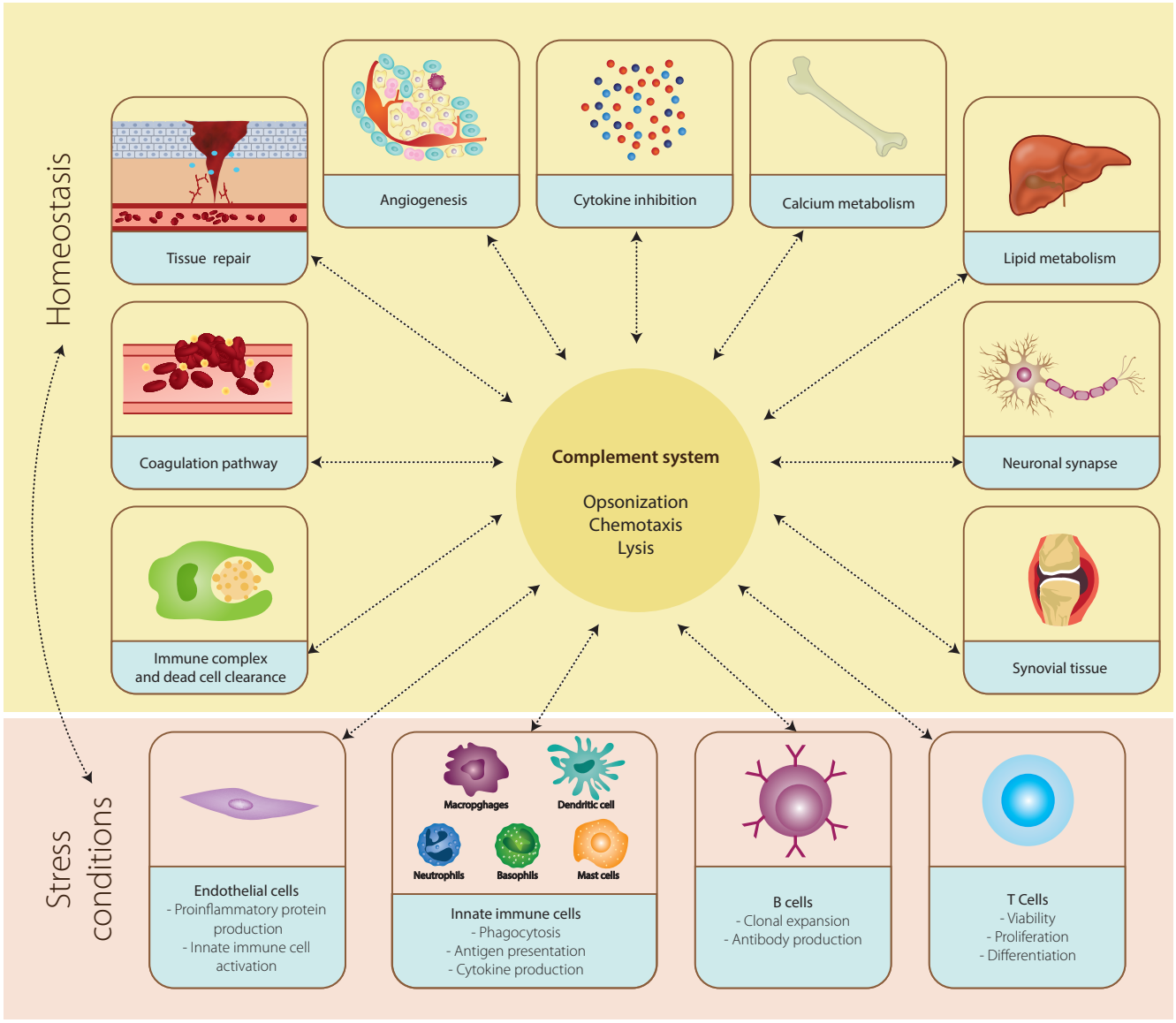

Figure 1. Role of complement in the homeostasis and in conditions of stress. Adapted from reference (3).

dependent on the external factors. The activation occurs sequentially manner and can be divided into four main steps: 1. initiation, 2. C3 convertase activation and amplification, 3. C5 convertase activation, and 4. terminal pathway activity or membrane attack complex (MAC) assembly. Moreover, the progression of the cascade and the action of the effector molecules are strictly controlled at each level by multiple regulators and inhibitors proteins which are present on normal host tissues (2). The cascade can also be activated by other means such as coagulation system components (18).

The classical pathway (CP) is activated by antibodies once those bind to antigens. When this complex is formed the fraction fields (Fc) of IgM, IgG3 and IgG1 interact with the collagen-like tail of C1q. Some pathogens, polyanonic molecules, C reactive protein (pentraxin), DNA, RNA and apoptotic bodies can also activate the CP (19,20).

C1 complex is made up of three proteins (C1q, C1r, and C1s). Likewise, when C1 complex is activated at least two of its six sites in the globular domains should bind to the Ig linked to the pathogen. After this binding, C1q goes through conformational changes that lead to the activation of C1r and cleavage of C1s. At this point, there is also cleavage of C4 and C2 by C1s. The C4b fragment produced by cleavage binds to the cell membrane of the pathogen and allows the binding of C2a. After that C1s cut C2 into C2a and C2b thus forming the C4b2a complex (classical C3 convertase). C4b2a cuts C3 to make C3a and C3b. This step is common to all three complement pathways and is the one all of them have to take to activate complement cascades (21). C4b and C3b bind to the target cell surface through a covalent binding reaction (22). In addition, the C reactive protein recognizes PAMP (Pathogen-associated

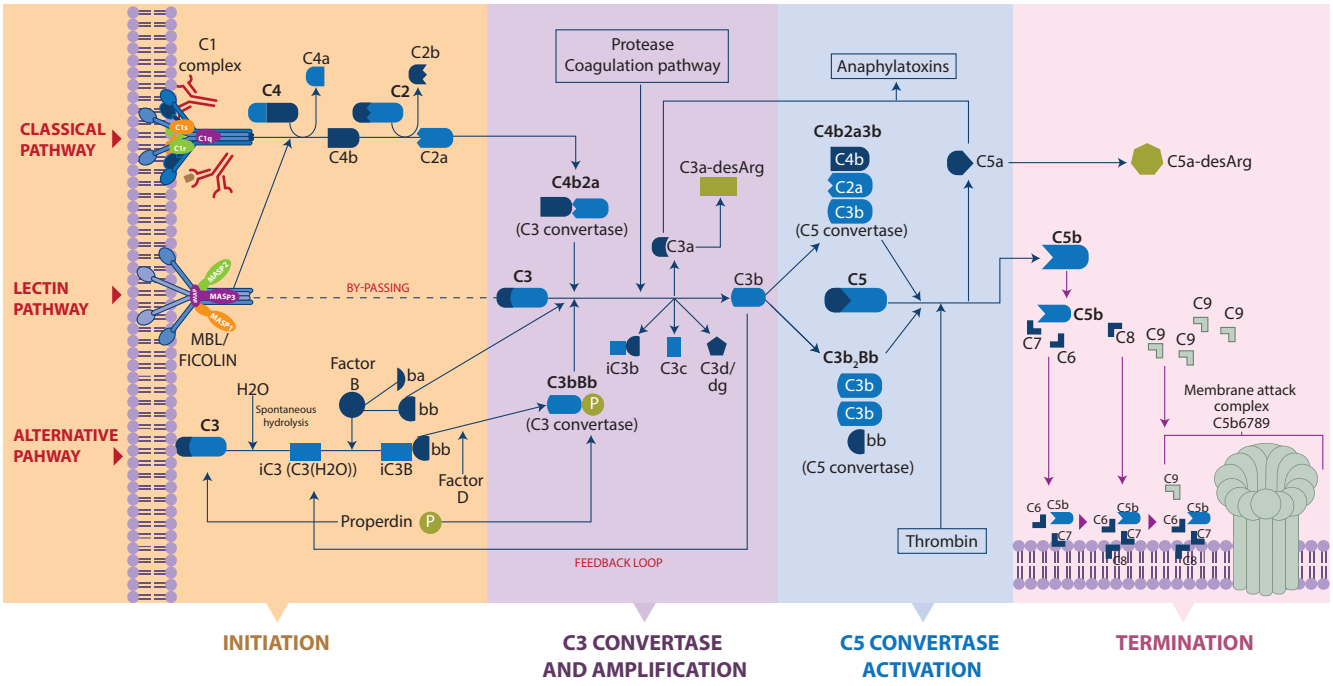

Figure 2. The complement cascade.

molecular patterns), DAMP (Damage- associated molecular patterns) on apoptotic and microbial cells and binds to C1q to activate CP. Factor H-related protein 4 (CFHR-4) recruits monomeric C reactive protein from necrotic cells to facilitate activation of CP by C1q (3).

The lectin pathway (LP), like the CP, used the same C4b2a convertase and differed in its recognition and activating factors. Moreover, this pathway does not require antigen-antibody complex. The LP is activated by the binding of C-type lectin and MBL (mannose-binding lectin), or proteins termed ficolins (L-ficolin, H-ficolin and M-ficolin) to some carbohydrates that are rich in mannose and beta glycans respectively. MBL and ficolins may form a complex with MBL-associated serine proteases (MASPs1, 2, 3), and with a smaller molecule called MAP19. MBL and ficolins are structurally similar to C1q, but unlike the latter, MBL is a well-characterized receptor of the collectin family. It was given this particular name because it has a collagenous domain with a calcium-dependant lectin domain. MBL is synthesized in the liver and secreted into the plasma as a component of the acute-phase response. Therefore, MBL can bind to common carbohydrate PAMPs from bacteria, viruses, fungi and parasites. Once it is activated, MBL-MASP complex leads to C4 cleavage by MASP2 and C2 cleavage by MASP1 and MASP2, which results in C3 convertase (14-23). A new mechanism in vitro called "by-passing" has been described and this occurs when target-bound MBL directly actives C3 in the absence of C4 and C2 (24).

While the CP and LP are activated exclusively by exogenous material, the alternative pathway (AP) is also constantly active at low levels in the host, an phenomenon called "C3 tickover" (25). In this process C3 suffers structural changes by spontaneous hydrolysis of the thioester bond, thus resulting in iC3 or C3 (H2O)). At this point, the presence of factor H is fundamental because it inhibits the progression of AP. However, if the iC3 does not bind to the surface of target cells, it remains in the fluid phase and could easily be inactivated. Once the iC3 binds to factor B fraction Bb, it forms iC3B, which is cleaved by factor D to make C3bBb complex (C3 convertase of AP). At the same time, this complex is stabilized by properdin (Factor P) (26), a protein which is released by active neutrophils, macrophages and T cells. This protein stabilizes the C3 convertase and can also initiate the activation of AP by the recognition of PAMPs, DAMPs or apoptotic cells (27).

C3 is a key mediator in the function of all three pathways activation. It circulates as a two-chain molecule (α and β) which is held together by multiple inter-and intra-chain disulphide bonds (20). Once C3 is cleaved by a C3 convertase, it is divided into C3b and C3a. The latter fragment plays a role in the innate immunity cells and has antifungal and antimicrobial activity. C3b, in turn, coats the microbial or apoptotic cell body surface and the cascade can progress. If the cascade progress, C3b binds to the previous C3 convertase and results in C5 convertase (C4b2a3b from CP and LP, and C3b2Bb from the AP). During the cleavage of C3b, iC3b, C3c, C3d/dg are produced and they have several functions (Table 1). Furthermore, C5 convertase cleaves C5 into C5a and C5b. C3a and C5a are anaphylactic peptides (activate neighboring cells to release inflammatory mediators). C5b, turn, starts the terminal pathway (2). In the AP, the Bb portion can also cleave C3 and thus generate C3b and C3a. C3b can bind random-

COMPONENT	ALTERNATIVE NAME	CHROMOSOME	MW (kDa)	FUNCTION AND COMMENTS
Components which active a cascade				
C1q		1p36.12	410	Part of the C1q complex, recognizes surface-bound IgG, IgM, other molecules which initiate activation of CP
MBL	Man-nose-binding lectin	10q11.2	26	Recognizes carbohydrate patterns and initiates the LP
Ficlon-1	M-Ficolin	9q34	35	Recognizes carbohydrate patterns and initiates the LP
Ficolin-2	L-Ficolin	9q34.3	34	Recognizes carbohydrate patterns and initiates the LP
Ficolin-3	H-Ficolin	1p36.11	33	Recognizes carbohydrate patterns and initiates the LP
Properdin	Factor P; CFP	Xp11.4	51	Recognizes PAMP, DAMP or apoptotic bodies which initiate the activation of AP. Also stabilizes C3 convertase
Components which continue the cascade				
C1r		12p13	80	Part of C1 complex, cleaves C1s
C1s		12p13	77	Part of C1 complex, cleaves C2 and C4
MASP1 (MBL-associated serin protease 1)		3q27	79	Binds to MBL/Ficolins. Cleaves C2, may cleave MASP-3 and C3. Apparently involved in coagulation cascade, cleaved fibrinogen, and Factor XIII
MASP2 (MBL-associated serin protease 2)		1p36	75	Binds to MBL/Ficolins. Cleaves C2 and C4
MASP3 (MBL-associated serin protease 3)		Unknown	Unknown	Unknown (binds to MBL/Ficolines, but does not cleave C4 or C2)
C2a		6p21.3	68	Larger fragment formed by cleavage of C2 during activation. Component of the C3 convertase and C5 convertase
C2b			30	Smaller fragment formed by cleavage of C2 during activation
C4a		6p21	8	Smaller fragment formed by cleavage of C4 during activation. Anaphylatoxin
C4b			192	Larger fragment formed by cleavage of C4 during activation. Component of the C3 convertase and C5 convertase. Recognized by CR1 forfunctionssuchasopsonin and activation regulator
Factor Ba	CFB	6p21.3	30	Smaller fragment by cleavage of Factor B
Factor Bb			63	Larger fragment formed by cleavage of Factor B. Component of the C3 convertase and C5 convertase in the AP
Factor D	CFD	19p13	27	Cleaves iC3b to form C3 convertase in the AP
Proteins common on the three pathways				
C3a		19p13.3	8	Smaller fragment formed by cleavage of C3 during activation. Anaphylotaxin with chemiotactic function and effects on microorganisms

COMPONENT	ALTERNATIVE NAME	CHROMOSOME	MW (kDa)	FUNCTION AND COMMENTS
C3a-desArg			8	Inactivated derivate of C3a. Involved in the chemiotactic effect on eosinophils and has effects on microorganisms
C3b			178	Larger fragment formed by cleavage of C3. Component of the C3 convertase in AP and C5 convertases on the all pathways. Recognized by CR1 for functions such as opsonin and activation regulator
iC3b			176	Fragment formed by the degradation of C3b through binding to CR3, CR4, and CR1 to function as opsonin
C3c			138	Degradation caused by cleavage of C3b
C3d/dg			33-38	Degradation caused by cleavage of C3b containing the c3b covalent binding sites. Binds to CR2 to regulate B cell function
C5a		9q33	11	Smaller fragment formed by cleavage of C5 during activation. Anaphylotaxins with chemiotactic effects on neutrophilsand monocytes. Also induces apoptosis
C5a-desArg			11	Inactivated derivate of C5a. Involved as chemiotactic effect on neutrophils and monocytes. Alsoinvolved in lipid metabolism
C5b			185	Larger fragment formed by cleavage of C5. Component of the MAC
C6		5p13	10	MAC Membrane insertion
C7		5p13	93	MAC Membrane insertion
C8		1p32	65	Induces pore formation by MAC
C9		5p14	63	MAC forms lytic pore by multiple C9 molecules

Table 1. **Components of the complement cascade.** Abbreviations: *MW*: molecular weight; *CP*: Classical pathway; *AP*: Alternative pathway; *LP*: Lectin pathway; *PAMP*: Pathogen-associated molecular patterns; *DAMP*: Damage- associated molecular patterns; *MAC*: Membrane attack complex. Adapted from reference (3).

ly and covalently to cell or macromolecular surfaces. Once this factor is deposited, it can attract more factor B, which will be cleaved by properdin to form C3 convertase. If more C3b binds to this convertase, this will produce C5 convertase, which in turn will activate and assemble MAC. All of the process described above is called a feedback loop (26).

C3 may also be activated by independent pathways such as proteases that are released by neutrophils and macrophages. However, other factors such as kallikrein, plasmin, elastase and factor XIIa can activate it (18). In contrast, thrombin cleaves C5 and generates C5a in the absence of C3 (28).

Once C5b is released, it binds to C6 to form the complex C5bC6. This binds with the target cell surface to establish the place where the other components of MAC will join it. Furthermore, C5bC6 also binds to C7 on the target surface to induce the C8α and β membrane insertion and thus create pores in the phospholipid bilayer in the target cell. C8α binds to C9and starts the polymerization of multiple C9 molecules to make pores with a diameter of 10nm. Finally, the C5b6789 complex is called MAC and it will cause the target cell to undergo osmolar changes by the entry of water, ions and other molecules. This will lead to cell lyses, and productions of cytokines, prostaglandins and other proinflammatory molecules (14).

During the complement cascade, small peptides named anaphylatoxins (ATs) are liberated. These peptides are C3a (77 amino acids) and C5a (74 amino acids) and have antimicrobial properties though C5a is more potent than C3a. The ATs are regulated by carboxypeptidase N (CPN) which is a plasma protease that cuts off the C-terminal arginine and yields an inactive form of C3desArg and C5desArg (29).The functional response is mediated by a superfamily of G-protein-coupled receptors: C3aR, C5aR (CD88) and C5L2 (C5a receptor-like 2, known as gpr77). These ATs can also bind to the fmlp-receptor (N-formyl-methionine-leucine-phenylalanine), ChemR23, and the chemokine receptors (CXCR1 and CXCR2) (10).

The ATs are very important in inflammatory responses because they enhance cytokine production (30), induce vasodilatation by the relaxation of smooth muscles, and induce permeability in small blood vessels.

C5a is the most powerful AT to induce chemiotaxis of macrophages, neutrophils, basophils, mast cell, and activated B cells and T cells. When ATs interact with basophils and mast cells, the latter two produce histamine. However, in addition to their pro-inflammatory properties, they are involved in tissue regeneration and tissue fibrosis (10).

THE COMPLEMENT REGULATORY FACTORS

The CS has regulatory factors that maintain the immunological homeostasis. Alterations in these regulatory factors can cause tissue damage triggered by all pro-inflammatory molecules.

The cascade has a passive control activation generated by C4b and C3b active sites which have a short half-life in contrast with the C4b2a complex (CP and LP) and C3bBb complex (AP) which are unstable. Control is also handled by regulatory proteins which are located in serum (fluid-phase) and on the surface of the endothelial and immune host cell (Tables 2-3). These are in constant contact with complement proteins and express multiple inhibitory regulatory proteins: membrane co-factor protein (MCP; also known as CD46), decay-accelerating factor (DAF, also known as CD55), complement receptor 1 (CR1; also known as CD35), and protectin (CD59). Another protein which regulates the action of CS is the factor H (the same one that acts on AP) that interacts with the endothelial cell surface and with components of the coagulation cascade (31,32).

The regulatory factors have four important functions in the cascade (Figure 3). (I) The C1 inhibitor (C1inh) blocks the initiation of the cascade by preventing the onset of CP and LP and binding to C1r, C1s and MASP1-2. It also inhibits AP activation but the location is still unknown (33). In addition, LP may also be inhibited by sMAP (MAp19) and MAP-1 (MAp44) which bind to MBL and ficolins (3). (II) Multiple regulatory factors act downstream once the cascade is activated and just before C3 convertases are assembled. At this point, there is an inhibition of C3, C4 and iC3 by factor I and its co-factors such as C4b-binding protein (C4BP) in the CP and LP and factor H in the AP. Note that factor I only acts when it is coupled with its co-factors (34). CR1 and MCP are also involved at this point and act as cofactors with

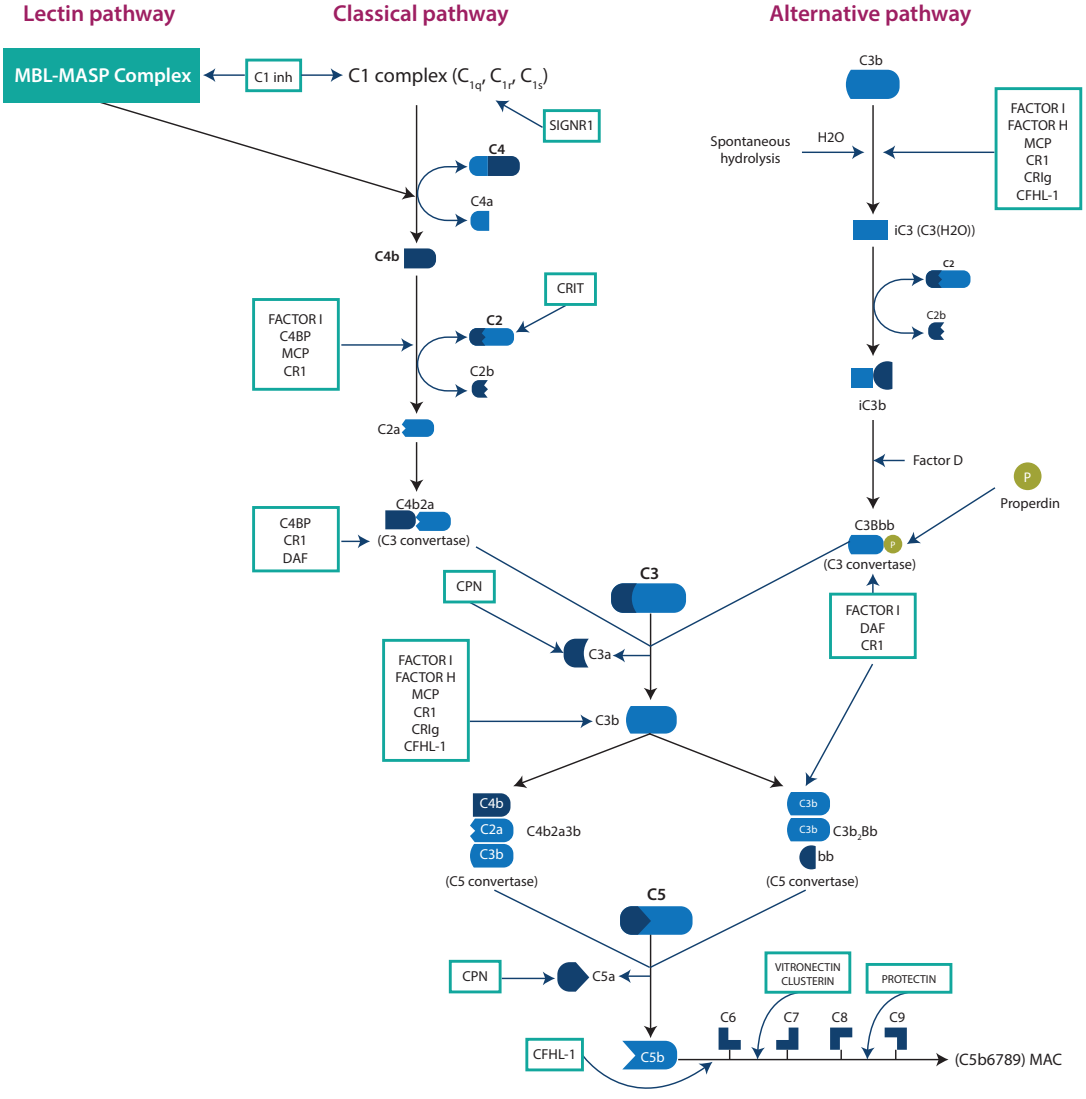

Figure 3. The regulatory factors. Abbreviations: C4BP:C4-binding protein; MCP: Membrane Cofactor protein; CR1:Complement receptor 1; CRIT:C2 receptor inhibitor trispanning; CPN: Carboxypeptidase-N; CRIg: Complement receptor of the immunoglobulin family; CFHL1: Complement Factor H-like protein 1.

REGULATOR	ALTERNATIVE NAME	LOCALIZATION	CH	MW (kDa)	POINT OF ACTION	CELL SURFACE OR EXPRESSION	FUNCTION
C1Inibhitor	Serpring 1	Serum	11q12	55	CP/LP/AP		Blocks C1r, C1s, MASP2, serine protease, C3b, and coagulation factors
Factor I	CFI	Serum	4q25	65	CP/LP/AP		Degrades C3b, C4b, and iC3 in presence of C4BP, factor H, and MCP
C4-binding protein	C4BP	Serum	1q32	67	CP/LP	Acquired on surface	Cofactor for CFI degrades C4b and accelerates C3 convertase decay in CP
Membrane Co-factor protein	MCP; CD46	Surface	1q32	43	CP/LP/C3b	All cells except erythrocytes	C3 degradation, Cofactors CFI and CFH, and effector for T cell maturation
Factor H	CFH	Serum	1q32	139	AP/C3b	Acquired on surface	Cofactor for CFI, and accelerates C3 convertase and C5 convertase decay in AP
Complement Factor H-like protein 1	CFHL-1; Reconectin	Serum	1q31	38	AP/C3b/TP		Cofactor for CFI, and acceleration C3 convertase and C5 convertase decay in AP.Inhibits binding of C5 to C6 in TP. Also is associated with lipid metabolism
Decay accelerating factor	DAF; CD55	Surface	1q32	41	C3 convertase	GPI anchor expression by most cell types, including erythrocytes, epithelial cells and endothelial cells	Accelerates C3 convertase decay and C5 convertase in AP
Carboxypeptidase-N	CPN	Serum	1q24.2	52	AT		Inactivator C3a and C5a
Vitronectin	S-protein; S40	Serum	17q11	54	TP		Prevents assembly of MAC, binds to C5b-C7
Clusterin	SP-40; Apolipoprotein J	Serum	8p21	52	TP		Prevents assembly of MAC, binds to C7-C9. Also involved in lipid metabolism in transportation of cholesterol, HDL, APOA1, and lipids
Protectin	CD59	Surface	11p13	14	TP	GPI anchor expression by erythrocytes and most nucleated cells, including renal cells	Prevents assembly MAC, binds to C8-C9

Table 2. **Regulatory factors of complement system.** Abbreviations: *Ch*: Chromosome; *MW*: molecular weight; *CP*: Classical pathway; AP: Alternative pathway; *LP*: Lectin pathway; *TP*: Terminal pathway; *MAC*: Membrane attack complex; *APOA1*: Apolipoprotein A-1; *GPI*: Glycosylphosphatidylinositol; *HDL*: High-density lipoprotein. Adapted from reference (2).

factor I to inactivate iC3 and C4b (35). Therefore, CR1 is an immune adherence receptor for iC3/C4b and together, they remove immune complex (36). CR1 on red cells can transport immune complex coated by CS to the spleen and liver where they are depurated by tissue macrophages. Furthermore, in the CP and LP another regulatory factor known as complement C2 receptor inhibitory trispanning (CRIT) was recently discovered. This factor binds to C2 and prevents its cleavage by C1s (37).There are other regulatory factor that inhibit iC3 such as factor H-like protein 1 (CFHL-1) (38) and complement regulator of the immunoglobulin superfamily (CRIg) which are expressed in macrophages and Kupffer cells (39). (III) Regulatory factors are also involved in the inhibition of C3 and C5 convertase amplification by destabilizing the enzyme complex. As was mentioned before, C3 convertases are the main factor in the amplification on the cascade in the three pathways. However, this convertase is inhibited by C4BP, CR1, and the DAF in the CP and LP. In contrast, the

C3 convertase in AP is inhibited by factor H, DAF, and CR1 which also inhibit C5 convertase at the same time. Another factor that can be inhibited is C3b. This inhibition is caused by the action of regulatory factors such as factor H, MCP, CR1, factor I and CRIg. As was mentioned above, ATs are important in pro-inflammatory processes and these proteins will be inhibited by CPN (40). (IV) Regulatory factors such as vitronectin (S-protein), clusterin (SP-40, also known as apolipoprotein J), CFHL1, and protectin play an important role during assembly of MAC. The two first factors interact with C5b-7 complex (41,42) while CFHL1 binds to C5b/C6 (43), and protectin binds with C8/C9 and inhibits the formation of the lytic pore (44).

THE COMPLEMENT RECEPTORS

The complement activation products C1q, C3a, C4b, and C3b are recognized by specific receptors on cell surfaces that control cell functions (Table 3). Complement receptor 1 (CR1, also known as CD35) is the principal immune adherence receptor on erythrocytes, and it allows the binding and bloodstream clearance of complement-coated immune complexes. This receptor is also expressed in follicular DCs (fDCs) to retain antigen within lymphoid follicles. Moreover, when CR1 is recognized by C1q, C4b, C3b, and iC3, it promotes phagocytosis (36). Another receptor is complement receptor 2 (CR2, also known as CD21) which interacts with C3b and C3d on B cells, fDCs, tonsils, and the lymphoid node. Just as in the case of CR1, CR2 allows the antigen to be transferred into the lymphoid node and to be retained in germinal centers in order to preserve the immunological memory (6,20,45). Another receptor is complement receptor 3 (CR3, known as CD11b-CD18). It is present in NK cells, PMN, microglia, and phagocytes. In the last one on the list, it interacts with an iC3-coated target cell to promote its elimination in secondary lymphoid tissue. CR3 captures and transports the antigen coated with C3 to B cells (46). Just as in the case of CR3, complement receptor 4 (CR4, known as ITB2 and CD11c/CD18) is present in phagocytes and has a similar function (20). There are other receptors which are present only in DCs and microglia cells such as SIGNR1 which binds to C1q and inhibits the assembly of C3 convertase (47). Furthermore, we can find other types of receptors, which are specific to the aforementioned ATs (C3aR, C5aR, and C5L-2). These are present in several inflammatory cells and smooth muscle cells and their purpose is to promote inflammatory reaction. C5L-2 acts as a decoy receptor (48,49).

THE COMPLEMENT SYSTEM AND ADAPTIVE IMMUNITY

Complement system and B cells. The B cells are involved in several functions: to produce antibodies, lead to neutralization and opsonization of pathogens, and provide immunological memory about infection (See chapter 6). The CS plays key roles in multiple stages of B-differentiation (6). Those stages are achieved by complement receptors, CR1 and CR2, that also bind to opsonins to improve the phagocytic system. Note that CR2 interacts with humoral adaptive immune system three different ways. The first one is by forming CR2-CD19-CD81 complex, which interacts with BCR and thus results in the reduction of B cell clonal expansion. The second is when CR2 is present on fDCs to capture C3d-opsonized antigen and present it to naïve or antigen-engaged B cells in the germinal center of the lymph node, thus generating effectors and memory B cells. And the last one is enhance the delivery of antigen to fDCs (50). Moreover, the ATs such as C3 and C5, apparently help in the expression of B cells, which promotes the trafficking and migration of various B cells populations (14).

Complement and T cells. T cells are activated by interaction with antigen presenting cells (APCs) in lymph nodes. The activation is dependent of microorganisms and environmental factors (first and second signal) (See Chapter 7 and 10). Once activated T cell migrate to inflammatory sites and continue the immunological response. The CS can modulate T cells directly or indirectly though the alteration of immunomodulatory cells (APCs) (51).

The role of CS on T cells has been suggested in murine models. C3-deficient mice were depleted of CD4+ and CD8+ T cells in the presence of viral infections. In contrast, DAF deficient mice had enhanced T cell proliferation (14,52). Furthermore, murine models with complement deficiency attenuate T cell mediated autoimmunity and delay allograft rejection. Increased complement activation results in autoimmune disease and accelerated allograft rejection (53). Moreover, APCs and T cells express C3aR and C5aR which bind to complement proteins to enhance T cell viability, proliferation and differentiation (54,55).

It has been recently discovered that during the antigen presentation to T cells, there are a variety of signal expressions to induce C3a, C5a, factor B and factor I as well as promote the up regulation of C3aR and C5aR, and the transient down regulation of the complement regulator DAF on T cells and APCs. However, when there is an absence of APC activation, it will lead to a deficiency of ATs with a subsequent induction of Treg cells (56).

THE COMPLEMENT SYSTEM AND INFECTIOUS DISEASES

The CS has five specific functions to defend the body from bacteria. The first function has to do with the phagocytosis and opsonization of microorganisms through the binding of C3b and iC3 to complement receptors, which are expressed on phagocytes, and PMN. The second is the chemotaxis and activation of granulocytes by ATs which induce inflammation. The third function is to lyse foreign pathogens through MAC formation. This complex is only effective against pathogens without cell walls in which the best effector mechanism is opsonization (57). In the fourth function, CS will interact with B lymphocytes to produce antibodies that are important in the

activation of adaptive immunity (58). Finally, the last one regulates T cell activation and its effector function (14).

However, activation of CS in normal conditions is caused by bacteria invasion by three different pathways. On the first pathway, the antibody recognizes the bacteria and actives the CP. On the second, the LP is activated by sugar moieties on the bacterial surface while the AP is activated by the presence of carbohydrates, lipids and proteins on the bacteria.

REGULATOR	ALTERNATIVE NAME	CH	MW (KDA)	CELL SURFACE OR EXPRESSION	FUNCTION
Complement receptor 1	CR1; CD35; C3b/ C4b-recetpr	1q32	223	Nucleated cells, erythrocytes, monocytes, fDCs, B cells, and leukocytes	Binds to C1q, C4b, C3b, and iC3b. Clearance of immune complex, enhancement of phagocytosis, and regulation of three pathways. Cofactor for CFI
Complement receptor 2	CR2; CD21; Epstein barr virus receptor	1q32	1129	fDCs, B cells, and T cells	Regulation of B cell function, B cell co-receptor, and binds to C3b and C3d tagged immune complex. Also allows transfer of antigen into lymphoid node and retains antigen in germinal center and maintains of immunological memory
Complement receptor 3	CR3; CD11b/ CD18; αMβ2 integrin; Mac-1(macrophage-1 antigen)	16p11.2	127	Monocytes, macrophages, neutrophils, NK, eosinophils, myeloid cells, fDCs, CD4+, and CD8+T cells	Induces and enhances phagocytosis by interaction with iC3 through the ICAM-1 allowing immune cell adhesion. Also involved in transportation of antigen in secondary lymphoid tissue
Complement receptor 4	CR4; CD11c/ CD18;p150/95; αXβ2 integrin	16p11.2	127	Monocytes, macrophages, neutrophils, and NK	Induces and enhances phagocytosis by interaction with iC3b
Complement receptor of the immunoglobulin family	CRIg; VSIG4;Z93Ig	Xq12	44	Macrophages and Kupffer cells	Induces and enhances phagocytosis by interaction with iC3b and regulate the AP, and C3 and C5 convertase
SIGNR1	CD209	19p13	46	DCs and microglia	Binds to C1q and inhibits assembly of C3 convertase
C2 receptor inhibitor trispanning	CRIT	1q31			Binds to C2 and prevents cleavage by C1s
C3aR		11p13.31	53	Neutrophils, monocytes, eosinophils, APC, T cells, astrocytes, neurons, and glial cells	C3a binds to C3aR. Results in immune cell recruitment and proinflamatory signals
C5aR	CD88	19q13.3	39	Myeloid cells, monocytes, neutrophils, DCs, APC, T cells, endothelial cells, and renal tubular cells	C5a binds to C5aR. Results in immune cell recruitment and proinflamatory signals
C5L2	GPR77	19q13.33	36	Macrophages and neutrophils	Bind C5a/ C5a-desARag for Immune cells recruitment and proinflamatory signal and possibly acts as a decoy receptor
cC1qR	C1q receptor for collagen region; Calreticulin; Collectin receptor	19p13.3	48	B cells, endothelial cells, and DCs	Bound to C1q and induces phagocytic signaling via CD91
gC1qR	C1qbp (C1q-binding protein);p33	17p13.3	31	Macrophages and B cells	Binds to C1q, potential role in phagocytosis and signaling. Also modulates IL-12 on DCs
C1qRp	CD93+unknow mediator	20p11.21	68	B cells, endothelial cells, and DCs	Part of receptor complex that binds C1q and involved in phagocytosis
Complement factor H-related 1,2,3,4,5	CFHR 1,2,3,4,5	1q31-32	30-38	Myeloid cells and monocytes.	Might be involved in complement regulation and also in lipid metabolism

Table 3. **The complement system receptors.** Abbreviations: *Ch*: Chromosome; *MW*: molecular weight; *fDCs*: Follicular dendritic cells; *NK*: Natural Killer cells. Adapted from references (2, 3).

And the third one is when C3b coats the bacterial surface and enhances their recognition by neutrophils and macrophages to promote opsonization. Nevertheless, there are other mechanisms helping with bacterial opsonization such as activation of iC3 that interacts with CR3 and CR4 in phagocyte cells. In addition, C3b could bind to CR2 to produce antibodies which will enhance the immunological response and finally produce bacterial lyses by MAC assembly (59).

Even so, the bacteria have five ways to evade the immune response: a) It mimicking and recruiting complement regulators, b) enzymatic degradation of complement proteins, c) inhibition or modulation of complement proteins, d) inhibition of Ig before its interaction with complement, and e) blockage of MAC penetration (Table 4) (59).

The CS may also play a role in immune response to viral infections through recognition of several viral glycoproteins by MBL. The viruses that are reportedly capable of activating MBL are HIV, SARS, coronavirus, Ebola, dengue, West Nile and Marbug virus (60,61). In a murine model, the CS is required for enhancement of T cell response to viruses(60). However, the CS could be involved in humoral response through the complement receptor (CR1-2-3), which recognizes C3b on IgM, and IgG coated viruses. However, like bacteria, the viruses may evade the immune response by three mechanisms: a) entering host cells, b) modifying C1q or C3, and c) using the host complement regulatory proteins or virally produced proteins to prevent cell lyses (59).

Moreover, the three CS pathways (CP, LP and AP) can be activated in response to parasites. Mainly, LP will be mainly activated through PAMPS on the parasite surface during early infections while the parasites with the ability to evade activation of CS can still be detected by CP after the production of antibodies (62). Nevertheless, some parasites such as trypanosomes can prevent complement activation by the expression of complement receptors which inhibit of C3 convertase. Other strategies are the stabilization and inhibitionof C4b2a (62).

BACTERIA	TARGET PROTEINS OF CS
Borriela spp	Factor H, CFHL-1, CFHR-1 C4BP, C8, and C9
Enterococcus Faecalis	C3a, C3b and, C5a
Escherichia spp	C4BP, C1-Inh, and C5b6
Haemophilus influenzae	C4BP, factor H, CFHL-1, and Vitronectin
Neisseria spp	C4BP, factor H, CFHL-1, and Vitronectin
Pseudomonas spp	C1q, C3, factor H, and CFHL-1
Staphylococcus spp	IgG, C1q, C3, C3b, C3d,C3 convertase, C5, and C5desArg
Streptococcus spp	IgA, IgG, C1q, C3, C5a, C5b-7, C5b-8, properdin, factor H, CFHL-1, and C4BP
Yersenia spp	Factor H

Activation of CS can be achieved by fungal infections. The main factors that act against fungal infection are ATs, C3a and C3b. The C3a has antifungical activities and C3b is involved in the opsonization. Both factors deposit on the fungal surface and activate the CS to assemble the MAC (63). Recently, it was also found that Candida infection activates C5a to stimulate PBMCs to induce pro-inflammatory cytokines (IL-6 and IL-1β) (64). Note that the pathogens described above utilize the CS to increase their virulence. Some intracellular bacteria, viruses and parasites use cell-bound complement regulatory molecules and receptors to enter host cells (1,65).

Because the CS is fundamental in the response to pathogens, it will play an important role in septic shock. In such cases, AP and CP are activated to clear endotoxins. However, an alteration in LP may increase the risk of developing sepsis (66). Complement proteins such as C5a, C5aR and C5L2 will have a critical role in the development of sepsis and the activation of immune cells like NK and NKT (67,68). Moreover, a decrease in MASP-2 during early phase of septic shock might be correlated with mortality (69). In conclusion, alterations in CS proteins are associated with major susceptibility to infections but mainly encapsulated bacterial infections (Table 5).

THE COMPLEMENT SYSTEM AND DEAD CELL CLEARANCE

Another one of the anti-inflammatory activities carried out by the CS is to clear immune complexes in circulation and tissues, apoptotic bodies and cells modified by injury or hypoxia after virus infection or tumor-caused modification. This clearance is mediated by pattern recognition molecule (PRM), opsonins and receptors.

During apoptosis, the cell loses lipid bilayer asymmetry (flip-flop), thus exposing molecules such as phosphatidyl-serine, which in turn will activate pro-phagocityc signals (See chapter 13). If the apoptotic cells are not eliminated, they will continue to secondary necrosis. In this step, the cell cytoplasm swells and loses its plasma membrane integrity which triggers cell rupture. This will cause the exposure of intracellular contents including nucleus (HMGB1) and cytoplasm proteins. These will be recognized as foreign molecules and produce inflammation and/or autoimmune response (70). HMGB1 (High mobility group 1) is an alarmin that participes in chromatin architecture and transcriptional regulation. It is associated with induction of chronic autoimmune diseases (See chapter 9) (71,72).

Furthermore, apoptotic bodies which undergo secondary necrosis are recognized by complement proteins, especially CP. However, the other two pathways can be activated

Table 4. **Bacteria with ability to avoid the complement system.** Abbreviations: FHL-1Complement Factor H-like protein 1; Complement factor H-related-1; *C4BP*: C4-binding protein; *C1-Inh*: C1-inhibitor; Adapted from reference (58).

under the same conditions (73). The CS is activated by recognition of plasma proteins (histidine rich protein, IgG, IgM, pentraxine 3, serum amyloid P component, C-reactive protein, annexin A2 and A5) and thus binding to apoptotic cells or DNA/RNA from apoptotic cell surfaces (74,75). The CP, in turn, is activated by binding C1q to the surface of calreticulin (also known as collagen-tail C1q receptor, cC1qR) which forms a complex with the endocytic receptor protein CD91 (also known as α-2-macroglobulin or the LDL-related receptor protein, LRP) and other receptors such as CR1, CR2, CR3, CR4 and CRIg, thus promoting phagocytosis (74). Moreover, C1q elicits a specific macrophage phenotype for the removal of apoptotic bodies (76). Once the C1q binds to apoptotic cells, it amplifies the CP and recruits factor H, which inhibits the amplification of CP and C5 convertase formation, thus protecting host cell against unwarranted inflammation (3). A deficiency in C1q may lead to impaired immune complex formation and apoptotic clearance (77).

In contrast, the LP may be activated by MBL bound to late apoptotic and necrotic cells (74). MBL is found in apoptotic cells that express a terminal sugar from cytoskeletal proteins, which makes it possible for macrophages to recognize them and facilitate their phagocytosis (78). As was mentioned earlier in this chapter, LP may be also activated by ficolin B which marks apoptotic and necrotic cells for subsequent removal and maintenance of tissue homeostasis (79). AP, in turn, may participate in apoptotic cell removal by binding iC3 and CR3 to promote the opsonization and phagocytosis. This process is accompanied by IL-12 down regulation and a lack of oxidative burst in macrophages or co-stimulatory molecule expression that impairs maturation of DCs (3).

THE COMPLEMENT SYSTEM AND DISEASES

The CS is involved in several diseases because of alterations in regulatory proteins and deficiencies in complement proteins. Complement disorders are autosomal recessive except for MBL, factor I deficiency, and C1-INH deficiency, which are autosomal dominant, and deficiency of properdin which is X-linked recessive (80). Genetic defects such as single nucleotide polymorphism (SNP) can result in generation of dysfunctional protein or complete gene deletion. Some diseases associated with complement deficiency or alterations are shown in Table 5 (2,7,26,81-105).

THE COMPLEMENT SYSTEM AND ATHEROSCLEROSIS

Atherosclerosis physiopathology is complex and several factors are involved in lipid deposition on vessel wall. Inflammatory proteins such as CS proteins, pentraxines, and cytokines produced by immune cells are involved in this process. The CS protein involved in this mechanism is C5L2. This protein is essential in glucose uptake and lipid and triglyceride clearance by induction of C3desArg (3). Moreover, adipocytes secrete factor D, factor B and C3 which stimulate insulin or lipids. This leads to a high turnover of AP and to generation of C3a (3). C1q, C3, C4 and MAC are present in atherosclerotic lesions while a murine models shows that a deficiency of regulatory proteins (protectin) accelerates atherosclerosis (59). Other studies have shown that CS facilitates macrophage extravasations and foam cell formations, which release proinflammatory factors and enhances atherosclerosis. All this is also related to the CS activation by the coagulation system (59). Note that pathologies associated with alterations in CS such as SLE have an increased risk of developing atherosclerosis (106). That is why complement proteins has also been proposed as biomarkers of atherosclerosis (107) (See chapter 38).

THE COMPLEMENT SYSTEM AND ISCHEMIA-REPERFUSION INJURY

An injury is associated with an interruption of the blood flow (ischemia or hypoxia) and, then, with the subsequent restoration (reperfusion). This mechanism is known to occur in myocardial infarction, stroke, transplantation or vascular surgery. During this catastrophic event, there is an increase in the generation of reactive oxygen species (ROS) and activation of various cell types such as endothelial cells and leukocytes. All of this together increases the production of apoptotic bodies which produce neo-epitopes that will be recognized by antibodies with subsequent activation of CS. The ATs produced by CS activation are involved in neutrophil activation and infiltration, thus resulting in more inflammation, cell injury and necrosis. In animal models with ischemia-reperfusion injuries treated with inhibitors such as anti-C5 antibodies and C5a receptor, it was shown an improvement was shown in early graft functions after transplantation (108). Furthermore, CS is activated during acute stroke, mainly within LP. That is the reason patients with decreased levels of MBL, which causes them to express low levels of C3, C4, and C-reactive protein, have better functional outcomes than patients with normal MBL (109).

THE COMPLEMENT SYSTEM AND NEURODEGENERATIVE DISEASES

Neuronal cells such as astrocytes and microglia have the ability to synthesize complement proteins while neuronal stem cells express receptors, which migrate and differentiate in response to CS. The CS has functions in the regulation of the neuronal development and synapse elimination (110).

Unfortunately, neurodegenerative diseases do not have a known etiology, but recently it has been suggested that neuron death is preceded by aberrant synaptic functioning and massive synapse loss. Based on this hypothesis the CS, mainly the CP pathway, may play an important role in neurodegenerative disease. This is based on the enrollment of CS in the synapse elimination by recognition of C1q and induction of phagocytosis mediated by C3b or iC3. Therefore, alterations in this process result in neuron

DISEASE	DEFECTS OR DEFICIENCIES	EFFECTS ON ALTERATION	CLINICAL CHARACTERISTICS	REF.
Hereditary Angioedema (HEA)	C1-Inhibitor	C1-Inh involved in regulating the activation of the CP, LP, controlling activation of CS through kallikrein, thrombin, FXIa, FXIIa. Also it can inhibit in FS, inhibit tPA, and plasmin. Alterations of C1-inh trigger uncontrolled activation of these cascade systems, which unleashes the production of bradykinin and this, in turn, causes angioedematous attacks.	Acute attack of facial, laryngeal, genital, or peripheral swelling or abdominal pain secondary to intra-abdominal edema	(81-83)
Atypical hemolytic uremic syndrome (aHUS)	Factor H, MCP, Factor I, C3, Factor B, Trhombomodulin, CFHR1, CFHR3, and CFHR5.	Abnormal proteins have reduced binding to C3b, heparin, and endothelial cells.	Microvascular occlusive disease characterized by hemolytic anemia with erythrocytes fragments, thrombocytopenia and acute renal failure	(84,85)
Paroxysmal nocturnal Haemoglobinuria (PNH)	DAF and protectin	AP apparently involved in tickover that initiate hemolysis hemolysis spontaneously. Hemoglobinuria at night may be due to the slightly lower blood pH found during sleep which accelerates the tickover. Erythrocytes are incapable of modulating physiologic complement activation on their surface. This triggers complement-mediated intravascular anemia.	Thrombophilia, hemolytic anemia, and bone marrow failure	(26,86-87)
Membranoproliferative glomerulonephritis, type II (MPGN II)	C3 convertase, C5 convertase, proteins of MAC, Factor H, CFHR5, and properdin	Unknown	Increase mesangial cellularity and matrix with thickening of glomerular capillarity walls secondary to deposition of complement and immune complex. C3 staining and the absence of C1q, C4, and Ig on the immunofluorescence.	(88,89)
Age-related Macular degeneration	C2, C3, Factor H, Factor B, CFHR1, and CFHR3	Unknown	Two types: dry and wet. Dry usually causes some degree of visual impairment and sometimes progresses to severe blindness while wet only affects 10-15% of the population and rapidly progress to blindness if left untreated	(2,90)
Infections	Factor H, Properdin, MBL, C1q, C1r/s, C4, and C2.	Neisseria Meningitidis	Meningitis, recurrent otitis, and pneumonia	(91-94)
	Factor I, and MAC proteins	*Neisseria Menengitidis* and *streptococcus pneumoniae*	Respiratory tract infections	(2,80,95)
Chronic Rhinosinusitis	MBL	Unknown	Difficulty breathing, swelling around the eyes and face, and headache.	(96)
Leukocyte adhesion deficiencies	CR3/CR4	Receptors are integrins and essential for leukocyte adhesion to endothelial cell	Recurrent bacteria and fungi infections	(80,97)
Preeclampsia	MCP and Properdin	Unknown	Hypertension, proteinuria, and edema	(98)
Systemic lupus erythematosus	C1q, C1r, C1s, C2, C3, C4, DAF, CR1, protectin, factor H, CFHR, and MBL		See chapter 25	(80,99-102)
Rheumatoid arthritis	C1q	Alterations in the clearance of apoptotic bodies and the cytokine inhibitions (See figure 4)	See chapter 24	(7,103)
Type 1 diabetes	MBL	Increased activation of CP and osteoclastogenesis	See chapter 29	(104)
Devic´s diseases	DAF, MCP, CR1, and protectin	Unknown	Optic neuritis, myelitis, CNS involvement beyond the optic nerves and spinal cord is compatible with neuromyelitis optica	(105)

Table 5. Diseases associated with deficiences or defects in complement proteins. Abbreviations: *CP*: Classical pathway; *AP*: Alternative pathway; *LP*: Lectin pathway; *MAC*: Membrane attack complex; *CS*: Coagulation system; *FXIa*: Activated factor XI; *FXIIa*: Activated factor XII; *FS*: Fibrinolytic system; *tPA*: tissue plasminogen activator; *CFHR1-5*: Complement factor H-related 1-5; *MCP*: Membrane Cofactor protein; *DAF*: Decay accelerating factor; *MBL*: Mannose-binding lectin; *CR3*: Complement receptor 3; *CR4*: Complement receptor 4.

loss and neurodegenerative progression such as Alzheimer's disease, glaucoma, Parkinson's diseases, multiple sclerosis and schizophrenia (110). In the case of Alzheimer's disease, amyloid-beta peptides are known to accumulate in the extracellular milieu to form amyloid plaques. As result, these plaques are recognized by C1q and they induce CP triggered neuronal damage (110).

THE COMPLEMENT SYSTEM AND OSTEOARTHRITIS

Osteoarthritis is characterized by breakdown of articular cartilage in the synovial joint. Therefore, CS is fundamental in the pathogenesis of the disease as was shown in several experimental studies in which it is observed complement protein deposits in the synovial (fluid and tissues) and diminished levels of regulatory proteins are observed (111). In MAC deficient mice models, the importance of MAC for the development of osteoarthritis is shown (112).

THE COMPLEMENT SYSTEM AND ALLERGY

Allergy is normally associated with Th2 response. However, CS (primarily AP) is involved in the progression of the disease just as it is in inflammatory processes. Some studies in mice models have shown the importance of endogenous factor H in regulating airway inflammation (113). High levels of ATs, in turn, have been detected in high levels in bronchoalveolar lavage fluid form asthmatic patients (26). In addition to being potent chemioattractants to PMN and producing proinflammatory factors, these molecules also contribute to smooth muscle cell contraction and mucus production which enhance vascular permeability. At the same time, ATs inside airways are involved in the balance between mDCs (myeloid DCs) and pDCs. However, alterations in this relation are associated with deregulation in Th cell response and high Th17 response, therefore enhancing the development of allergies (114). There are different experimental models in which inhibition of C3, C5, C3aR and C5aR during the allergic phase decreases the allergy symptoms (114). Because of their importance in allergy pathophysiology, the complement proteins have been proposed as biomarkers (115) and therapeutic targets (116).

THE COMPLEMENT SYSTEM AND CANCER

CS has a dual role in the development of cancer. During the carcinogenesis process, the CP and AP are activated by recognition of cell debris formed by necrosis and apoptosis. Moreover, CP can be activated by Ig production in order to act against tumor proteins by the host cell. In addition, LP can be activated by MBL-mediated recognition of mannose-containing carbohydrates expressed on the tumor cell surface. Furthermore, MAC has dual role in cancer pathogenesis because it induces cell lyses and also protects from apoptosis, thus promoting cell survival (117). All the proteins mentioned above are detectable in the tumor environment. These include, for example, thyroid carcinoma, adenomas, colon, kidney, gastric, and breast cancer. It is also found in ascitic fluid from ovarian cancer patients (117). CS is involved in several mechanisms within tumor cells. First of all, tumor cells are resistant to CS action, especially that of MAC and CR3. This resistance is due to production of regulatory proteins such as CR1, CD46 and DAF which are important in controlling C3 activation (59). Secondly, ATs bind to receptors on the tumor cells and they induce the production of IL-6, thus resulting in cell cycle progression and apoptosis mechanism inhibition. MAC can also induce these mechanisms (See chapter 13). The last mechanism is tumor cell production of C5a which binds to C5aR on myeloid-derived suppressor cells. This results in increased reactive oxygen and nitrogen species that prevent the activation of CD4+, CD8+, NK and stimulate tumerogenesis and angiogenesis (59) Studies have shown that treatment directed toward C5aR slowed tumor progression (118).

THE COMPLEMENT SYSTEM AND AUTOIMMUNE DISEASES

Innate immune alterations are crucial for development of autoimmune diseases (ADs), but complement pathway alteration has also been associated. Systemic lupus erythematosus (SLE) is the most studied. However, others have been associated such as Sjogren´s Syndrome (SS), antiphospholipid syndrome (APS), rheumatoid arthritis (RA), vasculitis, multiple sclerosis and dermatomiositis.

SLE is characterized by loss of tolerance to nuclear self antigens originated from dead cell nuclei and associated proteins (See Chapter 25). Alterations in complement pathways, mainly CP, are associated with susceptibility to SLE. It is still not clear what the exact mechanisms involved in SLE pathogenesis are. However, six hypotheses have been proposed to explain the role of the complement in this disease (Figure 4). The first hypothesis is the deficiency in clearance of apoptotic bodies. This alteration may be achieved by C1q deficiencies, thus resulting in disruption of apoptotic body removal which leads to the production of self-antibodies that work against intracellular structures (nuclear, double stranded DNA, and histone antibody). That is the reason why C1q deficiencies increase susceptibility to infections in SLE patients. Therefore, vaccination is recommended for these patients. Moreover, studies have shown that ficolins and MBL are also important in apoptotic body clearance. The second hypothesis is the impairment of immune complex handling by alterations of other CP proteins (Table 5). The third one is how alterations on CS trigger inefficient elimination of self-reactive B cells which in presence of T cells could produce autoantibodies. In normal conditions, self-antigens are coated with CS and delivered to specific B cells, thus producing elimination of self-reactive cells. In the fourth hypothesis, CS is involved in regulation of cytokine production, thus alterations in C1q could not inhibit IFN-α which,

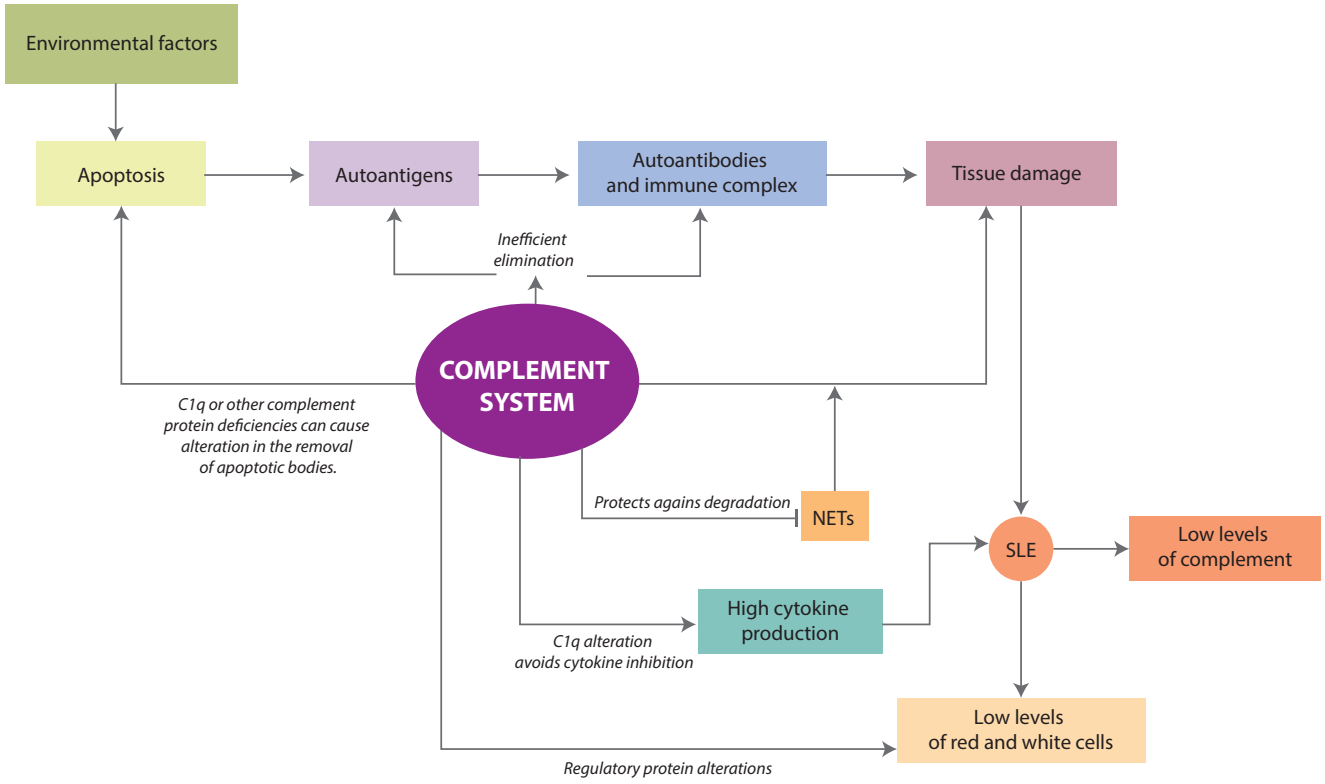

Figure 4. Role of complement in the pathogenesis of Systemic lupus erythematosus (SLE). Adapted from reference (99).

which is a very important cytokine in the pathogenesis of SLE. The five hypotheses suggest that complement components might be targets of autoantibody responses. This hypothesis is based on patients with high levels to anti-C1q antibodies which go against C1s and C1 inhibitor. Indeed, in nephritic lupus anti-antibodies could join to C1q presented in the kidney and cause severe tissue damage (99,119). Finally, the last one important mechanisms in which CS is involved in SLE pathogenesis is through activation of neutrophils which may produce neutrophil extracellular traps (NETs).(See chapter 13) (120). In addition, alterations in regulatory proteins (DAF, protectin, CR1) are attributed to lower levels of white and red blood cells. Apparently, this regulatory proteins are involved in the maintenance of blood cells homeostasis (121).

As we know, patients with SLE have hypocomplementemia which may be caused by genetic alterations, protein intake in response to self-antigens, or protein sequestration in tissues (119).

Another ADs is APS. In this disease the CS role is mediated by increased production of ATs and MAC which results in endothelial cell activation, monocyte tissue factor expression and platelet aggregation. It has been shown in murine models that blockage of the C5activation prevents complication of APS. As has been shown, mice deficient in C3, C5, C6 or C5aR are resistant to APS antibody-induced trombophilia and endothelial cell activation. APS pregnancy complications in murine C4, factor B, C3, C5 and C5aR deficient models are implicated in placental injury (122).

In RA patients, the CS in the synovial tissue plays an important role in its pathogenesis. C1 genetic variants and deficiency of regulatory proteins have been associated with the risk of developing RA (103,123) (Table 5). Apoptotic granulocytes are found in the synovial tissue which produces immune complexes that are recognized by CS proteins. This causes a pro-inflammatory response, activation of the three pathways of CS, and consumption of complement components. The presence of anti-CCP, type II collagen and IgA in the immune complex activate CP and AP (99).

At the same time, cartilage proteins such as osteomodulin and fibromodulin could bind to CS, thus this proteins bind to collagen fibers in cartilage and interact with C1q, resulting in CP activation and increased osteclastogenesis. In contrast, other proteins such as decorin, biglycan and cartilage oligomeric matrix protein (COMP) are inhibited by C1 activation. An alteration in LP, in turn, is associated with high severity of RA and an increased risk of erosive disease. However, COMP also binds to MBL, thus inhibiting the LP and activating CP by properdin and C3. COMP-C3 complex has been detected in patients with RA and it has been proposed a biomarker of the disease (99,124).

SS has also been related to CS. C4BP is associated with SS and is increases during periods of activate disease (125). Moreover, other regulatory proteins such as protectin, MCP, DAF

and clusterin have been observed in gland biopsies of patients with SS (126). CS is also involved in other ADs such as systemic vasculitis. The mice deficient in C5, factor B, C5aR are protected for ANCA-associated glomerulonephritis development. The final common pathway is known to be activated in this disease because levels of C5a, C5aR, and MAC are elevated in plasma and urine (127). Therefore, high levels of these proteins could reflect the severity of the disease (128). Moreover, the activation of neutrophil is caused by C5a and this interaction is mediated by cytokines or coagulation factors which are able to activate the AP (127). As is the case with LES, the NETs are important in the pathogenesis of this disease (129).

Other proteins are involved in other ADs. CTRP family proteins or C1q and tumor necrosis factor-related proteins belong to a group of 15 members expressed in several tissues (brain, eyes, lung, heart, liver, placental, muscle, prostate, ovary, etc). Furthermore, adiponectin (a member of the CTRP family) has several functions such as regulating of immune cell activity, insulin metabolism, cancer cell proliferation, angiogenesis, etc. Therefore, variants in CTRP members such as CTRP6 have been associated with high susceptibility to type 1 diabetes and vitiligo (130).

THE COMPLEMENT SYSTEM AND THERAPEUTIC TARGET

As we have seen throughout this chapter, the CS is involved in several homeostasis mechanisms and pathological conditions. Hence, the CS could be an important therapeutic target for the treatment of diseases associated with defects or deficiencies in this system (Table 5). It could also be an important target in the pathogenesis of ADs, cancer, allergy, atherosclerosis, etc. Therefore, these treatments could function as complement components inhibitors, enhancers of regulatory protein activity or supplements for complement proteins in deficiency diseases.

At present, only drugs that inhibit the complement on two key points: C1 and C5 have been approved for clinical use (20).

Eculizumab is a recombinant and fully humanized hybrid IgG2/IgG4 monoclonal antibody. It is specific to C5, thus inhibiting C5a generation and MAC formation. Currently, it is used for PNH, aHUS and C3 glomerulopathy (131). It has also been proposed to treat preeclampsia and HELLP syndrome (132),acute antibody-mediated transplant rejection (133), asthma (116) cold aglutinine disease (134), and myasthenia gravis (135). The most common adverse effect from its use is Neisseria infections (136). Moreover, a single-chain version of the C5-specific humanized monoclonal antibody is named Pexelizumab and it has been used in coronary disease complications but without any statistically significant differences (137).

C1 inhibitor drugs as Cinryze, Berinert and Rhucin have been utilized in HEA and they control bradykinin generation (20).

A complement-regulatory activity is achieved by immunoglobulins (IVg). Interestingly, they inhibit complement deposition on target cells when activation is triggered by antibodies through the CP. The complement proteins intercepted by IVg are C3b, C4b, and ATs. This treatment is used in ADs as Kawasaki disease, idiopathic thrombocytopenic purpura and SLE (20).

There are more drugs currently under development. These act to inhibit C3, ATs, factor B and regulatory proteins (DAF, MCP, CR1).

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5 INTRODUCTION TO T AND B LYMPHOCYTES

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INTRODUCTION

The T and B lymphocytes (T and B Cells) are involved in the acquired or antigen-specific immune response given that they are the only cells in the organism able to recognize and respond specifically to each antigenic epitope. The B Cells have the ability to transform into plasmocytes and are responsible for producing antibodies (Abs). Thus, humoral immunity depends on the B Cells while cell immunity depends on the T Cells. In the present chapter, the processes of ontogeny are summarized for each type of lymphocyte together with their main characteristics, the different subpopulations described to date, the signaling mechanisms employed for their activation, and their main functions based on the immunological profile that they present.

From the morphological point of view, T and B lymphocytes are indistinguishable since they are both small cells (8-10 microns in diameter) and each possesses a large nucleus with dense hetero-chromatin and a cytoplasmic border that contains few mitochondria, ribosomes, and lyzosomes. When they are activated by the antigenic stimulus, they may enlarge, thus increasing their cytoplasm and organelle number. Lymphocytes present receptors for antigen (Ag) recognition (TCR and BCR respectively) with different specificities on their surfaces. The genes that encode for these structures undergo a series of DNA recombinations, which provides them with immense phenotypic diversity.

T-LYMPHOCYTES (T CELLS)

ONTOGENY

The process of development and maturation of the T Cells in mammals begins with the haematopoietic stem cells (HSC) in the fetal liver and later in the bone marrow where HSC differentiate into multipotent progenitors. A subset of multipotent progenitors initiates the transcription of recombination activating gene 1 and 2 (RAG 1 and RAG2) and become lymphoid-primed multipotent progenitors and then common lymphoid progenitors (CLP). Only a small subset of pluripotent cells migrates to the thymus and differentiates into early thymic progenitors (ETP). The thymus does not contain self-renewing progenitors; and therefore, long-term thymopoiesis depends on the recruitment of thymus-settling progenitors throughout the life of the individual (1). These progenitors must enter the thymus to become gradually reprogrammed into fully mature and functional T Cells. The T Cell's distinct developmental steps, as illustrated in Figure 1, are coordinated with the migration of the developing thymocytes towards specific niches in the thymus that provide the necessary stage-specific factors that are needed for further differentiation.

The ETP are multipotent and can generate T Cells, B Cells, Natural killer cells (NK), myeloid cells, and dendritic cells (DC). ETP represent a small and heterogenous subset, have the ability to proliferate massively, and can be identified by the phenotype Lin^{low}, $CD25^{-}$, Kit^{high} as well as by their expression of Flt3, CD24, and CCR9 (1). These cells, which are attracted by the chemokines CCL19 and CCL21, enter the thymus via the corticomedullar junction. In the stroma of the thymus, the ETP encounter a large number of ligands for the Notch receptors as well as growth factors such as Kit-ligand and IL-7 which trigger and support the differentiation and proliferation of these cells in the initial stages of T Cell development (2). Moreover, the expression of Notch-1 receptors and their interaction with Delta-like ligands is essential for the differentiation of the T Cells in the thymus and for the inhibition of the non-T Cell lineage development (3).

Within the thymic cortex, ETP differentiate into double negative (DN) cells that do not express either CD4 or CD8 (i.e., $CD4^{-}$ and $CD8^{-}$). Some authors consider the ETP a DN1 cell that

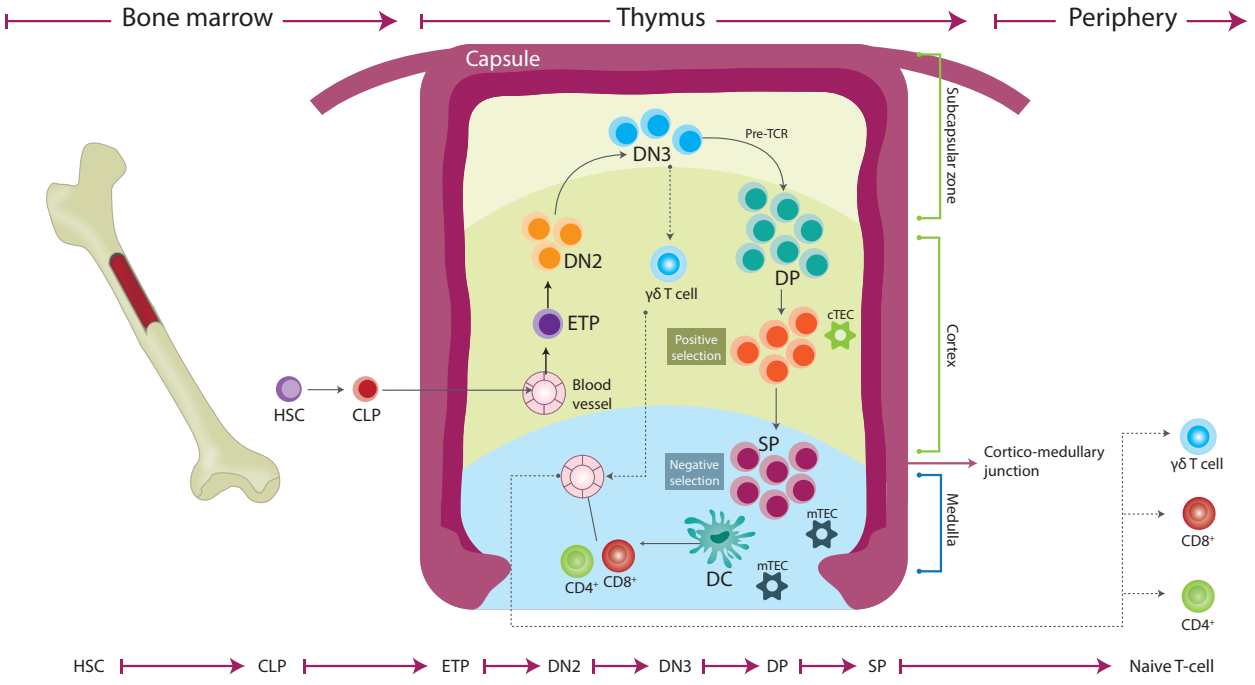

Figure 1. Overview of T Cell development and maturation. Adapted from Rothenberg *et al*. (4). Abbreviations. *HSC*: Haematopoietic stem cells, *CLP*: Common lymphoid progenitors, *ETP*: Early thymic progenitors, *DN*: Double negative; *DP*: Double positive, *SP*: Single positive, *DC*: Dendritic cells, *cTECs*: Cortical thymic epithelial cells, *mTECs*: Medullary thymic epithelial cells.

later differentiates into DN2 when it acquires the $CD25^+$ and $CD44^+$ receptors. At this stage of development, the cells lose the B potential and begin to express proteins that are critical for the subsequent T Cell receptor (TCR) gene rearrangement such as RAG1 and RAG2. They also begin to express proteins necessary for TCR assembly and signaling as CD3 chains, kinases, and phosphatases such as LCK, ZAP70, and LAT (4). DN3 cells can take two divergent routes of differentiation. A cell can either express the αβ chains of the TCR and follow the process of selection to generate $CD4^+$ or $CD8^+$ T Cells or express the γδ chains to generate a subpopulation of γδ lymphocytes with special functional characteristics (5,6) (Table 1).

The expression of the β chain of TCR, at the DN3 stage, cascades the simultaneous expression of the CD4 and CD8 molecules and thus, the cells convert into double positives (DP), which constitutes the largest population of cells in the thymus (4,7). At this stage of maturation, the DP cells enter a control point known as positive selection to select the cells with functional TCRs that bind to self-peptides with intermediate affinity and avidity. For this, the epithelial cells of the thymic cortex "*put* the DP *cells to the test*" by presenting their own peptides in the context of the class I (HLA-I) and class II (HLA-II) HLA molecules. Only a fraction (1%-5%) of the DP cells, that express a TCR with intermediate affinity for these Ags persists by survival signals. DP cells incapable of binding HLA-I or HLA-II undergo apoptosis. Positive selection allows the differentiation of the DP thymocytes towards *a single positive* (SP) population that is restricted to HLA (i.e., DP cells that recognize HLA-I differentiate into $CD4^-CD8^+$ and those that recognize HLA-II differentiate into $CD4^+CD8^-$) (8, 9). Subsequently, SP cells enter the medulla of the thymus where a second control point known as *negative selection* takes place. At the medulla, positively selected thymocytes are exposed to a diverse set of self-antigens presented by medullary thymic epithelial cells (mTEC) and DC. mTECs use a special epigenetic mechanism to give rise to what is often referred to as promiscuous gene expression which contributes to the low expression of many genes including tissue-restricted self-antigens. SP cells with a high affinity or avidity for binding self peptides presented on HLA-I or HLA-II are eliminated by apoptosis, thus assuring the destruction of potentially autoreactive cells (9). Cells that survive negative selection mature and become naïve T Cells given the fact that they have not been primed by Ag for which they express a specific TCR. Naïve T Cells leave the thymus and migrate continuously to the secondary lymphoid organs to be primed and differentiate into effector cells with specialized phenotypes.

T CELL RECEPTOR (TCR) COMPLEX

During the maturation process, T Cells acquire a receptor called TCR that recognizes a specific Ag. TCR is a multiprotein complex composed of two variable antigen-binding chains, αβ or γδ, which are associated with invariant accessory proteins (CD3γε, CD3δε, and CD247 ζζ chains) that are required for initiating signaling when TCR binds to an Ag (10).

CHARACTERISTIC	αβ T CELLS	γδ T CELLS
Antigen-receptor configuration	CD3 complex + αβ TCR	CD3 complex + γδ TCR
Theoretical receptor number	~10^{15}	~10^{20}
Antigen recognition	Peptide + MHC	Protein and non-protein
MHC restriction	Yes	Rare
Phenotype	CD4+ or CD8+	Most are CD4–CD8–; iIELs are CD8(αα)+
Frequency in blood	65–75%	1–5% (25–60% in gut)
Distribution	Blood and lymphoid tissues	Blood, epithelial and Blood and lymphoid tissues
Effector capability	CTLs (CD8+) and cytokine release	CTLs and cytokine release
Function	Immune protection and pathogen eradication	Immunoregulation and immunosurveillance

Table 1. Characteristics of αβ T cells and γδ T cells. *CTLs*, cytotoxic T lymphocytes; *iIELs*, intestinal intraepithelial T lymphocytes; TH cell; *TCR*, T Cell receptor. Adapted from Carding *et al.* (5).

The αβ-TCR does not recognize Ag in its natural form but recognizes linear peptides which have been processed and presented in the HLA-I or HLA-II context. The peptides presented by HLA-I molecules are small (8-10 aminoacids) and have an intracellular origin while those presented by HLA-II molecules are longer (13-25 aminoacids) and are generally of extracellular origin. Nevertheless, the αβ-TCR of NKT cells and the γδ-TCR can recognize glycolipids and phospholipids presented by CD1 molecules.

TCR α and β chains are very polymorphic, which favors the recognition of a great diversity of peptides. Each chain has a variable (V) and a constant domain (C) with a joining segment (J) that lies between them. The β chain also has an additional diversity segment (D). Each (V) domain has three hypervariable sectors known as CDR-1, -2, and -3 (*complementarity-determining regions)* and is capable of generating an inmense pool of combinations to produce different TCR specific for an Ag. CDR3α and β regions bind to the central region of the peptide presented. This region represents the most diverse region of the TCR and is considered to be the main determinant of specificity in Ag recognition. CDR1α and β also contribute to peptide recognition and bind to it through the amino and carboxy-terminal motifs respectively. TCR regions that come into contact with HLA mainly correspond to CDR-1 and CDR-2 (10). TCR associates with a molecule called CD3, which is composed of three different chains: gamma, delta, and epsilon (γ, δ, ε). These chains are associated as heterodimers γε and δε. TCR is also associated with a homodimer of ζ chains (CD247) that has a long intracytoplasmic portion and participates in the downstream transductional activation signals. Both the CD3 chains and the ζ chains that associate with TCR possess tyrosine-based activation motifs (ITAMs) in their intracytoplasmic moeities, which are phosphorylated to initiate T Cell activation (11).

TCR gene rearrangement is essential during T Cell development. Multiple gene segments dispersed in the genomic DNA must bind and transcribe to produce a functional TCR. This process occurs independently for each chain beginning with the recombination of genes for the β chain (12). Genes that code for the TCR chains in humans map to four *loci*: TCRA and TCRD on chromosome 14 and TCRB and TCRG on chromosome 7. The locus for the β chain has 42 gene segments for the region (V), 2 for (D), 12 for (J), and 2 for (C) while the locus for the α chain has 43 gene segments for the region (V) and 58 for (J) (13) (Figure 2). Somatic recombination of these gene segments occurs at the DN2 and DN3 stages of T Cell development and is mediated by the gene products RAG-1 and RAG-2. Nuclease and ligase activity, as well as the addition or elimination of nucleotides, generates the great variety of TCR present in our organism at the moment of birth. It is estimated that the diversity of TCR in humans may reach 2 x 10^7 (13).

ACTIVATION OF THE NAÏVE T CELLS

T Cell activation and differentiation will only be sucessfull if three signals are present: i) interaction of the TCR with the peptide presented by the HLA molecule, ii) signaling through co-stimulatory molecules, and iii) participation of cytokines that initiate clonal expansion (14).

Additionally, the cytokine microenvironment that accompanies the activation defines the type of response that will be generated later.

Ag recognition and signal transduction pathways in T Cells

Constant migration of the naïve T Cells towards the secondary lymphoid organs is essential in order for each one to encounter its specific Ag presented by an antigen-presenting cell (APC) (15). For this to occur, the naïve T Cells constitutively express L-selectin, an adhesion molecule which acts on the initial binding of T Cells to the high endothelial postcapillary venules located in the lymph nodes, tonsils, and aggregated lymphatic follicles. Only the specialized endothelial cells in the post-capillary veins allow constant passage of the T Cells from the blood towards the lymph nodes or Peyer's patches given that the latter two constitutively express the addressins PNAd *(peripheral node addressin*) or MAdCAM-1 (*mucosal addressin cell adhesion molecule-1*) respectively.

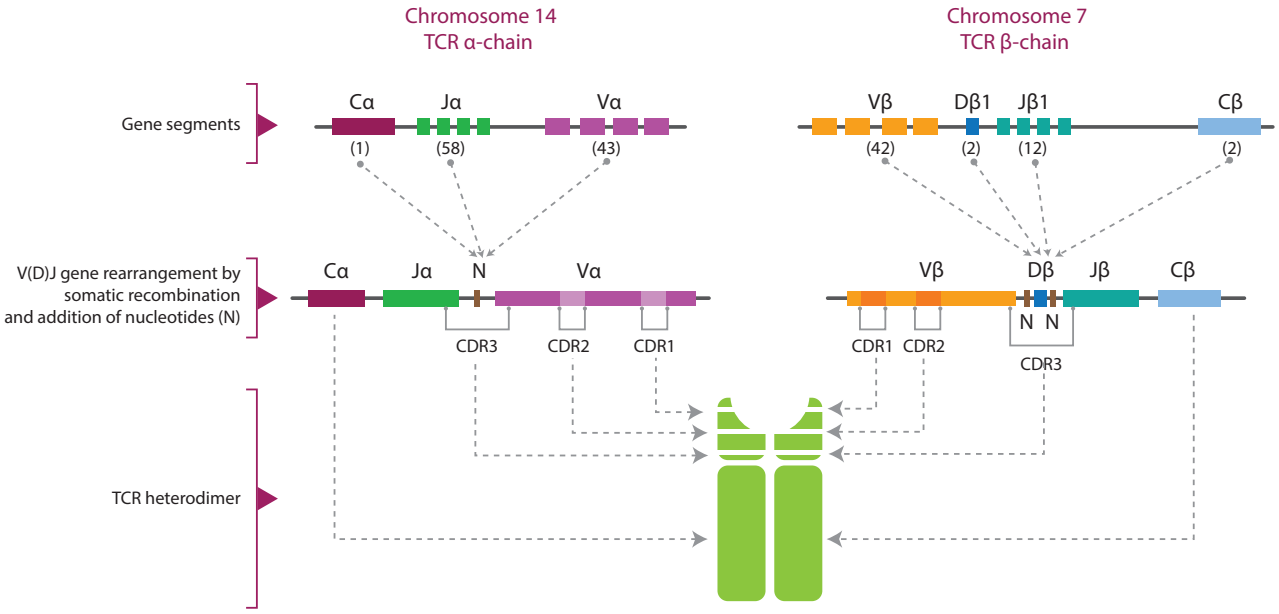

Figure 2. TCR generation by somatic recombination. Adapted from Turner *et al.* (13). Abbreviations. *TCR*: T Cell receptor; *C*: constant gene segment, *V*: variable gene segment, *D*: diversity gene segment, *J*: junctional gene segment, *N*: addition of non-template-encoded nucleotides, *CDR*: complementarity-determining regions, (#) Numbers inside the parenthesis represent de number of gene segments identified in the human TCR loci.

Both interact with the L-selectin of the lymphocytes. The endothelial cells of the rest of the vasculature restrict or impede binding of lymphocytes unless their receptors are induced by inflammation mediators (16).

In the lymph nodes, T Cells establish temporary contact with a great number of dendritic cells (DC) but only halt and bind to those which present an Ag which is compatible and specific to their receptor (15).

T Cells within lymph nodes migrate at high speeds of about 11-14 μm per minute. This is in contrast to DCs which transit through lymph nodes at speeds of about 3-6 μm per minute and then stop. This allows DCs to constantly establish new contacts with T Cells. In the absence of Ag, T Cells do not stop, but in the presence of an Ag, the duration of the interaction with the DC may be transitory (3 - 11 min) or stable (several hours) depending on the affinity for the Ag (15). Stable unions are favored by the high presence of peptides in the DC, highly antigenic ligands, mature DC, and expression of molecules such as ICAM-1 (15).

Antigen recognition by TCR induces the formation of several "*TCR microclusters*" that accompany the reorganization and approach of other membrane molecules and signaling proteins towards the contact zone with the DC. This contact zone between the T Cell and DC membranes is known as an *immunological synapse* and consists of a highly organized and dynamic molecular complex divided into three concentric zones known as the central, peripheral, and distal supramolecular activation clusters. The central region is composed of the TCR complex, co-stimulatory and co-inhibitory molecules, and co-receptors. These co-receptors are known as primary and secondary activation signals. The peripheral zone is mainly made up of the adhesion molecules LFA-1-ICAM-1 and CD2-LFA-3 that, due to their affinity, maintain and stabilize binding between the cells. The distal zone consists of F-actin and phosphatase CD45 (17).

After Ag recognition, a complex signaling process is initiated on the internal side of the membrane and in the cytoplasm for the subsequent activation of three essential transcription factors: NFAT, AP-1, and NF-κB. These signaling pathways are shown in a simplified diagram in Figure 3 and start when phosphatase CD45 activates the tyrosine-kinases, Fyn and Lck, which are associated with the ε chains of the CD3 and the co-receptors CD4/CD8 respectively (18). Once activated, these kinases autophosphorylate and phosphorylate the ITAM moieties of the ζ chains and CD3. Phosphorylated ITAMs attract the ZAP-70 molecule. Then, the binding of ZAP-70 to phospholipase C γ1 (PLC γ1) or LAT initiates two different cascades.

A first cascade is initiated when PLC-γ1 converts the phosphatidylinositol biphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). The IP3 diffuses into the cytoplasm and binds to the receptors of the endoplasmic reticulum, where it induces the release of Ca^{2+} deposits to the cytosol. The intracellular increase of Ca^{2+} stimulates the enzyme calmodulin, which is a serine/threonine-kinase. The activated calmodulin, in turn, activates calcineurin, a phosphatase that catalyzes the desphosphorylation of the nuclear transcription factor NF-AT to allow entry into the nucleus and activate the expression of several genes (e.g., IL-2, etc.) (19).

A second signaling cascade is initiated when ZAP-70 phosphorylates an adaptor protein known as LAT. LAT re-

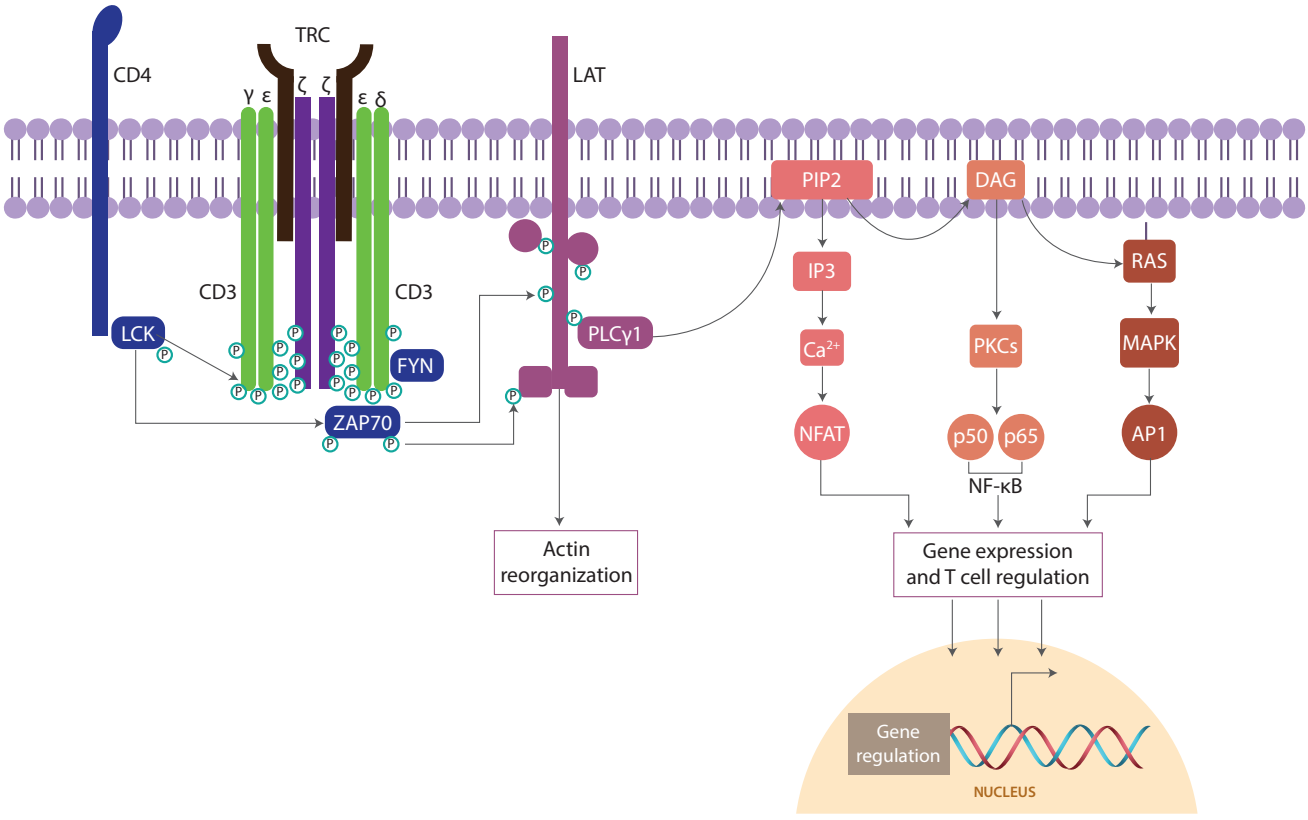

Figure 3. Overview of TCR signalling pathways. Adapted from Brownlie *et al.* (18). Abbreviations. *LCK*: lymphocyte-specific protein tyrosine kinase, *FYN*: a member of Src tyrosine kinases, *ZAP70*: ζ-chain associated protein kinase of 70 kDa, *LAT*: Linker for activation of T Cells, *PLCγ1*: Phospholipase C γ1, *PIP2*: phosphatidylinositol 4, 5-bisphosphate, *IP3*: Inositol trisphosphate, *NFAT*: Nuclear factor of activated T Cells, *DAG*: diacylglycerol, *PKC*: Protein kinase C, *NFkB*: nuclear factor kappa beta, *RAS*: family of GTP-binding proteins, *MAPK*: mitogen-activated protein kinase, *AP1*: activator protein 1 transcription factor.

cruits several proteins that allow transference of guanine nucleotides from GDP to GTP for the activation of some proteins called Ras. These initiate a cascade of phosphorylations resulting in the activation of mitogen activated protein kinases (MAPK). These MAPK are tasked with activating the transcription factor AP-1 which is composed of the proteins c-fos and c-jun. MAPKs allow dimerization of those proteins to initiate the transcription of genes (18).

The third signaling pathway is initiated with the production of DAG, which activates protein kinase C (PKC). Later, it gives rise to recruitment of the IKK complex which requires the proteins Carma1, Bcl10, and MALT1 for its activation. Activation of the IKK kinases permits the phosphorylation of the IκB inhibitors, which are then ubiquitinated and degraded. This releases NF-κB dimers that translocate to the nucleus and activate transcription of their target genes (20).

The transcription factors NF-AT, AP-1, and NF-κB enter the nucleus and induce the transcription of genes to initiate the secretion of IL-2; the expression of its high affinity alpha receptor (IL-2Rα); the expression of integrins that promote cellular adhesion; the expression of costimulatory molecules such as CD40L; and the expression of anti-apoptotic proteins (19, 20).

Co-stimulation

A co-stimulatory molecule is defined as a surface molecule that is not itself able to activate T Cells but which can significantly amplify or reduce the signaling induced by the TCR complex (21,22). The main T Cell co-stimulatory molecules and their respective ligands for the profesional and non-professional APC are shown in Table 2.

Positive co-stimulatory signals are known as the second activation signal and are indispensable for potentiating the production of IL-2 due to the induction of a sustained activation of the nuclear transcription factor NF-κB. Furthermore, interaction between these molecules initiates antiapoptotic signals that prolong T Cell life span and initiate the expression of adherence molecules as well as the production of growth factors and cytokines that promote their proliferation and differentiation.

Only CD28, CD27, and HVEM are expressed constitutively while the remaining co-stimulatory molecules are inducible and expressed only after activation. Constitutive co-stimulatory molecules have a positive regulatory effect (21, 22).

Although most of the co-stimulatory molecules have a monotypic binding (one ligand), some of them, e.g., CD28 and PD-1, interact with more than one ligand. Moreover, other molecules such as those of the SLAM family interact

T CELLS			ANTIGEN PRESENTING CELLS							
Professional							Non-professional			
Expression	Signal	Receptor	Ligand	DC	LB	Mac	End. cells	Epith. cells	Other*	Superfamily
Constitutive	Positive	CD28	B7-1(CD80), B7-2 (CD86)	+	+	+	(-)	(-)	(-)	CD28/B7
		CD27	CD70	+	+	+	(-)	+	+	TNF/TNFR
		HVEM	LIGHT	+	+	+	(-)	+	+	TNF/TNFR
Inducible	Positive	ICOS	ICOS-L	+	+	+	+	+	+	CD28/B7
		CD30	CD30L (CD153)	(-)	+	+	(-)	+	+	TNF/TNFR
		OX40 (CD134)	OX40L	+	+	+	+	(-)	+	TNF/TNFR
		4-1BB (CD137)	4-1BBL	+	+	+	(-)	(-)	+	TNF/TNFR
		SLAM (CD150)	SLAM (CD150)	+	+	+	(-)	(-)	(-)	Ig
Inducible	Negative	CTLA-4 (CD152)	B7-1(CD80), B7-2 (CD86)	+	+	+	(-)	(-)	(-)	CD28/B7
		PD-1	PD-L1, PD-L2	+	+	+	+	+	+	CD28/B7

Table 2. T Cell co-stimulatory molecules and their ligands. *Other cells include fibroblasts, myocytes, follicular dendritic cells, neurons and keratinocytes. '+' indicates positive function; '(–)' indicates negative function; *CTLA4*, cytotoxic T lymphocyte antigen 4; *HVEM*, herpes virus entry mediator; *ICOS*, inducible T cell co-stimulator; *PD1*, programed cell death 1; *SLAM*, signaling lymphocytic activation molecule. Adapted from Kroczek *et al*. (14).

homotypically with identical molecules. Almost all of the T Cell co-stimulatory molecules belong to the CD28/B7 superfamily or the TNF/TNFR family (21, 22).

There is a hierarchy in the downstream activation of these co-stimulatory molecules. For example, it has been observed that co-stimulation with CD28 significantly increases the induction of ICOS and OX-40 on the surface of the T Cell (22).

Clonal expansion

In response to antigen recognition and co-stimulatory signals, T Cells initiate the synthesis of IL-2 and express the high affinity receptor for it (IL2Rα or CD25) transitorily. CD25 binds to the other chains of the IL2R which are the β chain (CD122) and common γ chain (CD132). However, it does not participate in the signaling, but increases the affinity for IL-2 from 10 to 100 times (23).

IL-2 acts as an autocrine and paracrine growth factor. IL-2 activates blastogenesis or clonal expansion which gives rise to large numbers of T Cells with receptors identical to the original, able to recognize only the Ag that initiated its activation. IL-15 and IL-21 also participate in this process of clonal expansion (23).

T Cell activation and clonal expansion is followed by a death phase during which 90% of the effector cells are eliminated by apoptosis. The mechanisms which induce this phase of contraction or death include interactions Fas-FasL, TNF, and TNFR I and II as well as CD40-CD40L. In addition, molecules such as perforins, IFN-γ, and IL-2 regulate the contraction phase of the T Cells (24).

CD4⁺ T CELL SUBSETS

The differentiation of a CD4⁺ T Cell into distinct subpopulations or cell phenotypes is determined by the nature and concentration of the Ag, the type of APC and its activation state, the cytokine microenvironment that accompanies the antigenic presentation, and the presence and quantity of co-stimulatory molecules, along with other variables.

If the T Cell expresses CD4, it is converted into a T-helper cell (Th) which has a double function: to produce cytokines and to stimulate B Cells to generate Abs. Until recently, only four distinct phenotypes had been identified: Th1, Th2, Th17, and T-regulatory cells (Treg) each of which secretes a different cytokine profile. However, in the last few years, new T-helper subsets such as Th9, Th22, and follicular helpers (Tfh) have been identified. Figure 4 summarizes the main characteristics of these T Cell subsets, the factors that induce them, and the cytokines they produce.

Th1. The differentiation of the Th1 cells is induced by IL-12, IL-18, and type 1 IFNs (IFN-α and IFN-β) secreted by DC and macrophages after being activated by intracellular pathogens. IL-18 potentiates the action of IL-12 on the development of the Th1 phenotype. In general, the response mediated by Th1 depends on the T-bet transcription factor and the STAT4 molecule. These cells produce IFN-γ, IFN-α, IFN-β, and IL-2 and

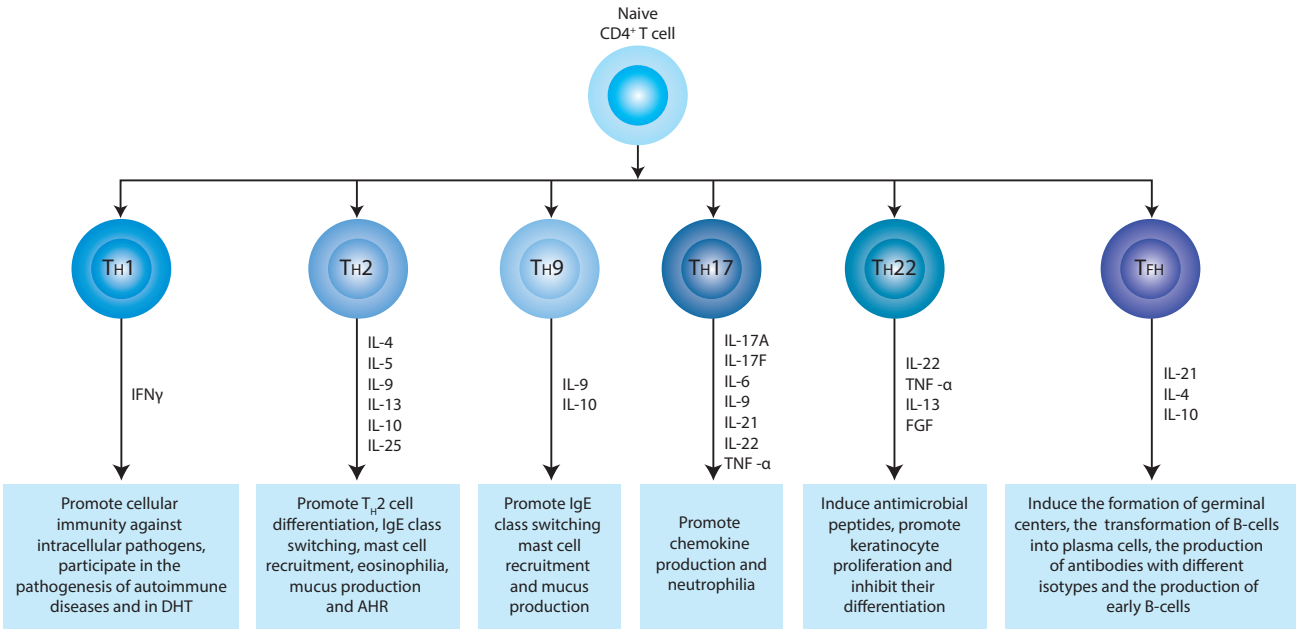

Figure 4. CD4 T Cell subsets. Adapted from Lloyd*et al*. (30). Abbreviations. *TH*: T-helper, *IFNγ*: interferon gamma, *DTH*: delayed type hypersensitivity, *TNF*: tumor necrosis factor, *FGF*: fibroblast growth factor, *AHR*: airway hyperresponsiveness.

express CXCR3 and CD161 (25). They stimulate strong cell immunity to intracellular pathogens as well as participate in the pathogenesis of the autoinmune diseases and in the development of delayed type hyper-sensitivity. In Th1 cells, the IL-2 increases the expression of T-bet and IL-12Rβ2 which then promotes the sustainability of this phenotype (23).

Th2. A Th2 response is induced by extracellular pathogens and allergens. It is generated by the effect of the IL-4, IL-25, IL-33, and IL-11 secreted by mast cells, eosinophils, and NKT cells. These cytokines induce the intracellular activation of STAT-6 and GATA-3, which initiates the secretion of cytokines of the Th2 phenotype such as IL-4, IL-5, IL-9, IL-13, IL-10, and IL-25, as well as, the expression of CCR4 and ICOS (26, 27). Th2 cells induce immunoglobulin class switching to IgE, through a mechanism mediated by IL-4. The IgE, in turn, activates cells of the innate immune system such as basophils and mast cells and induces their degranulation and the liberation of histamin, heparin, proteases, serotonin, cytokines, and chemokines. These molecules generate contraction of the smooth muscle, increase vascular permeability, and recruit more inflammatory cells. Th2 cells also migrate to the lung and intestinal tissue where they recruit eosinophils (through the secretion of IL-5) and mast cells (through IL-9). This leads to tissue eosinophilia and hyperplasia of mast cells. When acting upon epithelial cells and the smooth muscle (through IL-4 and IL-13), the Th2 cells induce production of mucus, metaplasia of the Goblet cells, and airway hyper-responsiveness as observed in allergic diseases (26). In the Th2 cells, IL-2 induces the expression of IL-4Rα and keeps the loci of the IL-4 and IL-13 genes in an accessible configuration during the final stages of the differentiation of these cells, which helps to conserve this phenotype (23).

Th9. This subset of T-helper cells arises through the effect of TGF-β and IL-4. Th9 cells produce IL-9 and IL-10 and do not express cytokines or transcription factors of the Th1, Th2, or Th17 subsets (28). IL-9 promotes the growth of mast cells and the secretion of IL-1β, IL-6, IL-13, and TGF-β. Nevertheless, IL-9 is not exclusive to this cell subpopulation. It is also produced by Th2, Th17, Treg, mast cells, and NKT cells (29). In allergic processes and infections by helminthes, the IL-9 stimulates the liberation of mast cell products and, through IL-13 and IL-5, indirectly induces the production of mucus, eosinophilia, hyperplasia of the epithelium, and muscular contraction (30).

Th17. These cells are induced by the combined action of IL-6, IL-21, IL-23, and TGF-β. The IL-6 activates the naïve T Cell resulting in the autocrine production of IL-21 which in synergy with TGF-β induces the nuclear transcription factor (ROR)c and the production of IL-17A and IL-17F. IL-23 is essential for the survival and activation of Th17 after its differentiation and selectively regulates the expression of IL-17 (31).

The Th17 cells are mainly located in the pulmonary and digestive mucosa. They produce IL-17A, IL-17F, IL-6, IL-9, IL-21, IL-22, TNF-α, and CCL20. IL-17, in synergy with TNF-α, promotes the expression of genes that amplify the inflammatory process. IL-17 binds to its receptor in mesenchymatous cells such as fibroblasts, epithelial cells, and endothelial cells to promote the liberation of chemokines and inflammation mediators such as IL-8, MCP-1, G-CSF,

and GM-CSF (31). IL-17 and IL-22 also induce the production of defensins. The inflammatory environment generated by Th17 cells is associated with diseases that have an important inflammatory component such as rheumatoid arthritis, systemic lupus erythematosus (SLE), bronchial asthma, and transplant rejection (32).

Th22. This T Cell subset is generated by the combined action of the IL-6 and TNF-α with the participation of plasmacytoid DC. Th22 cells are characterized by the secretion of IL-22 and TNF-α. The transcripcional profile of these cells also includes genes that encode for FGF (*fibroblast growth factor*), IL-13, and chemokines implicated in angiogenesis and fibrosis. The main transcription factor associated with this phenotype is AHR. In the skin, IL-22 induces antimicrobial peptides, promotes the proliferation of keratinocytes, and inhibits their differentiation which suggests a role in the scarring of wounds and in natural defence mechanisms (33). The Th22 cells express CCR4, CCR6, and CCR10 which allows them to infiltrate the epidermis in individuals with inflammatory skin disorders. They participate in Crohn's disease, psoriasis, and the scarring of wounds (34).

Follicular helper T Cells (Tfh). These cells were discovered just over a decade ago as germinal center T Cells that help B Cells to produce antibodies. The development of these cells depends on IL-6, IL-12, and IL-21. They are characterized by the sustained expression of CXCR5 and the loss of CCR7, which allows Tfh cells to relocate from the T Cell zone to the B Cell follicles that express CXCL13. There, they induce the formation of germinal centers, the transformation of B Cells into plasma cells, the production of antibodies with different isotypes, and the production of memory B Cells (35).

Among all the T-helper cell subsets, the Tfh express the TCR with the highest affinity for Ag and the greatest quantity of costimulatory molecules such as ICOS and CD40L. Furthermore, they express the transcription factor BCL-6 and cytokines such as IL-21, IL-4, and IL-10 which induce the differentiation of B Cells and the production of Ab (35).

Regulatory T Cells (Treg). Regulatory T Cells represent 5% to 10% of CD4+ T Cells in healthy adults. They constitutively express markers of activation such as CTLA-4 (CD152), α receptor of IL-2 (CD25), OX-40, and L-selectin (36). These are considered anergic in the absense of IL-2 which makes them dependent on the IL-2 secreted by other cells. By their mechanism of action and origin, they represent a heterogenous population of cells that can be divided into two: natural Treg cells of thymic origin and induced Treg cells differentiated on the periphery (37).

The natural Treg cells are $CD4^{+}CD25^{high}$ and constitutively express the transcription factor $FOXP3^{+}$ which is essential for their development. The $CD4^{+}CD25^{-}FOXP3^{-}$ cells can differentiate into Treg cells in the presence of IL-10 and TGF-β and for interaction with immature DC. In contrast, the differentiation of Treg cells is inhibited when mature DC produces IL-6.

The production of Treg cells is essential in preventing autoimmune diseases and avoiding prolonged immunopathological processes and allergies. They are also essential for inducing tolerance to allogenic transplants as well

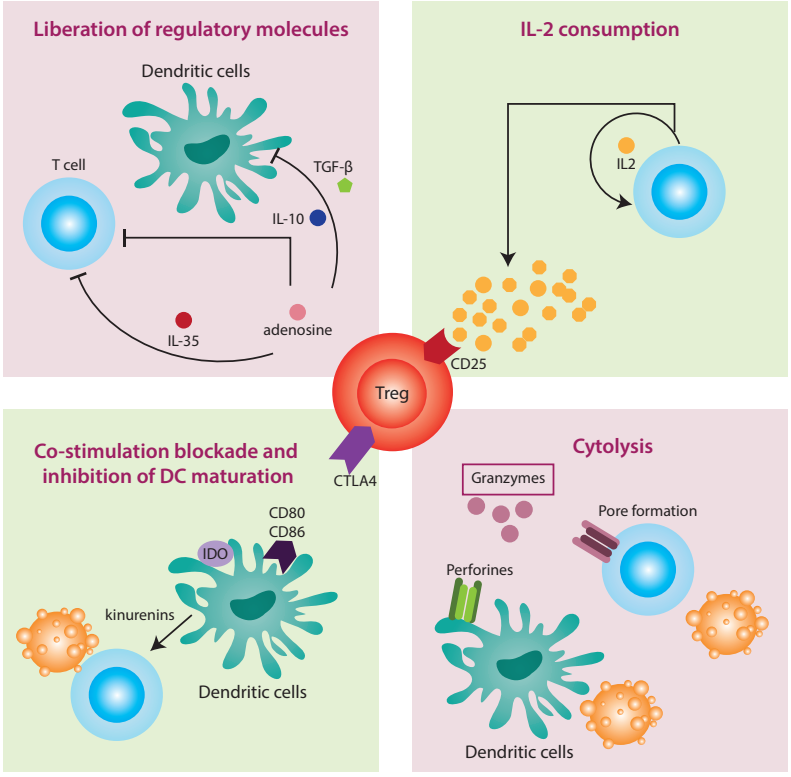

Figure 5. Mechanisms of action of T regulatory cells. Abbreviations. *TGFβ*: Transforming growth factor beta, *CTLA4*: cytotoxic T-lymphocyte antigen 4.

as tolerance of the foetus during pregnancy. They supress the activation, proliferation, and effector function of a wide range of immune cells including autoreactive CD4 or CD8 T Cells which escape negative selection in the thymus, NK cells, NKT, LB, and APC. Like a double-edged sword, Treg cells also supress antitumoral responses, which favors tumor development (37).

Action mechanisms of Treg cells are depicted in Figure 5. These mechanisms can be broadly divided into those that target T Cells (regulatory cytokines, IL-2 consumption, and cytolysis) and those that primarily target APCs (decreased co-stimulation or decreased antigen presentation). Major mechanisms by which Treg cells exert their functions include (36, 38):

- Liberation of regulatory molecules such as IL-10, TGF-β, IL-35, and adenosine which inhibit the secretion of specific cytokines of Th1, Th2, and Th17 phenotypes.
- Liberation of granzymes and perforins that induce apoptosis of the effector cell.
- Competition for IL-2. Treg cells do not produce IL-2, but they express high CD25, the IL-2 receptor α chain, and have the ability to compete with effector T Cells for IL-2. This results in a state of privation of proliferation signals of the effector cells and apoptosis.
- Downregulation of APC maduration and co-stimulatory function. CTLA-4 on the surface of Treg cells downregulates or prevents the upregulation of CD80 and CD86, the major costimulatory molecules on antigen-presenting cells.

Additionally, Treg cells induce the production of an enzyme in the DC called IDO (*indolamine deoxygenase*) that degrades tryptophan, transforming it into proapoptotic metabolites called kynurinines.

- Transference of cAMP by gap junctions, which exert immonosuppressive functions.

The clinical importance of these cells is shown in patients with mutations of the FOXP3 gene who develop an immunodeficiency linked to chromosome X which presents with pluriglandular and intestinal manifestations known as IPEX. This autoimmune multiorgan disease requires a bone marrow transplant in early infancy (37).

CD8⁺ T CELLS

When a CD8⁺ T Cell develops its effector functions, it is converted into a cytotoxic T Cell able to attack cells directly and destroy those that are malignant or infected with virus (39). In order to exert this function, a cytotoxic T Cell induces apoptosis in its target cells by the liberation of cytolytic granules or by the expression of ligands for death receptors such as FasL (CD95) (40).

The cytolytic granules contain pore-forming proteins called perforins or cytolysins, proteases known as granzymes or fragmentins, granulolysins which participate in the degradation of membrane lipids, inhibitors of perforins that protect the cytotoxic T Cell from autolysis (calreticulin, catepsin G), and FasL.

Once the immunological synapse between the cytotoxic T Cell and the target cell has been established, the content of these granules is liberated. Perforins polymerize in the plasma membrane and produce pores, which act as channels that allow water entry and generate an osmotic disequilibrium in the cell. Furthermore, they facilitate the passage of granzymes to the cytosol and to the nucleus of the target cell which favors their proteolytic action on the mitochondria and fragmentation of the DNA (41).

Cytotoxic T Cells also liberate IFN-γ and TNF-α, which are important in the defence against viral infections and in controlling the proliferation of tumoral cells (39).

B LYMPHOCYTES (B CELLS)

ONTOGENY

The first stages of B Cell development take place in complex microenvironments created by the stromal cells of the bone marrow known as "niches" from which come the stimuli and factors required to initiate a series of cell signals. These, in turn, activate transcription factors that induce, or repress, the expression of different target genes that modulate cell survival, proliferation, and differentiation. IL-7 is critical to the development of the B Cells and is produced by the cells of the stroma.

Thus, and as shown in Figure 6, the development of the B Cells initiates from a haematopoietic stem cell (HSC). This transforms into an early lymphoid progenitor (ELP) and, then, becomes a common lymphoid progenitor (CLP) from which is derived, on one hand, natural killer cells (NK) and dendritic cells (DC) and, on the other, common lymphoid progenitor-2 (CLP-2), which is responsible for the B Cell lineage. This is considered the first stage of the immature B Cells (42-44). A prerrequisite for the development of the B Cells in bone marrow is the absence or supression of protein Notch-1 (N1) signaling, which is necessary for T Cell development (45).

During the differentiation of the B Cells, a process of gene recombination is structured initially that codes for segments V (*Variable*), D (*Diversity*), and J (*Joining*) of the heavy chain (chain H) together with that of the genes for segments V and J of the light chain (chain L) of the membrane-bound immunoglobulin (mIg). This recombination process is initiated by the complex of proteins RAG1- RAG2 that generate the rupture of the double chain of DNA between segments of genes and specific recognition sites that are also known as "*recombination signal sequences*." This process leads to the generation of B Cells that express a wide repertoire of mIg. This will form the B Cell receptor (BCR) which is able to recognize more than 5×10^{13} different Ags (44,46).

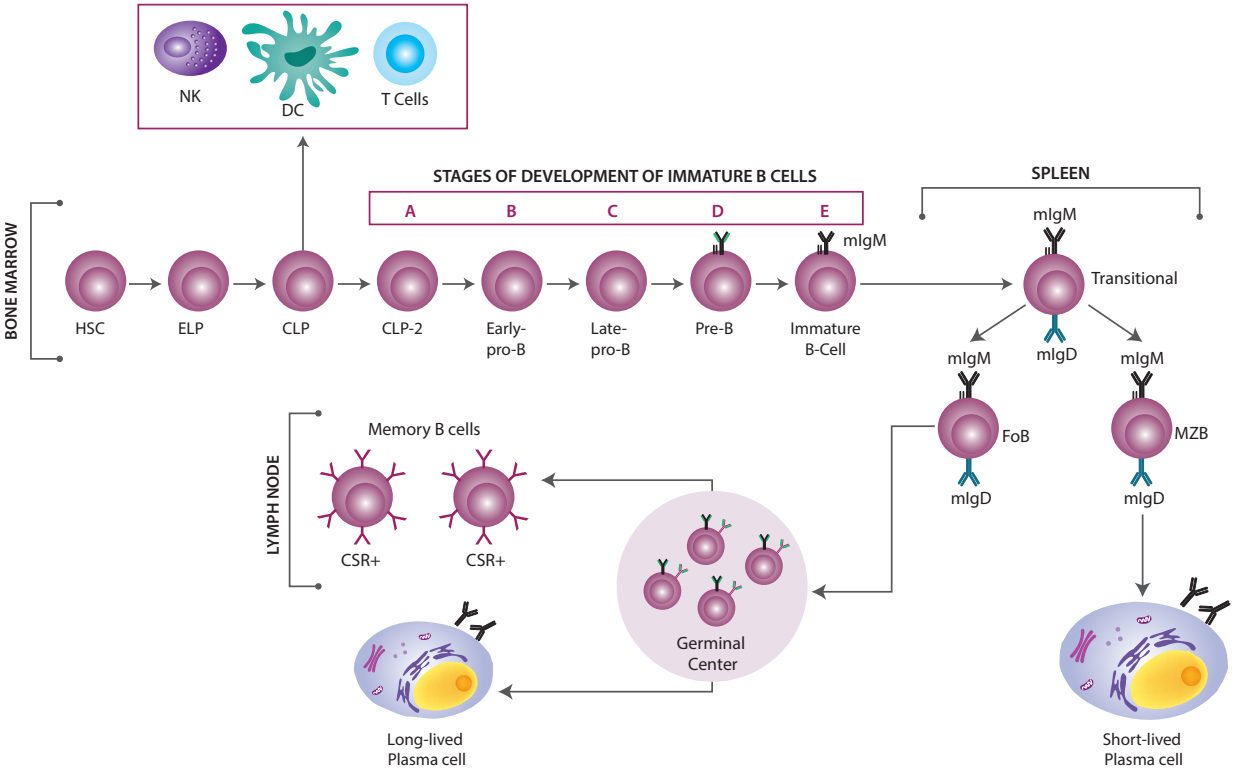

Figure 6. B Cell development and B Cell subsets (see text for details). Abbreviations. *HSC*: haematopoietic stem cell, *ELP*: early lymphoid progenitor *CLP*: common lymphoid progenitor, *CLP-2*: common lymphoid progenitor-2, *NK*: Natural Killer cell, *DC*: Dendritic Cell, *CSR*: class-switch recombination, *mIgM*: membrane-bound immunoglobulin M, *mIgD*: membrane-bound immunoglobulin D, *FoB*: follicular B Cells, *MZB*: marginal zone B Cells.

Allelic Exclusion: During its development, the B Cell generates a wide diversity of BCR for the gene recombination process mentioned above. Although each cell has many allelic *loci* for the different BCR chains (two *loci* for the heavy chain and multiple loci for the light chain), each mature cell eventually expresses only a single type of receptor. This is achieved by restricting the gene expression of the BCR of a single allele in a process known as allelic exclusion, which involves a monoallelic activation and feedback inhibition (46).

The expression of *pre-BCR* is an important control point for the recombination of the heavy chain. Its product Igμ associates with a *surrogate light chain* (SLC), a heterodimer composed of two germline-encoded invariant proteins (VpreB and λ5) to thus produce the molecular complex known as pre-BCR in the B Cell precursors (Figure 7 Panel A) (44,46). In the absence of Ag, signaling through the pre-BCR occurs by the cross-reaction between the positively charged arginine residues present in the λ5-region of the SLC and negatively charged molecules in the stromal cells (47). Once this pre-BCR is expressed on the cell surface, it generates a signal that induces proliferation of the pre-B cells and, as a result, significantly increases their number and guarantees that they successfully carry out the heavy chain recombination. Furthermore, signaling through the pre-BCR is implicated in activation of the gene recombination for the light chain and is thus required for B Cell differentiation to continue (46). With respect to this, it is known that pre-BCR plays an essential role in controlling the development of determined secretory cells of auto-Abs and may represent an important factor in multifactorial autoimmune diseases (47, 48).

Based on the differential expression of a complex of surface markers, five stages (A, B, C, D and E) have been described for the development of immature B Cells that occur inside bone marrow (Figure 6). These stages are as follows: CLP-2 corresponds to stage A with expression of $B220^+$, KIT^-, $CD19^-$, $FLT3^+$, $CD24^{low/-}$; $CD43^+$, IgM^-; stage B corresponds to early Pro-B cell with expression of $B220^+$, KIT^+, $CD10^+$, $CD19^+$, $CD24^+$, $CD43^+$, $FLT3^-$, IgM^-; stage C to late Pro-B cell which, furthermore, expresses $BP1^+$. During stage D, the immature B Cell expresses a pre-receptor B (Igμ+SLC λ5 and VpreB) which converts the cell into Pre-B with expression of $B220^+$, $CD19^+$, $CD24^+$, CD25+, and CD43; and finally, stage E corresponds to the B immature cells with expression of $B220^+$, $CD19^+$, $CD24^+$, $CD43^-$, and IgM^+, which emerge from bone marrow and are guided towards the secondary lymphoid organs (spleen, lymphonodules, Peyer's patches, tonsils, and mucosal tissue) to continue their differentiation into transitional B Cells (type 1 and type 2). These, through the effect of molecules such as BAFF and APRIL, differentiate into *marginal zone B Cells* (MZB) or enter the germinal centers and

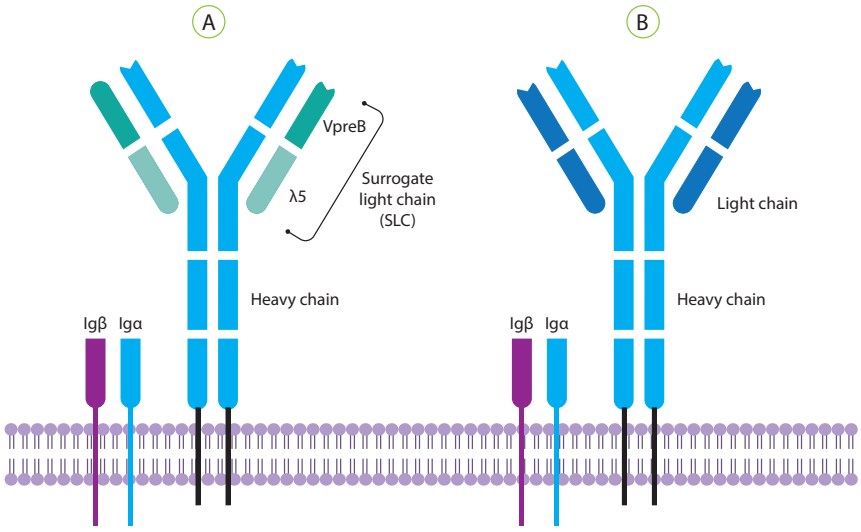

Figure 7. A) pre-B Cell receptor. B) B cell receptor. Abbreviations. *Igα*: Accesory Immunoglobulin alpha (CD79a), *Igβ*: Accesory Immunoglobulin beta (CD79b).

transform into *follicular B Cells* (FoB). Later, depending on the Ag stimuli they receive and the microenvironment of cytokines that surrounds them, each of these cells transforms into a *plasma Ab-secretory cell or memory B Cells* (42,44,49).

FORMATION OF THE GERMINAL CENTERS

Homing of B Cells in the spleen is regulated by expression of chemokines such as CCL21, CCL19, and CXCL13 produced by follicular dendritic cells (FDCs). These facilitate the movement of B Cells to the marginal zone or the follicules thereby giving rise to the formation of the germinal centers (GC). A GC is considered to be a specialized microenvironment of lymphoid tissue where an intense cell proliferation, somatic hypermutation, and selection by antigenic affinity occur. Here, the early development of B Cell differentiation is completed and cells undergo apoptosis (50).

During this process, Tfh cells activate the B Cells, which proliferate and create the first part of a germinal center within the follicle. At this stage, somatic hypermutation occurs (this is dependent on proliferation and the microenvironment of the germinal center although the exact factors that induce it are unknown). This process generates a progeny of B Cells with distinct receptors (almost identical but mutated in the variable zones). Some of these receptors do not recognize the presented Ag while others do with greater avidity. Where this maturation is happening, there are Ag-presenting DCs, and those B Cells that have increased their affinity for the Ag recognize it avidly and remain bound. This interaction is known as "*maturation by affinity*."

While the former happens, there is a change of BCR isotype through a process known as *class-switch recombination* (CSR), for which an intrachromosomal deletional rearrangement produces a change from the Cμ chain (which codes the constant region of IgM) to Cγ, Cα or Cε, encoding the constant regions of IgG, IgA, and IgE respectively (51). Finally, different B Cells are generated with a BCR that has a specific isotype and a modified affinity for and exit to peripheral circulation. Some of these cells convert into plasma cells and go to the bone marrow (increasing the Igs) while others remain within the same lymphoid organ. Many other B Cells become memory B Cells. The germinal center forms at least one week after contact with the Ag (50).

B CELL RECEPTOR (BCR)

The BCR is a macromolecular complex that is built in the membrane by *IgM/ IgD* with two additional *Ig* accessories denominated *Igα* (CD79a) and *Igβ* (CD79b) (Figure 7, Panel B). The membrane-bound immunoglobulins (mIgs) are glycoproteins with a basic monomer. Each of these is made up of four polypeptidic chains of which two are *heavy or H chains* with a molecular weight of approximately 65 kDa, and the others are *light or L chains* with a molecular weight of 25 kDa. Each L chain is linked to an H chain by a disulphur bridge. The H chains are linked to each other by at least one other disulphur bridge. These IgM or IgD monomers correspond to the extracellular segments of the BCR. However, the mIg also have another two segments, the transmembranal and the intracytoplasmic, which result from an extension of the carboxy-terminal portion of the two H chains. The two V domains, which form the active sites allowing each BCR to bind specifically to an antigenic determinant, are found in the amino-terminal (H and L) portions of each peptide chain of the BCR. The antigen-BCR interaction is a non-covalent reaction (52). The intracytoplasmic region of mIg, which presents only 3 aminoacids (lysine-valine-lysine), is very small and does not permit mIg to carry out the signaling process *per se*. The transmembranal segment of the C-terminal portion of the H chain of mIg consists of 25 aminoacids found close to the tyrosine kinase (PTK) enzymes which, in turn, are close

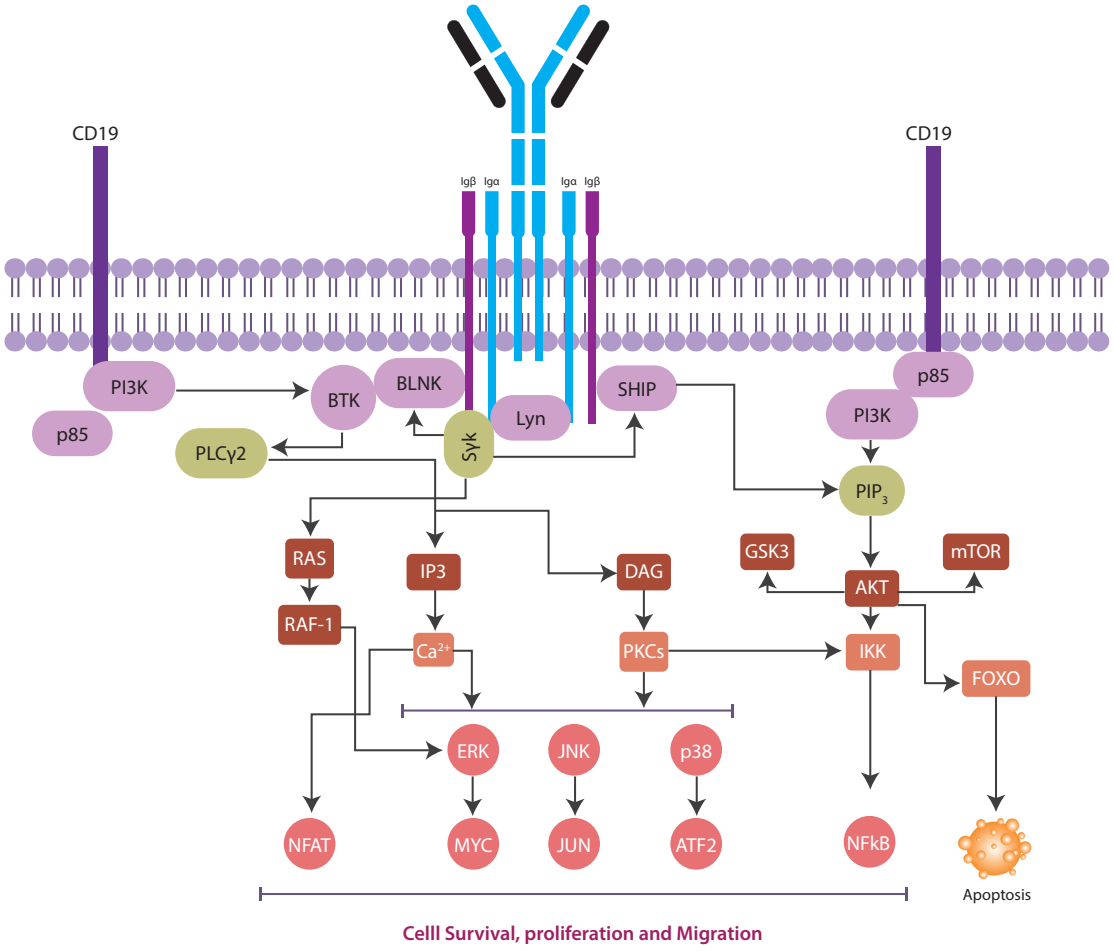

Figure 8. B Cell receptor signaling mechanisms. Abbreviations. *Lyn*: Tyrosine-protein kinase Lyn, *SHIP*: 5' phospholipid phosphatase SHIP-2, *Syk*: Spleen tyrosine kinase, *BLNK*: B Cell linker protein, *BTK*: Bruton kinase of Tec family, *PLCγ2*: phospholipase C gamma - 2, *PI3K*: phosphatidylinositol 3-kinase, *IP3*: Inositol 1,4,5-triphosphate, *DAG*: diacylglycerol, *GSK3*: Glycogen synthase kinase 3, *AKT*: a serine/threonine-specific protein kinase, *PKC*: Protein kinase C, *IKK*: inhibitor of IκB kinase, *PIP3*: phosphatidylinositol-3,4,5-triphosphate, *mTOR*: Mammalian target of rapamycin, is a serine/threonine kinase, *FoxO*: Forkhead box O transcription factor, *ERK*: Extracellular signal-regulated kinases, *JNK*: c-Jun N-terminal kinases, *NFAT*: transcription factor (nuclear factor of activated T cells), *MYC*: transcription factor, *ATF2*: Activating transcription factor 2, *NF-κB*: Nuclear factor-kappa B, transcription factor.

to the heterodimers (Igα and Igβ). The latter are responsible for the signaling process since they can provide the substrate for the tyrosinases in their ITAM regions (52).

SIGNALING MECHANISMS

Stimulation of the B Cells via antigenic BCR begins with the recognition and capture of the Ag through BCR molecules. This induces their aggregation and triggers the signaling process by activating the SRC family kinase (SFK) which then phosphorylates the ITAM moieties of the accessory chains Igα and Igβ. These carry out the same function as the ζ chain (CD247) to activate the TCR and produce lipid-raft-associated calcium-signaling module forms (Figure 8). This complex contains 3 classes of activated protein tyrosine kinases (PTKs): i) Lyn, Fyn, and Blk of the Src family; ii) Syk/ZAP70; and iii) of Bruton (Btk) of the Tec family

This initiates the formation of a macromolecular complex known as 'signalosome' composed of the BCR, the tyrosine kinases already mentioned, some adaptor proteins such as CD19 and BLNK (*B Cell linker protein*), signaling enzymes such as the phospholipase C gamma - 2 (PLCγ2), the phosphatidylinositol 3-kinase (PI3K), and molecules of the Vav family.

The signaling produced by the signalosome activates multiple signaling cascades which implicate other kinases, GTPases, and transcription factors such as NF-kB, Bcl6, NF-AT, FoxO, Jun, and ATF-2, etc. In order to study these signaling routes in greater detail, our recommendation is to consult the following web page: http://www.cellsignal.com/reference/pathway/B_Cell_Antigen.html

The activation of all these mechanisms gives rise to changes in cell metabolism, gene expression, and the organization of the cell cytoskeleton. The result of the response is determined by different factors or conditions such as: the

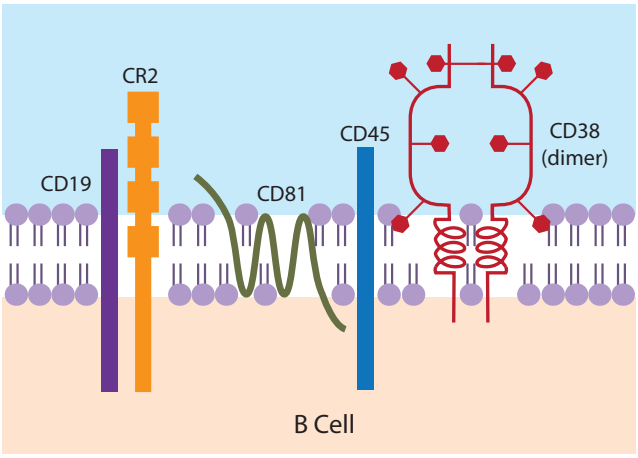

Figure 9. B Cell co-receptors. Abbreviations. *CD*: Cluster of differentiation, *CR2*: Complement receptor-2.

state of maturation of the B Cells, the nature of the Ag, the magnitude and duration of signaling through the BCR, and the signals of other receptors such as CD40, the receptor of IL-21, and BAFF-R. Thus, many other transmembranal proteins such as CD45, CD19, CD22, PIR-B, and FcγRIIB1 (CD32) modulate specific elements of the signaling BCR. During the *in vivo* processes, the B Cells are also activated by APCs, which capture and present the antigenic fractions on their cell surfaces. This type of B Cell activation by such cell membrane–associated Ags also requires a reorganization of the B Cell cytoskeleton. Thus, it is to be expected that the complexity of the signaling mediated by the BCR allows diverse biological effects to occur, including cell survival, tolerance, or apoptosis as well as proliferation and differentiation in Ab-producing cells or memory B Cells. (http://www.cellsignal.com/reference/pathway/B_Cell_Antigen.html) (53, 54).

CO-RECEPTORS OF THE BCR

During B Cell activation, another series of molecules participates to build a molecular complex that acts as a BCR co-receptor. These molecules can significantly increase the signaling Induced initially by the BCR and include: CR2 (CD21), CD19, CD45, CD38, and CD81 (Figure 9) (44, 55). The binding of CD21 with the Ags which are found to be opsonized with the complement fraction C3d facilitates grouping of the co-receptor with the BCR. The position of the CD21 allows the associated kinases to phosphorylate the tyrosine residues in the cytoplasmic domain of the CD19, and this binds to the tyrosine-kinases of the Src and the PI3-kinase family.

CO-STIMULATORY MOLECULES

Another important aspect in the activation of the B Cells is the presence of molecules which positively or negatively regulate the process. Together these are known as co-stimulatory molecules and some of them are described as follows.

B cell activating factor (BAFF) is a cytokine and member of the TNF family. It is produced by a wide variety of cells (neutrophils, monocytes, macrophages, DCs, and T Cells) (49). It is essential for the maturation and survival of the B Cells since it participates in the processes of differentiation and proliferation. To date, three BAFF receptors have been identified: i) BAFF-R, ii) TACI (*transmembrane activator, calcium modulator, and cyclophilin ligand interactor*), and iii) BCMA (*B Cell maturation factor*). Interaction with the BAFF-R is the most critical to making maturation possible and causing BAFF-R to act in combination with the BCR (44, 49). Elevated levels of BAFF have been observed in the sera of patients with SLE, Sjögren's Syndrome (SS), and rheumatoid arthritis (RA) (49).

APRIL (*A proliferation-inducing ligand*) is a BAFF homologue that binds to TACI and BCMA but does not interact with BAFF-R. In addition to its co-stimulatory function, APRIL improves the ability of B Cells to present Ag and to increase their survival time and also regulates tolerance. On the other hand, it promotes the proliferation and survival of malignant B Cells and other tumoral cells. As for BAFF, elevated levels of APRIL have been observed in the sera of patients with SLE (56).

CD40, a transmembranal glycoprotein type I receptor, belongs to the TNF receptor superfamily. It is expressed on a great variety of cells (e.g., monocytes, Ag-presenting cells, endothelial cells, smooth muscle, fibroblasts, keratinocytes, and platelets) and in all the stages of the B Cells. This receptor N-terminal extracellular domain includes several cysteines, has four subregions, and binds to its ligand (CD40L or CD154) through the 2nd and 3rd subregion. The interaction between the CD40 and its ligand, which is present in the T Cells, increases the expression of cytokines (IL-2, IL-6, IL-10, TNF-α, Lymphotoxin-α, and TGF-β), chemokines, metalloproteinases of the matrix, growth factors, and adhesion molecules. This allows signaling processes to occur through activation of several protein tyrosine kinases (PTK) such as ERK-1, ERK-2, p38, and JNK. This, in turn, permits the activation of several transcription factors such as NF-kB, AP-1, and NF-AT. These processes lead to maturation, differentiation, and cell proliferation of the B Cells with the subsequent production of Abs and, finally, the production of memory B Cells. Additionally, mutations in this molecule, or in its ligand, are responsible for the syndrome of hyperIgM linked to chromosome X. Although this CD40-CD154 interaction mediates many mechanisms of the humoral and cell immune responses, it is also implicated in a wide spectrum of chronic inflammatory and autoimmune diseases. The blockage of this signaling route is therefore considered to be a potential therapeutic mechanism for these pathologies (57).

Other co-stimulatory molecules: Additionally, and as is detailed in Table 2 , the B Cell expresses another series of molecules from the superfamilies CD28/B7 and TNF/TNFR

which interact directly with the T Cells as co-stimulatory molecules. Included among these are B7-1 (CD80), B7-2 (CD86), CD70, the ICOS ligand (ICOS-L), the CD30 ligand (CD30-L or CD153), the 4-1BB ligand (4-1BBL), SLAM (CD150), etc. (21).

MOLECULES OF ADHESION

B -cell mobilization requires adhesion mechanisms in which several types of molecules participate such as *chemokines*, *their receptors*, *the selectins*, and *integrins*.

Lymphoid chemokines are a group of chemokines/receptors that are expressed constitutively in the lymphoid tissue cells and aid in the recirculation of the lymphocytes. They include CXCL12, CXCL13, CCL19, CCL21, CXCR5, CCR7, and CXCR4 (16). The differential positioning of the B Cells in the GC or in the external part of the follicules is regulated by the EBI2 receptor coupled to protein G (also known as GPR183) which directs the B Cells to the perifollicular and interfollicular areas. This location of the B Cells mediated by EBI2 is important for the first stages of the Ab response (16). Molecules such as S1P (*Sphingosine 1 Phosphate*) and its S1PR1 receptor, in turn, control the exit of the B Cells from the lymphonodules (16, 58).

The Ab-secretory cells express high levels of integrins such as α4β1 and LFA-1 as well as ICAM-1, α5β1, and α6β1. The plasma cells in the intestine and the mammary glands express α4β7 (59, 60).

The selectins are another family of molecules that contribute to the adhesion and mobilization of the B Cells. The plasmocytes have a high expression of PSGL-1 (*P selectin glycol-protein ligand 1*) which recognizes the P and the E selectin (59). Another molecule that participates in the B Cell homing processes is the CD22, a member of the Siglec (*sialic acid-binding immunoglobulin-like lectin*) family which preferentially binds sugars with α2,6 -sialic acid radicals and is mainly present in mature B Cells (59).

B CELL SUBSETS

Mature B Cells can be divided into several subsets based on their location, cell surface phenotype, Ag specificity, and activation routes.

The transitional B Cells are considered to be the first stages of development of the B Cells once they leave the bone marrow to migrate to the secondary lymphoid organs. The lymphocytes $CD20^{+}$, $CD21^{\pm}$, $CD23^{\pm}$, IgM^{++}, and $IgD^{\pm}$ $CD38^{++}$ are designated B Cell transitional type-1 (T1) and differentiate from type 2 ($CD20^{+}$, $CD21^{++}$, $CD23^{\pm}$, IgM^{++}, and IgD++CD38±). The transitional B Cells T2 can evolve intomarginal zone B Cells or GC (44,61,62).

The Follicular B Cells (FoB) or B-2. These are generated directly in the bone marrow and reach the follicules of secondary lymphoid organs and the circulation. They are considered to be resting (naïve) cells and constitute the largest subpopulation of B Cells. Their differentiation is influenced by a great variety of factors including chemokines, BCR signaling, and some Ags. They participate in T-dependent (TD) immune responses since they can use the BCR to engulf the Ag, process it, and present it to the Ag-specific T Cells (63).

The Marginal zone B Cells (MZB). This type of cell is located as a sentinel in the marginal zones of the spleen which correspond to the interphase between the circulation and the splenic lymphoid tissue. These B Cells also inhabit the inner wall of the subcapsular sinus of the lymph nodes, the epithelium of tonsillar crypts, and the subepithelial dome of intestinal Peyer's patches. In humans, they present the following phenotype: $IgM^{high}IgD^{low}CD1c^{+}$ $CD21^{high}CD23^{-}CD27^{+}$. MZB express high levels of TLR (similar to macrophages, DCs, and granulocytes) phenomena that allow them to play a role of a bridge between innate and adaptive immune responses. MZB have the ability to rapidly respond to an Ag-specific stimulus by using both T-independent (TI) and dependent (TD) mechanisms and to transform into plasma cells that secrete IgM, IgG1, IgG2 (for both, TD and TI pathways), IgA1 (on the TD pathway), and IgA2 (on the TI pathway) low affinity Abs (51).

B1 B Cells. These are the first B Cells to form in the fetal liver. They subdivide into B1a and B1b with the former expressing the glycoprotein of membrane CD5, which is absent in the latter. Both express CD9 and CD45RA markers, are involved in type TI immune responses, are found in the peritoneal and pleural cavities, and are the main source of circulating Abs. As with the MZB, the B-1 responds rapidly to Ag-specific stimuli and transforms into plasma cells. Their numbers have been observed to increase in experimental studies and in humans with autoimmune diseases.

Seven sub-populations of mature peripheral B Cells have been identified in human tonsils based on the expression of two surface markers (CD38 and IgD). This has made it possible for a TD model for the differentiation of mature B Cells to be proposed. The subpopulations suggested are: i) B- cell mature naïve (Bm); ii) B- cell mature 1, Bm1 ($CD38^{-}$ IgD^{+}); iii) Bm2 ($CD38^{+}$ IgD^{+}). - These three would be activated by their specific Ag in the extra-follicular areas through interaction with interdigital DC and Ag-specific T Cells. Once activated, the three can be transformed into Bm2' founder cells of GC ($CD38^{++}$ IgD^{+}), and then differentiate into Bm3 centroblasts ($CD38^{+++}$ IgD^{-}). These Bm3 cells are selected during their differentiation into Bm4 centrocytes ($CD38^{++}$ IgD^{-}) as a function of their BCR affinity. Finally, these cells differentiate into either memory B Cells ($CD38^{+}$ IgD^{-}), Bm5 cells (CD38-IgD-), or high affinity plasma cells (Figure 10) (62).

Plasmocyte or Ab-secretory cells. These differentiate from an activated B Cell which, in the presence of IL-2 and IL-10, stops expressing surface molecules such as CD19, CD20,

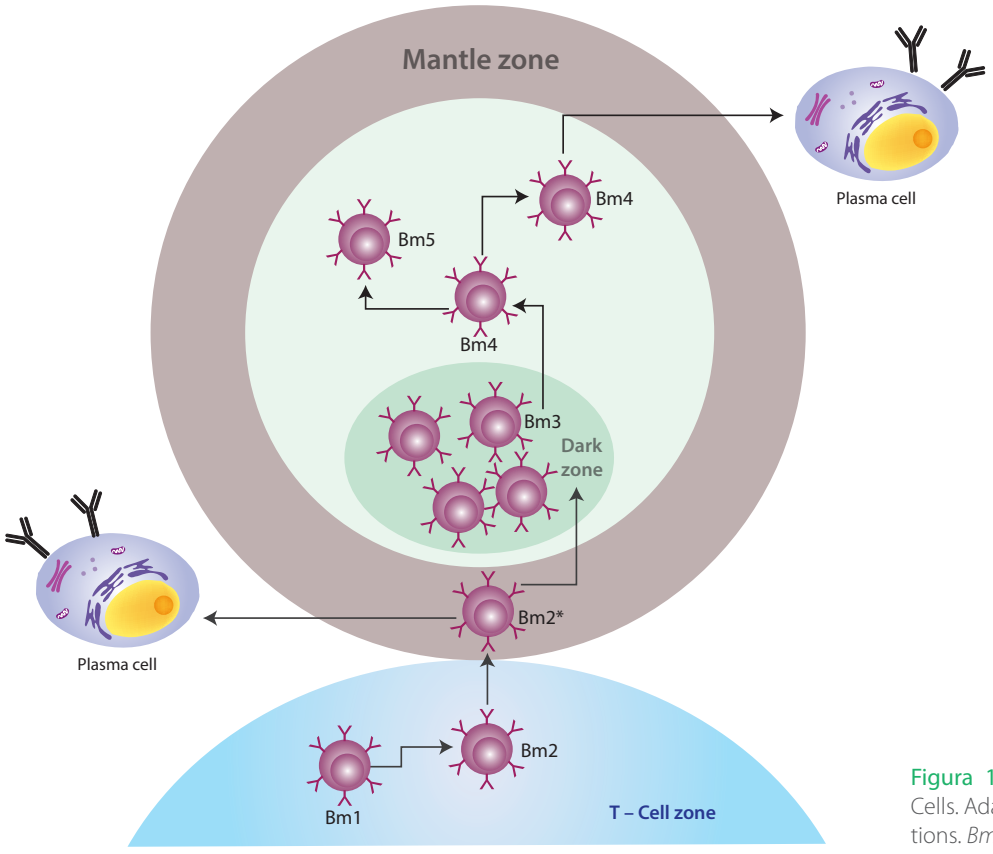

Figura 10. Sub-sets of peripheral mature B Cells. Adapted from Cornec *et al* (62). Abbreviations. *Bm*: B Cells mature.

CD22, HLA class II molecules, and their BCR. These cells also lose the ability to divide. At the same time, they undergo a series of cellular modifications, e.g., an increase in their cytoplasm due to enhanced growth of the endoplasmic reticulum that is required to harbor the high number of ribosomes for robust production of Abs. They also stop expressing CXCR5 and CXCR7 and increase CXCR4 which causes them to lose contact with the DC and forces the TFH cells to migrate from the GC to the medullar cords of the ganglia (44).

Two classes of plasma cells are known: **Short-lived cells**, which are located in the medulla of the ganglia and, later, quickly exit to the circulation and seek the site where the Ag enter to initiate, *in situ*, the production of specific IgM type Abs. **Long-lived cells** migrate to a special niche in the bone marrow following the expression of *SDF-1* by the stromal cells. Within this niche, an extended production of IgG type Abs, which can be used to mount a prolonged or permanent defence against the Ag that originally activated the B Cells, is initiated. The prolonged survival of these latter cells is due to the effect of IL-16. Development of short- or long-lived plasma cells depends on the expression of the transcription factor Blimp-1 (*B lymphocyte-induced maturation protein-1*) (44).

Memory B Cells. There are several subsets of memory B Cells that are classified based on their origin, the differential expression of CD27, and the isotype of the mIg being expressed. Three different origins for the cells have been described: i) the spleen, ii) the germinal center, and iii) the intestine lamina propria outside the GC. In the spleen, they present $CD27^{-}IgG^{+}$ markers. At the GC, they are $CD27^{+}IgM^{+}IgD^{-}$ and change from mIg to $CD27^{+}IgG/IgA^{+}$. Last of all, those generated in the intestine express $CD27^{-}IgA^{+}$ (44, 64).

Regulatory B Cells (Bregs). B Cells also liberate a wide variety of cytokines and, as with the T Cells, can be classified according to the profile of cytokines that they produce. Thus, the Bregs are a functional sub-set of B Cells, and they contribute to the maintenance of the fine equilibrium required to guarantee tolerance. Bregs restrict the excessive inflammatory responses that are produced during autoimmune diseases or that can be caused by unresolved infections. IL-10 is fundamental to the function of Bregs since they inhibit pro-inflammatory cytokines (IFN-γ, IL-17), reduce the expression of the MHC clase II molecules and support the differentiation of Tregs (65). It has also been reported that CD40-CD154 interaction is an essential activation pathway for the Bregs. With regard to the Breg surface markers, there is considerable controversy and the consensus is that there is no single marker, or even set of markers that make identification of this type of regulatory cell possible. Among the markers reported in these types of cells are: $CD1d^{high}$, $CD5^{+}$, $CD19^{+}$, $CD24^{high}$, CD27variable, CD38 variable, CD138 variable, IgM^{high}, and $IL\text{-}10^{+}$ (65).

INNATE B CELL HELPERS

It has also been demonstrated that B Cells receive additional help from other cells besides the T-helper cells. These include: the iNKT, DCs, epithelial cells, macrophages, and diverse granulocytes, including neutrophils, eosinophils, basophils, and mastocytes (51, 66-68).

The *iNKT* cells express a TCR invariant Vα14+ that recognizes soluble glycolipids, e.g., α-galactosylceramide, presented by DCs or subcapsular macrophages in the CD1d context. Soluble glycolipids improve the expression of CD40L and IFN-α, which stimulates the maturation of DCs in the efficient antigen-presenting cells. These interact with the TFH cells to form active CGs with the consequent generation of long-living plasma cells that produce IgG (66, 67).

The *B Cell helper neutrophils (NBH)* occupy the perimarginal zone of the spleen in the absence of inflammation or infection. They interact with perifollicular B Cells and MZB through the liberation of APRIL, BAFF, CD40L, IL-6, and IL-21 in response to stimuli by cytokines and microbial products. This interaction results in CSR processes by which the plasma cells generated stop expressing IgM and start to produce IgG and IgA (68).

In general, we can say that these innate immune cells can stimulate and help the response of Abs to both TD and TI processes. For the former, these cells make use of helper signals for B Cells in the GC and in the central lymphoid sites such as the bone marrow. On top of this, theTI type responses take place on the surface of the mucosae and in the marginal zone of the spleen to give rise to a rapid response from natural Abs (66, 67).

TYPES OF IMMUNE RESPONSES MEDIATED BY ABS

Traditionally, the humoral immune response mediated by Abs is classified based on whether or not the B Cells receive help from the T Cells, i.e., if they are TD or TI responses of the thymus (69).

One charactistic of the TD response is the induction of follicular GCs in which the Tfh cells select B Cells with high affinity BCRs by somatic mutation and cause them to differentiate into memory B – cells. In contrast, the TI response may be provoked by microbial ligands, which are classified as type TI -1 or by extensive crosslinking of the BCR with the Ag, which is known as type TI -2 (69).

Recent studies describe the existence of two new mechanisms that participate in the B- cell response, e.g., the B Cells can also receive TD type help but from cells of innate immune system. Some examples are those induced from iNKT cells which are classified as TD - 2. Furthermore, an innate TI-3 type response has been described that involves myeloid cells such as B Cell helper neutrophils (N_{BH}) (68), monocytes, eosinophils, mastocytes, and basophils (70). Much remains to be discovered with regard to the steps in the human B - cell response, including which cells and molecules are involved in the new mechanisms described above. The authors of the present chapter, therefore, recommend that those readers who are interested in a deeper understanding of this subject keep abreast of the reports that confirm and clarify such mechanisms.

ANTI-B CELL THERAPIES

Given the accelerated pace of new discoveries in B- cell biology, a better understanding of the cell and molecular mechanisms implicated in the development, differentiation, and functioning of these cells will reveal potential therapeutic targets and allow the design of anti-B Cell treatments which can resolve the cell alterations responsible for diverse pathologies.

It is already known that the B Cells play a fundamental role in the pathogenesis of RA by the production of autoantibodies and secretion of chemokines/cytokines, and that they may serve as Ag-presenting cells. Thus, several receptors, co-stimulatory molecules, parts of the signaling cascade, and pro-inflammatory mediators of the B Cells are considered to be promising target molecules for the treatment of RA, SLE, multiple myeloma, and other diseases (Table 3) (71,72).

TARGET	EXAMPLES OF DRUGS	DISEASES
BCR related		
Syk	Fostamatinib (R788)	RA (phase III)
BTK	GDC-0834	RA (phase I)
p38 MAPK	Pamapimod	RA (phase II)
	BMS-582949	RA, psoriasis (phase II)
	SB-681323	RA, ARDS, COPD (phase II)
	SD0006	RA (phase I)

Table 3. Anti-B Cell Therapies. *RA*: rheumatoid arthritis, *ARDS*: acute respiratory distress syndrome, *COPD*: chronic obstructive pulmonary disease, *SLE*: systemic lupus erythematosus, *MS*: multiple sclerosis. *Syk*: Spleen tyrosine kinase, *BTK*: Bruton kinase of Tec family, *MAPK*: Mitogen-Activated Protein Kinases, *BAFF*: B cell-activating factor, *APRIL*: A proliferation-inducing ligand, *IL-6R*: Interleukin -6 receptor, *IL*: Interleukin, *IFN-α*: Interferon alpha, *CCL2*: Chemokine (C-C motif) ligand 2 is also referred to as monocyte chemotactic protein-1 (MCP-1), *CCL7*: Chemokine (C-C motif) ligand 7, it was previously called monocyte-specific chemokine 3 (MCP3), *CCR2*: C-C chemokine receptor 2, *ICOS*: Inducible T cell costimulator, *TLR 7*: Toll like receptor 7. Adapted from (71,72).

TARGET	EXAMPLES OF DRUGS	DISEASES
Cytokines		
BAFF	Belimumab	SLE (approved)
	Blisibimod (A-623)	SLE (phase II/III)
	Tabalumab (LY2127399)	SLE, RA, MM (phase III)
	Briobacept (BAFFR-Ig)	RA (phase I)
BAFF/APRIL	Atacicept (TACI-Ig)	RA, SLE (phase II/III), MS, RA (phase II)
IL-6R	Tocilizumab	RA (market)
IL-21	NNC114-0005 (fully human anti–IL-21 mAb)	RA (phase I)
IFN-α	Rontalizumab	SLE (phase II)
	MEDI-545	SLE (phase II)
CCL2	Proteolytically	RA
CCL7	Processed antagonistic CCL2 variant	RA
CCR2	Anti-CCR2 antibodies	RA
Other		
CD20	Rituximab	RA (approved), SLE (phase III)
	Ofatumumab	RA (phase III), MS (phase II)
	Ocrelizumab	RA, SLE, MS (phase III)
	Veltuzumab	RA (phase II)
	TrU015	RA (phase II), SLE (phase I)
CD22	Epratuzumab	SLE (phase III)
CD19	Anti-CD19 antibodies	RA
Proteasome	Bortezomib	RA
Proteasome subunit LMP7	Selective inhibitor of LMP7	RA
CD79 α/β	Anti-CD79 α/β antibodies	RA
CD40 / CD40L (CD154)	Anti-CD40 antibodies	RA
ICOS/ICOSL	ICOS/ICOS-L inhibitor	RA
TLR7/9	TLR7/9 inhibitor	RA

Table 3. Anti-B Cell Therapies. *RA*: rheumatoid arthritis, *ARDS*: acute respiratory distress syndrome, *COPD*: chronic obstructive pulmonary disease, *SLE*: systemic lupus erythematosus, *MS*: multiple sclerosis. *Syk*: Spleen tyrosine kinase, *BTK*: Bruton kinase of Tec family, *MAPK*: Mitogen-Activated Protein Kinases, *BAFF*: B cell-activating factor, *APRIL*: A proliferation-inducing ligand, *IL-6R*: Interleukin -6 receptor, *IL*: Interleukin, *IFN-α*: Interferon alpha, *CCL2*: Chemokine (C-C motif) ligand 2 is also referred to as monocyte chemotactic protein-1 (MCP-1), *CCL7*: Chemokine (C-C motif) ligand 7, it was previously called monocyte-specific chemokine 3 (MCP3), *CCR2*: C-C chemokine receptor 2, *ICOS*: Inducible T cell costimulator, *TLR* 7: Toll like receptor 7. Adapted from (71,72).

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6 B LYMPHOCYTES IN AUTOIMMUNITY

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INTRODUCTION

B lymphocytes are the effectors of humoral immunity, providing defense from pathogens through different functions including antibody production. B cells constitute approximately 15% of peripheral blood leukocytes and arise from hemopoietic stem cells in the bone marrow (BM). It is here that their antigen receptors (surface immunoglobulin) are assembled. In the context of autoimmune diseases defined by B and/or T cell auto-reactive that upon activation lead to chronic tissue inflammation and often irreversible structural and functional damage; B lymphocytes play an essential role by not only producing auto-antibodies but also functioning as Antigen-Presenting Cells (APC) and as a source of cytokines. In this chapter, we describe B lymphocyte functions in autoimmunity and autoimmune diseases from ontogenesis to targeted therapies in this group of pathologies.

B CELL DEVELOPMENT AND THE B CELL RECEPTOR FORMATION

Different populations of B cells result in pre-immune pools where each cell in these quiescent populations expresses a B cell antigen receptor (BCR) with a unique specificity. When the BCR come in contact with their specific antigen, several intracellular signals are generated leading to activation, differentiation, and formation of plasma cells and memory B cells. This last subset of B cells maintains protective antibody levels and mediates the response to subsequent antigen challenges. As the mechanisms leading to maturing and antibody production are complex, the alterations of some of these populations or critical steps have been associated with immunodeficiency and autoimmune diseases. Table 1 summarizes the most important features of each of the subpopulations (lineages) of B lymphocytes (adapted from 1).

B CELL DEVELOPMENT

This process begins from stem cells present in the BM which, depending on the different stimuli received, will generate B lymphocytes. They are derived from the early lymphoid progenitor, which passes to the common lymphoid progenitor. This produces, first of all, the natural killer (NK) cells and dendritic cells, and, secondly, the common lymphoid-2 progenitor (LCA-2) that is responsible for the B cell lineage, which is considered the first stage of immature B lymphocytes. Development of the B cell lineage depends on BM stromal cells that produce mainly IL-7 but also the Fms-like tyrosine kinase 3 (Flt3-L) and on the action of several transcription factors such as PU.1, IKAROS (IKAROS family zinc finger 1), E2A, EBF (early B cell factor 1), PAX5 (paired box gene 5), and IRF8 (interferon regulatory factor 8) (2-5). In the BM, B cells pass through several distinct developmental stages. During this, they acquire their antigen specificity, follow a program of differential surface antigen expression and sequential heavy and light chain gene rearrangement, forming the BCR (initially IgM), that determine the cell maturation stage. Reaching the immature stage, B cells exit the BM and complete their development to the mature or naive stage, which is signaled by the appearance of IgD in addition to IgM on the cell surface. This development sequence occurs in the absence of any contact with exogenous antigen, a stage known as antigen-independent B cell development (2-5).

B CELL RECEPTOR DEVELOPMENT

Immunoglobulin molecules are composed of 2 identical 50 kd heavy chains and 2 identical 25 kd light chains (6). The genes encoding immunoglobulins are assembled from segments in a manner that is entirely analogous to the process for T cell receptor genes. The light and heavy chain loci are each composed of a series of V (variable) gene elements, followed by several D (diversity) segments (for the heavy

DIFFERENTIATION	SUBSET	SURFACE PHENOTYPE
Progenitor subsets (Bone marrow)	Pro-B	B220loCD43+
		AA4.1+
	Pre-B	B220loCD43-, AA4.1+preBcR+
	Immature (23-)	B220lo, sIgM+, sIgD-, CD23-
	Immature (23+)	CD19+, B220+, sIgM+, sIgD-, CD23+
Transitional subsets (Spleen)	T1	IgMhiCD23-, B220intAA4.1+
	T2	IgMhiCD23+, B220+AA4.1+
	T3	IgMloCD23+, B220+AA4.1+
Mature primary subsets	Follicular zone	IgMloCD23+, B220hiAA4.1-
	Marginal zone	CD19+IgMhiIgDlo CD23+ CD21+
	B1	CD43+ CD23- CD5+
T-independent responses	Early antibody-forming cells/short-lived plasma cells	B220loCD19+slg+iclghi
T-dependent responses	Early antibody-forming cells/short-lived plasma cells	B220loCD19+slg+iclghi
	Germinal center	B220+CD19+GL7+
	Long-lived plasma cells	B220loslg-iclg+
	Memory	B220+slg+IgD-
Natural antibodies	Peritoneal B1a and B1b	CD43+ CD23- CD5+

Table 1. Characteristics of primary B cell subsets and their progenitors. Modified from reference (1).

chain gene only), some J (joining) segments, and C (constant region) exons. Heavy chains (H) are assembled from 4 segments (VH, D, JH, and CH). Light chains (L) are assembled from 3 segments (VL, JL, and CL) (Figure 1). The genes for 9 different heavy chain types (IgM, IgD, IgG 1-4, IgA1-2, and IgE) are located on chromosome 14, and those for 2 light chain types (κ or λ) are on chromosome 2 and 22 respectively. The variable portions (V) of the H and L chains are in juxtaposition, and this creates the antigen-binding portion of the immunoglobulin molecule. These V regions contain 3 highly variable sub-regions, or hypervariable sequences, which produce the antigen-binding domain of the molecule. The amino-terminal portions of the chains vary in amino-acid sequence from one antibody molecule to another. The carboxyl terminal portions are constant in each subclass of antibody. The H chain constant regions form the Fc domain of the molecule and are responsible for most of the effector functions of the immunoglobulin molecule.

The development process of different subsets of B cells (7) begins when the recombinase enzyme complex catalyzes the fusion of one DH region gene to a JH region gene with the deletion of the intermediate DNA sequences. This recombination occurs on both chromosomes. Next, the recombinase joins one VH region genes to the rearranged DHJH gene. The enzyme terminal deoxynucleotidyl transferase (TdT) is expressed, adding random nucleotides to the sites of VHDHJH joining and enhancing the diversity of amino acid sequences. The rearranged VHDHJH element forms the most 5′ exon of the H chain gene and is

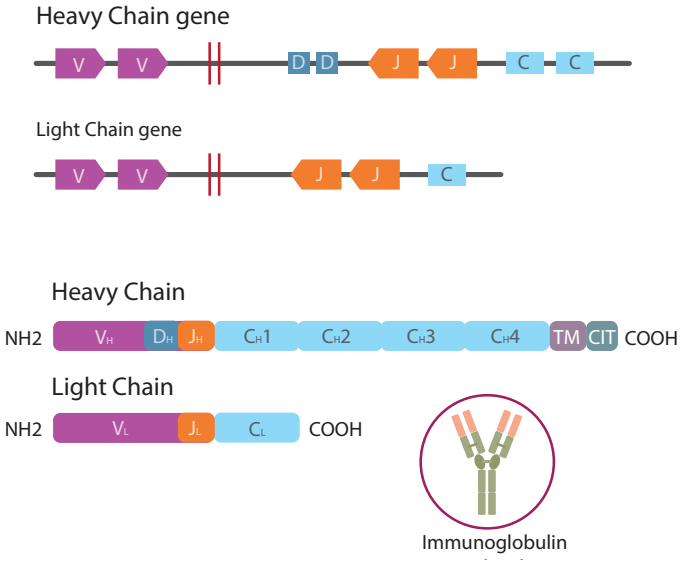

Figure 1. Schematic representation of the components of the H and L chains of immunoglobulins. The light and heavy chain loci are each made up of a series of V (variable) gene elements, followed by several D (diversity) segments (for the heavy chain gene only), some J (joining) segments, and C (constant region) exons. Heavy chains (H) are assembled from 4 segments (VH, D, JH, and CH); light chains (L) are assembled from 3 segments (VL, JL, and CL).

followed downstream by exons encoding the constant (C) region (initially μ chain), that pairs with an L chain and produces IgM. When the VHDHJH element is followed downstream by exons encoding the C region for the δ chain, it produces IgD. These events occur as a result of alternative RNA splicing. Finally, if the rearrangement of VH, DH, and JH elements yields an H chain transcript and encodes a functional H chain protein, this heavy chain is synthesized and pairs in with 2 proteins (called λ5 and VpreB), which act as a surrogate light chain, and results in the expression of a pre-B cell receptor. Once a functional heavy chain is produced, the cell down regulates the TdT gene and initiates an L chain rearrangement. It begins first with a κ element and, if this rearrangement is unsuccessful, continues with a λ element. A Vκ element rearranges to a Jκ element and produces a light chain, which, if it is functional, pairs with the H chain to make an immunoglobulin protein. Once a functional IgM and IgD are synthesized, the pre-B cell evolves into an immature B cell. The fully mature B cell receptor includes additional transmembrane proteins designated Igα and Igβ that activate intracellular signals after receptor binding to antigen (8,9). At that point, the mature B cell passes to peripheral lymphoid tissues (Figure 2).

B CELL CLASSIFICATION ACCORDING TO THEIR ONTOGENIC STATE

As mentioned above, B cells originate in the BM. As soon as they have productively rearranged their immunoglobulin genes, pro-B cells proceed to the pre-B cell stage. On their arrival in the spleen, immature B cells give rise to type-1 (BT1), type-2 (BT2), and possibly type-3 **transitional B cells** (10). As transitional B cells, they are pushed into migrating from the BM to secondary lymphoid organs (SLO).

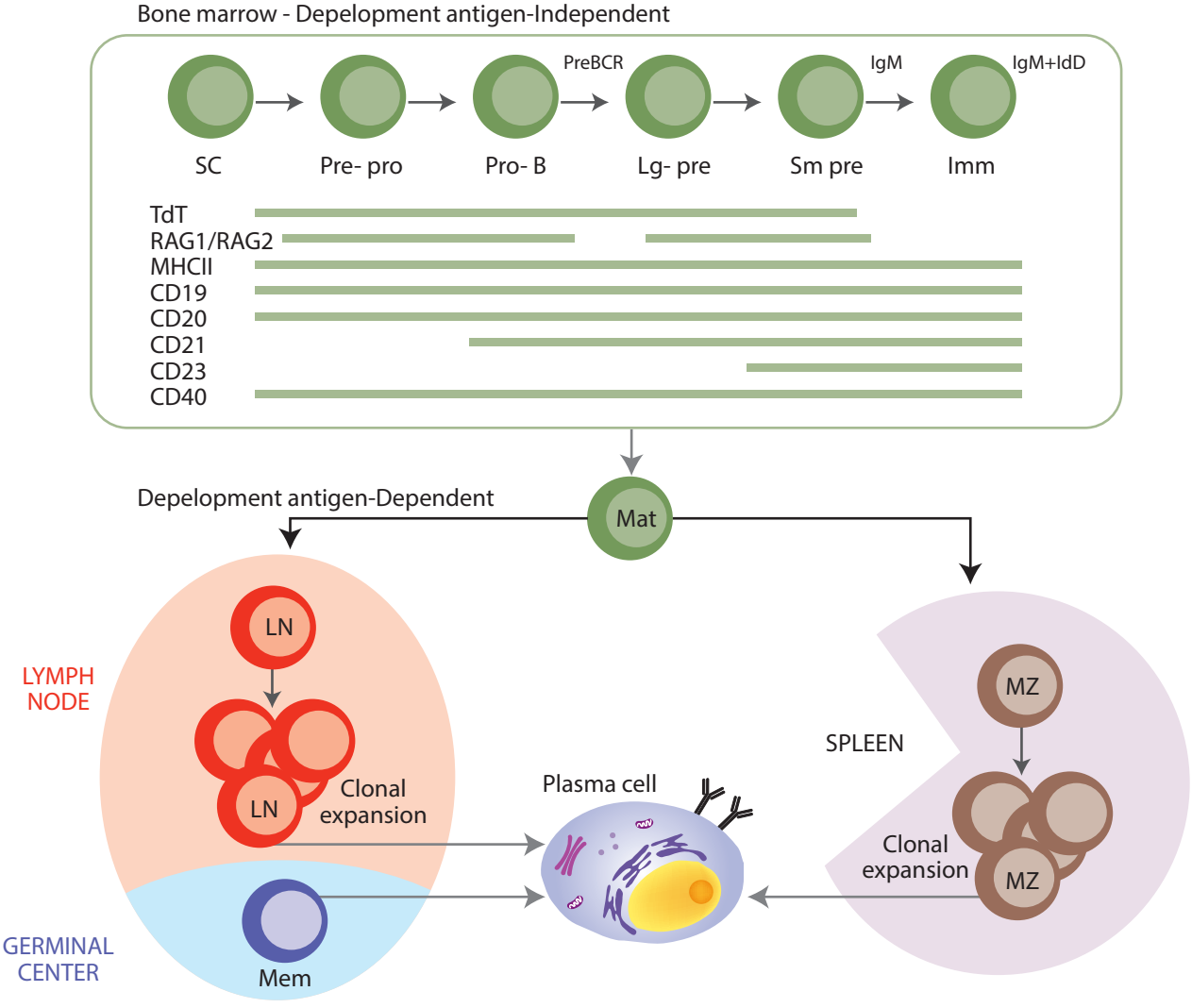

Figure 2. B- cell receptor development and differentiation.

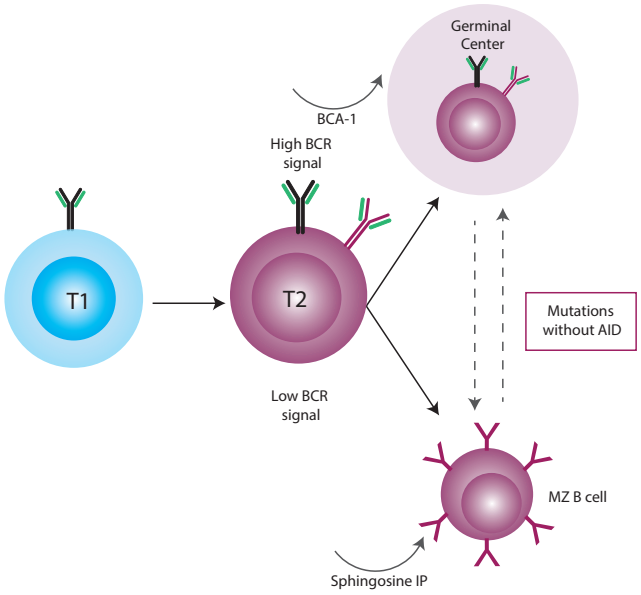

Figure 3. B cell classification based on their ontogenic state. From the transitional type 1 (T1) and T2 B cells, two options depend on the B cell receptor (BCR)-evoked signal and the downstream Notch 2 proteins: germinal center (GC) B cells driven by the B cell-attracting (BCA)-1 chemokine (or CXCL13) and MZ B cells with mutations but without activation-induced cytidine deaminase (AID). Modified from reference (103).

Although T1 cells undergo apoptosis in response to BCR engagement, they require signaling via the B cell activating factor belonging to the tumor necrosis factor (TNF) family receptor (BAFF-R, TNFRSF13) to mature to the T2 stage (11). T2 cells are only present in the spleen and reside in the follicles, whereas T1 cells are found in the red pulp and outer periarterial lymphatic sheath (PALS) (12).

There, they continue maturing and are further selected by antigens. As BT1, they present as CD20+CD5+CD10+/-CD21+/-CD23+/-IgM+IgD+/- and CD38+, but once they have evolved to type 2 (BT2), they become CD20+CD5+/-CD21++CD23+/-IgM++IgD++ and CD38+/-. T2 B cells differentiate into either circulating lymphocytes that get organized as Germinal Centers (GCs), or non-circulating lymphocytes that populate the Marginal Zone (MZ). Progression of T2 B cells towards MZ or GCs may be determined by the quality of BCR-evoked signals and the subsequent expression of the Notch proteins (13). Alternatively, MZ B cells with mutated immunoglobulin genes, but without activation-induced cytidine deaminase (AICDA), may have passed a germinal center (GC) response (14). Finally, the expression of sphingosine 1-phosphate receptor 1 on the B cells may overcome the recruiting activity of the B cell-attracting chemokine (BCA)-1 to the GCs (15), and thereby retain B cells within the MZ (16) (Figure 3). The main CD molecules expressed by B cells are summarized on Table 2.

MIGRATION OF B CELL INTO THE GERMINAL CENTERS

Organization of the B cell follicles and surrounding T cell zones is achieved by the secretion of chemokines by distinct stromal cell subsets. Of these subsets, follicular dendritic cells (FDCs) are essential to retain immune complexes and produce B-lymphocyte chemoattractants (BLC/CXCL13). FDC maintenance requires continual membrane expression of lymphotoxin α1β2 (LTα1β2) trimer as well as TNF secretion by B cells, and LTβR and TNF-R1 expression on FDCs (17). The MZ demarcates the perimeter of the white pulp of the spleen and contains a subset of B cells that likely arises from the transitional B cell compartment (18). MZ B cells are strategically located to respond to blood-born antigens and can rapidly differentiate into antibody-producing cells in the red pulp. Upon an encounter with antigens, follicular B cells migrate to the border regions of the PALS/cortex to present bound peptide and costimulate T cells. Reciprocal B cell activation is mediated by engagement of CD40 and provision of cytokine support. CD40-dependent B cell activation is required to undergo proliferative expansion and differentiation in the GC, where somatic hypermutation and enhanced immunoglobulin class switch recombination (CSR) occur. The architecture of the GC is divided into distinct regions: rapidly dividing B cells or centroblasts in the 'dark zone' of the GC give rise to centrocytes which occupy the 'light zone'. The light zone is thought to be the site of B cell selection by FDC-bound antigens that are processed and presented by B cells to primed T cells of the follicular helper CD4+ (Tfh) subtype.

B cell maturation in the GC is accompanied by somatic hypermutation of antibody variable region (V) genes, which provides the molecular basis for the production of

NAME	CELLULAR REACTIVITY	STRUCTURE
CD19	Pan-B cell, FDCs?	Ig superfamily
CD20	Mature B cells	MS4A family
CD21	Mature B cells, FDCs	Complement receptor family
CD22	Mature B cells	Ig superfamily
CD23	Activated B cells, FDCs, others	C-type lectin
CD24	Pan-B cell, granulocytes, epithelial cells	GPI anchored
CD40	B cells, epithelial cells, FDCs, others	TNF receptor
CD72	Pan-B cell	C-type lectin
CD79a,b	Surface Ig+ B cells	Ig superfamily

Table 2. Cell surface CD molecules that are preferentially expressed by B cells. *FDCs*: follicular dendritic cells; Ig: immunoglobulin.

B cells bearing high-affinity antigen receptors. These B cells are thought to have a competitive advantage when antigen becomes limiting and GC structures atrophy. B cells unable to bind antigen or receive sufficient T cell help die *in situ* by apoptosis and are cleared by macrophages, whereas antigen-selected B cells that leave the GC become memory B cells or plasmablasts by a process that is not fully understood. Long-lived plasma cells are actively retained in the BM responding to stromal derived factor / CXCL12 as well as survival factors such as interleukin-6 (IL-6), BAFF, and a proliferation-inducing ligand (APRIL). The trafficking of B cells in the lymphoid organs and target tissues is a regulation mechanism of B cell activation and differentiation (19-21).

B cells can act as an antigen delivery system that transports blood born antigens into the FDC network region of the spleen (16). This regulates the GC formation where high affinity antibody-forming B cell differentiation occurs. These migratory responses are extremely dynamic and involve ongoing shuttling of the B cells between the different anatomic sites and the GCs. Chemotactic responses play a key role in orchestrating the cell-cell interactions in the GCs. This process involves ongoing shuttling of the antigen-carrying B cells between the MZ and the GCs. In animal models of autoimmunity, the migration of MZ precursor B cells is promoted by high levels of interferon (IFN)-α produced by plasmacytoid dendritic cells in the marginal sinus that antagonize the activity of the S1P1 chemokine receptor. In contrast, within the GCs, interleukin-17A (IL-17A) upregulates the expression of regulators of G protein signaling (RGS) in B cells to desensitize the G protein-coupled receptor (GPCR) signaling pathway of CXCL12 and CXCL13 chemokines (22-24). This provides a prolonged stable interaction of B and T cells in the GC that induces high levels of AICDA and, as a result, enables the development of pathogenic autoantibody-producing B cells (Figure 4).

MATURE B CELLS

Peripheral B cell maturation, homeostasis, and antigen-dependent differentiation are complex processes occurring in distinct anatomic locations. As B cells egress from the BM, further maturation into follicular or MZ B cells is dependent upon the effects of the cytokine BAFF. B cell compartmentalization and cell– cell interactions in the SLO require expression of membrane-bound LTα/β trimers and TNF, whereas T cell-dependent B cell differentiation requires engagement of CD40 (TNFRSF5) by CD40L on activated CD4+ T cells. CD30 (TNFRSF8) is expressed on activated B cells and has been found to be required for efficient memory B cell generation. CD27 is also implicated in B cell memory.

The development stages of GC B cells are based on the relative expression of IgD and CD38 on mature B (Bm) lymphocytes (25) from naïve cells leaving the bone marrow (Bm1) to memory B cells activated and differentiated by their specific antigen (Bm5). The development starts with CD38−IgD+ naïve Bm1 that progresses into CD38+IgD+ antigen activated Bm2, of which some become CD38++IgD+ Bm2' GC founder cells. These differentiate into CD38++IgD− Bm3 centroblasts and Bm4 centrocytes (Figure 5). Two types of B cells arise from GC reactions: CD38+IgD− early memory B cells that mature locally into CD38−IgD−Bm5 memory B cells, and CD38++IgD− plasmablasts, which were first described by Odendahl *et al*. (26). The latter return to the BM, where they differentiate into long-lived plasma cells. A few cells of each subset escape into the circulation from GCs.

B CELL DERIVED CYTOKINES

Interleukin 7 (IL-7) is important in B cell functioning. This cytokine plays several important roles during B cell development including aiding in the specification and commitment of cells to the B lineage, the proliferation and survival of B cell

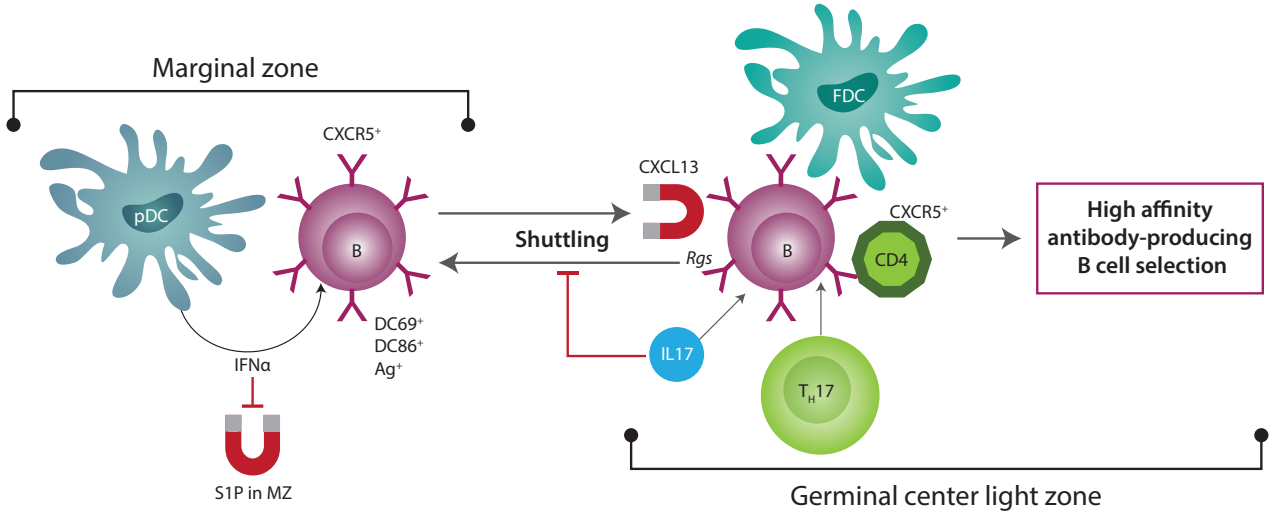

Figure 4. Chemotactic responses play a key role in orchestrating the cell-cell interactions in the Germinal centers.

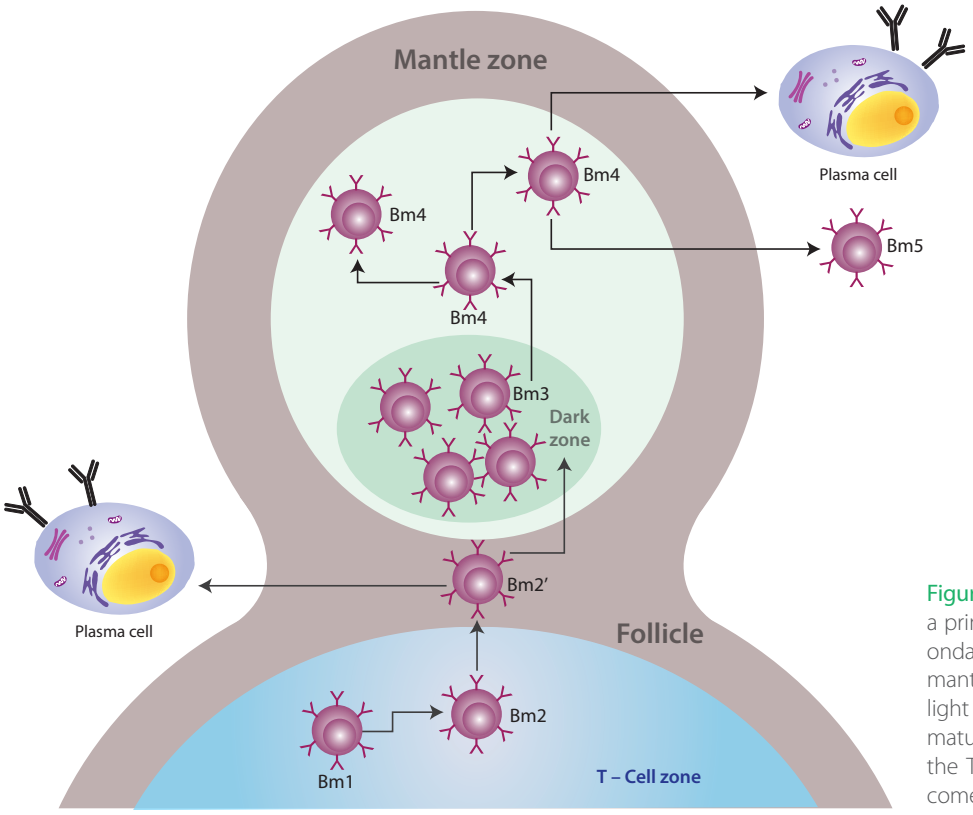

Figure 5. Germinal center (GC) changing a primary lymphoid follicle (LF) into a secondary LF. The GC is surrounded by the mantle zone, which is comprised of the light and dark zone, and populated by mature B (Bm)-cells evolving from Bm1 in the T cell area through plasma cells that come back to the bone marrow.

progenitors, and maturation during the pro-B to pre-B cell transition (27). Regulation and modulation of IL-7 receptor (IL-7R) signaling is critical during B lymphopoiesis because excessive or deficient IL-7R signaling leads to abnormal or inhibited B cell development (28). IL-7 works together with E2A, EBF, Pax-5, and other transcription factors to regulate B cell commitment while it also works to regulate immunoglobulin rearrangement by modulating FoxO protein activation and Rag enhancer activity. Suppressors of cytokine signaling (SOCS) proteins are inhibitors of cytokine activation and, in B cells, function to fine tune IL-7R signaling. This ensures that appropriate IL-7 signals are transmitted to allow for efficient B cell commitment and development (29).

Recent discoveries have unveiled new insights into B cell-derived cytokines, including IFN- γ and Interleukin (IL)-4 that modulate the response (30). They are likely to serve as effectors of some B cell functions. Given the kinetics of B cell generation and the cytokine profile of B lymphocytes, T helper (Th) 1 phenotype may be imprinted by B effector (Be) 1 cells through the expression of IL-2 and IFN-γ by B cells. This is sustained by an IFN- γ /IFN- γ receptor autocrine loop. Conversely, Th2 cells induced naïve B cell polarization into Be2, which produces IL-4 and IL-6 in the absence of GATA-3. In fact, the Th1/Th2 cytokine balance changes with the progress of the immunopathological lesions on autoimmune diseases such as primary Sjögren's syndrome (31). Distinct populations of serum cytokines have also been found (42) to differentiate autoimmune disease patients from controls and one patient from another depending on the presence or absence of different organ involvement. B cell produced cytokines may be classified as pro-inflammatory (IL-1, IL-6, TNF-α, and LT- α), immunosuppressive cytokines (TGF- α and IL-10), or as hematopoietic growth factors (granulocyte/monocytes-colony stimulating factor and IL-17).

B CELL TRANSCRIPTION FACTORS

B cell development depends on several transcription factors. One of the most important transcription factors is Pax5. Pax5 restricts the developmental potential of lymphoid progenitors to the B cell pathway by repressing B-lineage-inappropriate genes while it simultaneously promotes B cell development by activating B-lymphoid-specific genes. Therefore, Pax5 controls gene transcription by recruiting chromatin-remodeling, histone modifying, and basal transcription factor complexes to its target genes (33). Moreover, Pax5 contributes to the diversity of the antibody repertoire by controlling VH-DJH recombination. It does this by inducing contraction of the immunoglobulin heavy-chain locus in pro-B cells, which is likely mediated by PAIR elements in the 50 region of the VH gene cluster. Importantly all mature B cell types depend on Pax5 for their differentiation and function. Pax5 thus controls the identity of B lymphocytes throughout B cell development. Consequently, conditional loss of Pax5 allows mature B cells from peripheral lymphoid organs to develop into functional T cells in the thymus via differentiation to uncommitted progenitors in the BM. Pax5 has also been implicated in some diseases including human B cell malignancies.

B CELL TOLERANCE MECHANISMS AND THEIR ROLE IN AUTOIMMUNITY

B CELL TOLERANCE

This mechanism is essential for maintaining non-responsiveness to thymus-independent self-antigens such as lipids and polysaccharides. B cell tolerance is also important in preventing the development of antibody responses to protein antigens. Both central and peripheral mechanisms are implicated in B cell tolerance. In the central tolerance, the immature B lymphocytes that recognize self-antigens in the BM with high affinity are deleted or active mechanisms to change their specificity by receptor editing. This fate is defined by the strength of BCR signaling: a strong BCR signal by binding with high affinity to an autoantigen will lead to deletion or receptor editing while an intermediate binding affinity will permit B cells to survive and continue to the periphery (34).

Receptor editing is a major mechanism of central tolerance in B cells. Immature B cells in the BM that encounter multivalent self-antigens revert to pre-B stage, and continue to rearrange κ and, if necessary, λ light chain genes, and generate newly generated B cells that have a novel light chain that is no longer self-reactive. Immature B cells with novel light chains that are no longer part of a self-reactive BCR migrate to the periphery as BT1cells where they mature into newly generated IgM and IgD expressing recirculating BT2 cells and, then, into mature recirculating B cells.

If a mature B cell recognizes self antigens in peripheral tissues without specific helper T cell response, this cell may be functionally inactivated by anergy mechanisms or die by apoptosis. The AICDA is required for B cell tolerance in humans. This enzyme is required for CSR and somatic hypermutation. Patients with AICDA deficit develop primary immunodeficiencies and autoimmune complications. Single B cells from AICDA-deficient patients show an abnormal Ig repertoire and high frequencies of auto-reactive antibodies (35).

B CELL RECEPTOR EDITING

When the B cell differentiation is ongoing, its receptor presents a phenomenon known as receptor editing, which is the process of antibody gene rearrangement to have a functional BCR and inhibit further rearrangement (allelic exclusion). If a T lymphocyte produces a self-reactive receptor, different mechanisms are initiated to induce the apoptosis of this self-reactive cell (negative regulation). However, B cells have a second chance at escaping this negative regulation by "editing" the specificities of their receptors with additional antibody gene rearrangements. This editing affects both H and L chains. Furthermore, edited B cells are not simply endowed for life with a single, invariant antigen receptor, because an edited B cell which initial immunoglobulin gene is not inactivated during the editing process, may exhibit two specificities (36).

The BCR editing process initiates with the allelic exclusion. This is the phenomenon in which B cells usually express a single kind of antibody H chain and L chain and it is typically enforced at the genetic level with only one allele being productively rearranged. A series of epigenetic mechanisms, including replication timing, DNA methylation, histone modification, nucleosome positioning, and heterochromatization, appear to control H and L chain locus accessibility and which allele is first rearranged (37). These mechanisms regulate accessibility to recombination machinery and activate feedback inhibition of the rearrangement between H chain and L chains. Once the H chain protein is completed, L chain rearrangements initiate. This process is regulated by isotypic exclusion, a phenomenon in which B cells usually express a single L chain isotype (either κ or λ, not both) and is explained by two properties of L chain rearrangement: first, the κ or λ rearrange at different times during B cell development and second, the B cells which express λ often have both κ alleles deleted. Based on the analysis of cell lines in mouse and man, it was clear that κ chain nearly always rearranges before λ chain (38,39).

Another process identified is the secondary rearrangement of H and L chains. In Heavy chain, the mechanism is mediated by DH-JH rearrangement, DH-DH fusion, and VH replacement, all of which contribute to the elongation of the Third Complementarity Determining Region (CDR3) and promote autoreactivity. During DH-JH rearrangement, a DH gene upstream of the existing DH-JH rearrangement recombines with a JH gene downstream of the DH-JH rearrangement and replaces it by a leapfrogging deletional rearrangement. In a DH-DH fusion, the recombination process links a 5′ DH segment to a preceding DH-JH rearrangement rather than to a 3′ JH gene. DH-DH fusion occurs more frequently in murine lupus than in non-autoimmune strains of mice (40,41). Finally, during VH replacement, the conventional 23 Recombination Signal Sequence (RSS) of an upstream murine VH undergoes RAG-dependent deletional rearrangement with the cryptic RSS of an existing downstream VH gene which is part of an existing VDJ rearrangement on the same allele. This rearrangement results in replacement of all but the very 3′ end of the previously rearranged VH with a new VH. Secondary rearrangement, which would consist of either deletion or inversion of the chromosomal DNA between the recombining gene segments, can also occur at the κ locus. These rearrangements are apparently part of an important physiological process underlying failed allelic exclusion and might occur to edit the specificity of an autoreactive B cell receptor (Figure 6).

CONTROL OF RECEPTOR EDITING

Receptor editing has a genetic control and has been studied in several models. Pre-B cells expressing IκB show evidence of receptor editing which is consistent with a role for NFκB (42). PLCγ2 is present in higher quantities in immature B

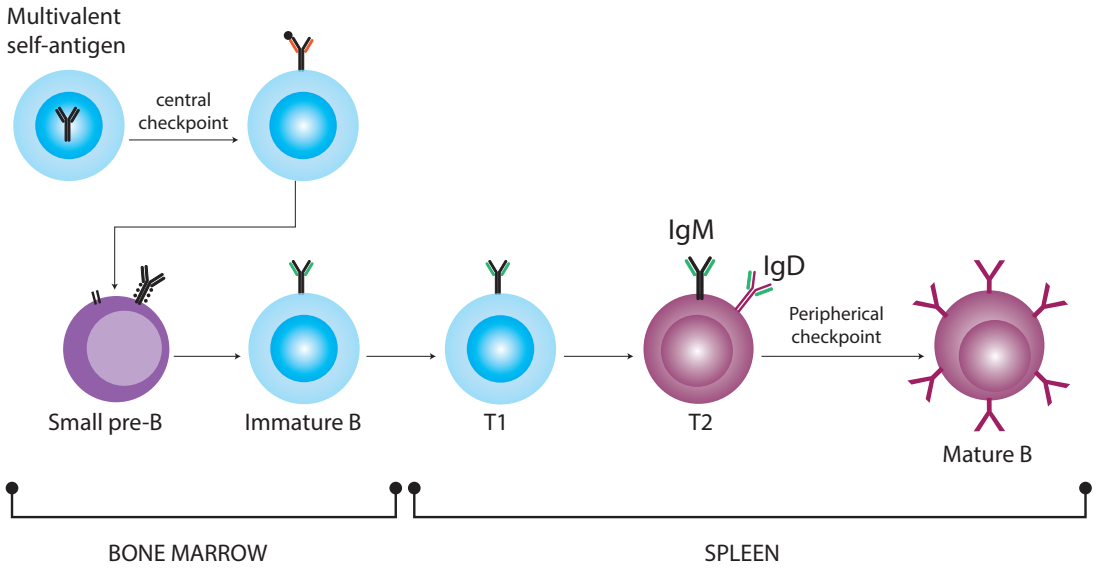

Figure 6. Receptor editing as a major mechanism of central tolerance in B cells. Receptor editing is a major mechanism of central tolerance in B cells. Immature B cells in the bone marrow that encounter multivalent self-antigens revert to the small pre-B stage, continue to rearrange k and, if necessary, l light chain genes, and generate newly B cells that have a novel light chain that is no longer self-reactive. Immature B cells with novel light chains that are no longer part of a self-reactive B cell receptor then migrate to the periphery as T1 B cells where they mature into newly generated IgM and IgD expressing recirculating T2 B cells and, then, into mature recirculating B cells.

cells, showing increased phosphorylation in response to BCR crosslinking, and probably induces the expression of rag2 in these cells. However, other data show down-regulation of rag induced by PLCγ2 and thus terminate receptor editing. Immature B cells can be induced to edit by BCR crosslinking while transitional B cells cannot. This may be due to an altered signaling pathway through PLCγ2 (43,44). The mechanisms that suppress editing and their potential role in autoimmune diseases are under research.

B CELL AND AUTOIMMUNITY

Classically, the immune mechanisms implicated in the development of autoimmune diseases have been categorized into two broad sets of diseases: one set in which the pathological process is driven by T cells, and the other in which the humoral B response mediates the disorder by producing auto-antibodies that are able to bind tissue self-antigens or by forming immune complexes. In recent years, with the new knowledge about the immune response, this approach –dividing autoimmune diseases into T cell and B cell mediated diseases—has dramatically changed. It is now recognized that T lymphocytes facilitate adaptive immune B responses, and B cells play a reciprocal role during CD4 T cell activation in autoimmune diseases.

For instance, most disease-related autoantibodies are IgGs that are somatically mutated, and this suggests that helper T cells drive the autoimmune B cell response (45). In addition, B cells have been shown to be important mediators of some autoimmune diseases. These are classically described as T cell mediated and include rheumatoid arthritis (RA), multiple sclerosis (MS), and Type 1 diabetes mellitus (T1D). In diseases in which specific autoimmune T cell clones drive the process of inflammation, auto-antibody synthesis may represent a marker for the expansion of auto-antigen specific B cells that capture and present auto-antigen peptides to T cells. As mentioned before, the central tolerance mechanisms are crucial in preventing B cell mediated autoimmune diseases. For instance, the strong BCR signal from binding with high affinity to an autoantigen will lead to deletion or receptor editing of the high affinity. This concept has been demonstrated in several autoimmune animal models, including a double-transgenic mouse model carrying not only the heavy chain against the myelin oligodendrocyte glycoprotein (MOG) autoantigen but also the light chain. The authors demonstrated that B cells expressing solely the MOG-specific Ig H-chain differentiate without tolerance. On the other hand, double-transgenic B cells expressing transgenic Ig H and L-chains are subjected to receptor editing (46,47).

If the signaling potential of the BCR is affected, for example, by over-expression of CD19 or PTPN22 polymorphisms (described in several autoimmune diseases), the self-reactive B cells will not be deleted and may reach the periphery (48,49). These mechanisms lead to the increase of self-reactive B cells on the periphery and, as a consequence, the possibility of developing autoimmune diseases. Thus, leaky central tolerance increases the risk for subsequent development of autoimmune disease, but additional factors (genetic, hormonal, environmental, etc.) control this progression from autoimmunity to autoimmune disease.

B CELL FUNCTIONS IN AUTOIMMUNITY

B cells do not simply produce auto-antibodies. In fact, B lymphocytes are uniquely endowed to drive autoimmunity as APC because they can bind native self-proteins through their BCR, process them, and present them to T lymphocytes. To demonstrate the antigen-presenting effect of B cells in autoimmunity, several models and observations have been used. For example, in the murine experimental allergic encephalomyelitis (EAE), B lymphocytes are dispensable when disease is induced by MOG peptides but absolutely required for disease to develop if mice are immunized with MOG protein (50). In MOG-specific TCR and BCR double transgenic mice, self-reactive B cells cause severe EAE by presenting endogenous MOG protein to self-reactive T cells rather than by autoantibody production (51,52). In the transgenic mIgM.MRL-FASlpr mouse, whose B lymphocytes cannot secrete antibodies but can present antigen, lupus develops spontaneously and T cell activation is comparable to MRL/lpr controls (53). Likewise, NOD mice with a mutant IgM heavy chain that cannot be secreted demonstrate that increased insulitis and spontaneous diabetes may occur in the absence of antibody production but require antigen presentation by B cells (54).

The ability of B cells to bind auto-antigens through their BCR allows them to act as potent APCs at very low protein concentrations. In the MOG-specific TCR and BCR double transgenic mice, antigen specific B cells process and present MOG protein to T cells at concentrations that are 100 fold lower than B cells with other BCR specificities. Other functions of B cells are cytokine and chemokine synthesis and ectopic lymphoid neogenesis in autoimmune diseases.

AMPLIFICATION OF THE AUTOIMMUNE RESPONSE BY EPITOPE SPREADING

B cells bind to a specific epitope in antigens via their BCR. After the initial recognition, protein and even protein complexes can be internalized and processed for antigen presentation. The protein may, however, contain several other epitopes besides the epitope originally recognized by the BCR, which can fit in the binding grooves of the MHCII molecules in the B cell. As a consequence, the B cells can present not only the original epitope but also other epitopes of the same protein or protein complex to T lymphocytes and thereby trigger different T cell specificities (55). This phenomenon, known as epitope spreading, allows self-antigens that were not the initial targets of autoreactive lymphocytes at the onset of autoimmunity to become antigens at later stages (56). This phenomenon is described in almost all immune diseases and is frequently associated with disease progression (56). Epitope spreading may trigger the clinically manifested autoimmune disease. As a representative example, the SJL/J mice immunized with protelipid (PLP) proteins develop T cell responses specific to different epitopes in the molecule. These distinct T cell responses contribute to the relapse phases of the EAE and can initiate disease upon secondary adoptive transfer to naïve animals (57). Epitope spreading also occurs in the NOD mouse model of spontaneous diabetes. In this model, T cell responses and antibodies to Type 1 Diabetes (T1D), autoantigens, GAD65 and GAD67 isoforms of GAD are observed in mice at 4 weeks of age. At 6 weeks of age, T and B lymphocyte responses for other β cell antigens-peripherin, carboxypeptidase H, and Hsp60-are also detected. By 8 weeks of age, responses to all former antigens are enhanced. The initial GAD specific reactivity in this model coincides with the onset of insulitis whereas the progression of insulitis to β cell destruction with age correlates to the epitope spreading of B and T cells (58). Temporal progression of autoreactivity to autoimmune disease by epitope spreading also occurs in human autoimmune diseases. In childhood T1D diabetes, insulin autoantibodies (IAA) are the first autoantibodies detected. IAA-positive children that sequentially develop antibodies to other β cell antigens such as GAD and protein tyrosine phosphatase-like proteins IA-2, usually progress to T1D. In contrast, children that remain positive for only IAAs rarely develop the disease (59). In RA, several reports have shown that the number of antibody specificities increases over time. Like T1D patients, healthy individuals with a broad anti-citrullinated peptide antibody (ACPA) profile have a higher risk of developing arthritis (56,60). The number of positive antibodies in Systemic Lupus Erythematosus (SLE) serums also increases over time until the onset of clinical symptoms as demonstrated in the classic article about autoimmune diseases prediction by Arbuckle *et al* (61).

THE EFFECTS OF THE CYTOKINE BAFF IN B CELL TOLERANCE

The cytokine BAFF (for B cell Activating Factor Belonging to the TNF family) has emerged since 1999 (62) as one of the critical factors controlling B cell maturation, tolerance, and malignancy. BAFF plays a key role in B cell differentiation, survival, and activation (62). BAFF, also known as B-lymphocyte stimulator (BLyS), is a cytokine that prevents apoptosis of autoreactive B cells [20]. The BAFF family consists of two

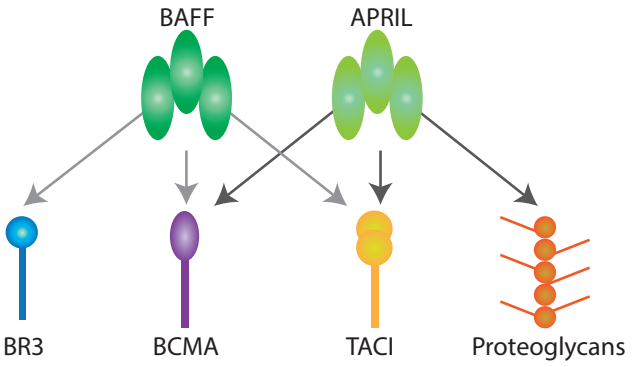

Figure 7. BAFF and APRIL receptors. BAFF binds chiefly to BAFF-R (BR3) but also to BCMA and TACI. APRIL, in turn, interacts with TACI and BCMA, but not with BR3. In addition, APRIL binds to proteoglycans expressed in membranes of lymphoid and non-lymphoid cells.

ligands, a proliferation-inducing ligand (APRIL), and BAFF; and three membrane receptors, BCMA (B cell maturation antigen), TACI (transmembrane activator, calcium modulator, and cyclophylin ligand interactor), and BAFF-R (also known as BR3). The interactions between ligands and receptors vary: thus, BAFF interacts chiefly with BR3 but can interact with all three receptors, whereas APRIL can interact with TACI and BCMA, but not with BR3 (63). BAFF enhances B cell survival, drives B cell maturation especially at the early transitional stages, and discontinues humoral tolerance by rescuing autoreactive B cells from apoptosis (64). Figure 7 shows the different receptors for BAFF and APRIL.

DOUBLE-TRANSGENIC MICE EXPRESSING BOTH HEL AND ANTI-HEL B CELL RECEPTOR

As mentioned before, to avoid the generation of pathogenic auto-antibodies, self-reactive lymphocytes have to be deleted or anergised at successive immune checkpoints during B cell development and maturation. Because immunoglobulin gene rearrangement is a random mechanism, 50–75% of the newly generated B cells in the bone marrow have a self-reactive B cell receptor (BCR). However, the development of autoimmune disease is rare, affecting up to 5% of the population. Consequently, effective mechanisms exist for preventing immune activation of self-reactive lymphocytes. BAFF is known for its role in the survival of mature B cells (62). Based on its receptor expression profile, BAFF has no effect on B cell tolerance in the BM but does act at the periphery (Figure 8). BAFF certainly plays a major role in B cell tolerance after the BT1 immature B cell stage. Whether or not BAFF can influence self-reactive BT1 cell elimination is unclear. However, BAFF is certainly needed for the survival of BT2 cells and downstream B cell subsets. BT2 cells, which express high levels of BAFF-R, are indeed dependent on BAFF because of their propensity for apoptosis (65), and B cell ontogenesis is stopped at the T1 stage when BAFF or BAFF-R are lacking (66). One of the most informative systems for studying B cell tolerance is the double transgenic (Tg) mouse model which expresses the anti-hen-egg lysozyme (HEL) BCR and HEL simultaneously. When HEL is expressed as a cell surface molecule, self-reactive B cells are deleted or undergo additional immunoglobulin gene rearrangements by the receptor editing mechanisms. When HEL is expressed as a soluble protein (sHEL), self-reactive B cells can migrate into the periphery where their fate depends on their ability to compete with non-self-reactive B cells. Without competition, self-reactive BT2 cells persist in an anergic state. In the presence of competition, self-reactive BT2 cells need the cytokine BAFF to sustain their survival and maturation. Because BAFF levels are limited under normal conditions, these self-reactive B cells undergo apoptosis. Thus, if double Tg mice for sHEL/anti-HEL are treated with antagonist for BAFF, survival of sHEL self-reactive B cells is dramatically decreased (67). In contrast, when BAFF is overexpressed, sHEL self-reactive BT2 cells survive and colonize

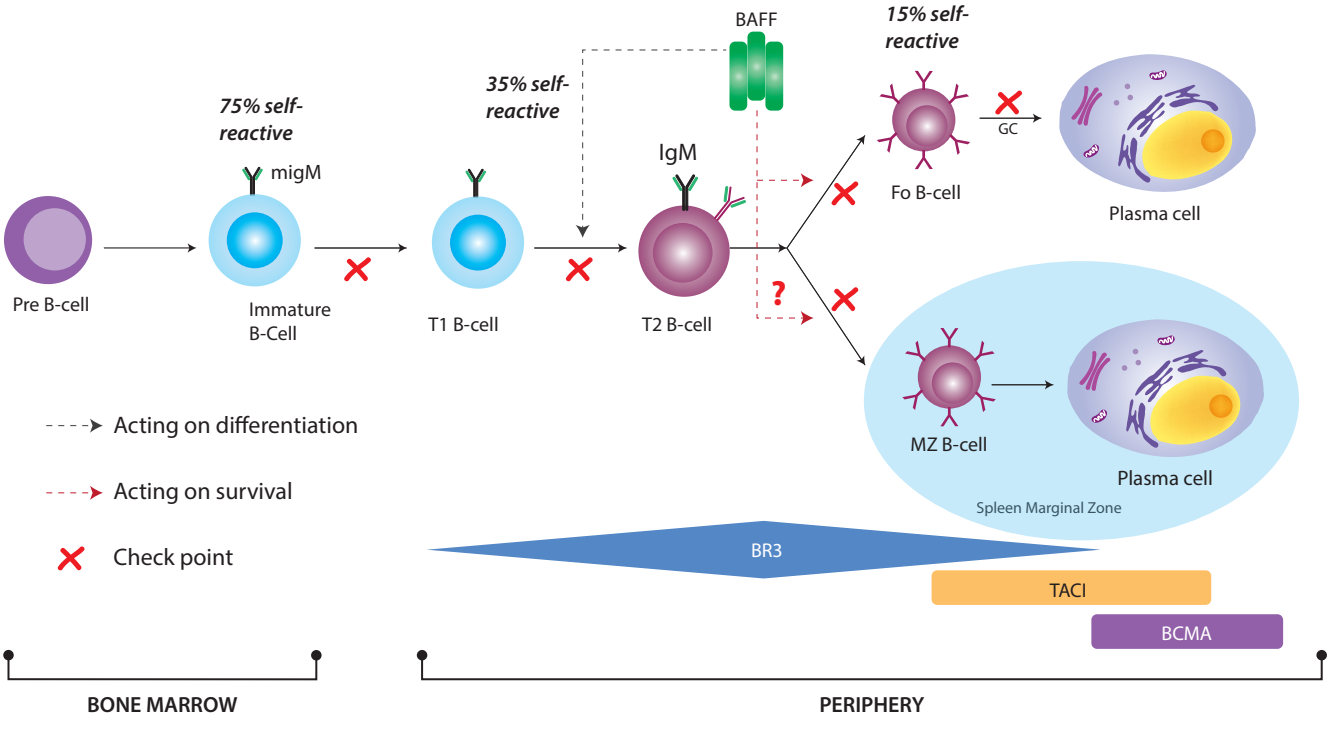

Figure 8. BAFF receptor cell surface expression and self-tolerance during B cell ontogenesis. Data indicate the proportion of self-reactive B cells at specific B cell stages before or after check points as determined in the anti-HEL/HEL transgenic models. *Fo*: follicular, *GC*: germinal center; *Imm*: immature; *MZ*: marginal zone; *Pre*: precursor; *T1 or 2*: Transitional type 1 or 2.

follicles and MZ in the spleen (68). Of note, when anti-HEL B cells compete with normal B cells in the animal, excessive expression of BAFF no longer prevents the escape of self-reactive B cells. In this scenario, self-reactive cells are eliminated at a much earlier maturation stage (T1), a stage when B cells express little BAFF-R and as such are unable to sense excessive BAFF production that can only efficiently rescue BT2 cells.

BAFF-TRANSGENIC MICE

BAFF-Tg mice constitute an effective model for autoimmunity. Overproduction of BAFF in these mice leads to B cell proliferation, auto-antibody production, and, ultimately, development of kidney failure similar to SLE-associated symptoms. Moreover, aging BAFF-Tg mice also present a primary Sjögren's syndrome (pSS)-like disease, in which they demonstrate inflammation and destruction of salivary glands (SGs) (64). In addition to the attendant polyclonal hypergammaglobulinemia, BAFF-Tg mice develop elevated titers of multiple auto-antibodies, including antinuclear antibodies, anti-double-stranded DNA, rheumatoid factors, circulating immune complexes, and immunoglobulin deposits in kidneys. Some B cell subsets such as BT2 cells, follicular (Fo) B cells, and MZ B cells rise. Moreover, without stimulation, a high number of GCs are found in the spleen and the lymph nodes. Finally, lymphocytes infiltrating the SG are essentially MZ-like B cells. Note that BAFF-Tg mice develop the same pSS manifestations when T cells are removed (69), but in this instance, BAFF exacerbates Toll-like receptor activation of B cells. An alternative model for the development of SS apart from T cells has since been proposed (70).

CD 22 IN ITS IMPLICATIONS IN AUTOIMMUNITY

Another important B cell molecule which has an effect on autoimmunity development is the CD22. B cell responses are initiated by antigen binding to the BCR and are modified by a broad repertoire of activating and inhibitory transmembrane co-receptors expressed on the B cell surface (71,72). In this context, the multifunctional BCR co-receptor, CD22, is interesting since it plays a critical role in establishing and modulating the antigen receptor signaling thresholds for B cell activation (73). CD22, as part of the BCR complex, can modulate the intensity, quality, and duration of homeostatic and BCR-induced signals in an inhibitory or stimulatory capacity through ligand dependent and -independent mechanisms (74,75). Based on substantial mouse model data, it appears that the predominant effect of CD22 is inhibitory (76). CD22 is a 135-kDa B-lymphocyte restricted type-I transmembrane sialoglycoprotein of the immunoglobulin superfamily (77). It appears intracellularly during the late pro-B cell stage of ontogeny but shifts to the plasma membrane with B cell maturation. CD22 is expressed at low levels on immature B cells and at higher levels on mature IgM+, IgD+ B cells. However, it is absent on differentiated plasma cells. It is strongly expressed in follicular, mantle, and marginal-zone B cells but is weakly present in germinal B cells (78). As previously mentioned, for the immune system to function effectively, it is essential to mount an appropriate humoral response against potential pathogens while avoiding autoimmunity and reactivity to self antigens (79). Understanding the function of CD22 may, therefore, suggest methods for modulating humoral immunity and aid in discovering treatments for autoimmunity (80).

To regulate B lymphocyte functions and migration, the interaction of CD22 with α2,6-linked sialic acid ligands is important. This binding is necessary for its negative regulatory functions (81). Cell lines expressing CD22 without sialic acid-binding activity are hyper-responsive to BCR stimulation (81).

Recent studies in mouse models have suggested a role for defects and loss of functionality in CD22 in the pathogenesis of autoimmune disease, including SLE. B cells obtained from CD22-deficient mice have been shown to be hyper-responsive to receptor signaling and demonstrate increased Ca2+ fluxes on BCR ligation, which increased serum titers of IgG anti-dsDNA autoantibodies. These antibodies were of multiclonal origin, were somatically mutated, and had high affinity (82).

Epratuzumab is a novel humanized antihuman CD22 IgG1 monoclonal antibody that binds to the extracellular domain of CD22 and induces modest but significant intracellular phosphorylation. Epratuzumab reduces total blood B cells by about 35–40% and has preferential effects on naïve and transitional B cells (83,84). Epratuzumab treatment has been used with moderate clinical success in SLE and primary Sjögren's syndrome (85).

A NEW CONCEPT IN AUTOIMMUNITY REGULATORY B CELLS

A functional B cell subset, called regulatory B cells, has recently emerged as an important factor for maintaining immune tolerance. This subtype restrains the excessive inflammatory response that occurs during the development of autoimmune diseases. The main regulatory B cell function is mediated by the IL-10 production that inhibits pro-inflammatory cytokines and supports regulatory T cell differentiation. The regulatory B cells were named in 2002 by Bhan *et al* (86), when they demonstrated that IL-10 producing B cells can suppress inflammatory responses in experimental autoimmune encephalomyelitis, collagen-induced arthritis, and autoimmune colitis (87,88).

In the murine models, regulatory B cells have also been shown to directly inhibit T cell proliferation through cell–cell contact. This may even lead to anergy, or apoptosis of T cells (89,90) and the modulation of the inflammatory response. In this regard, CD40 engagement on B cells appears to be a requisite for the induction of functional B regulatory cells in mice. Stimulation of CD40 brings about

DIRECT B LYMPHOCYTE TARGETING
CD-20 antigen
Rituximab (chimeric monoclonal antibody)
Ocrelizumab (humanized monoclonal antibody)
Ofatumumab (human monoclonal antibody)
Veltuzumab (humanized monoclonal antibody)
TRU-015 (engineered protein)
CD-22 antigen
Epratuzumab (humanized monoclonal antibody anti-CD22)
Indirect B lymphocyte targeting

INDIRECT B LYMPHOCYTE TARGETING
BAFF
Belimumab (LimphoStat B: fully human monoclonal antibody anti-BAFF)
BAFF receptors
Anti-BR3
Atacicept (Fusion IgG with the extracellular domain of TACI receptor)
Briobacept/BR3-Fc (Fusion IgG with the extracellular domain of BAFF receptor-BR3)

Table 3. Potential targets in B lymphocytes and the therapeutic molecule for the treatment of autoimmune diseases. BAFF: B lymphocyte Activator Factor belonging to the TNF family; TACI: transmembrane activator and calcium modulator and cyclophylin ligand interactor.

the development of B cells with suppressive properties. Furthermore, signaling in the absence of CD40 makes B cells unable to regulate inflammatory response (87,91).

The murine phenotypic nature of B regulatory cells is still a matter for debate. Two distinct IL-10-producing B cell subpopulations associated with regulatory functions have been identified. One has been recognized as transitional-marginal zone precursor B cells expressing a high level of CD21, CD23, CD24, IgM, and CD1d, designed as transitional type 2 (T2)-like cells (92-94). The second—described as CD1dhi, CD5+, and CD19hi B cells—has been called "B10" cells since IL-10 is the main cytokine produced by these cells (95). Recent studies have suggested that human B cells can also regulate inflammatory responses (96). These cells have been studied primarily in autoimmune diseases, including SLE and multiple sclerosis, for which functional as well as numerical defects of these cells have been described (94,97-99). Knowledge about these cells is increasing rapidly, but much remains to be understood regarding the biology of B regulatory cells in murine models and humans.

B CELL TARGETED THERAPIES

Several B cell molecules can be targeted to treat autoimmune diseases (Table 3). The most widely studied target for achieving B cell depletion in autoimmune disease is the CD20 antigen (human B cell-restricted differentiation Antigen), a hydrophobic transmembrane protein with a molecular weight of approximately 35 kDa found on pre-B and mature B cells (100,101) as well as in over 90% of the B cells in NHL (102). Another therapeutic approach is the inhibition of BAFF effects on B cell. This inhibition can be done by anti-BAFF or anti-BR3 monoclonal Abs, as well as BR3 or TACI decoy fusion proteins. Selective BAFF blockers prevent BAFF from interacting with its receptors, leaving APRIL available to interact with TACI and BCMA. Drugs in this class include anti-BAFF Ab (Belimumab or Lymphostat B) and a fusion protein consisting of human Ig Fc and of the extracellular BR3 domain (Briobacept, for BAFF-R-Ig). Non-selective BAFF blockers abolish the interactions of both BAFF and APRIL with all their receptors. To date, there is a single drug in this class which is human Ig Fc fused to the extracellular TACI domain (Atacicept, TACI-Ig). Differences in the distribution of the forms of BAFF could denote the potential of patients to respond or to resist to BAFF antagonist therapy. Treatment of B cells with TACI agonist Ab inhibits proliferation *in vitro* and activation of a chimeric receptor containing TACI intracellular domain induces apoptosis. These results demonstrate also the critical requirement for TACI in regulating B cell homeostasis. The therapeutic effects of anti-BAFF therapy with Belimumab have been demonstrated in patients with SLE.

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7 T LYMPHOCYTES IN AUTOIMMUNITY

Alejandro Ruiz-Argüelles

INTRODUCTION

T cells play a dual role in autoimmunity which will be described separately for mere didactical purposes. On one hand, T cells play a crucial role in the maintenance of tolerance to self, while on the other, autoreactive T cells play an important effector role in the cell and tissue damage that encompasses autoimmune disease (1-3).

GENERATION OF T CELL RECEPTORS

It is impossible to comprehend the mechanisms of tolerance maintenance and breakdown without understanding the origin of the diversity of the T cell receptors (TCR) (Fig 1).

Like immunoglobulin heavy and light chains, T cell receptor α and β chains each consist of a variable (V) amino-terminal region and a constant (C) region. The organization of the TCRα and TCRβ loci is shown in figures 2 and 3. The organization of the gene segments is broadly homologous to that of the immunoglobulin gene segments. The TCRα locus, like the Loci for the immunoglobulin light chains, contains V and J gene segments (Vα and Jα). The TCRβ locus, like that for the immunoglobulin heavy-chain, contains D gene segments in addition to Vβ and Jβ gene segments.

The T cell receptor gene segments rearrange during T cell development to make complete V-domain exons. T cell receptor gene rearrangement takes place in the thymus. Essentially, however, the mechanics of gene rearrangement are similar for B and T cells. The T cell receptor gene segments are flanked by heptamer and nonamer recombination signal sequences that are homologous to those flanking immunoglobulin gene segments and are recognized by the same enzymes. All known defects in genes that control V(D)J recombination affect T cells and B cells equally, and animals with these genetic defects lack functional lymphocytes altogether. A further shared feature of immunoglobulin and T cell receptor gene rearrangement is the presence of P- and N-nucleotides in the junctions between the V, D, and J gene segments of the rearranged TCRβ gene. In T cells, P- and N-nucleotides are also added between the V and J gene segments of all rearranged TCRα genes, whereas only about half the V-J joints in immunoglobulin light-chain genes are modified by N-nucleotide addition and these are often left.

The main differences between the immunoglobulin genes and those encoding T cell receptors reflect the fact that all the effector functions of B cells depend upon secret-

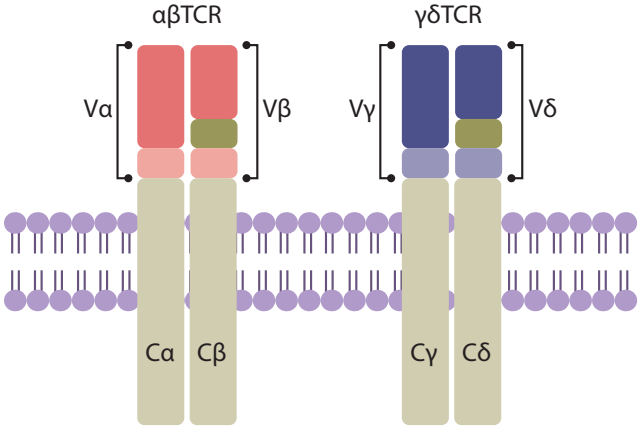

Figure 1. T cell antigen receptors (TCR) are composed of heterodimers. Each chain contains a constant (Cα and Cβ, or Cγ and Cδ) and a variable region (Vα and Vβ, or Vγ and Vδ). Variable regions of α and γ chains are encoded by two gene regions (V and D), while variable regions of β and δ chains are encoded in three gene regions (V, D and J). Constant regions have an hydrophobic domain that is responsible for the transmembrane anchorage of the cell receptor.

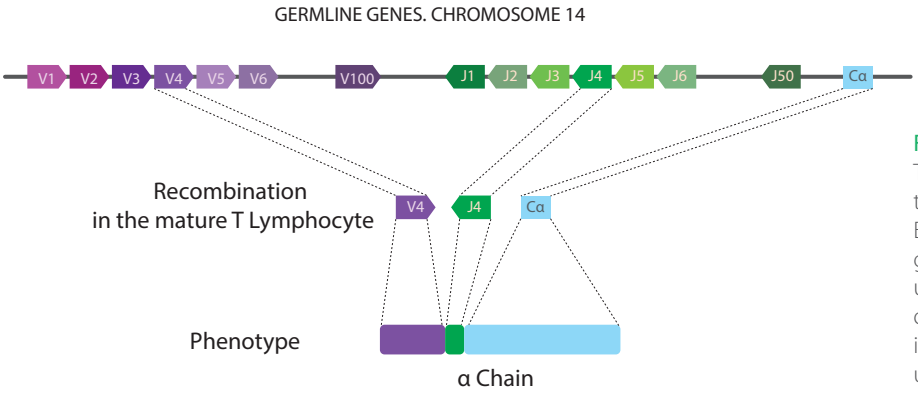

Figure 2. Genes encoding the α chain of the TCRαβ are mapped in chromosome 14, in three segments of the germline: V, J and C. Each mature T lymphocyte rearranges one gene out of each V and J segments and the unique C gene. This recombination is the one that is finally expressed phenotypically in that T cell clone. The γ chain of the TCRγδ uses a similar mechanism.

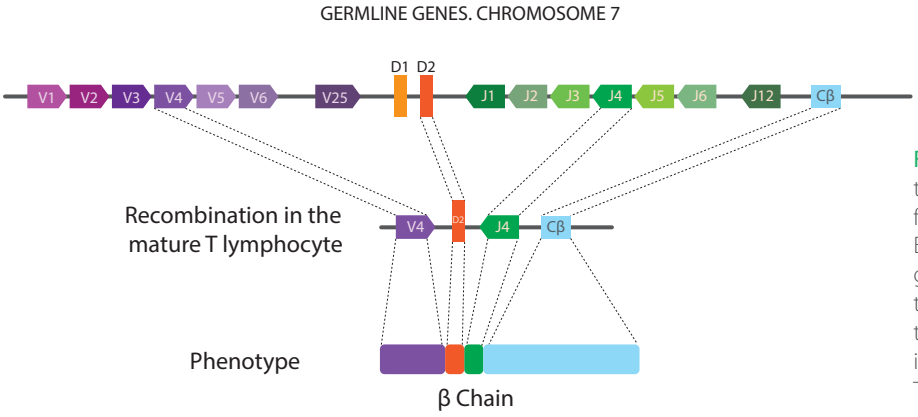

Figure 3. Genes encoding the β chain of the TCRαβ are mapped in chromosome 7, in four segments of the germline: V, D, J and C. Each mature T lymphocyte rearranges one gene out of each V,D and J segments and the unique C gene. This recombination is the one that is finally expressed phenotypically in that T cell clone. The δ chain of the TCRγδ uses a similar mechanism.

ed antibodies whose different heavy-chain C-region isotypes trigger distinct effector mechanisms. The effector functions of T cells, in contrast, depend upon cell-cell contact and are not mediated directly by the T cell receptor, which serves only for antigen recognition. Thus, the C regions of the TCRα and TCRβ loci are much simpler than those of the immunoglobulin heavy-chain locus. There is only one Cα gene and, although there are two Cβ genes, they are very closely homologous and there is no known functional distinction between their products. The T cell receptor C-region genes encode only transmembrane polypeptides.

The extent and pattern of variability in T cell receptors and immunoglobulins reflect the distinct nature of their ligands. Whereas the antigen-binding sites of immunoglobulins must conform to the surfaces of an almost infinite variety of different antigens and thus come in a wide variety of shapes and chemical properties, the ligand for the T cell receptor is always a peptide bound to an MHC molecule. The antigen-recognition sites of T cell receptors would therefore be predicted to have a less variable shape with most of the variability focused on the bound antigenic peptide occupying the center of the surface in contact with the receptor.

In spite of differences in the sites of variability, the three-dimensional structure of the antigen-recognition site of a T cell receptor looks much like that of an antibody molecule. In an antibody, the center of the antigen-binding site is formed by the CDR3s of the heavy and light chains. The structurally equivalent third hypervariable loops (CDR3s) of the T cell receptor α and β chains, to which the D and J gene segments contribute are also form the center of the antigen-binding site of a T cell receptor. The periphery of the site consists of the equivalent of the CDR1 and CDR2 loops, which are encoded within the germline V gene segments for the α and β chains (4-5).

T cell receptor loci have roughly the same number of V gene segments as do the immunoglobulin loci, but only B cells diversify rearranged V-region genes by somatic hypermutation. Thus, diversity in the CDR1 and CDR2 loops that comprise the periphery of the antigen-binding site will be far greater among antibody molecules than among T cell receptors. This is in keeping with the fact that the CDR1 and CDR2 loops of a T cell receptor will mainly contact the relatively less variable MHC component of the ligand rather than the highly variable peptide component.

The structural diversity of T cell receptors is mainly attributable to combinatorial and junctional diversity generated during the process of gene rearrangement. The TCRα locus contains many more J gene segments than either of the immunoglobulin light-chain loci: in humans, 61 Jα gene segments are distributed over about 80 kb of DNA, while immunoglobulin light-chain loci have only five J gene segments at most (table 1). Because the TCRα locus has so many J gene segments, the variability generated in this region is even greater for T cell receptors than for immunoglobulins. This region encodes the CDR3 loops in immunoglobulins and T cell receptors that form

GENE SEGMENT	α/β TCR		γ/δ TCR	
	vα	vβ	vγ	vδ
Variable (V)	~100	~50	5	10
Diversity (D)	NO	2	NO	2
Joining(J)	~60	12	4	2

Table 1. **Numbers of human T cell receptor gene segments.**

the center of the antigen-binding site. Thus, the center of the T cell receptor will be highly variable, whereas the periphery will be subject to relatively little variation.

When analyzing the generation of antibody diversity, it is clear that somatic hypermutation increases the diversity of all three complementarity-determining regions of both immunoglobulin chains. Somatic hypermutation does not occur in T cell receptor genes, so variability of the CDR1 and CDR2 regions is limited to that of the germline V gene segments. All the diversity in T cell receptors is generated during rearrangement and is consequently focused on the CDR3 regions.

Why T cell and B cell receptors differ in their abilities to undergo somatic hypermutation is not clear, but several explanations can be suggested on the basis of the functional differences between T and B cells. Because the central role of T cells is to stimulate both humoral and cellular immune responses, it is crucially important that T cells not react with self proteins. T cells that recognize self antigens are rigorously purged during development, and the absence of somatic hypermutation helps to ensure that somatic mutants recognizing self proteins do not arise later in the course of immune responses. This constraint does not apply with the same force to B cell receptors as B cells usually require T cell help to secrete antibodies. A given B cell whose receptor mutates to become self reactive would, under normal circumstances, fail to make antibodies due to lack of self-reactive T cells to provide this help.

A further argument is that T cells already interact with a self component, namely the MHC molecule that makes up the major part of the ligand for the receptor, and thus might be unusually prone to developing self-recognition capability through somatic hypermutation. In this case, the converse argument can also be made: because T cell receptors must be able to recognize self MHC molecules as part of their ligand, it is important to avoid somatic mutation that might result in the loss of recognition and the consequent loss of any ability to respond. However, the strongest argument for this difference between immunoglobulins and T cell receptors is the simple one that somatic hypermutation is an adaptive specialization for B cells alone because they must make very high-affinity antibodies to capture toxin molecules in the extracellular fluids.

IMMUNOLOGIC TOLERANCE

Tolerance is different from non-specific immunosuppression and immunodeficiency. It is an active antigen-dependent process that responds to the antigen. Like the immune response, tolerance is specific, and like immunological memory, it can exist in T cells, B cells or both. Also like immunological memory, tolerance at the T cell level is longer lasting than tolerance at the B cell level.

Induction of tolerance in T cells is easier and requires relatively smaller amounts of tolerogen than tolerance in B cells. Maintenance of immunological tolerance requires persistence of the antigen. Tolerance can be broken naturally (as in autoimmune diseases) or artificially (as shown in experimental animals, by x-irradiation, certain drug treatments, and exposure to cross reactive antigens).

Tolerance may be induced in all epitopes or only some epitopes on an antigen and tolerance to a single antigen may exist at the B cell level, T cell level, or at both levels.

MECHANISMS OF TOLERANCE

The exact mechanism of induction and maintenance of tolerance is not fully understood. Experimental data, however, point to several possibilities (1-17).

CLONAL DELETION

During development, T and B lymphocytes come across self antigens and these cells undergo clonal deletion through a process known as apoptosis or programed cell death (Fig 4). For example, T cells that develop in the thymus first express neither CD4 nor CD8. They next acquire both CD4 and CD8, called double-positive cells, and express low levels of αβ TCR. Such cells undergo positive selection after interacting with class I or class II MHC molecules expressed on the cortical epithelium. During this process, cells with low affinity for MHC are positively selected (6). Unselected cells die by apoptosis, a process called "death by neglect". Next, the cells lose either CD4 or CD8. The T cells then encounter self-peptides presented by self MHC molecules expressed on dendritic cells. Those T cells with high affinity receptors for MHC plus self-peptides undergo clonal deletion which is also called negative selection through induction of apoptosis. Any disturbance in this process can lead to the escape of auto-reactive T cells that may trigger autoimmune disease. Likewise, differentiating early B cells when they encounter self-antigen, cell associated or soluble, undergo deletion. Thus, clonal deletion plays a key role in ensuring tolerance to self antigen (5-9).

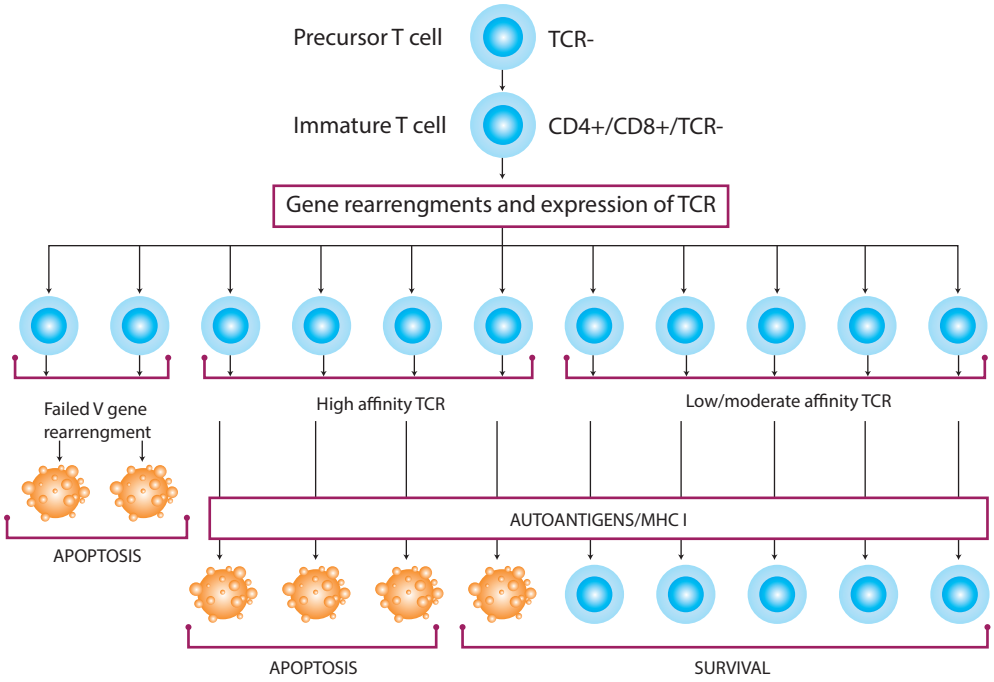

Figure 4. During the intrathymic maturation n of T lymphocytes, several selection processes occur. When mature lymphocytes begin to rearrange and express TCR molecules, some fail to construct functional receptors and undergo active cell death. From those who succeed to build functional TCR molecules, the ones bearing TCR displaying high-affinity for autoantigens, undergo apoptosis when contacting with their complementary self-antigens (negative selection). The remaining ones, showing low or moderate affinity TCR for self antigens, survive to become part of the active repertoire of T cells in the adult life (positive selection).

Peripheral tolerance: Clonal deletion is not a fool proof system and often T and B cells fail to undergo deletion. Therefore, these cells can potentially cause autoimmune disease once they reach the peripheral lymphoid organs. Thus, the immune system has devised several additional check points so that tolerance can be maintained.

Activation-induced cell death: Upon activation, T cells not only produce cytokines or carryout their effector functions but also die through programed cell death or apoptosis. In this process, the death receptor (Fas) and its ligand (FasL) play a crucial role. Thus, normal T cells express Fas but not FasL. Upon activation, T cells express FasL which binds to Fas and triggers apoptosis by activation of caspase-8. The importance of Fas and FasL is clearly demonstrated by the observation that mice with mutations in Fas (lpr mutation) or FasL (gld mutation) develop severe lymphoproliferative and autoimmune disease and die within 6 months while normal mice live up to 2 years. Similar mutations in these apoptotic genes in humans leads to a lymphoproliferative disease called autoimmune lymphoproliferative syndrome (ALPS).

CLONAL ANERGY

Auto-reactive T cells when exposed to antigenic peptides on antigen presenting cells (APC) that do not possess the co-stimulatory molecules CD80 (B7-1) or CD86 (B7-2) become anergic (nonresponsive) to the antigen. Also, while activation of T cells through CD28 triggers IL-2 production, activation of CTLA4 leads to inhibition of IL-2 production and anergy. Also, B cells when exposed to large amounts of soluble antigen down-regulate their surface IgM and become anergic. These cells also up-regulate the Fas molecules on their surface. An interaction of these B cells with Fas-ligand bearing T cells results in their death via apoptosis (10-12).

CLONAL IGNORANCE

T cells reactive to self-antigen not represented in the thymus will mature and migrate to the periphery, but they may never encounter the appropriate antigen because it is sequestered in inaccessible tissues. These cells may die out for lack of stimulus. Autoreactive B cells that escape deletion may not find the antigen or the specific T cell help and thus not be activated and die out (13-14).

ANTI-IDIOTYPE ANTIBODY

These are antibodies that are produced against the specific idiotypes of other antibodies. Anti-idiotypic antibodies are produced during the process of tolerization and have been demonstrated in tolerant animals. These antibodies may prevent the B cell receptor from interacting with the antigen. The so called idiotype network is an intriguing and

amazing concept, for it implies that self recognition of idiotypes by other antibodies/receptors is a mechanism that prevents autoimmune disease: in simple terms, controlled autoimmunity prevents uncontrolled autoimmunity (18).

REGULATORY T CELLS

Recently, a distinct population of T cells has been discovered called regulatory T cells. Regulatory T cells come in many flavors, but the most well characterized include those that express CD4+ and CD25+. Because activated normal CD4 T cells also express CD25, it was difficult to distinguish regulatory T cells and activated T cells. The latest research suggests that regulatory T cells are defined by expression of the forkhead family transcription factor Foxp3. Expression of Foxp3 is required for regulatory T cell development and function. The precise mechanisms through which regulatory T cells suppress other T cell functions is not clear. One of the mechanisms includes the production of immunosuppressive cytokines such as TGF-β and IL-10. Genetic mutations in Foxp3 in humans leads to development of a severe and rapidly fatal autoimmune disorder known as Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. This disease provides the most striking evidence that regulatory T cells play a critical role in preventing autoimmune disease (19-22).

Elimination of $CD25^+$ T cells, which constitute 5-10% of peripheral $CD4^+$ T cells in normal naive mice, leads to spontaneous development of various autoimmune diseases. These immunoregulatory $CD25^+CD4^+$ T cells are naturally unresponsive in vitro to TCR stimulation, and, upon stimulation, suppress proliferation of $CD25^-CD4^+$ T cells and CD8+ T cells. The antigen concentration required for stimulating $CD25^+CD4^+$ T cells to exert suppression is much lower than that required for stimulating $CD25^-CD4^+$ T cells to proliferate. The suppression, which results in reduced IL-2 production by $CD25^-CD4^+$ T cells, is dependent on cellular interactions on antigen-presenting cells and antigen non-specific in its effector phase. Addition of high doses of IL-2 or anti-CD28 antibody to the in vitro T cell stimulation culture not only breaks the anergic state of $CD25^+CD4^+$ T cells, but also abrogates their suppressive activity. Importantly, the anergic/suppressive state of $CD25^+CD4^+$ T cells appeared to be their basal default condition, since removal of IL-2 or anti-CD28 antibody from the culture milieu allows them to revert to the original anergic/suppressive state. Furthermore, transfer of such anergy/suppression-broken T cells from normal mice produces various autoimmune diseases in syngeneic athymic nude mice. These results taken together indicate that one aspect of immunologic self-tolerance is maintained by this unique $CD25^+CD4^+$ naturally anergic/suppressive T cell population and its functional abnormality directly leads to the development of autoimmune disease.

Additionally, a team led by Dr. John O'Shea of NIH's National Institute of Arthritis and Musculoskeletal and Skin Diseases, set out to investigate the development of Th17 cells. These helper T cells make a cytokine called IL-17 that causes inflammation. While helpful for fighting infections, Th17 cells have also been linked with several autoimmune disorders. Past studies have found that a combination of 3 cytokines—interleukin-1-beta (IL-1-beta), interleukin-6 (IL-6) and transforming growth factor beta (TGF-beta)—drive Th17 cell development. However, TGF-beta also drives the development of other T cells (regulatory T cells) that dampen the inflammatory response and help prevent autoimmunity. To further explore Th17 development, the scientists exposed immature mouse T cells to a variety of cytokines in culture. Later it was shown that replacing TGF-beta with another cytokine, IL-23, worked just as well. Gene expression analysis found that Th17 cells from TGF-beta cultures had higher levels of an anti-inflammatory cytokine called IL-10. In contrast, Th17 cells developed with IL-23 showed higher levels of IL-18R1, a molecule known to be involved in the development of experimental autoimmune encephalomyelitis (EAE), a mouse disease that resembles human multiple sclerosis. The researchers next looked at the ability of both sets of Th17 cells to cause autoimmune disease. They transferred the cells into mice engineered to lack mature T cells of their own. Mice that received Th17 cells from IL-23 cultures showed more severe symptoms of EAE. This shows that Th17 cells behave differently depending on which cytokines drove their development, with IL-23 producing Th17 cells that are more likely to cause autoimmune disease. These findings shed light on Th17 development, revealing 2 kinds of Th17 cells with distinct functions. The discovery of IL-23 as a driving force in Th17 development points to potential new targets for therapies against autoimmunity.

TERMINATION OF TOLERANCE

Experimentally induced tolerance can be terminated by prolonged absence of exposure to the tolerogen, by treatments which severely damage the immune system (x-irradiation) or by immunization with cross reactive antigens. These observations are of significance in the conceptualization of autoimmune diseases. Several mechanisms have been proposed to be operative in the pathogenesis of autoimmune diseases, against a backdrop of genetic predisposition and environmental modulation. It is beyond the scope of this chapter to discuss each of these mechanisms exhaustively, but a summary of some of the important mechanisms have been described, as follows:

a. T cell-B cell discordance A normal immune response is assumed to involve B and T cell responses to the same antigen, even if we know that B cells and T cells recognize very different structures: conformations on the surface of a molecule for B cells and pre-processed peptide fragments of proteins for T cells. All that is required is that a B cell recognizing antigen X endocytoses and processes a protein Y (normally =X) and presents it to a T cell.

b. T cell Bypass A normal immune system requires the activation of B cells by T cells before the former can produce antibodies in large quantities. This requirement of a T cell can be bypassed in rare instances, such as infection by organisms producing super-antigens, which are capable of initiating polyclonal activation of B cells, or even of T cells, by directly binding to the β-subunit of T cell receptors in a non-specific fashion.

c. Molecular Mimicry An exogenous antigen may share structural similarities with certain host antigens; thus, any antibody produced against this antigen (which mimics the self-antigens) can also, in theory, bind to the host antigens, and amplify the immune response. The idea of molecular mimicry arose in the context of rheumatic fever, which follows infection with Group A beta-haemolytic streptococci. Although rheumatic fever has been attributed to molecular mimicry for half a century no specific antigen has been formally identified. Moreover, the complex tissue distribution of the disease argues against a cardiac specific antigen. It remains entirely possible that the disease is due to an unusual interaction between immune complexes, complement components and endothelium. A similar case seems to operate in vitiligo (see below).

d. Idiotype Cross-Reaction Idiotypes are antigenic epitopes found in the antigen-binding portion (Fab) of the immunoglobulin molecule. There is evidence that autoimmunity can arise as a result of a cross-reaction between the idiotype on an antiviral antibody and a host cell receptor for the virus in question. In this case, the hosT cell receptor is envisioned as an internal image of the virus, and the anti-idiotype antibodies can react with the host cells.

e. Aberrant B cell receptor-mediated feedback A feature of human autoimmune disease is that it is largely restricted to a small group of antigens, several of which have known signaling roles in the immune response (DNA, C1q, IgGFc, Ro, small nuclear ribonucleoproteins, mitochondria, etc). This fact gave rise to the idea that spontaneous autoimmunity may result when the binding of antibody to certain antigens leads to aberrant signals being fed back to parent B cells through membrane bound ligands. These ligands include B cell receptor (for antigen), IgG Fc receptors, CD21, which binds complement C3d, Toll-like receptors 9 and 7 (which can bind DNA and nucleoproteins) and PNAR. More indirect aberrant activation of B cells can also be envisaged with autoantibodies to acetyl choline receptor (on thymic myoid cells) and hormone and hormone binding proteins. Together with the concept of T cell-B cell discordance this idea forms the basis of the hypothesis of self-perpetuating autoreactive B cells. Autoreactive B cells in spontaneous autoimmunity are seen as surviving because of subversion both of the T cell help pathway and of the feedback signal through B cell receptor, thereby overcoming the negative signals responsible for B cell self-tolerance without necessarily requiring loss of T cell self-tolerance (21, 22).

f. Dendritic cell apoptosis Immune system cells called dendritic cells present antigens to active lymphocytes. Dendritic cells that are defective in apoptosis can lead to inappropriate systemic lymphocyte activation and consequent decline in self-tolerance.

g. Cytokine Dysregulation Cytokines have been recently divided into two groups according to the population of cells whose functions they promote: TH1 and TH2 cells. The second category of cytokines, which include IL-4, IL-10 and TGF-β, seem to have a role in prevention of exaggeration of pro-inflammatory immune responses (22).

h. Epitope modification or Cryptic epitope exposure this mechanism of autoimmune disease is unique in that it does not result from a defect in the hematopoietic system. Instead, disease results from the exposure of cryptic N-glycan linkages common to lower eukaryotes and prokaryotes on the glycoproteins of mammalian non-hematopoietic cells and organs. This exposure of phylogenically primitive glycans activates one or more mammalian innate immune cell receptors to induce a chronic sterile inflammatory state. In the presence of chronic and inflammatory cell damage, the adaptive immune system is recruited and self–tolerance is lost with increased autoantibody production. In this form of the disease, the absence of lymphocytes can accelerate organ damage, and intravenous IgG administration can be therapeutic. Although this route to autoimmune disease may underlie various degenerative disease states, no diagnostics for this disease mechanism exist at present, and thus its role in human autoimmunity is currently unknown.

i. Epitope spreading or epitope drift When the immune reaction changes from targeting the primary epitope to also targeting other epitopes. In contrast to molecular mimicry, the other epitopes need not be structurally similar to the primary one.

ROLE OF CO-STIMULATORY SIGNALS IN MAINTENANCE AND BREAKDOWN OF SELF-TOLERANCE

The T cell-specific cell-surface receptors CD28 and CTLA-4 are important regulators of the immune system. CD28 potently enhances those T cell functions that are essential for an effective antigen-specific immune response (1-5), and the homologous CTLA-4 counterbalances the CD28-mediated signals and thus prevents an otherwise fatal overstimulation of the lymphoid system (6-9). Here we report the identification of a third member of this family of molecules, inducible co-stimulator (ICOS), which is a homod-

imeric protein of relative molecular mass 55,000–60,000 (Mr 55K–60K). Matching CD28 in potency, ICOS enhances all basic T cell responses to a foreign antigen, namely proliferation, secretion of lymphokines, upregulation of molecules that mediate cell–cell interaction, and effective help for antibody secretion by B cells. Unlike the constitutively expressed CD28, ICOS has to be de novo induced on the T cell surface, does not upregulate the production of interleukin-2, but superinduces the synthesis of interleukin-10, a B cell-differentiation factor. In vivo, ICOS is highly expressed on tonsillar T cells, which are closely associated with B cells in the apical light zone of germinal centres, the site of terminal B cell maturation. Therefore, it appears that an imbalance amongst co-stimulatory signals might be another additional mechanism leading to tolerance rupture (17-19).

In summary, the role of T cells in the generation of immune reactivity to self is rather complex, but some issues are clear and might be considered robust enough to be accepted:

a. The mechanisms of the generation of the diversity of the T cell receptors –as in B cells- allow for the random construction of self-recognition structures, therefore, autoreactive T cells develop in normal subjects.

b. Many autoreactive T cells are deleted –anatomically or physiologically- from the active repertoire in the healthy individual.

c. Non-deleted autoreactive T cells are kept quiescent by several cellular, humoral and combined mechanisms.

d. Failure or imbalance of such regulatory mechanisms might lead to self-tolerance rupture and lead to autoimmune reactivity or autoimmune disease.

e. Genetic and environmental factors play crucial roles in the pathogenesis of autoimmune disease.

According to several clinical and experimental observations, the critical issues that can trigger and abnormal immune response to a self antigen comprise:

a. The maturation stage of the autoreactive clone.

b. The affinity of the autoantigen for its receptor(s).

c. The nature of the autoantigen.

d. The concentration of the autoantigen.

e. The tissue distribution of the autoantigen.

f. The expression pattern of the autoantigen.

g. The availability (or not) of co-stimulatory signals.

PATHOGENETIC ROLE OF T CELLS IN AUTOIMMUNITY

Helper T cells, according to their cytokine-secreting profile, have been divided into two major groups TH1 and TH2 cells. The former produce pro-inflammatory cytokines and lead effector immune mechanisms to be mainly or exclusively mediated by cytotoxic T and accesory cells such as NK cells and macrophages, while the latter produce cytokines that drive immunoglobulin production and isotype switch, leading to exhuberant antibody mediated responses. The balance –or imbalance- of TH1 and TH2 cells determines the immunologic scenario and hence the clinical picture of immune mediated reactions or disease. Additionally, one cell type and the other are reciprocally inhibitory, therefore. It is valid to say that TH1 cells inhibit antivbody production, while TH2 cells might be considered as anti-inflammatory. When self-tolerance is broken, it is possible that the functional predominance of one or the other TH cell subgroups determines the type and intensity of the immune response to a self antigen. In animal models bearing TH1 mediated diseases, it is possible to down-regulate the self-directed response through the administration of autologous activated TH2 cells. It is worth to emphasize that the immune "deviation" towards TH1 or TH2 predominance is affected by genetic factors; thus the administration of a given antigen, at the same dose and through the same pathway, into two genetically different animals, leads to an overt inflammatory reaction in one of them, while in the other the reaction might be predominantly or exclusively mediated by antibody synthesis and secretion (12-14).

In human beings, it has been shown that there is genetic susceptibility to respond with a clear predominance of one of these cell subtypes towards the same bacterial or parasite antigens which, in turn, can define the efficacy of the immune response, the severity of the infection and the clinical picture. Such is the case, as an example, of lepromatous leprosy, the disseminated and most severe form of infection by M leprae, which is characterized by cutaneous anergy and TH2 type predominace, in contrast to the tuberculoid form of leprae, a localized and more benign form of infection, where TH1 responses are predominant.

It is therefore plausible to assume that the role of effector T cells in autoimmune disease is twofold: cooperation in antibody synthesis, and cell mediated tissue damage. There are examples of experimental and human diseases where these two functions are clearly involved in the pathophysiology of the morbid process.

The development of an immune response to self antigens drives naive T cells to differentiate into subsets of CD8(+) and CD4(+) effector cells including TH1, TH2, cells and the more recently described TH17, and regulatory T cells.

Rheumatoid arthritis is an autoimmune disease that engages an uncontrolled influx of inflammatory cells to the joints, eventually leading to joint damage. The role that effector T cells play in the local or systemic maintenance of,

or protection against, inflammation and subsequent joint damage is now becoming better understood through the use of animal models (23).

Autoreactive CD8+ T cells are emerging as important players in several animal models of autoimmunity; their roles in the human autoimmune diseases are only beginning to be understood. Autoreactive CD8+T cells can act as pathogenic effector cells and mediate tissue damage, while other autoreactive CD8+ T cells may have regulatory properties and serve to protect self against autoimmune attacks.

Recent advances made in the field include a better understanding of their activation, mechanism of action, negative regulation, and antigenic specificities. Importantly, advances have been made in the development of strategies that detect these antigenic specificities in human patients and at risk individuals. Together, these findings foster hope for the development of novel antigen-specific therapeutic strategies that target the CD8+ T cell compartment of autoimmune processes.

In the case of T cell mediated autoimmune disorders,-such as Multiple Sclerosis (MS) and Type 1 Diabetes (T1D), CD4+T cells were traditionally thought to be the key effectors of tissue damage. This was due, in part, to the fact that disease susceptibility and/or resistance are strongly associated with certain MHC class II alleles. More recently, it has become evident that cytotoxic CD8+T cells also play major roles as effectors of autoimmunity. This is not surprising because, unlike most CD4+ T cell specificities, CD8+ T cells can directly recognize and kill antigen-expressing cell types.

Activated CD8+ T cells can kill target cells via the Fas/Fas ligand (FasL) pathway, or by releasing cytolytic granules at the effector/target cell junction. Activated cytotoxic T lymphocytes (CTLs) can also cause tissue damage by secreting high levels of pro-inflammatory cytokines, such as TNFα and IFNγ. CD8+ T cells have been shown to contribute to the pathogenesis of several animal models of autoimmunity, notably type 1 diabetes (T1D) in the nonobese diabetic (NOD) mouse, and experimental autoimmune encephalomyelitis (EAE), a model of demyelinating central nervous system (CNS) diseases. Interestingly, and in addition to their role as pathogenic effectors, subsets of CD8+ T cells have also been identified as negative regulators of autoimmune responses in several models. Recent findings on the roles of autoreactive CD8+ T cells in autoimmunity, with a focus on these two types of organ-specific autoimmune disorders, are summarized.

PATHOGENIC ROLE OF AUTOREACTIVE CD8+ T CELLS

CD8+ T cells in type 1 diabetes (T1D). T1D is a prototypic T cell-dependent autoimmune disease characterized by a CD4+ and CD8+ T cell-dependent autoimmune process that specifically targets the pancreatic beta cell. Epidemiological studies have shown that inheritance of certain human MHC class I alleles, such as HLA-A*0201, increases the genetic susceptibility for T1D when expressed in the context of certain MHC class II alleles. Importantly, significant CD8+ T cell infiltration was observed within the pancreas of recently-diagnosed diabetic patients, as well as in diabetic patients transplanted with pancreatic grafts from healthy monozygotic twins or HLA identical siblings. There is also extensive evidence from studies using murine models of T1D that CD8+ T cells play crucial roles in the pathogenesis of T1D. NOD mice lacking the β2-microglobulin or the CD8α genes are T1D resistant. Furthermore, transfer of diabetes from young female NOD mice into NOD. severe combined immunodeficent (scid) recipients using splenic lymphocyte subsets is most efficient when CD4+ and CD8+ T cells are transferred together. Studies of beta cell-specific TCR-transgenic mice have demonstrated that autoreactive CD8+ T cells have pathogenic activity.

In NOD mice, CD8+ T cells are found in the earliest lymphocyte infiltrates of pancreatic islets. The presence of a population of CD8+ T cells recognizing an insulin-derived epitope in the islets of 3-4 week-old NOD mice has been informed. These cells wane with age and are progressively replaced by another population of CD8+ T cells that recognize an epitope consisting of residues 206-214 of the beta cell antigen islet-associated glucose-6-phosphatase catalytic subunit-related protein (IGRP206-214). IGRP206-214-reactive CD8+ T cells are highly diabetogenic, and are prevalent in the islets and circulation of pre-diabetic NOD mice and undergo cyclic expansion and contraction prior to the onset of overt diabetes. Another unique feature of the IGRP206-214-reactive CD8+ T cell subset is that it undergoes a process of avidity maturation during disease progression, whereby low-avidity clonotypes are progressively replaced by their higher-avidity,more pathogenic counterparts. This process is developmentally controlled, such that during early stages of the disease, the high-avidity clonotypes are kept in check by both central and peripheral tolerance. At the level of the target organ, on the other hand, pancreatic islet inflammation shelters these clonotypes from peripheral tolerance and fuels their local expansion, allowing them to undertake the diabetogenic process.

CROSS-TOLERANCE AND AUTOREACTIVE CD8+T CELLS

Cross-presentation of self-antigens to CD8+T cells may lead to another outcome: T cell tolerance, also referred to as “cross-tolerance”. Cross-tolerance may occur in the thymus, or in the periphery. Negative co-stimulatory molecules of the CD28 family have been shown to be critical for peripheral cross-tolerance. One well-studied example is the CTL-associated antigen 4 (CTLA-4). Ligation of CTLA- 4 on activated T cells by CD80 and CD86 on DCs serves to down-regulate/terminate T cell responses. Loss of CTLA-4 results in lymphoproliferative disease and multiorgan autoimmunity, and in vivo blockade of CTLA-4 augments autoimmune responses in EAE. Programmed death-1 (PD-1) is another

inhibitory receptor that is upregulated on activated T cells, B cells, and myeloid cells.

The interaction of PD-1 with its ligands, PD-L1 and PD-L2, on multiple cell types, including dendritic cells (DC), macrophages, lymphocytes, and parenchymal cells such as pancreatic beta cells, results in a negative signal that aims to terminate T cell immune responses. Recent studies have shown that the interaction of PD-1 on CD8+T cells and PD-L1 on DCs and pancreatic beta cells is important for inducing and maintaining peripheral CD8+T cell tolerance to self antigens. It was also shown that abrogation of the PD-1/PD-L1 interaction fostered CD8+T cell-mediated autoimmunity, including type 1 diabetes in NOD mice. There is some evidence suggesting that whereas PD-L1 expression in PLN DCs negatively regulates the priming of diabetogenic T cells early on in the disease process, PD-L1 expression in islet cells inhibits islet destruction at a later phase in diabetes progression.

There is now no doubt that, in autoimmunity, CD8+ cells plays important roles as both effectors of tissue damage and immunoregulators. Pathogenic CD8+ T cells mediate tissue damage through their cytotoxic activity, as well as through the secretion of cytokines that further fuel inflammation. However, when the suppressive activity of autoreactive T cells targets pathogenic immune cell types, it suppresses autoimmunity. Thus, advances in our understanding of the biology of autoreactive CD8+T cells, coupled to antigenic epitope discovery and characterization, may accelerate the discovery of novel antigen-specific therapeutic strategies to prevent and/or blunt autoimmune responses (24-27).

ANTIGEN DRIVEN-DENDRITIC CELL ACTIVATION IN AUTOIMMUNITY

For a long time it has been considered that triggering of autoimmune reactants, mainly autoantibodies, does not follow the regular pathway that non-self antigens do. Anti-DNA antibodies, for instance, are not known to be produced after DNA fragments are presented to T cells by MHC molecules in antigen presenting cells in patients with systemic lupus erythematosus, neither are rheumatoid factors believed to be produced after IgG molecules or immune complexes are presented to the immune system. For the vast majority of autoantibodies; it is believed that autoreactive clones are "freed" from regulatory mechanisms thus resulting in the spontaneous activation of such clones and the synthesis and secretion of their autoantibody products. Polyclonal activation, super-antigens, equivocal cooperation, and some other mechanisms have been mentioned and proposed, however, it is generally thought that specific antigen-driven responses are not involved in autoimmune diseases. However, some disease might follow a different pattern and behave, at the least at the triggering event, as an immune reaction directed to non-self antigens. We have recently shown that vitiigo skin lesions are infiltrated by large amounts of dendritic cells, which disappear after a few months of the progression of the disease, to be replaced by T cells that remain for years within active lesions. We believe that these findings support the notion that a primary non-autoimmune phenomenon causes the breakdown of melanocytes. This primary process that could be traumatic, physical or infectious, might result in the exposure and uptake of intracellular melanocyte-associated antigens by professional antigen presenting cells, and trigger –in individuals with genetic susceptibility- a "traditional" T cell dependent immune response towards previously hidden self-antigenic structures. The antibody response to such self-antigens might aggravate the condition by inducing apoptosis of melanocytes, and the resulting apoptotic blebs might, in turn, further stimulate self-antigen reactive cells. This process might close a vicious circle and self-perpetuate the progression of the disease (28).

In summary, T lymphocytes play a crucial role in the development of autoiimune diseases, for they are the main responsible of self-tolerance maintenance but, additionally, they actively participate in the mechanisms of cell and tissue damage in autoimmune mediated diseases. The difficulties to analyze the specificity of autoreactive T cells hinder the possibility to manipulate them for therapeutic purposes en the short term, however, the use of experimental models of autoimmune disease and the genetic manipulation of these animals, will soon change our understanding of the intimate mechanisms of autoimmune disease and possibly will gift us the opportunity to intervene.

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8 ARE AUTOIMMUNE DISEASES AUTOIMMUNE EPITHELITIS?

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ARE AUTOIMMUNE DISEASES CONSIDERED AUTOIMMUNE EPITHELITIS?

Epithelial tissues such as those in the skin and mucus or glands (endocrines and exocrines) are a frequent target of the immune response, which is responsible for the natural history and development of autoimmune diseases. Therefore, it is not surprising that autoimmune thyroiditis, type 1 diabetes, and Sjögren's syndrome (3 of the most frequent autoimmune diseases) are organ specific diseases, and that those organs have a purely epithelial parenchyma (Table 1) (1). Moreover, a great number of autoimmune diseases have an epithelial component as is the case with psoriasis, pemphigus, autoimmune hepatitis, immune glomerulonephritis, autoimmune cholangitis, ulcerative colitis, and Crohn's disease.

If the fact that the epithelium is the tissue present on body surfaces is considered, and at the same time, that these tissues are the boundary between the exterior and the entrance for microorganisms, it will be obvious that epithelial tissue must play an important role in the discrimination between foreign and self molecules. Therefore, this tissue is essential for the initial immune response to what is foreign and the immune tolerance induction against self molecules. All these roles put the epithelium at risk as a target for autoimmune attack.

Hence, this chapter will describe some structural and functional characteristics of epithelial tissues which convert them into undeniable targets for autoimmune reactions with the subsequent development of autoimmune diseases.

EPITHELIUM AS THE MOST REPRESENTATIVE OF ALL TISSUES PRESENT WITHIN THE ANIMAL KINGDOM

Evolutionarily speaking, once unicellular eukaryote microorganisms (protozoa) evolved to multicellular microorganisms (metazoan), a single cell evolved to generate one or more layers composed of various cells which make up the covering epithelial tissue. Since then, epithelium has been the only tissue component to serve as an interface between the inner (self organisms) and the exterior (foreign organisms). Consequently, when these organisms evolved to more complex ones, the epithelium started to generate other tissues and with them create different organs (parenchyma) and the systems-organs known in the most evolved superior animals (mammals).

Everything mentioned above takes place during human ontogeny when two purely epithelial cells – the oocyte and spermatozoon – fuse into one: the zygote. This cell will then generate a blastula, gastrula, embryoblast, and the trophoblast. All of these are strictly epithelial in nature.

The embryoblast, which will give rise to the new human being, will take the shape of a bilaminar germ disk and then a trilaminar one, thus generating from the ectoderm an endoderm and, finally, a mesoderm. All of the above supports the idea that the epithelial cell is the source of a new human being because it is the primary representative and participant in the development of all body tissues from the perspective of the structural composition (phenotype) (Figure 1).

EPITHELIUM AS THE ENTRANCE POINT FOR MICROORGANISMS

The majority of microorganisms that infect an organism gained access through body surfaces such as skin and mucus. All these surfaces are covered by cell layers of epithelial tissue called covering epithelial tissue or simple epithelium. Some, like the skin, are resistant to foreign attack because they consist of various cell layers which originate in the lowest layer (the stratum basal or germinativum or stem cell. These cells proliferate, differentiate, and migrate to the surface where they finally suffer apoptosis in which

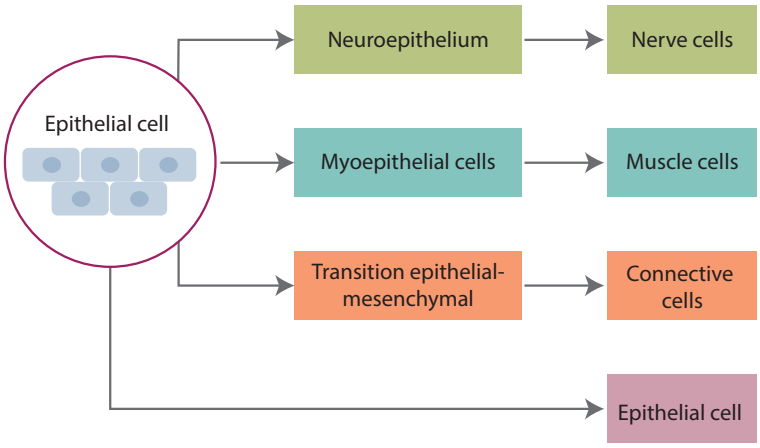

Figure 1. Phylogeny and ontogeny of four basic tissues from epithelial tissue.

they become the stratum corneum and shed, thus relieving the skin of many noxious substances that adhere to the external surface. In terms of evolution, the high rate of cell change in these tissues is a mechanism of protection from toxic substances, but also explains the harmful conseuqences of epithelial neoplasias such as carcinomas and adenocarcinomas.

In contrast, other tissues such as distal respiratory mucus are more vulnerable to attacks of microorganisms because they only have a single layer of simple columnar epithelial cells or, as in the case of alveoli, a layer of simple squamous epithelium. These types of epithelium do not easily stop the invasion of microorganisms. That is why distal respiratory tract and mucus infections especially are more frequent than cutaneous ones.

On any of these biological surfaces where there actually are commensal microorganisms, self and foreign antigens live together, most of the time, in homeostatic conditions. There, rather than discerning between self and foreign, epithelial cells eliminate whatever is hazardous or dangerous through the expression of pathogen associated molecular pattern receptors/danger associated molecular pattern receptors (PAMP/DAMP) as TLRs (Toll like receptor), NLR (*Nucleotide-binding and oligomerization domain-NOD-like receptors*) receptors, and RLR (*RIG-I-like receptors)* (2) (Figure 2). These receptors recognize pathogen self molecules such as lipopolysaccharides (LPS), peptidoglycans, nucleic acids, etc. and initiate a biological process which, with the presence of microbial antigens, will stimulate dendritic cells (DC) to mature them and start the immune response.

The expression of the PAMP/DAMP receptors by the epithelial cells has evolved from invertebrate organisms because they lived and interacted with different classes of pathogens. The above suggests that in addition to being an entrance point for microorganisms, the epithelium has the ability to differentiate between self and foreign. Hence, just as the epithelium functions as an entrance for the various microorganisms, it is a target of attack. In response to the pathogens, it releases alarmins such as IL-18, IL-33, and IL-1 beta, which are members of IL-1 family. All of them are related to the induction of Th1, Th2, and Th17 response respectively. Other key cytokines in the epithelial response that coordinate the immune response are IL-25 and TSLP, which points it toward a Th2 pattern. Epithelium has anatomically kidnapped antigens

Epitheliums are in limbo between the exterior and the inner of the body. This means that although they are an integral part of the body:

- They are separated from it by a basal membrane – specialized structural-functional underlying connective tissue – which attaches it to these tissues. This membrane is also a selective physiochemical barrier in processes such as diffusion.
- A lot of epithelial cells have a rich, apical glycocalyx which is also a selective physiochemical barrier.
- A lot of epithelial cells produce biomolecular glycoprotein interfaces (e.g., mucins from mucociliary barrier) and/ or lipidics (fat, surfactant) which also behave as an efficient physiochemical barrier.

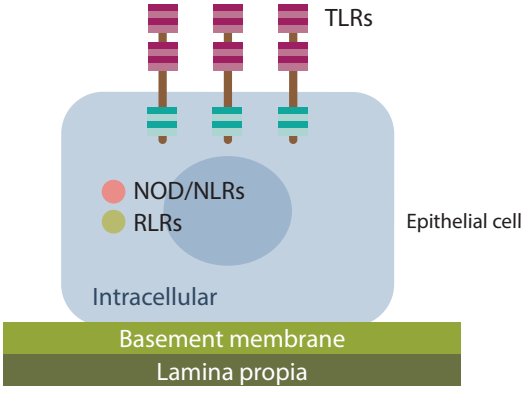

Figure 2. Pathogen associated molecular pattern receptors/danger associated molecular pattern receptors (PAMP/DAMP) in epithelial cells.

AUTOIMMUNE DISEASES	TARGET ORGAN	MAIN IMMUNOGEN	MAIN SOURCE	CAPILLARIES
Autoimmune thyroid disease	Thyroid	Thyroglobulin, Thyroid peroxidase, TSH-Receptor, Thyroidal iodide transporters Na+/I- symporter (NIS), Pendrin	Epithelium (Thyrocytes)	Fenestrated
Rheumatoid arthritis	Synovial joint	Cyclic citrullinated peptides/Joint antigens	Synovial membrane (Synoviocytes)	Fenestrated
Sjogren syndrome	Exocrine glands	Ro (SSA)/La (SSB) ribonucleoprotein (RNP) particle	Exocrine glandular epithelium	Fenestrated
Systemic lupus erythematosus	Renal corpuscle	Nucleosome	Epithelium	Fenestrated *
Multiple sclerosis	Encephalo	Central nervous system antigens	Central nervous system myelin (Oligodendrocyte)	Continuous
Type 1 diabetes	Endocrine pancreas	Proinsulin (PI), glutamic acid decarboxylase (GAD), tyrosine phosphatase-like islet cell Ag 2 (IA-2), zinc transporter 8 (ZnT8), islet glucose-6-phosphatase catalytic subunit-related protein (IGRP)	Pancreatic endocrine glandular epithelium (Beta-cells)	Fenestrated
Primary biliary cirrhosis	Liver	E2 subunits of oxo-acid dehydrogenases complex (PDC-E2, OGDC-E2, BCOADC-E2); E3BP and PDC-E1α subunits of Pyruvate dehydrogenase complex (PDC); gp210 and Nucleoporin 62 of Nuclear Pore Complex; Sp100 and Promyelocytic leukemia (PML) bodies of Multiple Nuclear dots	Epithelium (biliary epithelial cells of small bile)	Discontinuous
Myasthenia gravis	Muscle	AchNmR (Acetylcholine Nicotinic muscular-type receptor), MUSK (Muscle-specific tyrosine kinase), LRP4 (Low-density lipoprotein receptor-related protein 4)	Neuromuscular junction of striated skeletal muscle	Continuous
Vitiligo	Skin	SOX9, SOX10, PMEL (Premelanosomal protein), Tyrosinase, TYRP1 (Tyrosine related protein 1), DDT (D-Dopachrome tautomerase), Rab38, MCHR1 (Melanin-concentrating receptor)	Epithelium (Melanocytes)	Epithelium
Alopecia areata	Skin	Hair follicle structures	Epithelium (Hair foliccle cells)	Epithelium
Pernicious anemia	Stomach	Intrinsic factor receptor	Epithelium (Parietal cells of the gastric fundus oxyntic glands)	Epithelium
Addison disease	Adrenal gland	21-Hydroxylase	Epithelium (glandular epithelial cells of exocrine adrenal tissue)	Epithelium

Table 1. Most relevant histological features of major autoimmune diseases. *Renal glomerulus.

- They have a high tissue replacement rate in comparison to other tissues (apoptosis). As a result, this replacement functions as a barrier that prevents the perpetuation of potential toxins.
- Their waste products go directly (except for, obviously, endocrine epithelium) to a corporal surface, i.e., the exterior.
- There is a lack of blood and lymphatic vessels.
- They are fed by diffusion from connective tissues with vessels and its drainage is to the exterior of the organism. Therefore, a lot of antigens present within epithelial tissue do not usually go into lymphatic circulation unless trauma or inflammation has occurred. These antigens that are present within the tissue and to which the lymphocytes moving through the blood and lymphoid organs are usually not exposed are called anatomically kidnapped antigens. They abound within epithelium, and they are in greater quantities within barriers which hide them even more as is the case with the eye, testicles, etc.

Unlike other epithelial tissues, endocrine organs do not drain their products to the exterior but rather into the blood. Furthermore, once their cells die by physiological apoptosis, they must be cleaned out by neighboring cells or by macrophages and DC which are present within the surrounding connective tissue. Therefore, in the case of tissular damage, anatomically kidnapped antigens are released, are recognized by antigen presenting cells, and travel to secondary drainage lymphatic tissues (e.g., lymphatic tissue associated with skin and mucus - SALT, MALT-, and lymph nodes), where they initiate the immune response. This explains why autoimmune diseases are more frequent in endocrine organs (Table 1).

THE MAJORITY OF ORGANS AND TISSUES AFFECTED BY AUTOIMMUNE DISEASES HAVE FENESTRATE CAPILLARIES

All the endocrine organs, e.g., renal nephron, skin, synovial joints, and the liver have capillaries characterized by the presence of fenestrate-pores, which allow large molecules to cross through to the tissue to a greater or lesser degree (Table 1). This crossing is dependent on pore size and hemodynamic factors (rheological ones) such as the flow and intravascular pressure. The presence of these fenestrae makes it easier for molecules and viral or microbial particles to cross from the blood to the tissue. Additionally, during all inflammatory processes cytokines, chemokines, and other inflammatory molecules travel by lymphatic and blood vessels toward arterial circulation and from there to different organs and systems (3,4).

These molecules reach target organs involved in acute phase response such as the hypothalamus, liver, adipose tissue, muscle, bone, and organs with fenestrated capillaries, which are necessarily involves the inflammatory process. It is well known that viral infections that cause significant viremia, are usually associated with joint inflammation (reactive arthritis) manifested as arthralgia. Likewise, all viral infection induces interferon (IFN) production, which will disseminate by blood towards distant organs where it can start the inflammatory process (5).

EPITHELIAL TISSUES ARE THE ONES WHICH SUFFER THE MOST APOPTOSIS

Apoptosis – a form of programed cell death – (See chapter 13) is a process in which cells are eliminated whether because they have accomplished their functions, because it is a part of the functional cycle of an organ, because they are part of a transitory structure or even because they are the target of stress or infection. That is how through apoptosis interdigital membranes are eliminated during embryo development, endometrium is released during the menstrual phase, ovarian follicles suffer atresia, spermatozoa get older within the sperm tract, injured hepatocytes are released by toxins or pharmaceutical drugs, the effector lymphocytes are eliminated once they have eliminated their antigens, and the non functional lymphocytes depleted within the thymus. In other words, cells entering the cell cycle are the ones with a higher chance of suffering apoptosis. Therefore, epithelial tissues and immune system organs suffer more apoptosis than others (6).

Apoptosis is a very important source of auto-antigens which under normal conditions do not unleash immune responses, but under stress conditions (inflammation, trauma, and oxidative-reductive imbalance) may be a source of PAMP/DAMP and modified auto-antigens (e.g., citrullination) which would induce an immune response. It has been convincingly proven that the most important immunogen in Systemic Lupus Erithematosus (SLE) is nucleosome (structure for chromatin storage) which is released by epithelial cells suffering physiological apoptosis as their life cycle ends. Under normal conditions, the nucleosome is not exposed to the immune system because apoptotic cells are easily phagocytised and cleaned out by macrophages and DC. A similar process happens in Sjögren's syndrome and its known antigens (7,8).

However, in the genetic background of SLE, a defect in the physiological phagocytosis makes the macrophages insufficient under conditions of intense apoptosis. That being the case, the apoptotic molecules and their structures are exposed to the oxidative environment within the tissue and to a variety of post-traductional modifications. It is during this period that the nucleosomes are exposed to the immune system and thus produce anti-DNA-protein antibodies (e.g., histones) which will be deposited by electrochemical affinity within the tissue, particularly within the glomerular basal membrane of the kidney. All these together in addition to alteration within the signaling pathway (e.g., PTPN22) guarantee that this recognition will be pathologically effective and be perpetuated (9,10).

According to this, SLE is an autoimmune disease directed at first towards nucleosomes which are produced and released by epithelial cells in constant replacement and regeneration. Therefore, we should ask ourselves if SLE is a purely epithelial disease?

It is very likely that the explanation for the higher frequency of autoimmune diseases such as SLE is related to what was mentioned above since women have more epithelium tissue (breast glands and endometrium) which is highly sensitive during the menstrual cycle to the action of hormonal oscillations. Furthermore, the endometrium expresses Vitamin D receptors (VDR), which are deregulated by the presence of microbiota, and this mechanism is involved in the immunological deregulation associated with the autoimmune response (11).

EPITHELIUM AS A TARGET OF VIRAL INFECTION

Viruses as obligatory intracellular parasites require a host that frequently enters the cell cycle for replication and protein assembly. In other words, viruses have a trophism for highly cyclical cells.

Viruses have evolved to enter their targets and the best target for them is the surface epithelial cells. Therefore, epithelial cells have frequently become hosts for viral infection as is indicated by the expression of epithelial receptors for viruses and the high frequency of viral diseases compromising the epithelium. Moreover, the presence of viruses within epithelial cells is not innocuous and, in most cases, entails programed apoptotic death for the cell. In both situations, infected cells produce cytokines and chemokines, e.g., type I IFN which in addition to attracting inflammatory cells, activates them to initiate the immune response. Viral infection is an important stimulus for the recruitment of plasmocytoid dendritic cells (pDC) which are the main source of IFNα. The interferon produced within the infection sites and in the plasma by pCD generates systemic effects that cause tissular damage and

the expression of anatomically kidnapped antigens. Naturally, the main targets of interferon action are the epithelial tissues and those with fenestrated capillaries. As a consequence, new therapeutic molecules involving cytokines such as IFN have been associated with an increase in the frequency of autoimmune diseases as was clearly demonstrated by the association between IFNα used to treat hepatitis C and the higher risk of autoimmune thyroiditis (12,13). pDC population is under strict regulatory control, and this control includes negative feedback mechanisms managed by self IFN type I (14).

Finally, considering what was mentioned above, the participation of pDCs as mediators between viral infection and autoimmune disease is now becoming recognized in the pathogenesis of various autoimmune diseases, e.g., Sjögren's syndrome (12,13).

VIRAL INFECTION ASSOCIATED WITH AUTOIMMUNE DISEASE

A lot of autoimmune diseases have been related to viral infections as is the case with rheumatic arthritis, the Epstain Barr virus (Herpes virus type 4), type 1 diabetes, the Coxsackie virus, Sjögren's syndrome, Hepatitis C, and the association between this last virus and autoimmune thyroiditis (See chapter 19).

Viruses not only produce tissue damage and inflammation but they also induce the expression of extracellular antigens and raise the presentation of antigens on the surface of infected cells. This converts infected cells into targets for the IFN, lymphocytes T CD8, and natural killer (NK) cells. This cell lysis not only raises auto-antigen exposure but also the exposure of cryptic epitopes because of the use of the granzyme B enzyme, which is usually not used during the conventional processing of antigen, by the professional APC. That is why, viruses produce inflammation and the subsequent production of co-stimulators which expose and modify the conventional processing of intracellular auto-antigens with the subsequent production of cryptic epitopes (15,16).

EPITHELIAL CELLS ACT AS ANTIGEN PRESENTING CELLS

Nucleated cells express molecular complexes within their membrane produced by peptides created by the proteolitic degradation of their intracellular proteins and histocompatibility type I molecules (HLA). These HLA-antigen complexes avoid attacks by NK cells and, at the same time, they allow CD8 T lymphocytes to review the intracellular component in search of possible intracellular microbial antigens. Strictly speaking, all cells act as antigen presenting cells, but the name, professional antigen presenting cell, is reserved for T lymphocyte accessory cells which in addition to presenting intracellular antigen in HLA I, phagocytose, process, and present extracellular antigens in HLA II as well as in HLA I (cross-presentation). These cells are capable of expressing co-stimulator molecules such as B7 family molecules (e.g., CD80, CD86) and the tumoral necrosis factor family and its receptors (TNF/TNFR: CD40/CD40L system and OX40/OX40L system) which ensure the activation of T lymphocytes. Therefore, the title of professional presenting cell is reserved for CD, macrophages, and B lymphocytes acting as professional APC during primary and secondary immune responses. Within this context, epithelial cells (including endothelial cells) acquire an important role because they not only express HLA I constitutively but also HLA II under cytokine action, and furthermore, the co-stimulator molecules of B7 family and TNF/TNFR family convert them into authentic antigen presenting cells (17–27).

In the majority of organs which are primary targets for autoimmune disease, molecules related to antigen presentation in parenchyma epithelial cells have been identified. With an APC behavior, these epithelial cells reinforce the stimulation of the immune system, but at the same time they collaborate with the perpetuation of the established autoimmune disease (17,19-25,27).

EPITHELIAL CELLS SELECT THE REPERTOIRE OF T LYMPHOCYTES WITHIN THE THYMUS

The thymus is an organ which remains a major mystery. The epithelial nature of its stroma comes from its ectoderm and endoderm, embryo layers which are the origin of a major amount of diversity of diversity of epithelia (28-30). When the precursors? of T lymphocytes reach the thymus from the bone marrow (common lymphoid progenitor stem cells –CLP), they initiate the expression of antigen receptors (TCR). The TCR is a membrane heterodimer which enables the lymphocytes to recognize HLA-peptide complexes expressed on the antigen presenting cell surfaces (Figure 3). The genes codifying for both proteic chains of the receptor have evolved through the genetic duplication mechanism which has allowed them to diversify in mammals and reach approximately 10^{18} different specificities. This huge diversity within the TCR surpasses

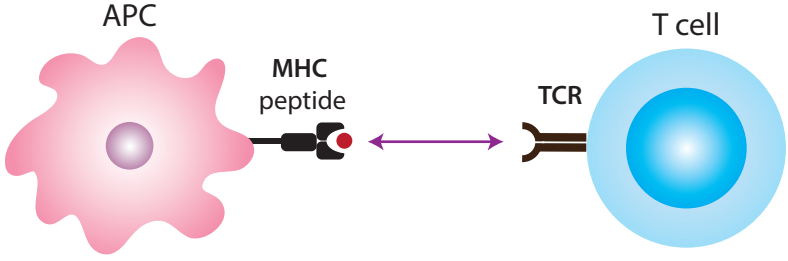

Figure 3. Peptide presentation to T cells through the interaction of HLA-peptide complex to the TCR.

the accumulated polymorphisms within HLA molecules by a very wide margin. The HLA in human beings do not surpass 2,000 and, in each individual, they reach a maximum of 22 (2 HLA A, 2HLA B, 2HLA C, 4 HLA DQ, 4 HLA DP, and 8 HLA DR). If we add to this the fact that every HLA molecule has a limited ability to attach to antigenic peptides, that it will not surpass 2,000 per molecule, it is easy to calculate that the majority of lymphocytes generated within the thymus cortex will not have any functionality in every individual. This means there are many more lymphocytes with their respective TCR than HLA-peptide complexes expressed in presenting cells (31-34). This indicates a need for positive selection to choose from this huge potential repertoire only those lymphocytes that are capable of seeing peptides attach to HLA molecules on the surface of their own presenting cells (20,26). This job is done by epithelial cells from the cortex (cTEC) of the thymus, which process their own constitutive proteins and present them together with HLA I and II molecules on their surface. Once the intrathymic T lymphocyte expresses its receptor, it activates a programed cell death mechanism which will be carried out in 3 days unless the cell interrupts it. This will only occur if the lymphocyte presents a receptor that is able to interact with the peptide-HLA complexes expressed by the cTEC (Figure 4.). This process, which is called positive selection, rescues functional lymphocytes for every individual from death because it has the ability to recognize peptides derived from epithelial antigens. In other words, the entire selected repertoire is autoimmune in nature and directed against self antigens or against epithelial antigens, even when their avidity is moderate. Once the positively selected cells go to the medulla, they run into other presenting cells—medullar epithelial cells (mTEC) and DC. The peculiarity of the former is that they ectopically express specific extrathymic antigens of the tissue due to the expression of protein encoding by AIRE gen (*autoimmune regulator*). This protein produces complexes with other proteins (*AIRE nuclear body*) for diverse functions and, they interact with promoter regions of various genes which, under normal conditions, are only expressed in tissues or organs other than the thymus (promiscuous antigen expression) (36,37).

Thymic processing also has its own particularities. Therefore, within epithelial reticular thymic cells from the cortex a large variety of immunoproteosomes (thymoproteosome). The immunoproteosome presents variations in its sub-unitary constitution in its quaternary structure (35). The proteosome presents 3 main catalytic activities: as a trypsine, chemotrypsine, and caspase. In the thymoproteosome, the structural variation for an alternative beta subunit (β5t) diminishes chemotrypsine activity and conditions the differential proteolytic catalysis of the antigenic epitopes

In addition, the DC reaches the thymus from the blood stream and peripheral tissues. They may or may not carry antigens captured from extrathymic tissues. Once they locate in the thymic medulla, they can phagocytose antigens reaching the thymus from peripheral tissues. These antigens are then processed and presented to the previously selected medullar thymocytes within the cortex. This second interaction between thymocytes and thymic APC seeks to eliminate auto-reactive T lymphocytes with high affinity directed against auto-antigens within the medulla (negative selection) or differentiate them from T regulatory lymphocytes (Tregs) which, once they leave the thymus and interact with self auto-antigens, suppress any chance of immune response against them (Figure 5.) (30-32,34).

Considering the fact that the most widely accepted thymic model selection is the avidity model. This model demonstrates

Note that the most widely accepted thymic model selection is the avidity model. This model demonstrates the average avidity of the different interactions between HLA-peptide complexes and the thousands of TCR (because one APC can present up to 100,000 HLA molecules with 200 different peptides). A positive selected lymphocyte in the cortex can recognize different epithelial peptides with different affinities (low, moderate, or high) due to the low or moderate avidity (Figure 4) (38). The low, moderate or high affinity auto-reactive thymocytes for epithelial cortical antigens go through the thymic medulla. Within the medulla, the affinity of the interaction between new HLA-peptide complexes and the TCR from medullar thymocytes is modified by changing the presentation of cortical peptides to medullar ones. Therefore, within the medulla there are auto-reactive lymphocytes of low, moderate, and high affinity for the new peptides brought to the thymus or expressed ectopically for mTEC (31-35).

DC cells from the thymus –particularly those interacting with thymic reticular epithelial cells from the Hassal corpuscles – convert some of these cells into Treg cells (CD4 and CD8) expressing the FOXP3 gen and the other cells are eliminated by negative selection (Figure 5): Thus, the autoimmunity risk is reduced (39-43).

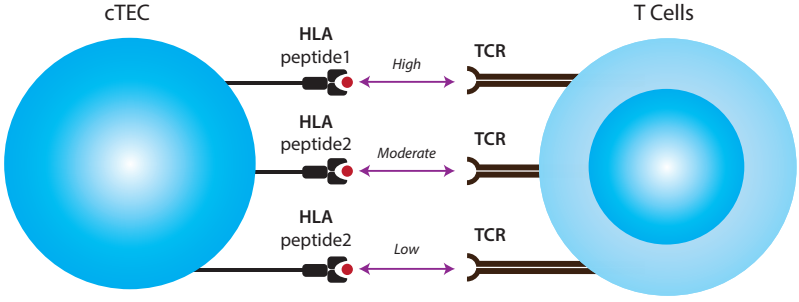

Figure 4. Thymocyte selection in the thymic cortex. *cTEC*: epithelial cells from the cortex of the thymus.

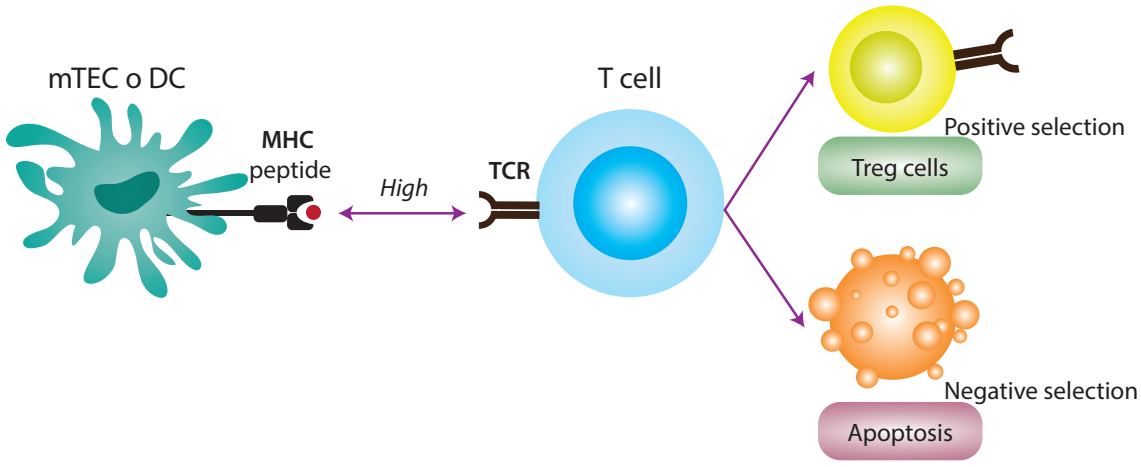

Figure 5. Thymocyte selection in thymic medulla. *mTEC*: medullar epithelial cells.

In summary, all lymphocytes migrating from the thymus to the periphery are auto-reactive to epithelial antigens. Eventually, they may react to other self antigens or foreign ones that were not expressed within the thymus. Therefore, self is epithelial and foreign is extrathymic. Deregulation of high affinity auto-reactive lymphocytes on the periphery generates a risk of loss of tolerance to self antigens, which are primordially epithelial.

An indirect conclusion of all this is that it is not at all unusual for a specialized epithelial cell in the thymus to respond to the selection profile of T lymphocytes interacting with epithelial cells on the periphery. It appears that this process is not random!

THE EPITHELIUM IS FREQUENTLY COMPROMISED BY AUTOIMMUNE POLYGLANDULAR SYNDROMES

Based on what was discussed above, it is easy to speculate that an disruption in the production of regulatory cells, for example, in IPEX (*Immunodeficiency, Polyendocrinopathy,* and *Enteropathy, X-Linked Syndrome*) (OMIM304790) or in the promiscuous thymic expression of antigens such as in APECED (Autoimmune-polyendocrinopathy-candidiasis-ectodermal-dystrophy) (OMIM240300) due to mutation in the FOXP3 genes (*forkhead box P3*) and AIRE respectively will translate into an increase in the lifespan of high affinity auto-reactive T lymphocytes. These can interact with non expressed peptides in the thymus that were positively selected based on their interaction with cortical epithelial antigens. All of the above explains why the compromise of endocrine epithelial organs is frequently found in these syndromes (24,44,45).

EPIGENETICS AND AUTOIMMUNITY: IS THE EPITHELIAL CELL AN EPIGENETIC TARGET? WHAT ARE THE IMPLICATIONS OF THAT?

There is more and more consistent evidence of how environmental modulation can alter gene expression patterns, thus altering the cell phenotype. This dynamic process is due to:

- Chemical modification of chromatine histones (acetylation, methylation, phosphorylation, ubiquitination, sumolyation, poliACP-ribosylation, biotylation, deamination, butylation, N-formylation, and prolinic isomerization).
- Methylation or demethylation of gene promoters, in cytosine nucleotide residue (in the context of CpG dinucleotides) or in adenine residue.

These chemical modifications are redundant in the differential inhibition or expression of genes, including in the filial progeny which is called transgenerational heritage (46). From the same genotype, due to epigenetic mechanisms, there will be an infinity of phenotypes which in the cell and tissue context is a way to guarantee the flexibility to adapt to noxious substances, physical and chemical stress agents, and endogenous or exogenous factors, e.g., infections, inflammation, nutrition-energetic alterations, metabolic-endocrine aberrations, etc.

In all honesty, which is the tissue which is ecologically exposed? What tissue is the border or limbo and, therefore, defenseless to an epigenetic regulation? The answer is: epithelial tissue. Therefore, the differential gene modulation caused by epigenetics in the epithelial cell (and not only in the immune cells) is, potentially, a key player in autoimmunity genesis (47). This epigenetic deregulation has been identified in such autoimmune diseases as psoriasis, autoimmune thyroiditis, and ulcerative colitis (See chapter 22) (48-50).

However, epigenetic alterations and their role in the autoimmunity process go beyond differential gene expression. Covalently modified histones exposed to the immune system on apoptotic cell surfaces and in the apoptosomes are better immunogens than native non modified histones as has been observed in SLE. Indeed, DNA-modified histone complexes are excellent innate response stimulators because they function as TLR9 ligands (51-54).

The impact radius of the epigenetic field in epithelial regulation continues to amaze. In fact it could participate in thymic selection since within the medullar thymic epithelium, AIRE generates complexes with various enzymatic factors associated with histone modifications (55).

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9 CYTOKINES, CHEMOKINES AND GROWTH FACTORS

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INTRODUCTION

The complex organization and functionality of multicellular organisms is guaranteed by cell communication, also called biosignaling. In terms of evolution, molecule systems (mediators of cell communication), cells, tissues, and organs specialized in the maintenance of homeostasis, e.g., hematoinmune system, the endocrine system, and the nervous system appeared in higher animals due to the need for an organized and orchestrated response. Hence, the mediators of cell communication within the endocrine system are the hormones, neurotransmitters (as well as neuromodulators, neurohormones, neuropeptides, and neurosteroids) in the nervous system, cytokines and colony-stimulating factors (CSF) in hematoinmune system, and morphogens (for example, notch's, wnt's, hedgegog's), and growth factors (GF's) in embryogenesis, tissue repair, and healing (1-12).

As was mentioned above, cytokines are important molecules for cell communication. The term cytokine (Greek –*cyto*, cell, and –*kinos*, movement) was proposed by Stanley Cohen in 1974 and refers to peptides, proteins, and glycoproteins which play a role in controlling the survival/death of cells, their growth and differentiation as well as the effector functions in tissues and immune cells.

The cytokines are small cell-signaling protein molecules with several functions, e.g., (Figure 1):

Intracrine actions: intracellular action by regulation of intracellular events within the cytoplasm and/or nucleus.
Autocrine: action produced within the cell through surface cell receptors.
Intercrine: communication between cells. This type of cell interaction can be classified into:

- Paracrine: signaling produced by soluble mediators through neighboring cells.
- Matricrine: cytokines are immobilized in the extracellular matrix (ECM) by its binding to proteoglycans, and they are then stored in an inactive form. These cytokines will be released by the action of proteases such as Metalloproteinases (MMPs) by a mechanism know as Protease-triggered matricrine (PTM). Glycocalyx, which is made of glycoprotein carbohydrate-motifs with proteoglycan on its surface, could play the same role.
- Cytokine secretion by exchange of membrane fragments between cells through mechanisms such as trogocytosis, formation of tunneling nanotubes (TNTs) and release, secretion, and transportation of microvesicles (MVs)/Exosomes.
- Juxtacrine: neighboring adjacent cells send signals through membrane-anchored mediators. The classic example is the action of the endothelium on smooth muscle of the tunica media of certain vessels. Some cytokines have the ability to bind to extracellular matrix soluble proteoglycans or to proteoglycan-cell surfaces (for example, CD44, Glypicans, Syndecans, Betaglycan/TGFBR3, inter alia), where this mechanism serves as a reservoir, or as an enabler of these mediators to act on specific receptors in a juxtracrine manner.
- Endocrine: this refers to the distal or systemic action which depends on secreted cytokine and its transportation within the blood.

Note that the autocrine, paracrine, juxtacrine, and endocrine actions are exerted by the binding to transducing signals (second messenger cascade) through specific cell surface receptors. Cytokines have limited biological half-lives and they act locally for the most part. Furthermore, they have overlapping actions characterized by a very broad range of functions, e.g., hematopoiesis, cell growth and differentiation, angiogenesis, tissue remodeling, wound

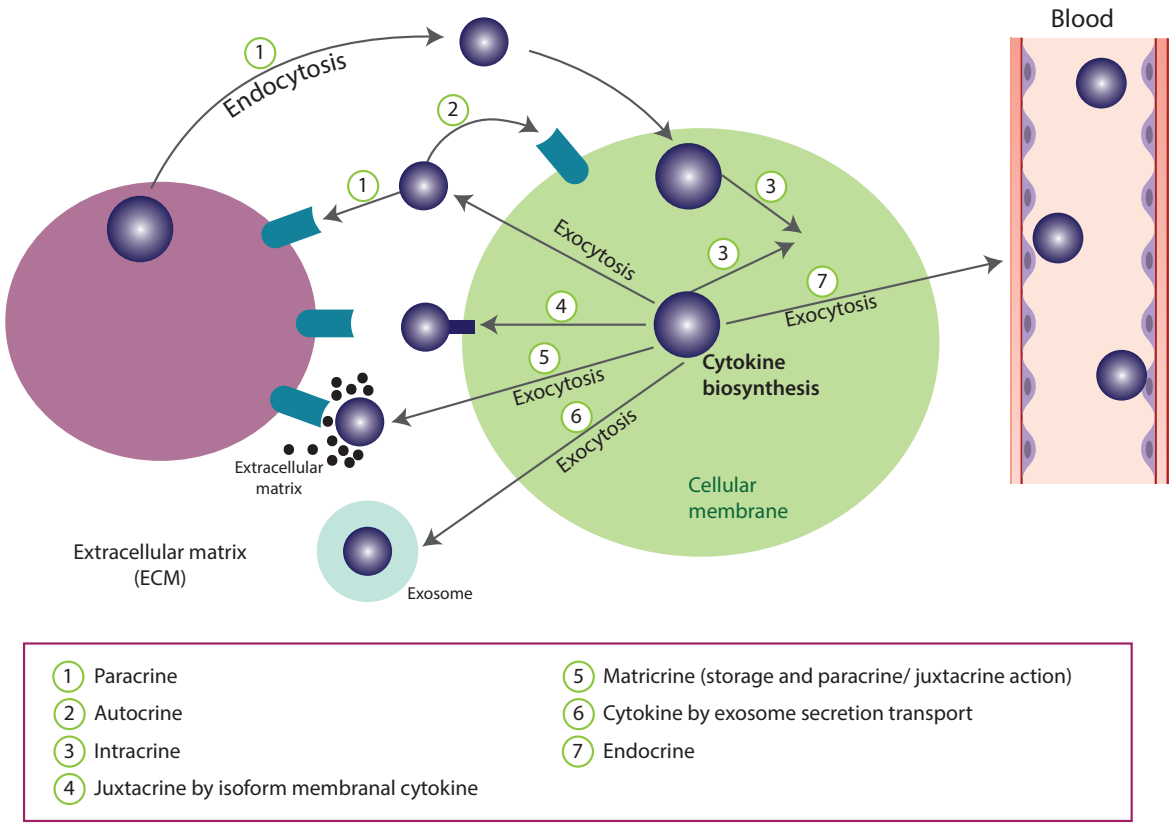


Figure 1. Basic mechanisms of cell communication.

healing, effector immune cell activity, and life/death decisions. Moreover, cytokines with endocrine action circulate in picomolar concentrations, but under the influence of strong immune activation circumstances, they can surge up to 1,000-fold (cytokinemia).

In immunological jargon, terms such as interleukins (IL's), monokines, lymphokines, haematopoietins, lymphopoietins, myelopoietins, leucopoietins, basophilopoietins, chalones, leucokines, macrophage-activator factors (MAF), macrophage inhibitor factors (MIF), histamine-releasing factors (HRF), endogenous pyrogens, tumor necrosis factors, and interferons were originally used to identify the cellular source, the target cell and/or their action-type. However, at present, it is cleared understood that these substances are produced by a multiplicity of cell populations, depending on whether the cell is in a physiological resting state, activated state, or in the pathological context of a specific scenario. The evolution of protein domain families is evident in the genesis and in the great diversity of different cytokines and cytokine-receptor families.

Chemokines (Chemoattractant cytokines) are a particular class of hematoimmune system cell communication mediators. They are a family of low molecular mass (8-14 kDa) proteins, mostly basic and structurally related, which exhibit a wide variety of immunological activities such as cell trafficking (1-12).

FUNDAMENTAL CLASSIFICATION

The nomenclature for genes and related diseases, which is used throughout this chapter is assigned by the Human Genome Organisation (HUGO), the Gene Nomenclature Committee (HGNC) by the National Human Genome Research Institute (NHGRI) (http://www.genenames.org/), and the Online Mendelian Inheritance in Man (OMIM) catalog, which is a registered trademark of Johns Hopkins. (http://omim.org/)

ARCHETYPICAL CYTOKINES SIGNALING THROUGH CLASICAL-CYTOKINE RECEPTORS

A. Type I helical Cytokine families signaling through Class I cytokine receptors (CRF1 family or Hematopoietin family)
1. IL-2 Family or Common gamma Chain Receptor Family: IL-2, IL-9, IL-15, IL-21, IL-4 subfamily (IL-4, IL-13), and IL7 subfamily (IL-7, TSLP)
2. Common beta Chain Receptor Cytokine Family: IL-3, IL-5, and Colony Stimulating Factor 2/Granulocyte Monocyte-stimulating factor (CSF2/GMCSF)
3. Prolactin family: PRL, GH Subfamily (GH1, GH2), Chorionic somatomammotropin Subfamily (CSH1, CSH2), Erythropoietin (EPO), Thrombopoietin (TPO), and Colony Stimulating Factor 3/Granulocyte-stimulating factor (CSF3/GCSF).

4. IL-6 Family: IL-6, IL-11, IL-31, LIF, Ciliary Neurothropic Factor (CNTF), Oncostatin M (OSM), and Cardiotrophin subfamily (CT1, CLC)
5. IL-12 Family: IL-12, IL-23, IL-27/30, and IL-35

B. Type II Cytokine families signaling through Class II cytokine receptors (CRF2 family or IL-10/IFN superfamily)
1. IL-10 Family: IL-10, IL-22, and IL-26
2. IL-19 Family: IL-19, IL-20, and IL-24
3. Type I IFN: IFN-α Family (IFN-α1, IFN-α2, IFN-α 4, IFN-α 5, IFN-α 6, IFN-α 7, IFN-α 8, IFN-α10, IFN-α13, IFN-α14, IFN-α16, IFN-α17, and IFN-α21)
4. Type II IFN: IFN-γ, and IFN-λ Family (IFN-λ1/IL-29, IFN-λ2/IL-28A, IFNλ3/IL-28B, and IFN-λ4)
5. IFN-β/ω: IFN-type I β/ω Family (IFN-β1 and IFN-ω1)
6. Tissue Factor-VIIa system

CYTOKINE FAMILIES SIGNALING THROUGH IMMUNOGLOBULIN(IG) SUPERFAMILY CYTOKINE RECEPTORS

A. Receptor tyrosine-kinase class III-ligands –RTKIII/PDGFR family: MCSF Family (CSF1/MCSF and IL-34), Flt3/Flk2, and Stem Cell Factor/KitL (SCF/KitL)
1. CSF1/MCSF
2. IL-34
3. FLT3LG (Fms-like tyrosine kinase 3 ligand)
4. Stem Cell Factor(SCF)/KitL

B. non-Receptor tyrosine-kinase(RTK)
1. IL-1 Family: IL-1s, IL-18, IL-33, IL-36, IL-37, and IL-38
2. IL-16
3. HMG1B (High Mobility Group 1B)

CYTOKINE TNF FAMILY SIGNALING THROUGH TNF RECEPTOR FAMILY

A. LTA/TNFSF1, TNF-α/TNFSF2, LTB/TNFSF3, OX40L/TNFSF4, CD40L/TNFSF5, FasL/TNFSF6, CD70/TNFSF7, CD30L/TNFSF-8, 4-1BBL/TNFSF-9, TRAIL/TNFSF10, RANKL/TNFSF11, TWEAK/TNFSF12, APRIL/TNFSF13, BLYS/TNFSF13B, LIGHT/TNFSF14, VEGI/TNFSF15, GITRL/TNFSF18, and EDA (Ectodysplasin).

B. Non TNF-ligand: Granulin/Epithelin (GRN) and Nerve Growth Factor (NGF).

CHEMOKINE SUPERFAMILY SIGNALING THROUGH CHEMOKINE RECEPTORS (SEVEN-TRANSMEMBRANE HEPTAHELICAL (SERPENTINE) RECEPTORS ASSOCIATED WITH G-PROTEIN TRIMERIC SYSTEM)

A. Chemokine CC Motif Ligand Family (CCL): CCL1, CCL2, CCL3 Subfamily (CCL3, CCL3L1, and CCL3L3), CCL4 Subfamily (CCL4, CCL4L1, and CCL4L2), CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, and CCL28

B. Chemokine CXC Motif Ligand Family (CXCL): CXCL1, CXCL2, CXCL3, CXCL4 Subfamily(CXCL4/PF4 and CXCL4L1/PF4V1), CXCL5, CXCL6, CXCL7/PPBP, CXCL8/IL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL16, and CXCL17

C. Chemokine XC Motif Ligand Family (XCL): XCL1 and XCL2

D. Chemokine CX3C Motif Ligand (CXC3L)

E. Other non-Chemokine ligands signaling through Chemokine receptors: Chemerin

ORPHAN AND OTHER CYTOKINE FAMILY MEMBERS

A. IL-17 Family: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E/IL-25, and IL-17F.

B. Other Cytokines: IL-14, IL-25/SF20, IL-32, MIF family (MIF1 and MIF2), Osteopontin (OPN), Thymic peptide family [Thymosin Family (Prothymosin-α, Thymosin-β4, Thymosin-β10, Thymosin-β15, and Parathymosin), and LEM Family (Thymopoietin)]

GROWTH FACTORS AND OTHER GLYCOPROTEIN HORMONES WITH IMMUNOLOGICAL FUNCTIONS

A. Insulin

B. Cardiac Natriuretic Hormones/Natriuretic Peptides (CNH/NPs)

C. TGF-β Superfamily members with immunological functions: TGF-β family (TGF-β1, TGFβ-2, and TGFβ-3), Activin family (Activin A, Activin B, and Activin AB), and GDF15/Mic1

MORPHOGEN FACTORS WITH IMMUNOLOGICAL FUNCTION

A. Notch Receptor/Notch Receptor Ligand System: Notch Receptor Family (Notch1, Notch2, Notch3, and Notch4), Notch Receptor Ligand Jagged Subfamily (Jag1 and Jag2), Notch Receptor Ligand Delta-like Subfamily (DLL1, DLL3, and DLL4)

B. Hedgehog (HH) system: SHH, IHH, DHH

C. Wnt system

ADIPOKINE FUNCTIONAL FAMILY

CYTOKINES AND CYTOKINE-RECEPTORS GENERAL CHARACTERISTICS AND PROPERTIES

General Cytokine characteristics and properties are:
- A functional network.

- Usually many cytokine families have evolved from common ancestral genes which, through mechanisms such as ancestral gene duplication, have created a wide variety of cytokines that often have the benefit of generating functional diversity.
- Some cytokines are produced constitutively while others are inducible. Many of them are produced as pre-pro-cytokine or pro-cytokines, which require convertase-dependent processing for their activation.
- Many have clearly infered redundant actions; as a result, some cytokines carry out similar functions.
- Many cytokines are immobilized in the extracellular matrix (ECM) by binding to proteoglycans (see Matricrine action example in the Introduction) (13-15).
- Many cytokines will have different effects depending on the concentration and on wheter or not their action is during acute or chronic immune response phase. For example, Prolactin (PRL) has a dual effect, so low PRL concentrations stimulate T-lymphocytes, whereas high concentrations are anti-inflammatory/immunosupresors.
- A number of them are pleiotropic; therefore, they act on a wide variety of cell types.
- They act on hematoinmune system cells as well as on almost any cell type in the body. Since they also function as metabolic modulators and response coordiantos aginst systemic stressors within all tissues, organs, and body systems, some cytokines exert systemic effects that go beyond their immunoregulator role.
- At any given time of life, a cell may have a wide variety of receptors to various cytokines.
- Certain cytokines are read through products.This means that they are proteins decoded from contiguous genes. For example, TNFSF12-TNFSF13 read through genes produce a cytokine called TWEAKPRIL. In other situations, the contiguous cytokine gene mRNA transcripts probably encode miscRNA, which is a term used for a series of miscellaneous small RNA. The miscRNA have a wide variety of functions, e.g., enzyme-like catalysis and processing after RNA synthesis. An example of this is the CCL15-CCL14 read through gene chemokines (non-protein coding).
- Some cytokines are capable of forming hybrids with other cytokines within the same or different families, which increases the response diversity, and signaling system versatility. These cytokines are called Hybridokines. For example, chemokine CXCL5-CCL5, or HGF/SF-IL7 heteromerization produces pre-pro-B cell growth-stimulating factor -PPBSF.
- Typically, cytokine production tends to be local (tissue) and limited in time (transient) unless there is a pathological background phenomenon.
- Many cytokines may be synergistic or antagonistic depending on the situation.
- Cytokines may regulate or modulate the production/activity of other cytokines.
- A cytokine can have a differential effect on the same cell depending on the cell basal state (standby or activation) as well as the cell type and variety of expressed receptor. Moreover, one different cytokine could modulate the effect of another cytokine.
- Some cytokines are pro-inflammatory, others anti-inflammatory (IL-2, IL-10, TGF-β family, IL-27, IL-35, and IL-37) or both (for example, IL-6), and others are regulatory, thus allowing tolerance to self - components as well as pathogen control and destruction with minimum tissue damage. For example, promoting the induction of immunocyte regulation (e.g., Treg, and Breg) (16).
- There is an association between the hematoimmune and skeletal systems–a phenomenon called Osteoimmunology. Numerous pro-inflammatory cytokines affect bone cells as in the case of IL-1, IL-6,TNF-α, IL-8, IL-11, IL-15, IL-17, and IL-32 which are osteoclastogenic cytokines. In contrast, IFN-γ, IFN-β, IFN-α, IL-4, IL-10, IL-13, IL-18, and IL-33 are anti-osteoclastogenic. There are other cytokines with dual roles such as IL-17, IL-12, and IL-23. Note that RANK-RANKL system is a member of the Tumor Necrosis Factor Superfamily-Tumor Necrosis Factor Superfamily Receptor system (TNFSF-TNFRSF) (17-19).
- Cytokines are key factors in reproduction physiology through regulation of specialized gonadal processes and during gestation (20).
- Many cytokines are endogenous molecules called alarmins. They are rapidly released after non-programed cell death, but they are not released by apoptotic cells from injured tissues or as a result of stress. These cytokines promote the adaptive immunity responses and restore homeostasis by promoting tissue reconstruction (21).
- Certain cytokines are rapidly inactivated by proteases such as the Neutral Serine Proteases (NSP) secreted by human polymorphonuclear neutrophils (PMNLs) and thus generate a negative feedback loop. So, for example, IL-2, IL6, and TNF-α are inactivated by human leucocyte elastase (HLE), proteinase 3 (PR3), or cathepsine G (Cat G). In other cases, proteases generate cytokine fragments which may function as antagonists of the active cytokines such as in the case of IL-2.
- The hematopoietic cytokines have pro- and anti-angiogenic effects. Pro-angiogenic cytokines are erythropoietin (EPO), Granulocyte-colony stimulating factor (GCSF), Granulocyte-macrophage colony stimulating factor (GMCSF), IL-1, IL-3, IL-4, IL-6, IL-8, IL-10, IL-15, and IL-17. Some of the anti-angiogenic cytokines are IL-2, IL-4, IL-12, and IL-13 (22).
- The genome of certain viruses encodes cytokine-like molecules, which are referred to as "virokines." They function as competitive inhibitors or competitive stimulators of host cytokine-receptors. So they subvert host immune responses and thereby favor the development of an infecto-pathogenic scenario. Some example are the vIL-6 (viral Interleukin 6) encoded by the Human Herpes Virus 8/Kaposi's Sarcoma Virus genome (HHV8/KSV), vIL-8 encoded by Marek's disease virus (MDV) genome, vIL-10/BCRF1 (Bam HI C fragment rightward

reading frame 1) encoded by Human Herpes Virus 4/Epstein-Barr virus genome, vIL-10/UL111a (open reading frame) encoded by Human Herpes Virus 5/Citomegalovirus genome, and vIL-17 encoded by Herpesvirus saimiri genome, inter alia. The herpes virus and poxvirus family genome encodes proteins that modulate chemokine activity, e.g., proteins with homology to chemokinesor secreted chemokine-binding proteins (CKBPs). These CKBPs competitively interact with chemokines and prevent chemokine interactions with chemokine-receptors or the extracellular matrix. (See section chemokine superfamily signaling through chemokine receptors seven-transmembrane–heptahelical (serpentine) receptors associated with g-protein trimeric system) (23).

- Certain cytokines are found within biological fluids and are considered biomarkers for disease.
- Cytokines have been used as contemporary therapeutic intervention targets. In some cases they have been used to block or inhibit their own receptors or signalings pathways. In other cases, they have been used to stimulate the immune response (primary immunodeficiency, secondary immunodeficiency, severe infections, cancer, and vaccine adjuvant) or hematopoiesis (recombinant DNA technology) (24).

Cytokines as well as other cell communication mediators are able to regulate various cell events within the cell, tissue, and system dynamics. Therefore, it is said that they can have many type of effects:

- Genotropic: effect on gene expression regulation.
- Metabotropic: metabolic processe regulation.
- Ionotropic: regulation of ion flow through cell membranes and related physiological processes.
- Redoxtropic: oxidation-reduction potential regulation in the cell, which is also related to the physiological and pathological role of antioxidant neutralization and free radicals from reactive oxygen, nitrogen, and sulfur species (ROS/ROI, RNS/RNI, and RSS/RSI respectively).
- Mitotropic, also know as mitogen cytokine: cell proliferative capacity regulation, but in addition to this function, they also regulate the entire cell cycle.
- Morphogenic/morphogenetic, also know as morphogen cytokine: they regulate ontogenetic development processes; commitment, proliferation and differentiation during hystogenesis; embryogenesis, fetogenesis, and organogenesis.
- Motitropic/motogenic, also called as motogen cytokine: cytoskeletal activity regulation, cell motility capability, and cell contractility regulation. Some of these molecules are involved in the migration processes during embryogenesis and fetogenesis, e.g., operating through gradients as motomorphogens.
- Trophotropic, or organotrophic factor, also known as trophogen cytokine: many cytokines such as growth factors favor cell and tissue trophism, which promotes their survival and proper function. Because of that, these cytokines are called trophogens.
- Cytoprotection (against harmful and stressful agents) and reparation/regeneration factors.
- Deathtropic: under certain circumstances, some cytokines are death cell signals (type I/apoptosis, type II/autophagy, type III/necrosis, or mixed/special states such as necroptosis, pyroptosis, and pyronecrosis, etc). The withdrawal of some cytokines also produces cell death, e.g., the case of certain growth factors.

Throughout this section of the chapter, general cytokine characteristics and properties as well as the different effects they have on the cells or tissues have been discussed. Therefore, the general characteristics and properties of the Cytokine-*receptors* will be dealt with next (25,26):

- The cytokine-receptors are (glyco) proteins signaling through different signal transduction mechanisms (Figure 2).
- Usually, many cytokine-receptor families have evolved from common ancestral genes. Hence, a wide variety of cytokine-receptors have been created through mechanisms of ancestral gene duplication, often with the benefit of generating functional diversity.
- In other cases, cytokine-receptors are obtained through secretion or exchange of membrane fragments between cells using mechanisms such as trogocytosis, formation of tunneling nanotubes (TNTs), and the release, secretion, and transportation of microvesicles (MVs)/Exosomes.
- Cytokine-receptors satisfy the general properties of receptors including: specificity, selectivity, induced fit (in previous scientific models, it is called lock-key model), desensitization/adaptation, signaling amplification, integration, saturability, and reversibility.
- The receptors are usually located in specialized membrane microdomains known as lipid rafts, detergent-resistant membrane-DRM, and caveolaes. These microdomains may be of various kinds depending on the variable presence of sphingolipids, glycosphingolipids, cholesterol, Glycosylphosphatidylinositol-proteins (GPI-linked), and certain specialized proteins such as caveolins (caveolin1, caveolin 2, and caveolin 3), Myelin and Lymphocyte protein (MAL) family members (MAL, MAL2, and MALL), and flotillins (FLOT1, and FLOT2). This is a way to centralize and coordinate the intracellular signaling platforms (receptor clustering). Moreover, note that there is even functional diversity when the receptor signals come either from inside of these domains or from outside of them (27,28).
- Certain cytokine-receptors are unimeric, and others are dimeric, oligomeric, or polymeric complexes. There is usually a cytokine-binding receptor and cytokine signaling-coreceptors in the dimeric, oligomeric or polymeric receptors.

- Some receptors are part of polymeric complexes, in which several transmembrane proteins are recruited from different families including: membrane proteoglycans (mPG) and CAMs (cell adhesion molecules) such as integrins. This is a key biological mechanism of receptor cross-talk (e.g., transphosphorylation) which generates diversity of cell responses to a single ligand, even when there is an intracellular signaling modulation.
- Some cytokine-receptors lack transmembrane and cytoplasmic domains. They function as scavengers, interceptors, and silent or decoy receptors and bind to ligands without inducing cell signaling. Many of these receptors are usually anchored to the cell surface via Glycosylphosphatidylinositol (GPI-linked). Some cytokine-receptors are kidnapper receptors acting via endocytosis, and some of them, in turn, are even in cytokine intracrine-pathways or cytokine-catabolisms.
- Many receptors undergo the internalization and degradation that is dependent on the proteasome-ubiquitin route. Endocytic proteins that contain ubiquitin-interaction motifs (UIMs) recognize the ubiquitylated receptors and direct them into clathrin-coated vesicles and, ultimately, into lysosomes. So, degradation is achieved through the action of the E3-ubiquitin-ligase Cbl member (Casitas B-lineage lymphoma proto-oncogene) family. In humans, four members of the Cbl-family are recognized: Cbl, CblB, CblC and Cbll1 (29).
- Some cytokine-receptors, particularly receptor tyrosine-kinase (RTK) type autocatalytic, are inhibited by the Sprouty (Spry) protein, which contains the SPRY/B30.2 homologous domain. Various human SPRY family members (SPRY1, SPRY2, SPRY3, SPRY4, SPRED1, SPRED2, and SPRED3) and ERBB receptor feedback inhibitor 1/Mitogen-inducible gene 6 protein (ERRFI1/Mig6) modulate the actions of RTKs and have inhibitory effects on signal transduction. Spry proteins in some cases are genetically induced by the same cytokines, and in other cases, they are phosphorylated as part of the signaling cascade. This promotes their inhibitory action, in both cases as a feedback negative (30).
- There are soluble versions of some receptors which are produced from mRNA splicing and/or a receptor ectodomain undergoing shedding Regulated Intramembrane Proteolysis (RIP). During RIP cleavage are involved enzymes called "Sheddases" e.g., the MMPs, ADAMs (A Disintegrin and Metalloproteinase), and the gamma-secretase/presenilin complex are involved. Other examples are IL-2R, TNFRII, CSF1/MCSF-R, CD44, NOTCH-Receptors, and IL-6R shedding by PMN-serine proteases. These soluble versions function in some cases as decoy-receptors. In other cases, they act as binding soluble receptors carrying cytokines towards membrane receptors, or as conveyors. Likewise, the cytosolic fraction receptor free or intracellular domain (ICD), functions as a transcription factor (31).
- Some receptors are not directly activated by binding their ligands. Activation occurs upon binding another ligand to its specific receptor. This phenomenon is called "Receptor transactivation," and it refers to the ability of a ligand to bind to its specific receptor and thus activate another ligand receptor. The mechanism involved during this process is transphosphorylation.
- Some receptors are anchored to the membrane and are recognized as entry routes for certain infectious pathogenic agents. An example of this is the HGF/SF-R (Hepatocyte growth factor/scatter factor-receptor), which is involved in the recognition and internalization of *Listeria monocytogenes*. A surface protein of this bacteria - internalin B (InlB) - interacts with the HGF/SF-R (Hepatocyte growth factor/scatter factor-receptor) and thus favros the entry of the bacteria. The chemokine-receptor Duffy

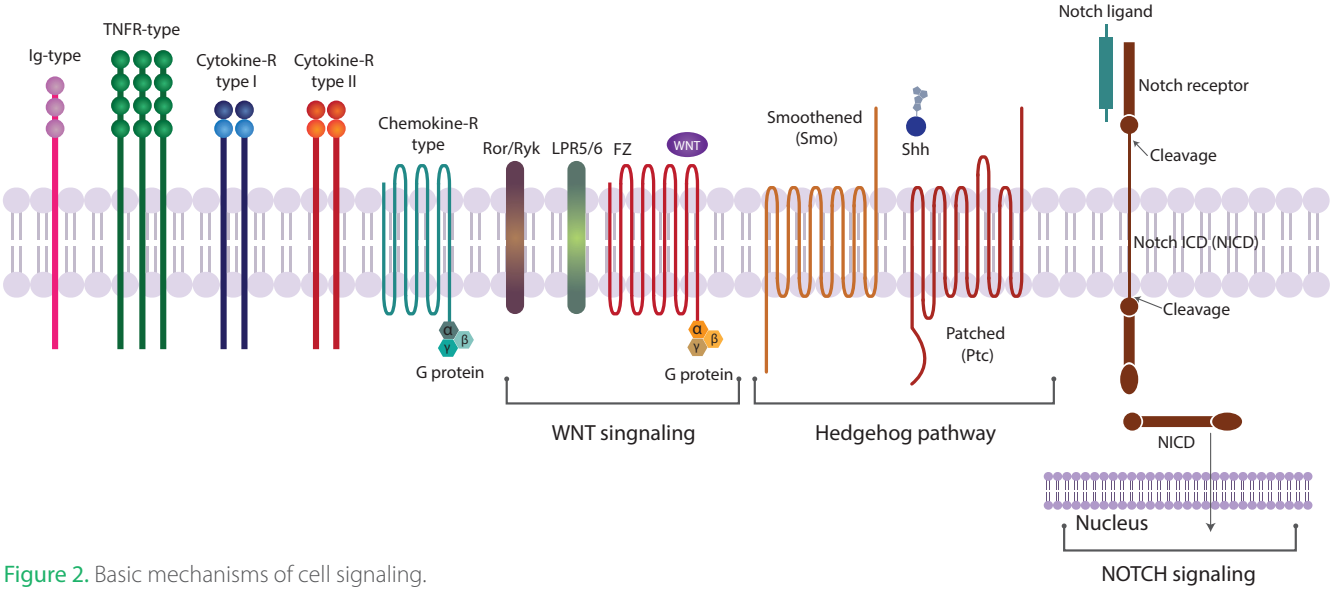


Figure 2. Basic mechanisms of cell signaling.

antigen receptor for chemokines (DARC) serves as the erythroid receptor for the human malaria parasite (*Plasmodium vivax*) and the monkey malaria parasite (*Plasmodium knowlesi*). Another example is the Human immunodeficiency virus (HIV)-coreceptors by chemokine family (See chemokine superfamily signaling through chemokine receptors (seven-transmembrane –heptahelical(serpentine) receptors associated with g-protein trimeric system).

- The cytokine-receptors suffer negative regulation by specialized inhibitory receptors, which belong to the Paired-receptor Superfamily. Some inhibitory receptors of this family are: SIRPα/CD172a, CD200R, CEACAM1/CD66a, 5 Leukocyte immunoglobulin-like receptor (LILR) members (LIR1/ILT2/CD85j, LIR2/ILT4/CD85d, LIR3/ILT5/CD85a, LIR5/ILT3/CD85K, and LIR8/CD85c), NKG2A/CD159a, DCIR/CLEC4A, CMRF35H/IREM1, PILRA, 12 Sialic acid-binding immunoglobulin-type lectin (SIGLEC) family members (SIGLEC1, SIGLEC2/CD22, SIGLEC3/CD33, SIGLEC4/MAG, SIGLEC5, SIGLEC6/CD327, SIGLEC7/CD328, SIGLEC8, SIGLEC9/CD329, SIGLEC10, SIGLEC11, and SIGLEC12), and 8 Killer cell immunoglobulin-like receptor (KIR) family members (KIR2DL1/CD158a, KIR2DL2/CD158B1, KIR2DL3/CD158B2, KIR2DL5A/CD158F, KIR2DL5B/CD158F2, KIR3DL1/CD158E, KIR3DL2/CD158K, and KIR3DL3/CD158Z). These inhibitory receptors have immunoreceptor tyrosine-based inhibitory–motifs (ITIM) in their cytoplasmic region that upon receptor triggering, recruit cytosolic tyrosine-phosphatases (32).
- The genome of certain viruses encodes cytokine-receptor-like molecules referred as "viroceptors," which function as competitive receptors of host cytokine-receptors. For example, herpes virus and poxvirus familiy genomes encoded a large number of proteins that modulate chemokine activity such as proteins with homology chemokine-receptors (See section chemokine superfamily signaling through chemokine receptors seven-transmembrane –heptahelical (serpentine) receptors associated with g-protein trimeric system) (33).
- Some receptors are part of the blood group system. The main example is the chemokine-receptor DARC (Duffy antigen receptor complex)/CD234.Therefore, variations in DARC generate the basis of the Duffy minor blood group system.
- Some receptors are expressed abnormally in target cells, tissues and organs involved in some diseases. Therefore, they can be considered pathophysiological and diagnostic biomarkers. The same happens with some soluble receptors when they are detected in biological fluids.
- Within the current biological therapy, the use of soluble receptors produced by recombinant DNA technology emerges. An example of this is the biopharmaceutical molecule Etanercept (Enbrel ®), which was produced by the fusion of the Tumor Necrosis Factor Receptor (TNFR) protein linked to the IgG1 Fc portion of an antibody (24).

CYTOKINES, CYTOKINE RECEPTORS, AND THEIR SIGNALING PATHWAYS SPECIFIC CHARACTERISTICS AND PROPERTIES

ARCHETYPICAL CYTOKINE FAMILIES SIGNALING THROUGH CLASS I CYTOKINE RECEPTORS (CRF1 OR HEMATOPOIETIN FAMILY) AND CLASS II CYTOKINE RECEPTORS (CRF2 OR IL-10/IFN SUPERFAMILY)

Type I cytokines have limited homology between the sequences of their family members. However, they are characterized by four α-helical bundle structures with an 'up-up-down-down' configuration. They can be further divided into short-chain and long-chain four α-helical bundle cytokines because of their α-helices length as well as some other structural and topological characteristics. Short-chain type I cytokines are those cytokines sharing the γc (gamma common chain) receptor, the βc (beta common chain) receptor, and also MCSF and SCF/KitL as atypical examples. Within of long-chain type I cytokines, there are cytokines sharing the gp130 common chain receptor, GH, Leptin, EPO, TPO, IL-12, PRL, and CSF3/GCSF. Unlike the type I cytokines, the type II cytokines have different structures and correspond to the members of the IL-10/IFN superfamily (1-12).

CRF1 family members are characterized by conserved extracellular domains of approximately 200 aminoacids, known as cytokine receptor homology domain (CHD), hematopoietin receptor domain (HRD), or D200. The CHD consists of two tandem fibronectin type III (FBN/FNIII) folds. It also contains two pairs of conserved cysteines (four conserved cysteines - C4) linked via disulfide-bonds, and it is arranged in a CX-(9–10)-CXWX-(26–32)-CX-(10–15)-C motif within the first FBN/FNIII fold. The second FBN/FNIII fold (proximal to the transmembrane domain) has a highly conserved tryptophan-serine doublet [(WS)2]=Trp-Ser-X-Trp-Ser motif (WSXWS-motif) in its carboxyl extreme. The C4 (WS) 2 motif represents a common signature to define Class I cytokine receptors. Aditionally, this family has other module domains including the extracellular Ig-like, a transmembrane (TM) domain, and conserved intracellular motifs such as Box1- and Box2-motifs. These last two motifs are associated with cytosolic tyrosine Janus Kinase (JAK)-docking. These conserved intracellular motifs are part of the "Intracellular Homology Region (IHR) sequences".

Jean-Louis Boulay, John J. O'Shea, and William E. Paul did a comparative study on molecular phylogeny of type I cytokines and proposed that they be classified into 5 groups based on certain characteristics:

Group 1: receptor chains have an extracellular domain consisting solely of a CHD. For example, erythropoietin receptor (EPOR), thrombopoietin receptor (TPOR), prolactin receptor (PRLR), and growth hormone receptor (GHR) chains. Each of them produces homodimers in the presence of their respective ligands.

Group 2: receptors with polypeptidic chains structurally related to the prototypical glycoprotein 130 (gp130), which

has an N-terminal Ig-domain and FBN modules between their CHD and transmembrane domains.
Group 3: receptor chains generally possess an N-terminal Ig domain in addition to the CHD. They are solubles and they have short intracellular regions.
Group 4: receptors chains consist solely of an extracellular CHD domain and long intracellular domains.
Group 5: receptor chains possess extracellular Ig-domains in addition to the CHD and have short intracellular regions.

Receptor chains from Groups 2 and 3 constitute the large IL-6R family receptor complexes, which variably share the gp130 as a common signal transducer. Group 4 and 5 receptor chains associate in order to generate the IL-2R and IL-3R family receptor complexes with IL-2Ryc and IL-3Rβc respectively. IL-2Rα and IL1-5Rα receptor chains are not members of the class I family receptors. Instead they contain a distinctive 'sushi domain', also known as Complement control protein (CCP) modules or short consensus repeats (SCR) (1-12,34).

However, for academic purposes, we proceeded to classify receptors and cytokine systems of this type as follows:

A. Family or Common gamma Chain Receptor Family: IL-2, IL-5, IL-9, IL-15, IL-21, IL-4 subfamily (IL-4 and IL-13), and IL-7 subfamily (IL-7 and TSLP).
B. Common beta Chain Receptor Cytokine Family: IL3, IL5, and CSF2/GMCSF.
C. Prolactin family: PRL, GH Subfamily (GH-1, and GH-2), CSH Subfamily (CSH-1 and CSH-2), EPO, TPO, and CSF3/GCSF
D. IL-6 Family: IL-6, IL-11, IL-31, LIF, CNTF, OSM, and Cardiotrophin subfamily (CT1 and CLC)
E. IL-12 Family: IL-12, IL-23, IL-27/30, and IL-35

Type II cytokines have different structures in comparison to type I. However, they retain the Box1/2 regions and they are classified as follows:
A. IL-10 Family: IL-10, IL-22, and IL-26.
B. IL-19 Family: IL-19, IL-20, and IL-24.
C. Type III: IFN- λ Family (IFN-λ1/IL-29, IFN-λ2/IL-28A, IFNλ3/IL-28B, and IFN-λ4).
D. Type I: IFN-α–IFN Family (IFN-α1, IFN-α2, IFN-α 4, IFN-α 5, IFN-α 6, IFN-α 7, IFN-α 8, IFN-α10, IFN-α13, IFN-α14, IFN-α16, IFN-α17, and IFN-α21).
E. Type II – IFN: IFN-γ.
F. Type I IFN-β/ω: IFN β/ω Family (IFN-β1 and IFN-ω1).
G. Tissue Factor – VIIa system.

JAK/STAT-SIGNAL AND NEGATIVE CONTROL MECHANISMS IN CYTOKINES FAMILIES SIGNALING THROUGH CLASS I CYTOKINE RECEPTORS AND CLASS II CYTOKINE RECEPTORS

JAKs and STATs

The CRF1 and CRF2 are heterocatalytic tyrokine-kinase receptors lacking cytosolic tyrosine-kinase domains. Therefore, they recruit cytosolic tyrosine-kinases called Janus kinase (JAK): JAK1, JAK2, JAK3, and TYK2. Once a cytokine binds to the receptor, it leads to dimerization or oligomerization of the cytokine-receptor after which, JAK are docked in proximity to the cytokine-receptor cytosolic region. Later, JAKs undergo autophosphorylation, thus producing phosphorylation of the cytokine-receptor and other JAKs. Tyrosine phosphorylation of cytokine-receptor cytosolic region creates sites for the recruitment of proteins containing Src – homology (SH2) domains. These domains consist of transcription factors called signal transducers and activators of transcription (STATs): STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. The STATs are hetero- or homodimerized and are, then, translocated to the nucleus (Figure 3) (35).

Control mechanisms

Several mechanisms ensure regulated cell responses to cytokines and prevent excessive activation. Among these mechanisms are the following:

- Receptor internalization, and in some cases, the subsequent ubiquitination and proteosomal degradation.
- The negative feedback loop has its effect by inducing gene transcription of cytokine signaling suppressor proteins (SOCSs): CISH, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, and SOCS7. SOCSs interact directly with JAKs and inhibit their enzymatic activity. In addition, they bind to cytokine-receptors and block STAT binding and phosphorylation. Other mechanisms of action work through binding to cytokine-receptors before inhibiting JAKs, or through SOCS proteins containing a SOCS-box and thereby allow the proteosomal degradation of the cytokine-receptor JAKs and STATs. Loss of SOCS expression (so far only found within epigenetic mechanisms) has been observed in multiple cancer types (36,37).
- Src-homology 2 containing tyrosine-phosphatase, non-receptor protein (SHP/PTPNs): SHP1/PTPN6 and SHP2/PTPN11. SHP2/PTPN11 has been shown to have a dual function, and in some cases, it stimulates the JAK/STAT pathway. Moreover, the membrane protein tyrosine-phosphatase CD45 also has been shown to dephosphorylate the JAKs. Other tyrosine-phosphatases that regulate the JAK/STAT pathway are: PTP1B/PTPN1, TC-PTP/PTPN2, and PTPRT. The SHPs can be directly activated by paired-receptor superfamily members (See cytokine and cytokine-receptor general characteristics) (32).
- Protein inhibitors of activated STATs (PIAS): PIAS1, PIAS2, PIAS3, and PIAS4. PIASs interact with STAT dimers and inhibit their transcription factor function (Figure 3) (38-40).
- A wide variety drugs to inhibit JAKs (Jakinibs) are currently being developed. They have potential use in cancer, allergic diseases, hematologic disorders, and autoimmunity, e.g., Tofacinib/Cp-690550 (Xeljanz), Ruxolitinib (Jakafi, Jakavi) and Lestaurtinib (38-39)

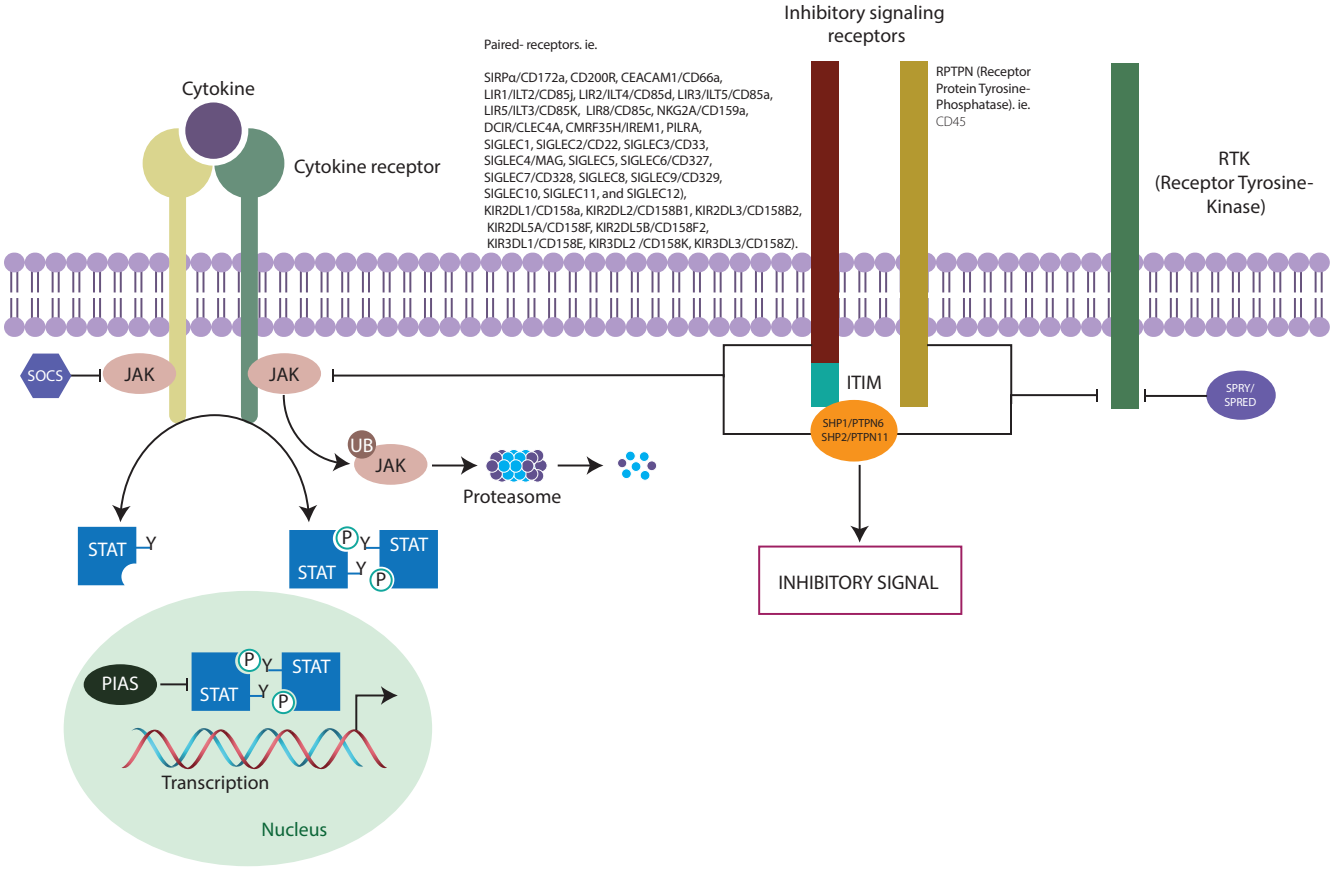


Figure 3. JAK/STAT-signalling and negative control mechanisms. For further information see text. Adapted from reference (35).

CYTOKINE TYPE I HELICAL FAMILIES SIGNALING THROUGH CLASS I CYTOKINE RECEPTORS (CRF1 FAMILY OR HEMATOPOIETIN FAMILY)

1. IL-2 Family or Common γ Chain Receptor Family: IL-2, IL-9, IL-15, IL-21, IL-4 subfamily (IL-4, andIL-13), and IL-7subfamily (IL-7, and TSLP) (Tables 1 and Figures 4–6) (1-12, 41-50)
2. Common β Chain Receptor Cytokine Family (IL-3, IL-5, CSF2/GMCSF) (Figure 7) (1-12, 51)
3. Prolactin family: PRL, GH Subfamily (GH1, GH2), Chorionic somatomammotropin Subfamily (CSH1, CSH2), Erythropoietin (EPO), Thrombopoietin (TPO), and Colony Stimulating Factor- 3/Granulocyte-stimulating factor (CSF3/GCSF) (Figures 8- 9) (1-12)

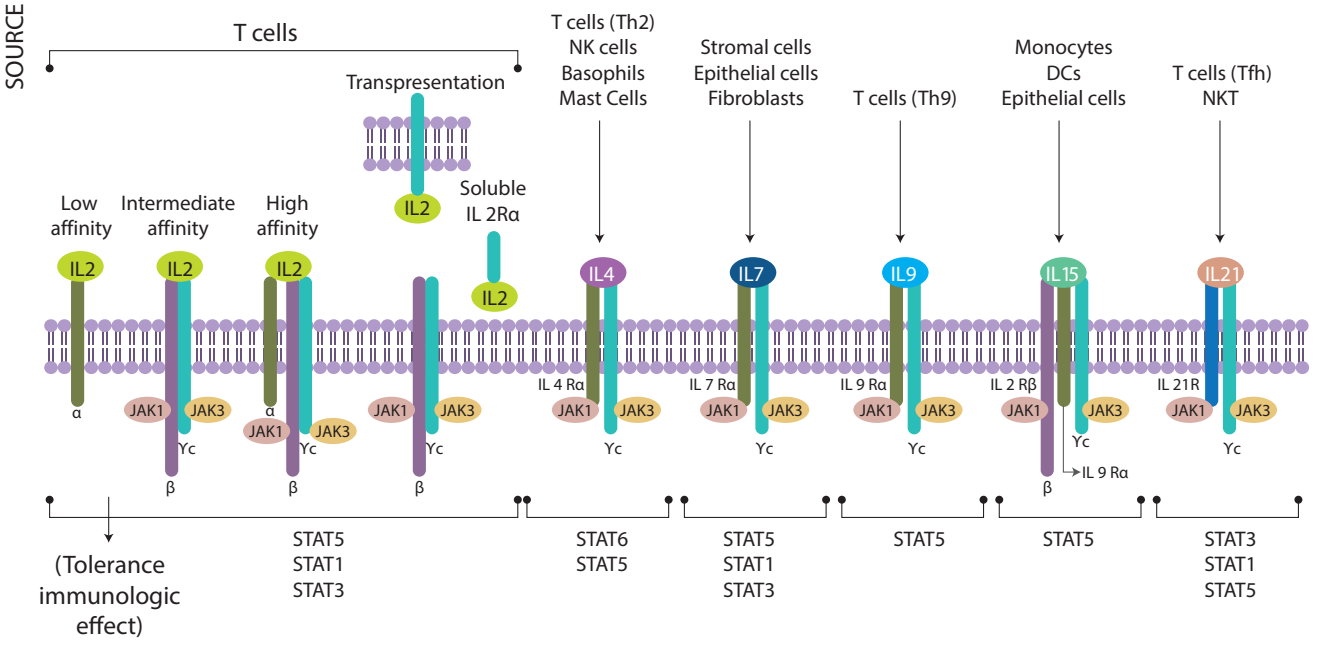
CSF3/GCSF

CSF3/GCSF is a secreted monomer glycoprotein of 207 aminoacids and 22,293 kDa. This cytokine is produced by all tissues and cells in the human body, and it is a fundamental factor for enhancing the proliferation and differentiation of committed myeloid progenitor cells as well as the maturation of myeloid progenitor cells to neutrophilic granulocytes. Furthermore, it is important for the physiology (intensifies multiple functions) and survival of these granulocytes. In physiological conditions CSF3/GCSF is produced at a very low level. Therefore, it is minimally detectable in blood plasma. CSF3/GCSF is rapidly eliminated by renal route, and its rise in serum levels is triggered by proinflammatory cytokines, bacterial PAMPs (Pathogen-associated molecular patterns) and myelo-suppression.

Note that there is a numerical control of neutrophil population in the IL-23 - IL1-7 - CSF3/GCSF axis, also known as the neutrostat feedback loop. Under normal conditions, a proportion of circulating neutrophils enters tissues where they become apoptotic and are engulfed by tissue phagocytes. It also inhibits IL-23 production and secretion by phagocytes, which leads to suppression of IL-17 production by T-lymphocytes (called Th17). IL-17, in turn, regulates systemic levels of CSF-3/GCSF through yet unclear mechanisms. During neutropenia conditions, there is a decrease in the proportion of neutrophils undergoing apoptosis within the tissues, which triggers the elevation of IL-23 production and the subsequent increase in IL-17production. At the same time, this will stimulate the production of CSF-3/GCSF (Figure 9) (52). Finally, CSF3/GCSF stimulates neutrophil production (stress granulopoiesis)

IL-2 FAMILY MEMBERS	PRODUCTION SOURCE	IMMUNOLOGICAL ROLES AND OTHER FUNCTIONS
IL-2	T cells, NKT, DCs, and mast cells	Modulates T cells differentiation in response to antigen into Th1 and Th2 response, while inhibiting Th17 response and Tfh differentiation Development and maintenance of Treg and activation-induced cell death Can stimulate B cells monocytes, NK, NKT and glioma cells Recombinant human des-alanyl-1, serine-125 human IL-2 "Aldesleukin"(Proleukin) is used in metastatic renal cell carcinoma, melanoma, and is an investigational immunomodulatory agent for treatment of several infectious diseases Daclizumab (Zenapax) are Basiliximab (Simulect) are humanized monoclonal antibody works by binding toIL 2Rα/CD25, and are used in prophylaxis of acute organ rejection in adult patients
IL-9	T cells and mast cells	Promotes Th17 development Th9 cells characterized in response to a balance of TGFβ and IL-4 stimuli B cells development and function Promotes mast cell growth and function Regulates hematopoiesis and also acts as an activator of megakaryocyte progenitor cells Has direct effects on airway epithelial and smooth muscle cells
IL-15	Fibroblasts, keratinocytes, epithelial cells, neuronal cells, monocytes, macrophages and DCs	Stimulates T cell and NK proliferation in IL-12-like manner thought common receptors Has little effect on Treg cells Protects neutrophils from apoptosis, modulates phagocytosis and stimulates secretion of IL-8 and IL-1R antagonist Acts in mast cells as a growth factor and an inhibitor of apoptosis Induces B cells proliferation and differentiation, and increase immunoglobulin secretion by plasma cells
IL-21	CD4+ T Cells predominantly Th follicular cells	Proliferation and functions of CD4+ and CD8+ T cells and negatively regulates the differentiation and activity of Treg. Differentiation of B cells into memory cells and immunoglobulin secretion by plasma cells (IgG 1 and 3) Enhances the activity of NK Differentiation, proliferation and activity of macrophages
IL-4 subfamily		
IL-4	T cells, mast cells, basophils and eosinophils	Promotes Th2 and inhibits Th1 and Th17 differentation Regulation of alternative macrophage activation B cell activation and induces the expression of class II HLA on resting B cells. Enhances secretion and cell surface expression of IgE and IgG1 Regulates the expression of the low affinity Fc receptor for IgE on both lymphocytes and monocytes
IL-13	T cells, mast cells, basophils, eosinophils, NKT, monocytes, macrophages and DCs	Promotes Th2 response B cells maturation and differentiation Up-regulates class II HLA and CD23, and promotes IgEisotype switching of B cells. Induces STAT 6 activation and epithelial cell changes such as mucus hypersecretion, airway inflammation, bronchial hyperresponsiveness, and tissues fibrosis Inhibits inflammatory cytokine production, with IL-2 regulating IFN-γ synthesis
IL-7 subfamily		
IL-7	B and T cells, endothelial cells, and secondary and tertiary lymphoid organs	Induces Th1 and Th17 response Overexpression is associated with ectopic lymphoid organs to induce LTβ and TNF-α and activate innate lymphoid tissues. Several cells produce IL-7 in inflammatory conditions (macrophages, dendritic cells, and fibroblasts) Promotes T cell-driven osteoclastogenesis and fibroblast activation Cofactor V(D)J rearrangement of the T cell receptor β (TCRB) during early T cell development. Pre-pro B cell growth-stimulating factor
TSLP	Epithelial cells (skin, gut, lung, keratynocites, thymus, breast, and corneal), corneoscleral tissues, fibroblasts, smooth muscle cells, B and T cells, mast cells, and DCs	Epithelial program of Th2-primed immune response (together with IL-25 and IL-33) by targeting mDCs, mast cells, eosinophils, and NKT cells Up-regulates the expression of class II HLA, CD54, CD80, CD83, and CD86 in mDCs May activate NKT to produce IL-13 Maternal Fetal tolerance

Table 1. IL-2 family and common gamma chain receptor family sources and functions.



Target cell	T cells B cells NK cells	T cells B cells NK cells Mast cells Basophils	T cells DCs	T cells Mast cells Eosinophils	T cells NK cells	T cells B cells NK cells DCs
Immunologic effect	· T cell proliferation ↑ Th1, ↑ Th2 and ↑Treg ↓ Th17 and ↓Tfh differentiation ·↑Effector and memory CD8+ T cell formation ↑B cell and áNK cell function · Activation induced cell death (AICD)	·↑B cell proliferation ·↑Th2 cell differentiation ·↑IgE and IgG4 class switch	·↑T cell development ·↑Memory CD4+ and ↑CD8+ T cell development	·↑Mucus production ·↑Mast cell production · Anti cancer effect	·↑NK cell development and function ·↑Memory CD8+ T cell development	↑Anti cancer activity of CD8+ T cells and NK cells ↑Tfh production ↑Th17 and NK cell differentiation ↑B cell differentiation to plasma cell and Ig production

Figure 4. IL-2, IL-7, IL-9, IL-15, and IL-21, receptors and signaling.

and enhances neutrophil release from the bone marrow by disruption of CXCR4-CXCL12/SDF1 signaling within bone marrow mature neutrophils.

The CSF3/GCSF – CSF/GCSFR system can also act on neuronal precursors and neuron cells in the brain and spinal cord as a neurotrophic factor, thus inducing neurogenesis to increase the neuroplasticity and to counteract apoptosis. In addition, CSF3/GCSFR is expressed in cardiomyocytes and endothelial cells. Moreover, there are CSF3/GCSF-producing carcinomas that arise in several organs, especially in the lung, stomach, liver, thyroid gland, gall bladder, urinary bladder, thymus, and cervix. These types of carcinomas are classified as poorly differentiated or undifferentiated, and they have poor prognosis.

At present, the recombinant human form of this cytokine (rhCSF3/GCSF: filgrastim, pegfilgrastim, and lenograstim) is used in supportive care after autologous stem cell transplantation and bone marrow transplantation, in the management of neutropenia from different origins (for example, congenital neutropenia, cancer neutropenia, febrile neutropenic syndrome, neutropenia post-chemotherapy, and radiotherapy), and in infectious disease prevention and management. Likewise, it is been currently being studied for its use in revascularization of ischemic heart disease, retinopathy, neurodegenerative diseases (amyotrophic lateral sclerosis and cerebral ischemia), and to increase the number of peripheral blood neutrophils in leukocyte donors.

1. IL-6 Family: IL-6, IL-11, IL-31, LIF, CNTF, OSM, Cardiotrophin subfamily (CT-1, and CLC) (Table 2 and Figure 10) (1-12, 56-65)
2. IL-12 Family: IL-12, IL-23, IL-27/30, and IL-35 (Table 3 and Figures 11-12) (1-12, 66-68).

CYTOKINE FAMILY SIGNALING THROUGH CLASS II CYTOKINE RECEPTORS (IL-10/IFN SUPERFAMILY)

1. IL-10 Family: IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26.
2. IL19 Family: IL-19, IL-20, and IL-24
3. Interferons (IFNs)

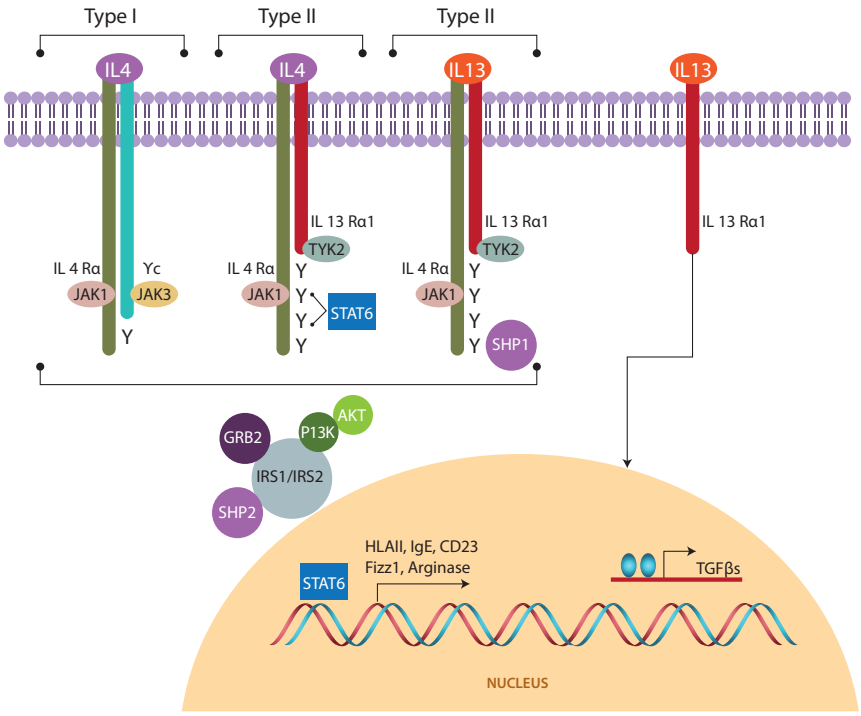


Figure 5. IL-4 subfamily(IL-4, IL-13), receptors and signaling. For further information see text. Adapted from reference (40).

IFNs are a group of glycoproteic cytokines with antiviral, anti-proliferative (anti-cancer), and immunoregulatory/immunomodulatory effects. Under physiological conditions, they do not have demonstrable levels in blood circulation. Instead, they only appear in blood at clinical and subclinical levels in response to an infection or in immune responses (Figures 13,14) (1-12,69-74).

IFNs are composed of type I IFNs (acid stables), type II IFNs (non-acid labile), and type III IFNs. Type I and type III IFNs are induced during viral infection, and they are involved in host defense against viruses. IFNAs are produced by leukocytes, particularly plasmocytoid dendritic Cells (pDCs). Type II IFNs, in turn, are involved in allergic response, in host defense against intracellular pathogens, in cancer control, and are mainly produced by Th1 lymphocytes.

The IFNs are classified as class II alpha-helical cytokines, and they share basic secondary structural elements with an overall architecture of five helical bundles. However, the IFNs share very limited functional homology as well as a conserved fold. This is undoubtedly reflected by their binding to diverse receptors (IFNRs).

There are nine identified mammalian type I IFN subtypes: IFN-α (13 known subtypes, all of them present in humans), and single forms of IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-τ, IFN-ν, IFN-ζ. All of them are monomerics. The type I IFN gene induction is primarily regulated by helix-turn-helix DNA-binding motif transcription factors know as IFN regulatory factors (IRF family members: IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, IRF-7, IRF-8, and IRF-9).

The human type I IFNs (IFN-α, IFN-β, and IFN-ω) interact with the IFNR complex, which consists of at least two subunits: IFNAR1 (low affinity) and IFNAR2 (high affinity). There are three IFNAR2 isoforms produced by exon skipping from the same gene, alternative mRNA splicing and differential polyadenylation sites usage. IFNAR2c isoform is a long transmembrane protein with a full intracellular region and full

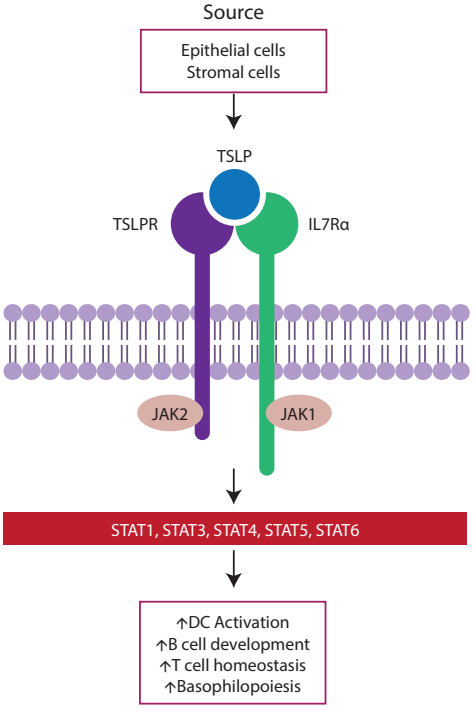


Figure 6. TSLP, receptor and signaling.

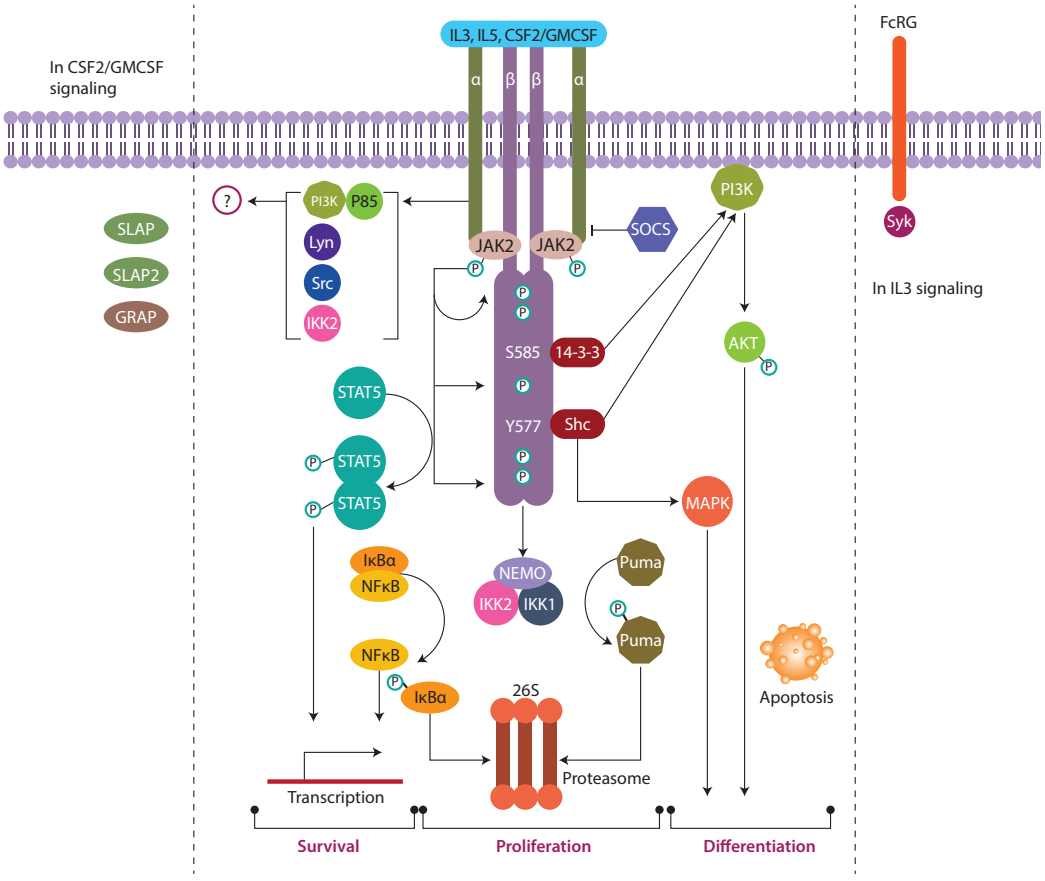


Figure 7. Common Beta Chain Receptor Cytokine Family (IL-3, IL-5, CSF2/GMCSF) signaling. For further information see text. Adapted from reference (41).

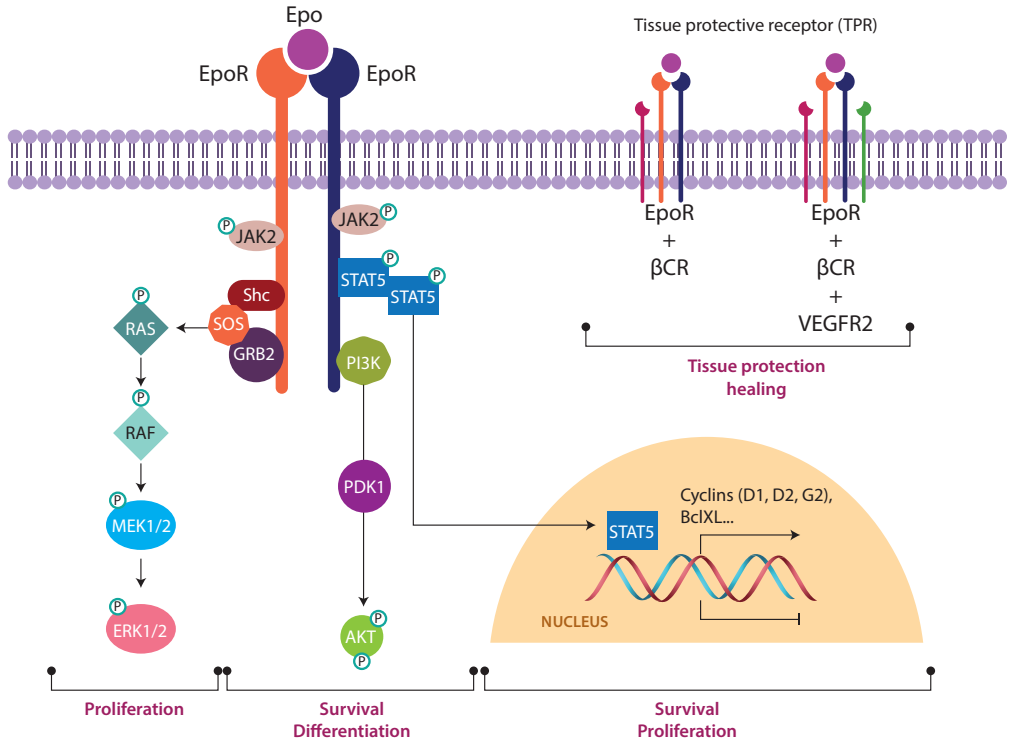


Figure 8. EPO-EPOR signaling.

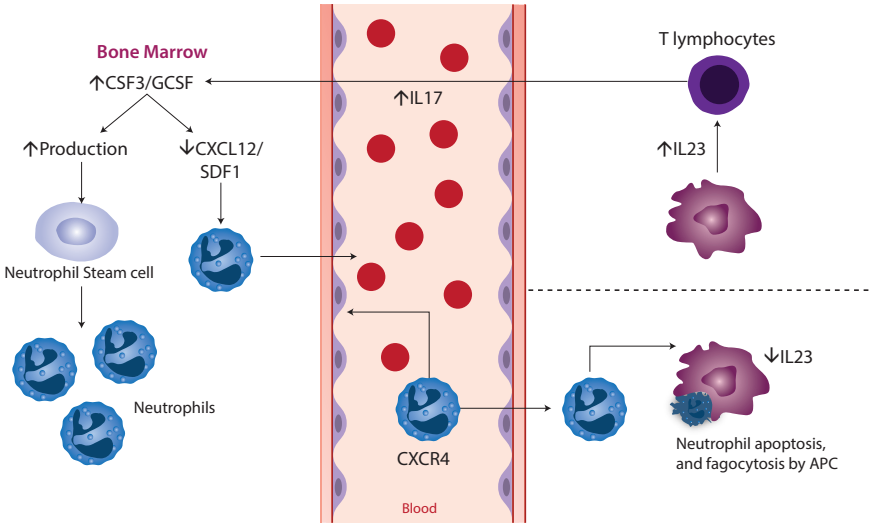


Figure 9. CSF3/GCSF and the Neutrostat feedback loop. Adapted from reference (52).

signaling ability while IFNAR2b is a short transmembrane isoform acting as dominant negative signaling regulator. Finally, IFNAR2a is a soluble truncated isoform exhibiting both agonistic and antagonistic properties in certain circumstances.

The IFNAR1 and IFNAR2 intracellular domains (particularly IFNAR2c) are associated with the cytosolic tyrosine-kinase JAKs, TYK2, and JAK1 respectively. The binding of type I IFNs to IFNARs results in the cross-activation of these JAKs, which then will phosphorylate STAT transcription factors (mainly STAT1 and STAT2, but also STAT3, STAT4, and STAT5). Recrutied and phosohorylated STATs generate two distinct transcriptional activator complexes called IFN α-activated factor (AAF) and IFN-stimulated gene factor 3 (ISGF3). Whereas AAF is a STAT1 homodimer, ISGF3 is a STAT1, STAT2, and IRF-9 heterotrimeric complex (also called p48 or ISGF3c). AAF and ISGF3 translocate into the cell nucleus and bind to specific DNA sequences in the promotors of specific genes, for example, the IFNG-activated sequence (GAS) and the IFN-stimulated response element (ISRE) respectively. Other signaling pathway components that have been identified are MAPKs, PI3K-PDK1-Akt pathway, NFkB, p53, and protein arginine-methyltransferase 1 (PRMT1).

IFN-γ is a type II IFN, and it is an anti-parellel homodimer cytokine that signals through the IFNR complex which consist of IFNRG1 and IFNRG2. IFNGR1 binds to JAK1 while IFNGR2 binds to JAK2, which then phosphorylates STATs transcription factors (STAT1, STAT2, STAT3, and STAT5). Other components of the IFNRG/IFN-γ signaling system that have been identified are MAPKs, PI3K-PDK1-Akt pathway, and NFkB complex. IFN-γ-IFNGRs also use the AAF transcriptional activator complex.

IFN-λ (IL-28A, IL28-B, IL28-C, and IL-29) are type III IFNs. They utilize the IFNλ receptor complex consisting of IFNLR1 and IL-10RB. While IFNLR1 is associated with JAK1, IL-10RB is associated with TYK2, which then phosphorylates STAT transcription factors (STAT1, STAT2, STAT3, STAT4, and STAT5). Furthermore, MAPKs and the PI3K-PDK1-Akt pathway are involved in the IFNL signaling pathway. IFN-λ-IFNLR use ISGF3 and AAF transcriptional activator complex.

IFN/IFNR signaling system results in the transcription of hundreds of specific genes (Interferon inducible protein –"IFI") such as the genes for eukaryotic translation initiation factor 2 –alpha kinase 2/double strand RNA (dsRNA), activated serine/threonine-protein kinase (EIF2AK2/PKR), 2',5'-oligoadenylate-synthetase family members (OAS1, OAS2, OAS3, and OASL), Mixovirus resistance GTPase protein family members (Mx1 and Mx2), iNOS, complex major histocompatibility II (CMHII), and the p53 tumor suppressor. PKR is under tight control, so it is inhibited by the 58-kDa PKR inhibitor –P58 (IPK). P58 (IPK) is inhibited through direct interaction with P52 (rIPK), which, in turn, results in PKR activity upregulation.

OAS catalyzes the synthesis of 2',5'-oligomers of adenosine (2-5As). These 2-5As bind to and activate Ribonuclease (RNASEL), which degrades viral and pathological/anormal cellular RNAs. This leads to cell protein synthesis inhibition and viral replication impairment. OAS is also implicated in the cell growth control, differentiation, and apoptosis.

IFN-γ induces the Death associated protein (DAP)/DAP-kinase (DAPK) system genes. They are positive mediators of apoptosis and autophagy. DAP family members (DAP1 and DAP3) are mediators in cell induced IFN-γ -death. DAPK family members (DAKP1, DAPK2, DAPK3/ZIPK, and DAPK related apoptosis inducing protein kinase 1 –DRAK1/STK17A and DRAK2/STK17B) are calcium/calmodulin (CaM)-regulated serine/threonine-kinases involved in numerous cell death mechanisms and pathways. Loss of DAPK expression (so far only found within epigenetic mechanisms) has been observed in multiple cancer types. DAPKs gene expression and apoptotic activity also rise in response to TGFBs and to stimuli activating p53 such as DNA-damaging agents (75).

IL-6 FAMILY MEMBER	PRODUCTION SOURCE	ROLE IN INFLAMMATION, AND OTHER FUNCTIONS
IL-6	Various types of lymphoid and non-lymphoid cells, such as T lymphocytes, B lymphocytes, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several cancer cells	- Potent inducer of the acute phase response together with IL-1 and TNF - Differentiation B cells into plasma cells(PC), and stimulation of PC activity.- Monocyte and T cells differentiation - Acts on hepatocytes, hematopoietic progenitor cells and cells of the CNS. Also acts as a myokine
IL-11	Various cell types that include epithelial, endothelial, keratinocytes, stromal, neuronal, bone marrow stromal cells, fibroblasts, osteoclasts, and other stromal cells	- T cell-dependent development of immunoglobulin-producing B cells. - Proliferation of hematopoietic stem cells and megakaryocyte progenitor cells. - Trophoblast-endometrial interactions during the establishment of pregnancy - Recombinant human IL-11[(Oprevelkin)Neumega] is used in patients undergoing chemotherapy for nonmyeloid malignancies that displayed severe thrombocytopenia
IL-31	T cells, monocytes, macrophages, DCs, mast cells, keratinocytes and fibroblast	Enhancement of inflammatory process in the skin induced by UV radiation -Stimulates Th2 response -Regulates hematopoiesis and cell proliferation
LIF	Expressed in the trophectoderm of the developing embryo, with its receptor LIFR expressed throughout the inner cell mass. Given the structure of the gene, it follows constitutive expression predominantly characterized by low production levels in certain tissues and inductive type expression preferentially in lung fibroblasts, various types of epithelial cells, muscle cells, mesangial cells and hematopoietic cells in the immune system	-Induction of hematopoietic differentiation in normal and myeloid leukemia cells. -Induction of neuronal cell differentiation -Regulator of mesenchymal to epithelial conversion during kidney development -Trophoblast-endometrial interactions during the establishment of pregnancy -Role in immune tolerance at the maternal-fetal interface.
CNTF	Cells of CNS and retina	-Survival factor for various neuronal cell types, has several functions such as neuroprotection, induces photoreceptor plasticity and axogenesis
OSM	Cells of CNS and epithelium	-Growth regulator. -Inhibits the proliferation of a number of tumor cell lines -Survival factor for various neuronal cell types -Hematopoiesis and liver development
CT1/CLC heterodimer	Cardiac myocytes, hepatocytes, megakaryocytes, endothelial cells, adipocytes, osteoclasts, and neuronal cells	-Is present in different conditions such as hypertension, congestive heart failure, myocardial infarction, valvular heart disease, metabolic syndrome, and chronic kidney disease. Promotes survival of motor neurons
Neuropoietin	(-)	Induces Th1 and Th17 response Overexpression is associated with ectopic lymphoid organs to induce LTβ and TNF-α and activate innate lymphoid tissues. Several cells produce IL-7 in inflammatory conditions (macrophages, dendritic cells, and fibroblasts) Promotes T cell-driven osteoclastogenesis and fibroblast activation Cofactor V(D)J rearrangement of the T cell receptor β (TCRB) during early T cell development Pre-pro B cell growth-stimulating factor

Table 2. **IL-6 Family sources and functions.**

IL-12 FAMILY MEMBER	PRODUCTION SOURCE	ROLE IN INFLAMMATION, AND OTHER FUNCTIONS
IL-12	Is produced by activated phagocytic cells (monocytes, macrophages, and neutrophils) and by dendritic cells	Differentiation of naive T cells into Th1 cells, stimulates the production of IFNγ and TNF-α from NK and NKT, mediates enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes
IL-23	Is produced by activated DCs and phagocytic cells, and expression is elicited by PAMPs	Together with IL-6 and TGF-β1, IL-23 stimulates naive CD4+ T cells to differentiate into Th17 cells
IL-27/30	Is produced by antigen-presenting cells	In adaptive immunity, IL-27 was shown to synergize with IL-12 to promote IFNγ production by CD4 T cells, CD8 T cells and NKT cells . IL27 is a early initiator of Th1 differentiation. In innate immunity, induces the production of IL-1, TNFα, IL-18 and IL-12 in monocytes, and IL-1 and TNFα in mast cells . IL-27 inhibits differentiation of Th17 cells
IL-35	Anti-inflammatory/immunosuppressive cytokine	Is produced by Treg stimulated(sometimes called iTreg35)

Table 3. **IL-12 Family sources and functions.**

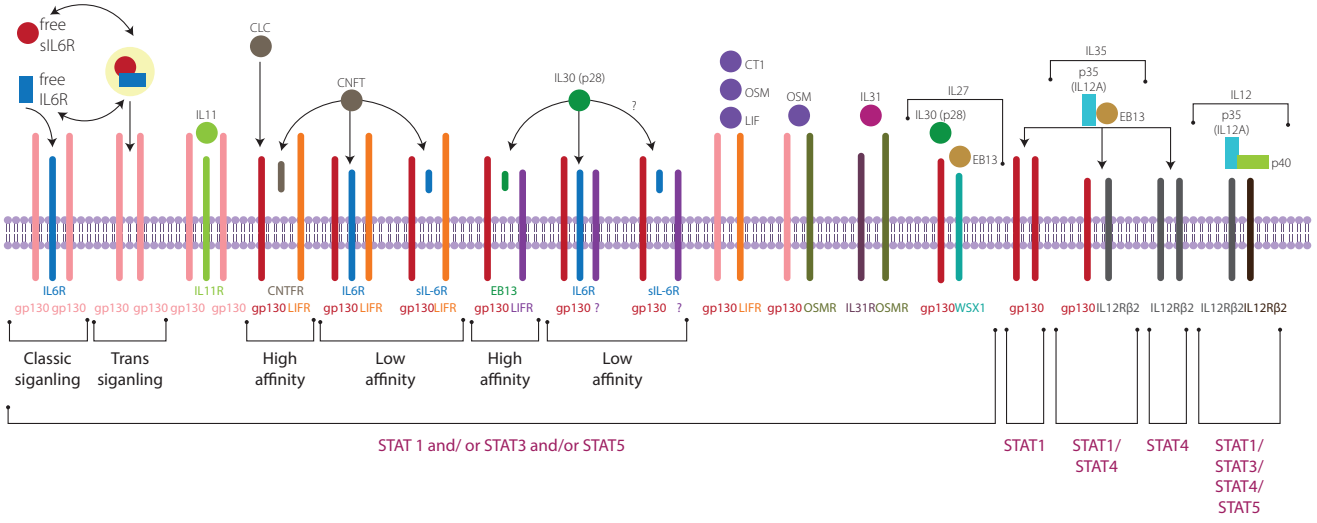


Figure 10. IL-6 Family, receptors, and signaling. Adapted from reference (58).

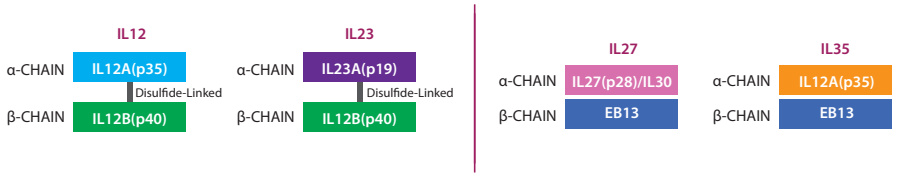
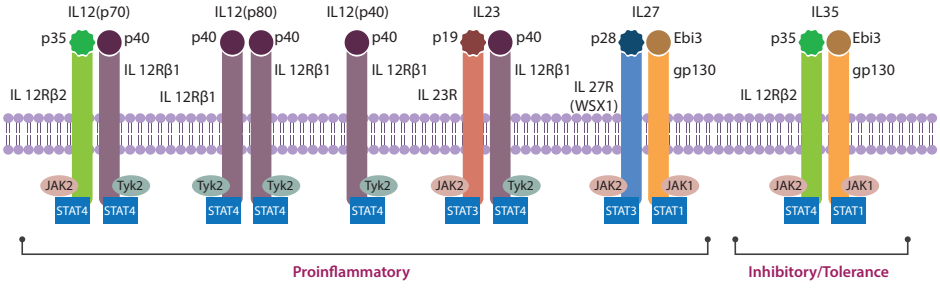


Figure 11. IL-12 Family structure.



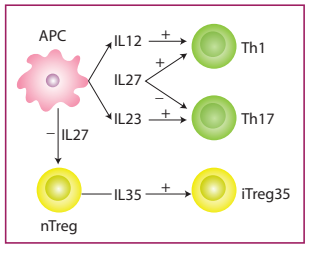


Figure 12. IL-12 Family, receptors, and signaling. Adapted from reference (66).

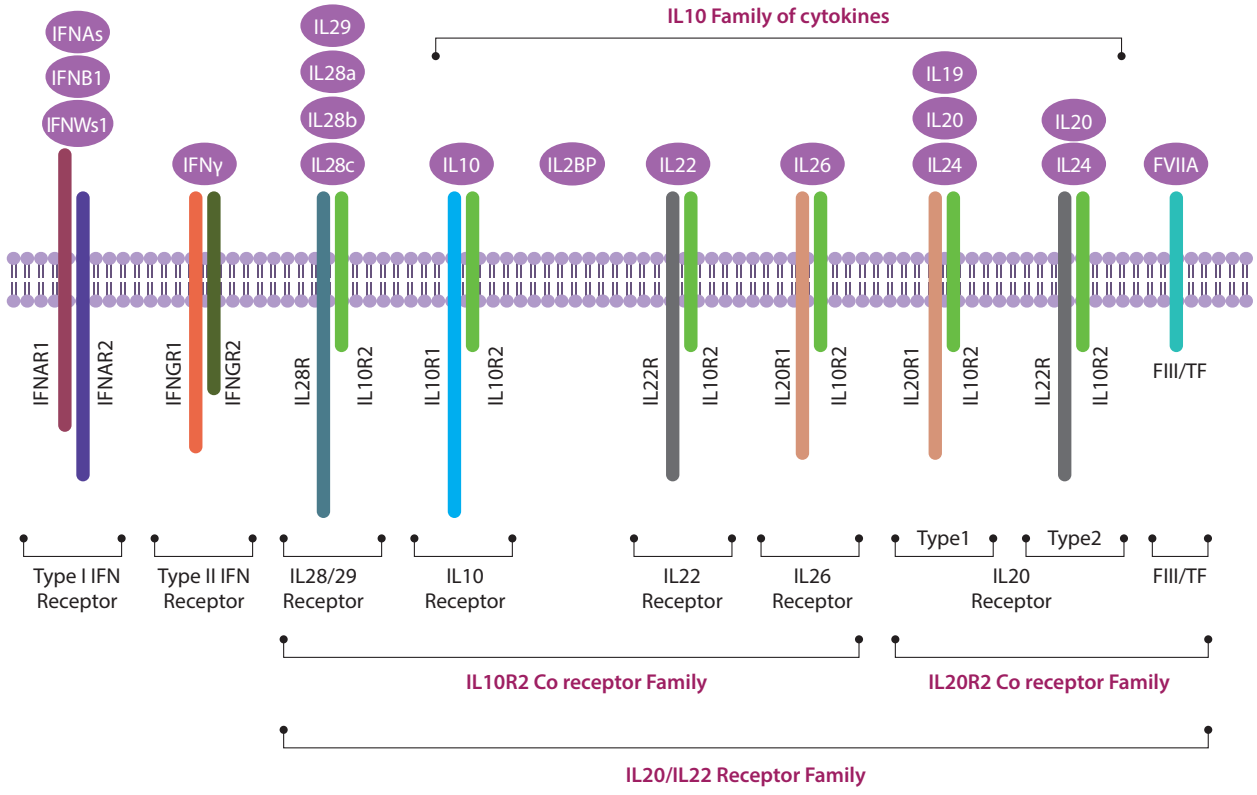


Figure 13. Cytokines families that signal through of Class II cytokine receptors (IL-10/IFN superfamily). Adapted from reference (74).

IFNs have a high therapeutic potential as does recombinant human IFN-α [IFN2b (INTRON A), Albinterferon (albIFN, trade name Albuferon), pegylated recombinant human IFN-α, inter alia]. The IFN-α are used in Chronic Hepatitis B Virus infection, Chronic Hepatitis C Virus infection, Condyloma acuminatum, treatment of HIV-related Kaposi's sarcoma, treatment of hairy cell leukemia, treatment of malignant melanoma, and treatment of follicular lymphoma. Recombinant human IFN-β [IFN-β1a (Avonex, Rebif) and -1b (betaferon)] is used in treatment of relapsing multiple sclerosis, and recombinant human IFN-γ [IFN-γ1b (Actinmune)] is used in Chronic granulomatous disease. It delays the profression in severe malignant osteopetrosis but has been shown to be ineffective and may increase mortality in patients with idiopathic pulmonary fibrosis (24).

CYTOKINE FAMILY SIGNALING THROUGH IMMUNOGLOBULIN CYTOKINE RECEPTOR SUPERFAMILY

RECEPTOR TYROSINE-KINASE CLASS III-LIGANDS–RTKIII/ PDGFR FAMILY: MCSF FAMILY (CSF1/MCSF AND IL-34), FLT3/FLK2, AND STEM CELL FACTOR/KITL (SCF/KITL)

The CSF1/MCSF (colony – stimulating factor – 1/macrophage – colony stimulating factor), IL-34, Stem Cell Factor/Kit Ligand (SCF/KitL), and Fms-like tyrosine kinase 3 ligand (FLT3LG) are cytokines signaling through the hematopoietic colony stimulating factor – 1 receptor (CSF1/MCSFR), Kit, and Flt/Flk2 receptors. All of them are from the Ullrich/Schlessinger class III receptor tyrosine kinase (RTK-III)/ PDGF-receptor family (Figures 13-14) (1-12, 74). They are characterized by five Ig-like extracellular domains.

- CSF1/MCSF
  The core functions of these cytokines include myelopoiesis, and the proliferation, differentiation, and functionality (phagocytic and chemotactic activity stimulation) of cells derived from the mononuclear phagocytic lineage such as monocytes, macrophages, microglia, osteoclasts (CSF1/MCSF released by osteoblasts as a result of parathyroid hormone endocrine stimulation), and DCs (1-12, 76-78). These cytokines are also required for normal male and female fertility. For example, they are expressed in female reproductive tract cells (placenta and endometrial glands during the menstrual cycle and in the terminal duct of the mammary gland at the end of the growth buds. CSF1 or CSF1R abnormal expression can promote cancer cell proliferation, invasion, and metastases formation in breast, ovarian, prostate, and endometrial cancers. The CSF1/MCSF appears to play a major role in the promotion and maintainance of HIV-1 reservoirs in infected individuals.

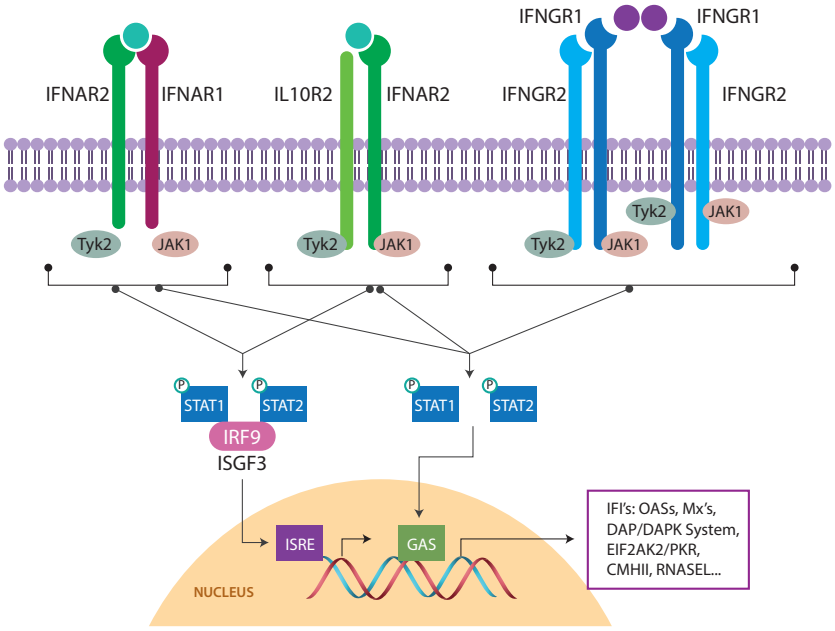


Figure 14. IFNs-IFNR system, and signaling.

HIV-1 infection induces production of CSF1/MCSF by macrophages, which, in turn, promote infection of other macrophages via an increase in CD4/CCR5 receptors as well as the increase in retrovirus gene expression.

The CSF1/MCSF is produced by Monocyte/macrophage lineage/DCs, granulocytes, endothelial cells, fibroblasts, and activated B and T cells. The CSF1/MCSF gene contains 10 exons and 9 introns, which expand lengthwise 20 kb. The active form of the protein is found extracellularly as a disulfide –linked homodimer, and it is thought to be produced by a membrane-bound precursor's proteolytic cleavage.

- The CSF1/MCSF gene is transcribed into 4.0, 3.7, 3.1, 2.6, or 1.6 kb mRNA isoforms via alternative splicing. The 1.6 and 3.1 kb transcripts encode 256 aminoacid proteins known as MCSF-α, MCSF short, MCSF cell surface, or MCSF slow-release isoform. The 3.7 kb transcripts encode 438 aminoacids protein called MSF-γ, MCSF intermediate, MCSF secreted, or MCSF soluble isoform. Finally, the 2.6 and 4.0 kb transcripts encode 554 aminoacid proteins known as MCSF-β, MCSF long or MCSF secreted fast released isoform. The CSF1/MSF is present in the circulation, predominantly as a proteoglycan (at 277 aminoacids or in its longer-form at 554 aminoacids). Note that when a proteoglycan moiety is added, pM-CSF is created.

The CSF1/MCSF homodimer binds to two CSF1/MCSFR molecules, thus facilitating receptor dimerization and tyrosine autophosphorylation by the intrinsic kinase region. These phosphorylated tyrosines recruit several proteins with SH2-domains and different signal transduction pathways such as: Src-family kinase member, phosphoinositide 3 – kinase pathway (PI3K–PDK1–PKB/Akt), JAK–STAT (JAK1, TYK2, STAT1, and STAT3), Phopholipase C gamma (PLCG), Grb2–SOS–Ras–MAPK, and Cbl-family member. The Cbl proteins allow the ubiquitin ligation to CSF/MCSF receptor and, as a result, produce the receptor downregulation from the cell surface by ubiquitination and proteasome activity.

- IL-34
  IL-34 is a secreted 242 aminoacid protein. It is detected predominantly in the sinusoidal epithelium within the red pulp of the spleen (1-12, 76-78). In addition, it is found in heart, brain, lung, liver, kidney, thymus, testis, ovary, small intestine, prostate, and colon (note that intestinal epithelial cells forming villi possess CSF1/MCSFR on the basolateral side of the cells). Two isoforms produced by alternative splicing have been identified. IL-34 activity is relegated to the production and function of microglial cells and dendritic Langerhans cells.

- FLT3LG (Fms-like tyrosine kinase 3 ligand)
  FLT3LG cytokine stimulates the commitment, proliferation, differentiation, and the survival of early hematopoietic cells (1-12, 79-81). It also synergizes with Colony stimulating factors (CSFs) and some interleukins and controls DC development. It is particularly important for the functionality of pDCs, CD8+ DCs, and their CD103 (Integrin alpha E-ITGAE) positive tissue counter parts. FLT3LG is ubiquitously expressed, and it has 2 well defined isoforms even though there are 3 known alternative splicing-mRNAs. The 2 isoforms are: FLT3LG isoform 1 (Single-pass type I membrane-bound protein) with 235 aminoacids and FLT3LG isoform 2 (soluble homodimer) with 178 aminoacids. The FLT3LG receptor – FLT3/FLK2 – is located primarily in hematopoietic and neural tissues.

FLT3/FLK2 – FLT3LG system signaling is frequently deregulated in hematological malignancies in which FLT3 stimulates the survival and proliferation of leukemic blasts. Moreover, FLT3 is expressed on the leukemic cells of 70 – 100% of acute myeloid leukemia (AML) patients, and activated mutations in FLT3 are observed in 30% of AML adult patients. The two leading types of mutations found in AML are: internal tandem duplications in the juxtamembrane domain (ITD) in 17-34%, and mutations in the tyrosine kinase domain (TKD) activation loop in 7%. FLT3 aberrant expression occurs in Myelodisplasic Syndrome and Chronic Myelomonocytic Leukemia at a lower frequency than AML, and they are not predictors of poor outcome.

FLT3/FLK2 is a 993 aminoacid protein with a weight of 112,903 kDa. It has a signaling cascade involving phosphorylation of the adapter protein SHC1 (SH2 and SH3 domain transforming protein 1) and the AKT1 serine-threonine-kinase, and the activation of the downstream effector mTOR (mammalian target of rapamycin). It also promotes activation of Grb2-Sos-Ras-MAPK (MAPK3/ERK1, and MAPK1/ERK2) signaling, and the phosphorylation of downstream cytosolic tyrosine–kinases (FES/FPS, FER, LYN, FGR, HCK, and SRC), pro-

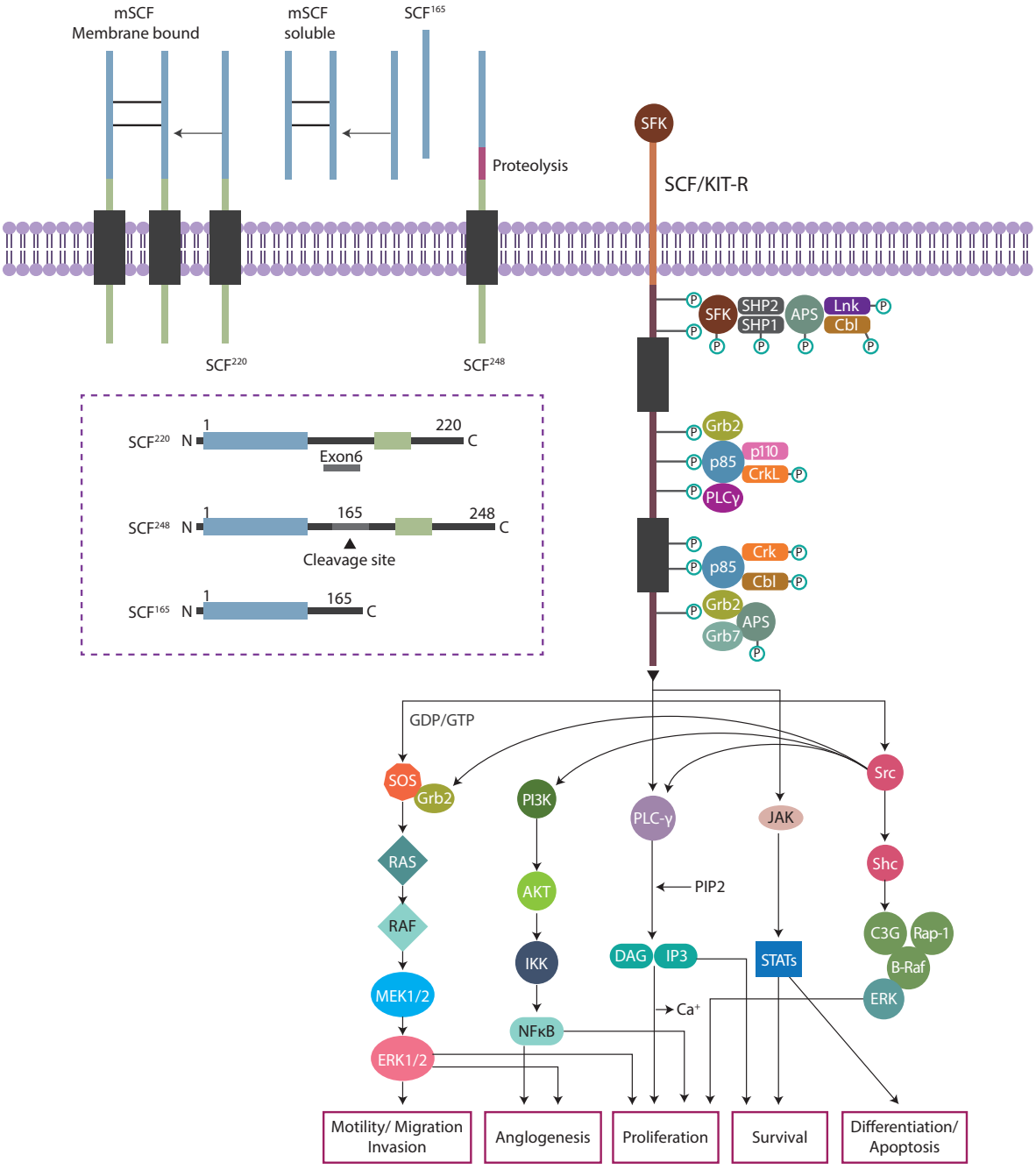


Figure 15. SCF/KitL-SCF/KitR system, and signaling. Adapted from references (84, 85).

tein tyrosine-phosphatases (PTPN6/SHP1, PTPN11/SHP2, PTPRJ/DEP1, PTP1B/PTPN1, PTPN12, and PTP4A3/PRL3), phospholipase PLCG1, STAT5A and/or STAT5B transcription factors, and FLT3-interacting zinc finger 1/Zinc finger protein 798 (FIZ/ZFP798).

- Stem Cell Factor (SCF)/KitL

"Kit" comes from "Kitten," because the v-kit oncogene was identified in 1986 as the transforming gene in the Hardy – Zuckerman 4 feline sarcoma virus (Figure 15) (1-12,82-85).

The SCF/KitL is a homodimeric N-glycosilated protein expressed by fibroblasts and endothelial cells throughout the body. It regulates proliferation, migration, survival, and differentiation of hematopoietic progenitors, mast cells, DCs, melanocytes, and germ cells. It is also expressed in granulose and sertoli cells, bone marrow stroma cells, keratinocytes, and mature granulocytes. SCF/KitL acts synergistically with various cytokines, especially IL-3, IL-7, colony stimulating factor 2/granulocyte-macrophage colony stimulating factor (CSF2/GMCSF), and Epo.

SCF/KitL is produced as two transmembrane isoforms (SCF/KitL220 and SCF/KitL248) due to alternative mRNA-splicing of exon 6. SCF/KitL 248 has exon 6 encoding for the cleavage of proteolytic sites and thus generates the soluble sSCF/KitL 165. This cleavage is generated by MMP9, chymase–1, ADAM17, and ADAM33. ADAM19, in turn, is a negative regulator of this process. However, six alternative mRNA have been identified. Hypoxia up-regulates the SCF/KitL expression through the hypoxia inducible factor 1 (HIF1), and, at the same time, SCF/KitL up-regulates HIF1. Ultraviolet B (UVB) light also induces SCF/KitL.

SCF/KitL-SCF/Kit-R system mediates cell survival, migration, and proliferation during hematopoiesis, pigmentation, fertility, gut movements (development and function of Cajal interstitial cells), vasculogenesis-angiogenesis, and cardiac stem cell differentiation. It is also associated with cardiomycyte regulation of terminal differentiation, maintainance of the integrity of lung tissue, proliferation of neuronal stem cells, and regulation of mast cell activity and DCs during the induction of Th2 and Th17 immune response.

SCF/Kit-R dysfunction is associated with cancer: Mastocitosis systemic (carrying active mutations), gastrointestinal stromal tumors (80-85% carrying active mutations), small cell lung carcinoma (overexpression and autocrine loop), acral melanomas (20-25% carrying active mutations), seminoma (25% carrying active mutations), gynecological tumors (ovarian serous adenocarcinoma, small cell carcinoma, and ovarian immature teratoma expressing autocrine loop), and acute myeloid leukemia (expression in 85% of activating mutations: 30% of inv(16), and 20-25% of t (8;21)). While active mutations in exon 17 are found in hematological malignancies and testicular carcinomas, exon 11 mutations are found in gastrointestinal stromal tumors (GIST). SCF/Kit-R system plays an important role during allergic-type inflammation, wich is why it is a new field of study for therapeutical interventions.

SCF/Kit-R non-selective inhibitor imatinib (INN) - mesylate is a 2-phenyl amino pyrimidine derivative, manufactured by Novartis as Gleevec in USA or Glivec in Europe, Australia, and Latin America. It is used to treat GIST. Another drug with a similar action that is under study is Dasatinib (Sprycel) by Bristol-Myers Squibb.

CYTOKINES FAMILIES SIGNALING THROUGH IMMUNOGLOBULIN CYTOKINE RECEPTORS SUPERFAMILY NON-RECEPTOR TYROSINE-KINASE (RTK)-CLASS

IL-1 FAMILY: IL-1S, IL-18, IL-33, IL-36, IL-37, AND IL-38

The IL-1 system in humans consists of 11 members and it represents one of the most pleiotropic and redundant cytokine systems. It has a myriad of physiological functions that go beyond mere immune activity (Table 4 and Figure 16) (1-12, 86-95). Primarily, the IL-1s are alarmins released by parenchymal cells and/or immune cells during stress or injury. Insults of various kinds such as inflammation, infection, autoinflammatory diseases, endocrine-metabolic stress (hypoglycemia, hyperglycemia), hypoxia/hyperoxia (ischemia, ischemia-reperfusion, free radicals), direct toxicity, ionizing radiation, hyperosmolarity, energetic failure, advanced glycosylated end-products (AGEs), and thermal injury can trigger the expression and release of IL-1s. The most representative members of this group are: IL-1α, IL-1β, IL-18, and IL1RA. They support a ubiquitous system response, where IL-1β is the major proinflammatory, and its action is systemic (endocrine) while IL-1R represents a natural antagonist.

At present, there are 11 well-recognized members of the family, which have a wide variety of functions, e.g., antiinflammatory mediators and natural antagonist corresponding to IL-1Ra and IL-36Ra.

The proinflammatory members of the IL-1 family stimulate the activation of innate and adaptative immunity, acute inflammation, and chronic inflammation. In innate immunity, IL1s induce activation and also gene expression of phospholipase A2 (PLA2), cyclooxygenase 2 (COX), inducible nitric-synthase (iNOS), and NADPH-oxidase system. The proinflammatories IL-1s, IL-6, and TNFs are part of the acute stress response which includes: hypothalamic activation as part of stress response, PGE2 endocrine production, acute phase plasma protein induction in the liver, metabolic catabolism, fever (PGE2 production by hypothalamic COX3), lowered pain threshold, vasodilation and hypotension, and activation of the glucocorticoid hypothalamic-pituitary endocrine axis.

IL-1β has been linked to a very particular type of programed cell death called "pyroptosis," which is a caspase 1-mediated macrophage cell death triggered by intracellular pathogen infection as part of host response. Some IL-1s are processed from their precursors by caspase 1 through a molecular platform knwn as inflammasomes. For example,

the IL-1β precursor is processed by caspase 1-activating inflammasomes composed by Pattern-Recognition Receptors (PRR) from the Nod-like Receptors (NLR) family. This family consists of NLRP1, NLR family pyrin domain (PYD) containing 3 (NLRP3/Cryopyrin), NLRP6, NLRP7, NLR family CARD domain-containing protein 4 (NLRC4), the DNA-sensing absent in melanoma 2 (AIM2), and RIG-1 receptors. The best described inflammasomes tody are made by NLR family PRRs, NLRP3, and NLRC4. The recruitment of caspase 1 requires two adapter proteins: CARDINAL/CARD8 and ASC/PYCARD.

Assembly of the inflammasomes with inactive pro–caspase 1 need a decrease in intracellular potassium, which depends on P2RX7 (purinergic receptor P2X ligand-gated ion channel 7) activation by ATP (its release is autophagy-dependent). After this, caspase 1 is activated by the inflammasome. P2XR7, in turn, recruits the Pannexina 1 (PANX1), which is a channel-forming glycoprotein, thus generating ATP-permeable mechano-sensitive channels. The cleavage of the IL-1β precursor by active caspase 1 can take place in the cytosol or in specialized secretory lysosomes. Furtheremor, a rise in intracellular calcium increment is required for IL-1β release outside the cell because this increase activates phosphatidylcholine-specific phospholipase C and calcium-dependent phospholipase A2.

There are 3 types of receptors: the heterodimers, the decoy-receptor (IL-1R2), and the inhibitory receptors (SIGIRR). Heterodimers consist of α subunit (IL-1R1, IL-18R1, IL-1RL1, and IL-1RL2) joining members and an IL1-beta chain IL-1β chain (IL-1RAP and IL-18RAP), and they function as co-receptors. Once heterodimers bind the IL1-members, they recruit the adapter protein MyD88 to the Toll-IL-1 receptor (TIR) domain. Next, the IRAK serine/threonine-kinases (IL-1 receptor-associated kinase), adapter protein TRAF6, and MAP3K7/TAK1 serine/threonine kinase are recruited. All of them may activate JNK-MAPK, IKK (I-kappa-B kinase), and p38MAPK pathways.

There are 2 other signaling systems used by the IL-1R. The first one is by activation of sphingomyelinases (SMPDs and Magnesium-dependent Sphingomyelin phosphodiesterases neutral membrane -nSMase) such as SMPD2, SMPD3, and SMPD4 and sphingomyelin phosphodiesterase 1 acid lysosomal (SMPD1/A-SMase or ASM). SMPDs catalyze sphingomyelin degradation in a phospholipase C-like hydrolysis reaction yielding ceramides and phosphocholine. Even though the TNFR1 activates neutral-SMPDs through adapter protein NSMAF/FAN (Neutral sphingomyelinase activation-associated factor/ Factor associated with N-SMase activation), there is still no evidence that the adapter protein is involved in IL1R-signaling. Free fatty acids such as araquidonic acid have been shown to activate nSMases at the same time that SMPD1/ASM is inhibited by ceramide-1-P (phosphate) and by polyphosphoinositides. The SMPD1/ASMase activation is triggered by the cytoplasmic domain of IL-1R1. This activates phosphatidylcholine-specific phospholipase C (PCPLC), thus producing diacylglycerol (DAG), which is a direct cofactor in SMPD1/ASMase activation within the endosomes. Also, it seems that P2XR7 indirectly activates SMPD1/ASMase through cytosolic-tirosine kinase Src-type, which is associated with the cytosolic region of the receptor-channel. Src-type ki-

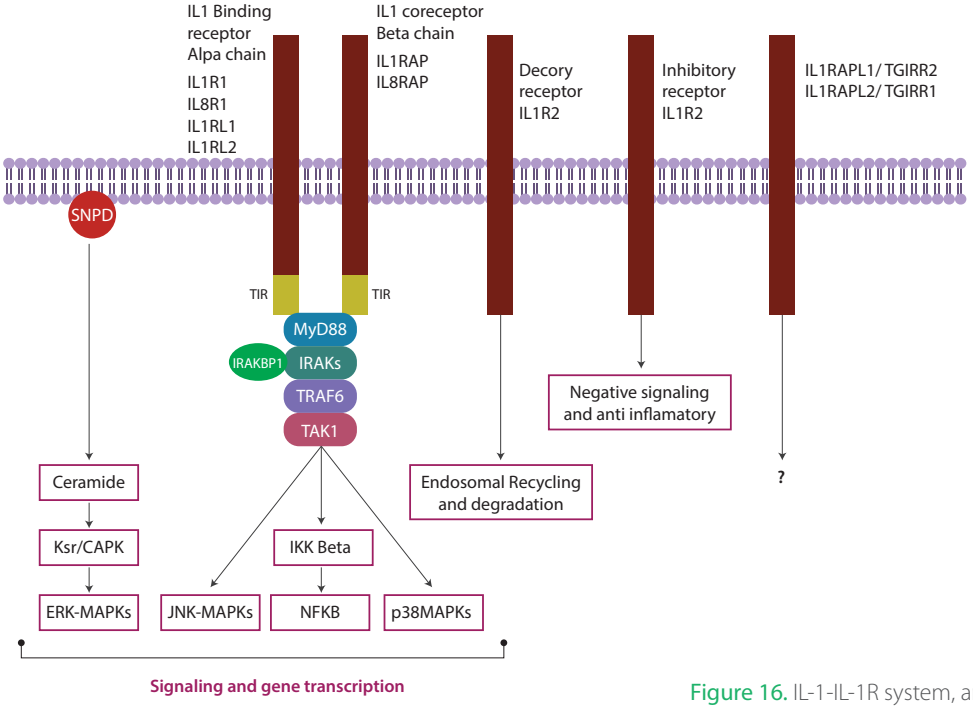


Figure 16. IL-1-IL-1R system, and signaling.

IL-1 FAMILY MEMBER	OTHER NOMENCLATURE	PRODUCTION SOURCE	RECEPTOR	CO-RECEPTOR	AGONIST/ ANTAGONIST	LOCALIZATION	ROLE IN INFLAMMATION, AND OTHER FUNCTIONS
IL-1F1	IL-1A	Ubiquitous, and formally significant production by the monocyte/ macrophage lineage/ dendritic cell	IL-1R1	ILRAP	Agonist	Extracellular, Cytosolic, Nuclear.	Proinflammatory. Pro IL-1a is an autocrine growth factor that regulates normal cellular differentiation, particularly in epithelial and ectodermal cells (for example: keratinocytes). Endocrine action. IL-1A precursor form can bind to their receptor and trigger signal transduction.
IL-1F2	IL-1B	Ubiquitous, and formally significant production by the monocyte/ macrophage lineage/ dendritic cell	IL-1R1	ILRAP	Agonist	Extracellular, Circulation andbody fluids	Proinflammatory. Processes and Secretion of IL-1B via the Caspase-1 Inflammasome. Non–caspase-1 extracellular processing of the IL-1B precursor depends of Cathepsin G, Chymases, Chymotrypsin, Elastase, Proteinase 3, and Granzyme A. In human T cells, IL-1B dependent Th17 differentiation is because of the intermediate production of prostaglandin E2 (PGE2) from macrophages. IL-1B plays a key role in Angiogenesis.
IL-1F3	IL-1Ra	Ubiquitous, and formally significant production by the monocyte/ macrophage lineage/ dendritic cell, particularly Macrophagues M2 and Dendritic Cell DC2	IL-1R1	(-)	Antagonist	Extracellular	Anti-inflammatory by antagonism with IL-1A and IL-1B.
IL-1F4	IL-18	Monocyte/ macrophage lineage/ dendritic cell(including microglial cells and osteoclasts), T cells and B cells, osteoblasts, keratinocytes, intestinal epithelial cells, corneal epithelial cells, glucocorticoid-secreting adrenal cortex cells, and astrocytes	IL-18R1	IL-18RAP	Agonist	Extracellular	Proinflammatory. Th1 paradigm (stimulates IFNG production). NK cell lineage growth, differentiation and activating factor. Processing and Secretion of IL-1β via the Caspase-1(or Caspase-4) Inflammasome. IL-18BP is a constitutively secreted protein that functions as a natural anti-inflammatory neutralizing the effects of IL-18.
IL-1F5	IL36Ra	Monocyte/ macrophage lineage/ dendritic cell, Placenta, uterus, skin (including psoriasis), brain, heart, kidney, B cells	IL-1RL2	(-)	Antagonist for IL-36(IL-36A, IL-36B and IL-36G)	Extracellular	Anti-inflammatory through the union to SIGIRR (single immunoglobulin and toll-interleukin 1 receptor (TIR) domain).
IL-1F6	IL-36A	Spleen, lymph node, tonsil, leukocytes, bone marrow, foetalbrain, keratinocytes(including psoriasis), monocytes, B cells, T cells	IL-1RL2	IL1RAP	Agonist	Extracellular	Proinflammatory. IL-36Ra is the specific receptor antagonist for IL-1RL2 and prevents the activity of IL-36A, IL-36B, and IL-36G.

IL-1 FAMILY MEMBER	OTHER NOMENCLATURE	PRODUCTION SOURCE	RECEPTOR	CO-RECEPTOR	AGONIST/ ANTAGONIST	LOCALIZATION	ROLE IN INFLAMMATION, AND OTHER FUNCTIONS
IL-1F7	IL-37	Isoforms IL37D and IL-37E, in testis and bone marrow. IsoformsIL37A, IL37B, and IL-37C in lymph-node, thymus, bone marrow, lung, testis, placenta, uterus, skin, colon,NK cell, mono-cytes, stimulated B cells, and keratinocytes. Brain only express isoform form IL-37A. Kidney only express isoform IL-37B. -Heart only express isoform IL-37C.	IL-18R1	?	Antagonist	Extracellular	Anti-inflammatory. IL-1F7 also binds to the IL-18–binding protein (IL-18BP), and on formation of a complex with IL-18BP, there is decrease in IL-18 activity, especially isoform IL37B binds to IL-18BP and enhances its IL18 inhibitory capacity.
IL-1F8	IL-36B	Bone marrow, tonsil, heart, placenta, lung, testis, colon, monocytes, B cells.	IL-1RL2	IL-1RAP	Agonist	Extracellular	Proinflammatory. IL-36Ra is the specific receptor antagonist for IL-1RL2 and prevents the activity of IL-36A, IL-36B, and IL-36G.
IL-1F9	IL-36G	Placenta, stimulated keratinocytes(including psoriasis),epithelial cells, squamous cell-epithelia of oesophagus.	IL1RL2	IL1RAP	Agonist	Extracellular	Proinflammatory. IL-36Ra is the specific receptor antagonist for IL-1RL2 and prevents the activity of IL-36A, IL-36B, and IL-36G.
IL-1F10	IL-38	Basal epithelia of skin, proliferating B cells in the tonsil.	?	?	Antagonist	Extracellular	?
IL-1F11	IL-33	Constitutive in human lung epithelium and smooth muscle cells, High Venules en-dothelial cells, induced by proinflammatory cytokines in fibroblasts, keratinocytes and at low level in macrophages and DC. Absent inresting/activa-ted macrophages, DC, T and B cells, NK-cell, and peripheral blood mono-nuclear cell(PBMC).	IL-1RL1	IL-1RAP	Agonist	Extracellular, Nuclear	Proinflammatory Th2-paradigm. IL-33 precursor form can bind to their receptor and trigger signal transduction. Antiinflammatory through the union to SIGIRR(single immunoglobulin and toll-interleukin 1 receptor (TIR) domain).

Table 4. IL-1 family members, receptors, localization and functions.

nases phosphorylate and activate p38MAPK cascade, thereby inducing SMPD1/ASMase migration from luminal lysosomal compartment to cell membrane.

Ceramide activates serine/threonine-kinase PKCζ (Protein-kinase calcium-dependent zeta) and Ksr/CAPK (Kinase suppressor of ras 1, Ceramide activated protein-kinase). Both of them activate other signaling pathways. Other ceramides are the PP (protein-phosphatases) such as PP1 and PP2A, which induce dephosphorylation of signaling substrates. Moreover, PP1 and PP2A are often called CAPP (Ceramide-activated protein-phosphatases).

The second signaling system has GTPase activity. Both IL-1RI and IL-1RAP contain a domain commonly found in GTPases, but their receptor functions as GTPase have not been demonstrated. However, there have been reports of increased GTP binding and hydrolysis in IL-1-treated cells.

Evolutionarily, note that IL-1R1 and TLR structures (including intron-exon organization) and functions differentiate the IL-1 receptor superfamily from plants and insects. IL-1s, in turn, belong to Fibroblastic-growth factor (FGFs) superfamily because of common gene duplication (some about 350 million years ago).

In human therapy, the IL-1RA is a novel drug for the treatment of severe inflammation associated with autoimmune alterations. Therefore, it is produced as a human recombinant [rhIL-1RA, -Anakinra-(Kineret)] for use as biological

therapy in Rheumatoid arthritis. Rilonacept/IL-1 Trap (Arcalyst), a dimeric fusion protein consisting of the ligand-binding domains of the extracellular portions of the human IL-1R1 and IL-1RAP and linked in-line to the Fc portion of human IgG1 that binds and neutralizes IL-1, is used for Gout. Canakinumab (Ilaris) is a human monoclonal antibody targeted at IL-1β. It is used in Cyopirin-associated periodic syndromes (CAPS), including familial cold autoinflammatory and Musckle-Wells syndrome, and in Chronic obstructive pulmonary disease (COPD).

IL-16

This cytokine was initially described in 1982, and its gene was cloned in 1994. IL16 has 3 distinct isoforms as result of alternative splicing: leukocyte IL-16 (isoform 1 or canonical version nPro-IL-16), neuronal IL-16 (isoform 2), and hemopoietic tissues (isoform 3, produced by alternative promoter usage and proteolytically processed to yield IL-16). The Precursor proteins are cleaved by caspase 3 to produce the biologically active carboxyl-terminal. This active region is composed of 121 amino acids and fragment aggregates (apparently homotetrameric), which are then secreted as bioactive IL-16 - an active immunomodulatory cytokine that binds to membrane receptor CD4. IL-16/CD4 can signal through p56lck cytosolic tyrosine-kinase and Maspardin/ACP33 (acidic cluster protein, 33 kDa), which is a negative regulator of this signaling pathway (Figure 17) (1-12,96-99).

Physiologically, IL-16 mRNA is present almost exclusively in lymphatic tissue and is constitutively expressed in T lymphocytes. In T-lymphocytes, IL16 mediates inflammation, activation, and growth, and it induces chemotaxis of Th1-lymphocytes and inhibites antigen-mediated Th2 inflammation. Further, IL-16/CD4 binding interaction cross-desensitizes CCR5 and CXCR4, thus impairing CCL4 and CXCL12 mediated T-lymphocyte chemoattraction. In addition, IL16 induces upregulation of IL-2Ra and IL-2Rb with the subsequent enhancement of IL-2 mediated T-lymphocyte cell proliferation. IL-15 mediated T cell proliferation is also enhanced. Migratory response is also stimulated in monocytes and eosinophils.

The IL-16 cytokine has been found to be a factor associated with the biology of various cancers such as colorectal cancer, hepatitis B Virus-related hepatocellular carcinoma, prostate cancer, and multiple myeloma. Note that gene polymorphisms have even been reported in some of these cancers.

IL-16 isoforms 1 and 3 may act as scaffolding/anchoring and/or trafficking proteins anchored to ion channels in the membrane. As a result, IL-16 signaling may ultimately modulate the function of ion channels by intracrine, autocrine, paracrine, and endocrine activity.

IL-16 inhibits HIV-1 replication through a mechanism not related to viral entry (IL-16 as competitive inhibitor of CD4) or through reverse transcription activity. However, it plays a role at the level of transcription. Here IL-16 isoform 3 has been found to interact with protein Tax of the Human-T-lymphotropic Virus 1 (HTLV1).

An inquiry into whether IL16 is an intracrine agent (cytokine production for intracellular action) and/or endocytosed through a system that includes CD4 or other

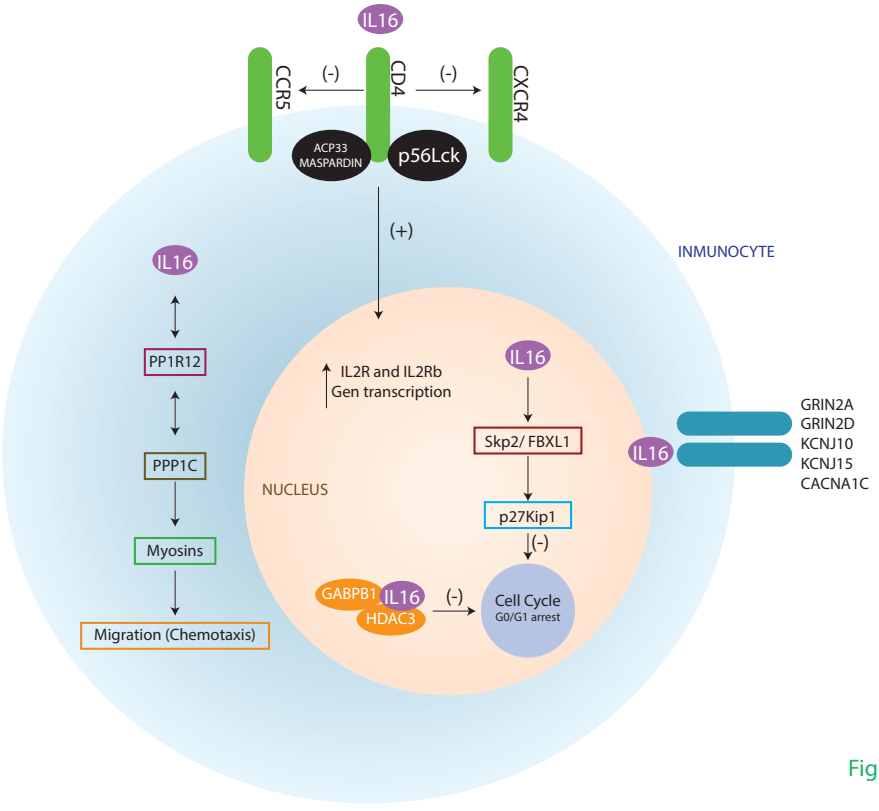


Figure 17. IL-16-CD4 system, and signaling.

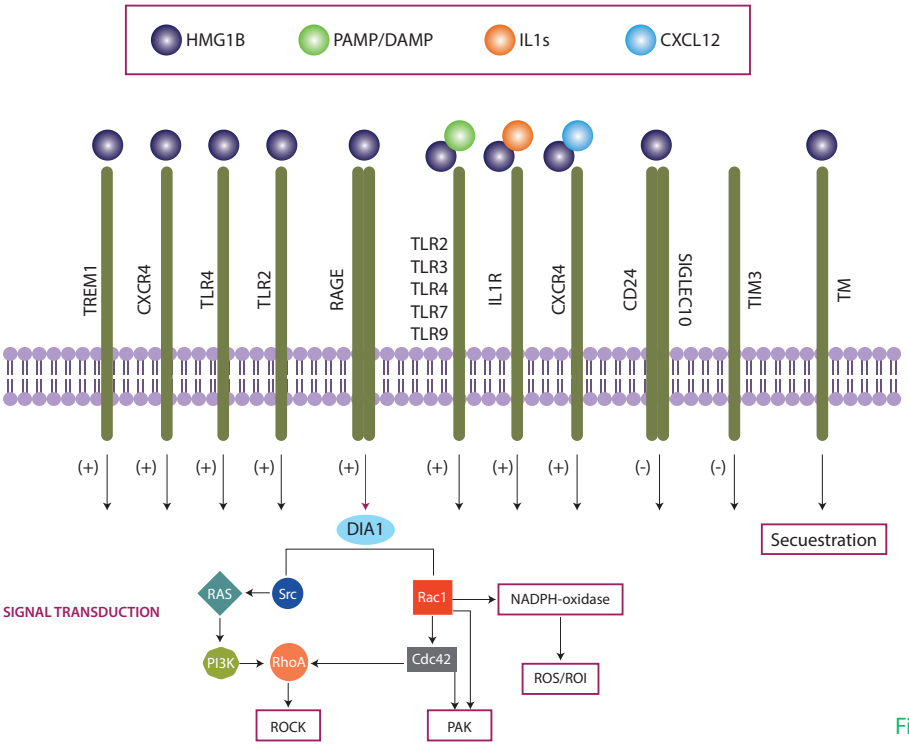


Figure 18. HMGB1 system, and signaling.

surface receptors could be worthwhile. This might explain some of their functions in correlation to their intracellular location: control of cytoskeleton, scaffolding, and cell cycle.

HMG1B (HIGH-MOBILITY GROUP BOX 1)

Structurally, HMGB1 is a polypeptidic chain of 25 aminoacids, with two positively charged DNA binding motifs, HMG-boxes A and B, and an acidic COOH-terminal (high amounts of aspartic or glutamic acid residues). HMGB1 in the cell nucleus is a non-histone-chromatin binding protein that binds to the minor groove of DNA, which is involved in the regulation of genetic transcription, stabilization of nucleosomes. and promoting the access of DNA to transcriptional factors such as NFKB, p53, and nuclear steroid hormone receptors. In addition, HMG1B works as a DNA chaperone and as such facilitates DNA replication, recombination, and repair. It also functions as a damage-associated molecular pattern (DAMP)-alarmin and a proinflammatory cytokine molecule in many physiological and pathological scenarios. This cytokine is actively secreted by macrophages, mature dendritic cells (mDCs), and natural killer cells (NK) and is passively secreted during injury, infection, and cell stress (Figure 18) (1-12,100-103). It is also produced from death cell processes (both apoptosis and necrosis) such as cell death associated molecular patterns (CDAMP). HMG1B also regulates autophagy specialized process through interaction with the protein BECLIN1 (coiled-coil, moesin-like BCL2 interacting protein). Therefore, HMB1 can be translocated from the nucleus to the cytosol and extracellular space.

CYTOKINES FROM TNF FAMILY SIGNAL THROUGH TNF RECEPTOR FAMILY, OTHER CYTOKINES WITH TNF-DOMAINS AND NON TNF-LIGANDS

This group of cytokines is composed of the cytokines from TNF family such as: LTA/TNFSF1, TNF-α/TNFSF2, LTB/TNFSF3, OX40L/TNFSF4, CD40L/TNFSF5, FASL/TNFSF6, CD70/TNFSF7, CD30L/TNFSF8, 4-1BBL/TNFSF9, TRAIL/TNFSF10, RANKL/TNFSF11, TWEAK/TNFSF12, APRIL/TNFSF13, BLYS/TNFSF13B, LIGHT/TNFSF14, VEGI/TNFSF15, GITRL/TNFSF18, AND EDA (Ectodysplasin). Moreover, this group consists of other cytokines with TNF-domains such as the C1QTNF Family and other non TNF-ligans such as Granulin/Epithelin (GRN) and nerve growth factor (NGF) (Figure 19) (1-12,104-105).

Since the discovery of TNF-α, it has been established that humans have 18 TNFSF genes and 31 TNFR receptor genes within the genome. Members of these superfamilies are widely distributed among all tissue and they play fundamental roles: regulation of immune responses-in many situations such as co-stimulation systems (i.e., CD40-CD40L and OX40-OX40L) or, in others, as co–inhibition systems (i.e., GITR-GITRL), hematopoiesis and morphogenesis, neoplasiogenesis, transplant rejection, septic shock, viral replication, bone metabolism (RANKL/RANK/OPG system), metabolic syndrome, and autoimmunity.

Since most of TNFSF members are expressed as cell surface proteins, it can be deduced that they are acting at the local level (tissue, paracrine). These members are oligomeric

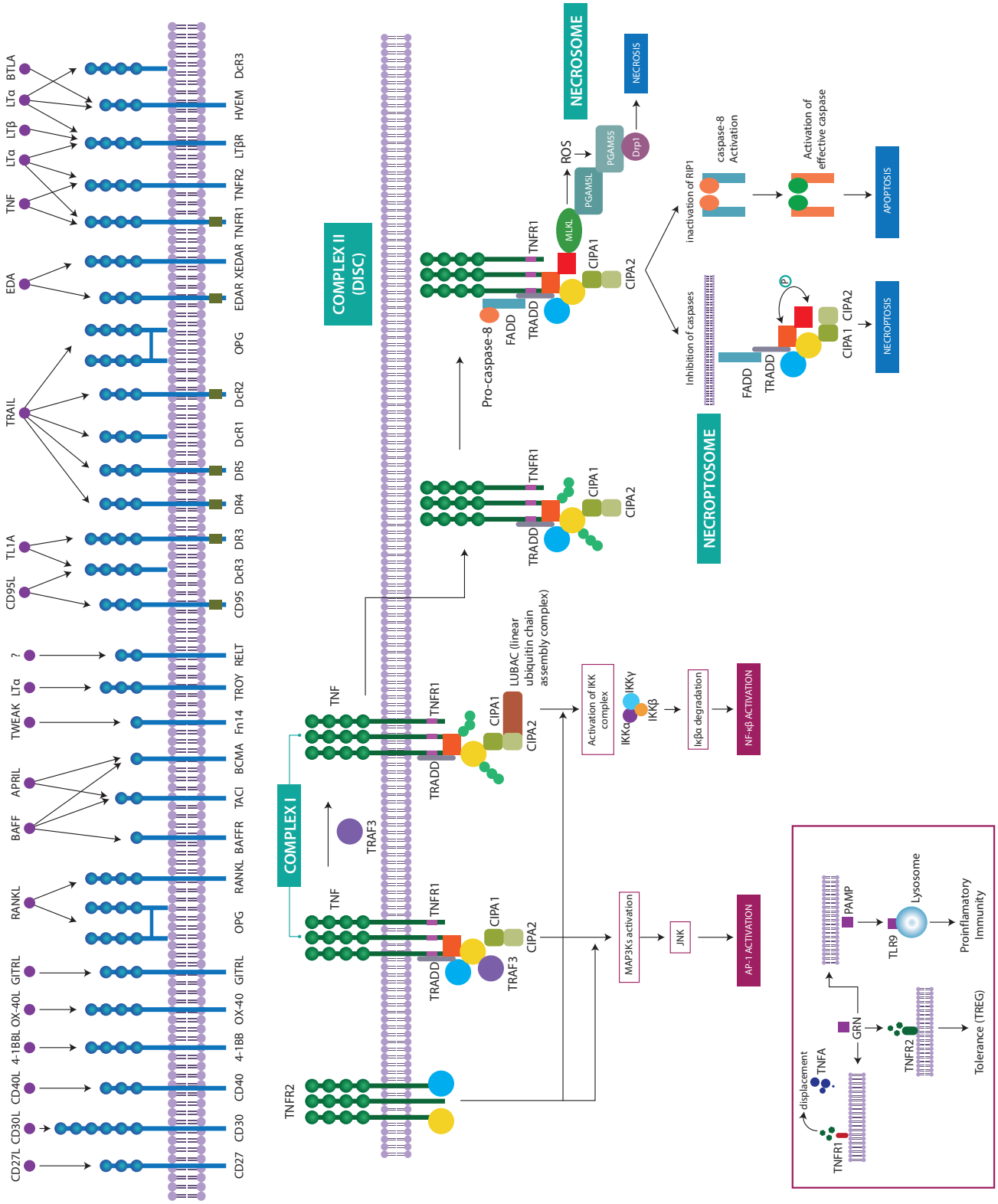


Figure 19. TNFSF-TNFRSF system, TNF-TNFR1/TNFR2 signaling, and TNF-TNFR1/TNFR2 regulation by Progranulin (GRN). Adapted from reference (104).

complexes composed of type I- or type III- transmembrane proteins, which contain a variable number of multiple extracellular cysteine-rich domains(CRD). Most of TNFSF members bind to a single TNFRSF receptor, but a few of them bind to more than one receptor, e.g., TRAIL binds to five receptors (DR4, DR5, DcR1, DcR2 and OPG). At the same time, TNFRSF members are classified into three structural/functional major groups:

- TNFRSF members that contain a death domain (DD) in their cytoplasmic region, such as: CD95, TNFR1, DR3, DR4, DR5, and DR6. The binding of TNFSF ligands to these receptors causes a downstream signaling through adaptor and effector proteins. These adaptor and effector proteins possess variable protein domains belong to the death domain (DD) superfamily such as DD, death effector domain (DED), caspase recruitment domain (CARD), or pyrin domain (PYD). These protein domains mediate homotypic interactions and the assembly and activation of signaling protein complexes involved in cell death and also in immune response. For example, TNFRSF members recruit TNFR-associated death domain (TRADD) and Fas-associated death domain (FADD), resulting in activation of the caspases and the subsequent cell death (apoptosis, necroptosis, or necrosis). TRADD and FADD promote the formation of an intracellular signaling platform which coordinates the death mechanism. There are numerous examples of specialized platforms such as death-inducing signaling complex (DISC), TNF complex II, Apoptosome, PIDDosome, toll-like receptor (TLR) complexes, RIG-I complex, and Ripoptosome. FADD recruits pro-caspase 8 by the homotypic interaction of the DED from both proteins. This signal is tightly regulated by antiapoptotic proteins such as cellular caspase 8, FADD-like apoptosis regulator/cellular FLICE-like inhibitory protein (CFLAR/c-FLIP), and TNFAIP3 interacting protein 2/FLICE-like inhibitory protein 1 (TNIP2/FLIP1). Another key component of this pathway is receptor (TNFRSF)-interacting serine-threonine kinase (RIPK family members: RIPK1, RIPK2, RIPK3, RIPK4, and dual tyrosine/serine-threonine kinase – DSTYK/RIPK5). RIPKs interact with TNFRSF and TRADD via death domain. TRADD acts as an adaptor protein to recruit RIPKs to the TNFRSF complex in a TNFSF-dependent process.
- In the necrosis pathway, RIPK1 interacts with RIPK3 to make the RIPK1-RIPK3 necrosome, resulting in the mutual phosphorylation of RIPK1 and RIPK3. After that, RIPK3 phosphorylates and interacts with mixed lineage kinase domain-like protein (MLKL) that recruits phosphoglycerate mutase family member 5 (PGAM5 with 2 splice variants -long and short: PGAM5L and PGAM5S). Consequently, RIPK3 phosphorylates PGAM5S which, in turn, dephosphorylates Dynamin-related protein 1 (DRP1) leading to its activation and subsequent necrosis. MLKL is also required for the generation of ROS/ROI, which then also triggers necrosis via the PGAM5/Drp1 cascade. PGAM5 interacts with the apoptosis and cell stress-associated kinase (ASK1/MAP3K5) and activates ASK1/MAP3K5 by threonine dephosphorylation, which results in downstream activation of MAPK-JNKs and p38MAPKs.
- TNFRSF members that contain one or more TNF receptor-associated factor (TRAF) interacting motifs (TIM) in their cytoplasmic region such as TNFR2, CD40, CD30, CD27, LTβR, OX40, 4-1BB, BAFFR, BCMA, TACI, RANK, NGFR, HVEM, GITR, TROY, EDAR, EDA2R, RELT, and Fn14. Ligands binding to these receptors induce recruitment of TRAF family members (TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, TRAF6, TRAF7, TRAFD1, and TANK) and activation/regulation of cell signaling pathways including the activation of nuclear factor-κB (NF-KB) transcription factor, Jun N-terminal kinases (JNKs), p38MAPKs, extracellular signal regulated kinase (ERK1/2MAPKs), and phosphoinisitide-3 kinase (PI3K). TRAFs are also involved in the signaling of IL-1 Receptor and IL-17 Receptor complex.
- TNFRSF members that do not contain functional intracellular signaling domains or motifs such as DcR1, DcR2, DcR3, and OPG. They can act as decoy-receptors to compete for ligand binding and block the signaling.

A wide variety of drugs acting on the TNF-TNFR systems for the treatment of cancer, autoimmune and allergic diseases are under development or in the clinical phase.

NON-TNFSF LIGANDS: GRN AND NGF

These non-TNFSF ligands signal through TNFRSF such as Nerve Growth Factor (NGF) and Progranulin (GRN).

-GRN/PGRN binds to TNFR1, competes and blocks TNFα-induced inflammatory and death cell pathways. The interaction of GRN with TNFR2 may trigger an unknown protective signal cascade and promote Treg cells. Furthermore, GRN assists in recruting PAMPs (i.e., CpG-ODNs) to TLR9 in the endosome in order to stimulate innate immunity (106).

-NGF is a neurotrophin family member, a protein of 13.2 kDa that exists as a non-covalently bound homodimer, and is released from the cell through a constitutive secretory pathway. NGF is generated by intracellular or extracellular cleavage from proNGF. NGF binds high-affinity receptor Ullrich/Schlessinger class VII RTK, called TrkA (induces receptor homodimerization), or low-affinity receptor TNFRSF16 (p75NTR/CD271). ProNGF is also biologically active and can bind to a complex of TNFRSF16 with sortilin family members, inducing apoptosis. Neurons and other neurosensory structures are a target of the inflammatory process, and they participate in the regulation of immune responses by actively releasing soluble mediators as the neurotrophins, forming what is termed as neurogenic inflammation.

NGF plays a role in the development and maintenance of the peripheral nervous system since it is critical in the pathogenesis of inflammatory pain. Loss of TrkA function by mutations leads to impaired NGF signaling and produces Congenital insensitivity to pain with anhidrosis (CIPA).

NGF acts as an angiogenic factor in the cardiovascular system and in endothelial cells. There, it plays an important role in the maintenance, survival, and function of the endothelium. NGF also participates in tissue repair processes as it influences skin and connective tissue repair.

NGF and its receptors are expressed by all of the different cell types in the immune system, including T lymphocytes, B lymphocytes, macrophages, neutrophils, basophils, mast cells, and bone marrow stromal cells. They regulate a wide variety of immune functions including proliferation, differentiation, and antibody synthesis of B lymphocytes; proliferation and expression of cytokine-receptors in T lymphocytes; differentiation, chemotaxis, and effector functions of macrophages and neutrophils; and platelet biology.

Mast cells in particular both a source and a target for NGF and this may provide a link between NGF action and its influence on atopic/allergic diseases, for example, asthma. NGF is detected in the bronchoalveolar lavage from asthma patients, and in lung epithelium, fibroblasts, and infiltrating cells (macrophages, T lymphocytes). All these lung cells in asthma as well as basophils, mast cells, and monocytes express TrkA. NGF promotes survival and activation of infiltrating cells and regulates bronchial smooth muscular innervation, which may be altered by increased NGF levels.

NGF is also overexpressed in allergic rhinitis and in many inflammatory and degenerative rheumatic diseases. However, it sometimes shows detrimental and sometimes regenerative activity, all of which indicates that there is a lack of research. Tanezumab is a monoclonal antibody against NGF and is used as a treatment for inflammatory pain in osteoarthritis and interstitial cystitis (107).

OTHER CYTOKINES WITH TNF-DOMAINS

C1q/TNF related protein (CTRP) family in humans is composed of 18 proteins: Adiponectin, C1qTNF1, C1qTNF2, C1qTNF3, C1qNTF4, C1qTNF5, C1qTNF6, C1qTNF7, C1qTNF8, C1qTNF9, C1qTNF9B, C1qTNF10/C1qL2, C1qTNF11/C1qL4, C1qTNF12/FAM132A (family with sequence similarity 132 member A), C1qTNF13/C1qL3, C1qTNF14/C1qL1, C1qTNF15/FAM132B, and C1qTNF15/Otolin1. CTRP cytokines share a common structure which includes four distinct domains: a signal peptide at the amino terminus, a short variable region, a collagenous -domain, and a carboxyl-terminal globular-domain, which is homologous to complement component 1q. CTRPs produce highly stable and biologically active homotrimers or heterotrimers. The 3D structure of the globular C1q domain is nearly identical to that of the carboxyl-terminal region of TNF homology domain (THD), a protypical feature of members of the TNFSF.

C1qTNF are widely expressed in organs, tissues, and cells, and they have important roles in physiology and pathophysiology. Recently, a start has veen made on studying those roles, and they appear to regulate the crosslinking between immunity, metabolism and vascular disease. Receptors for CTRPs, except for Adiponectin (AdipoR1 and AdipoR2), have not yet benn identified. Adiponectin is a CTRP member and, structurally and functionally, it is the best-described one (108).

CHEMOKINE SUPERFAMILY

Chemokines (CHEMOtactic cytoKINES) constitute a superfamily of proteins found in vertebrates, bacteria, and viruses. They are involved in immunoregulatory and inflammatory processes and originated from a single ancestral gene approximately 650 million years ago. There are 48 chemokine genes and 24 chemokine receptor genes in the human genome. This includes CMKRL1, which is a receptor for the cytokine/adipokine known as Chemerin. There is an additional receptor Formyl-peptide receptor 2 (FPR2) which is activated for CCL23 chemokine. This superfamily consist of by seven-transmembrane-heptahelical (Serpentine) receptors associated with G-protein. They are the following (Tables 5-6) (1-12,109-112):

A. Chemokine CC Motif Ligand (CCL) Family
- CCL1, CCL2
- CCL3 Subfamily: CCL3, CCL3L1, and CCL3L3
- CCL4 Subfamily: CCL4, CCL-4L1, and CCL4L2
- CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, and CCL28

B. Chemokine CXC Motif Ligand (CXCL) Family
- CXCL1, CXCL2, CXCL3, CXCL4 Subfamily (CXCL4/PF4and CXCL4L1/PF4V1), CXCL5, CXCL6, CXCL7/PPBP, CXCL8/IL-8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL16, and CXCL17

C. Chemokine XCL (XCL1, XCL2)-XCR system

D. Chemokine X3CL1-CX3CR system

E. Other non-chemokine ligands that signal through Chemokine receptors: Chemerin-CMKRL1 system

Chemokines are soluble, basic, heparin-binding proteins of approximately 70 aminoacids and 7-12 kDa with the exceptions of CX3CL1/fractalkine and CXCL16 that are membrane associated. Chemokines can be monomeric and are able to join together to produce dimers, tetramers, or multimers. The chemokines are grouped into four families: CXC (α), CC (β), CX3C (γ), and C (δ) (where X is any residue). Here classification is based on presence and relative positioning of the aminoterminal cysteines and the arrangement of cysteine aminoacids involved in the formation of disulfide-bonds. Chemokines have 4 cysteines which are conserved in all family members, except in XC where only two cysteines are conserved. The resulting overall cysteine organization is as shown below:

	AGONISTIC LIGAND	ANTAGONISTIC LIGAND	H (HOMEOSTATIC), I (INFLAMMATORY), AND D (DUAL FUNCTION)
CCR1	CCL3, CCL3L1, CCL3L3, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23	CCL26	I
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16, β-defensins (Bd2, Bd3)	CCL11, CCL26	I
CCR3	CCL2, CCL3L1, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL24, CCL26, CCL28, Asparaginyl-tRNA synthetase (AsnRS)	CXCL9, CXCL10, CXCL11, CCL18	I
CCR4	CCL17, CCL22, CKLF	(-)	D
CCR5	CCL3, CCL3L1, CCL3L3, CCL4, CCL4L1, CCL4L2, CCL5, CCL7, CCL8, CCL11, CCL13, CCL16, Histidyl-tRNA synthetase (HisRS) myositis autoantigen, mycobacterial Hsp70, Cyclophilin 18 by Toxoplasma gondii	CCL7, CCL26, CXCL11	I
CCR6	CCL20, β-defensin2(Bd2)	(-)	I
CCR7	CCL19, CCL21	(-)	H
CCR8	CCL1	(-)	H
CCR9	CCL25	(-)	H
CCR10	CCL27, CCL28	(-)	H
CXCR1	CXCL6, CXCL7, CXCL8, N-acetyl Pro-Gly-Pro[acPGP: collagen-breakdown product derived of metallo-protease-9 (MMP9) activity]	(-)	I
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, acPGP, MIF	(-)	I
CXCR3	CXCL4, CXCL4L1, CXCL9, CXCL10, CXCL11, CXCL13, interphotoreceptor retinoid binding protein(IRBP) uveits autoantigen, S-antigen (S-Ag) uveitis autoantigen	CCL11	I
CXCR4	CXCL12, MIF, Ubiquitin, HMG1B	Transactivator of transcription (Tat) by HIV1	H
CXCR5	CXCL13, interphotoreceptor retinoid binding protein(IRBP) uveits autoantigen	(-)	H
CXCR6	CXCL16	(-)	H
XCR1	XCL1, XCL2	(-)	D
CX3CR1	CX3CL1, CCL26	(-)	D
CMKLR1	Chemerin, Resolvin 1	(-)	I
FPR2	CCL23, Lipoxin A4, Serum Amyloid A(SAA), beta-Amyloid peptide Abeta42, mitochondrial and bacterial derived peptides, the neurotoxic prion peptide fragment 106–126, Helicobacter pylori peptide Hp (2-20), synthetic peptides such as WKYMVM and MMK-1	(-)	?

Table 5. **Specificity and selectivity of Chemokine-ligands and Chemokine-receptors.**

ATYPICAL RECEPTORS	CHEMOKINE LIGANDS
CXCR7	CXCL11, CXCL12, MIF
CCRL1	CCL19, CCL21, CCL25, CXCL13
CCRL2	CCL5, CCL19, Chemerin
DARC	CCL2, CCL5, CCL7, CCL11, CCL13, CCL14, CCL15, CCL17, CCL18, CCL22, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL11, KAI1/CD82
CCBP2	CCL2, CCL3, CCL3L1, CCL4, CCL4L1, CCL4L2, CCL5, CCL7, CCL8, CCL11, CCL12 CCL13, CCL14, CCL17, CCL22, CCL23, CCL24

Table 6. **Atypical Chemokine receptors/nonchemotactic, recycling of scavenger receptors (ACR/NCRSR).**

- C: _____C_____C_____
- 4CC:_____C___C_____C___C_____
- 6CC:_____C___C_____C_____C___C_____C_____
- Non-ELR-CXC:____CX__C_____C___C_____
- ELR-CXC:__ELR___CX__C_____C___C_____
- CX3C:_____CXXXC_____C___C_____

The description of CXC (alpha chemokines) and CC (beta chemokines) is based on the arrangement of the first 2 of the 4 conserved cysteines. While the 2 cysteines are separated by a single aminoacid in the CXC chemokines, in CC chemokines the first two cysteine aminoacids are juxtaposed or adjacent.

CXC chemokines are also subdivided into ELR and non-ELR types based on the presence or absence of an ELR (Glu-Leu-Arg) sequence adjacent and aminoterminal to the CXC motif. ELR-CXC share the same receptor (CXCR2), attract neutrophils, and promote angiogenesis. Non-ELR-CXC such as CXCL9, CXCL10, and CXCL11, attract lymphocytes, are angiostatic, not angiogenic, and utilize the same receptor CXCR4.

Chemokine CXCL16 is a transmembrane transmembrane protein that mediates bacterial phagocytosis by APCs through its chemokine domain. CXCL16 influences the uptake, subcellular localization, and cytokine profile induced by DNA oligonucleotides.

Within of CC chemokines, there are two distinct CC groups: the MCPs (monocyte chemoattractant protein) and the MIPs (macrophage inflammatory protein), whose members typically chemoattract for monocytes, macrophages, T cells, B cells, DC, NK, eosinophils, and basophils, but not neutrophils.

In the CXC and CX3C chemokines, one or three amino acid residues are inserted between the first two of four cysteine residues. The first and third cysteine aminoacids are absent in the XC chemokines that possess only one disulfide bond.

The C-chemokine known as lymphotactin (XCL1) binds to XCR1 and induces neutrophil and T and B cells migration. The CX3C-chemokine called fractalkine (CX3CL) is a membrane-anchored or shed ligand form and binds CX3CR1 on blood-derived neutrophils, monocytes, NK, and T lymphocytes.

CHEMOKINE FUNCTION CLASSIFICATION

The chemokines also have also been classified by their function as "H (homeostatic)," "I (inflammatory)," and "D (dual function)" chemokines:

- H-Chemokines: some examples are CXCL12, CXCL13, CXCL14, CCL18, CCL19, and CCL21.
- I-Chemokines: some examples are CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL16, CCL1, CCL2, CCL3, CCL3L1, CCL3L3, CCL4, CCL4L1, CCL4L2, CCL5, CCL7, CCL8, and CCL13.
- D-Chemokines: some examples are CCL11, CCL17, CCL20, and CCL22.

Chemokine H, I and, D effects are consistent with their function H, I and D function as Chemokine-Receptors. The specificity and selectivity of chemokine-ligands and chemokine-receptors are listed on Table 5, even non-chemokine ligands such as autoantigens, defensins, tRNA-synthetases, Macrophage Inibhitory Factors (MIFs), High mobility group 1B (HMG1B), Ubiquitin, retroviral products, bacterial products, inter alia.

MAIN CHEMOKINE FUNCTIONS

Chemokines regulate development, angiogenesis, and tissue repair through cell trafficking. A CXCR4-CXCL12 system is a key signal for the maintainance of hematopoietic stem cells in the bone marrow and allows mobilization of bone marrow-derived non-hematopoietic stem cells during tissue damage/injury. CXCR2 has shown a function myelosuppresive function which is not yet clarified.

The CCR9-CCL25 system plays a fundamental role in T cell development in the thymus. Transition from CD4+CD8+ DP thymocytes in the thymic cortex to CD4 or CD8 single-positive (SP) thymocytes in the thymic medulla is associated with upregulation of CCR4 and CCR7, chemokine receptors for CCL22, and CCL19, and CCL21, respectively. These are expressed on endothelial cells of medullary venules.

The CCR9-CCL25 system is involved in gut cryptopatch formation and subsequent positioning of intraepithelial lymphocytes.

CCR10 and its ligands, chemokines CCL27 and CCL28, are involved in the epithelial immunity. Here CCL27 is expressed predominantly on the skin by keratinocytes while CCL28 is expressed by epithelial cells of mucosal tissues. Cutaneous lymphoid antigen (CLA)+ T lymphocytes express CCR4 and CCR10.

Platelets release chemokines, e.g., CXCL4, CCL5, CXCL7, CXCL12, CXCL1, and CXCL5. Some chemokines are antimicrobial peptides with direct action, therefore, Thrombocidins designated as bactericides (Thrombocidin1 and Thrombocidin2) are byproducts of CXCL7.

Chemokines are associated with T-lymphocyte polarization. Hence Th1 cells express CCR5 and CXCR3, Th2 cells express CCR4 and CCR8, Th17 cells express CCR4 and CCR6, Treg cells express CCR4, CCR5, and CXCR3 while Th follicular cells (Tfh) and Treg follicular cells (Tfr) express CXCR5.

The NK cells, CD56dimCD16+ subset (high cytotoxic capacity and low cytokine production), express CXCR1 and

CX3CR1. NK cells, CD56brightCD16dim subset (large amounts of cytokines and but low killing capacity), express CCR7.

CXCL1, CXCL2, CXCL3, CXCL7, and CXCL8 induce basophil chemotaxis and histamine liberation.

Monocyte/Macrophage lineage expresses CCR1, CCR5, CXCR1, CXCR2 and CX3CR1. Macrophage differentiation is associated with downregulation of CCR2 and induction of CCR5 and CX3CR1.

CXCR4, CXCR5, and CCR7 systems are major regulators of lymphoid development and of adaptive immunity.

CXCL12 has shown chemopulsion activity, which favors the expulsion of leukocytes from tissues.

Some chemokines have roles in neurobiology and neuropathology (they have even been termed the Neurochemokines). For example, CXCR2 and its ligands have been implicated in several neuroinflammatory brain pathologies as well as in neutrophil recruitment and in the developmental positioning of neural cells. The expression of CX3CL1, CCL2, and CCL21 and their receptors CX3CR1, CCR2, and CXCR3 is altered in the spinal cord under neuropathic pain conditions. CCL2, CXCL12, CCL5/RANTES, CX3CL1/Fractalkine, CCL22, CXCL8/IL-8, and CXCL1 modulate the electrical activity of several neuronal populations such as hippocampal, cortical, cerebellar, hypothalamic, mesencephalic and dorsal root ganglia (DRG) neurons. The CXCR4-CXCL12 system is constitutively expressed in the brain and is important in the development of the central nervous system and peripheral nervous system. CXCR4 is found in neurons, astrocytes, microglia, bone marrow-derived cells as well as other neural progenitor cells. The CXCR4-CXCL12 system is up-regulated in the ischemic penumbra regions following ischemic stroke and in spinal cord injury (114).

In the pathological field, chemokines play roles in carcinogenesis, cancer metastasis, inflammation, and autoimmune diseases.

ATYPICAL CHEMOKINE RECEPTORS/NONCHEMOTACTIC RECYCLING OF SCAVENGER RECEPTORS (ACR/NCRSR)

There are atypical chemokine receptors/Nonchemotactic recycling of scavenger receptors (ACR/NCRSR) such as CXCR7, CCRL1, CCRL2, DARC, and CCBP2. They work in some cases as decoy-receptors which promote the degradation of chemokines. In certain scenarios, they signal, and in other cases, they have been involved in transepithelial transport (transcytosis) (Table 6) (113,114).

CHEMOKINES, VIROKINES AND VIRORECEPTORS

Herpes virus and poxvirus family genomes encode a wide number of proteins that modulate the chemokines activity. Therefore, they subvert host immune responses in order to develop an infecto-pathogenic scenario. Some examples of this scenario are proteins that are homologous to chemokines, proteins that resemble chemokine-receptors, or secreted chemokine-binding proteins (CKBPs). These competitively interact with chemokines and prevent chemokine interactions with chemokine-receptors or the extracellular matrix. For example, Human Herpes virus 5/Citomegalovirus (HHV5/CMV) encodes four G protein-coupled receptors - US27, US28, UL33, and UL78 – with high homology to human chemokine-receptors

CHEMOKINE RECEPTORS AND HIV/AIDS

CCR5 and CXCR4 serve as secondary CD4-co-receptors which facilitate HIV1 entry into target cells. Retroviruses that use the CCR5 are termed R5 HIV or macrophage tropic. Those that use CXCR4 are termed X4 HIV o T cell line tropic strains, and viruses that can use both co-receptors are called R5X4 HIV.

Drugs such as Maraviroc (Selzentry, or Celsentri), Vicriviroc (SCH 417690, SCH-D), and Cenicriviroc (TAK-652, TBR-652) are antiretroviral CCR5 receptor antagonist/inhibitor class and used in the treatment of HIV infection. Ibalizumab (TMB-355 or TNX-355) is a non-immunosuppressive monoclonal antibody that binds CD4 and inhibits the retroviral entry process.

There is no conclusive evidence that co-receptors other than CCR5 and CXCR4 can play important roles in HIV1 infection in vivo. However, a large array of alternative receptors have been identified which support viral entry, not only for HIV-1 but also HIV-2 and SIV (Simian immunodeficiency virus). The list is long and includes CCR1, CCR2b, CCR3, CCR4, CCR6, CCR8, APJ/AGTRL1, CXCR3, CXCR6, CXCR7, CX3CR1, CMKLR1/ChemR23, GPR1, GPR15, and FPRL1(110-113).

CHEMOKINE SUPERFAMILY MEMBERS OF UNKNOWN FUNCTION

Other proteins with unknown functions are:

- Chemokine-like factor (CKLF). CCR4 has been identified as the CKLF-receptor.
- Chemokine-like superfamily (CKLF-like) and related proteins for vesicle trafficking and membrane link (MARVEL), transmembrane domain containing (CMTM) protein family, e.g., CMTM1, CMTM2, CMTM3, CMTM4, CMTM5, CMTM6, CMTM7, and CMTM8.
- Family with sequence similarity 19 -chemokine (C-C motif)-like-(FAM19A) members such as FAM19A1, FAM19A2, FAM19A3, FAM19A4, and FAM19A5.

ORPHAN OR OTHER CYTOKINE FAMILY MEMBERS

IL-17 FAMILY: IL17-A, IL17-B, IL17-C, IL17-D, IL17-E/ IL-25, AND IL17-F. THE IL-17 CYTOKINES FAMILY IS MADE UO OF SIX MEMBERS: IL17-A, IL17-B, IL17-C, IL17-D, IL17-E (ALSO KNOWN AS IL-25), AND IL17-F.

The protypical member is IL17-A also called IL17. The IL17 receptor (IL17R) family consists of IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE. These IL-17R family members contain a fibronectin III (FNIII)-like domain in their extracellular

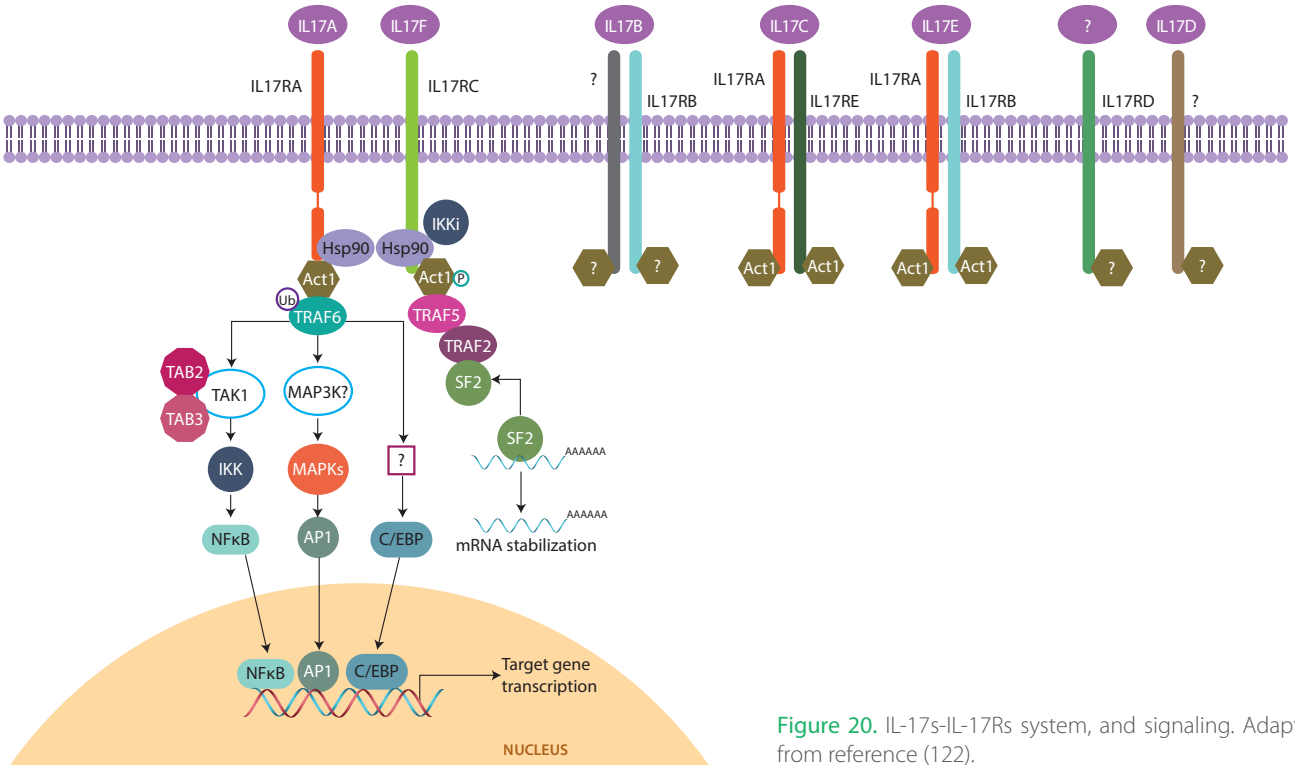


Figure 20. IL-17s-IL-17Rs system, and signaling. Adapted from reference (122).

region and a SEF/IL-17R (SEFIR) domain in their intracellular region (Figure 20) (1-12,120-123). IL-17 family members play critical roles in host defense (antibacterial and antifungal), as well as in the pathogenesis of allergy, autoimmune diseases, obesity/metabolic syndrome, and cancer, which explains why IL-17s activate several downstream signaling pathways that target NFKB, AP1, and C/EBPs transcription factors. Note that these transcription factors induce gene expression of antibacterial peptides, proinflammatory chemokines and cytokines, and MMPs. Moreover, IL17s stabilize mRNAs of genes induced by TNF-α.

IL-17 (particularly IL-17A and IL-17F) production and secretion define highly specialized lymphocyte populations known as Th17, nTh17, Tc17, iNKT17, and T γ/δ-17. They are likewise produced by NK cells, LTi cells, neutrophils, paneth cells, and by colonic epithelial cells. IL-17E/IL25 is characteristic of Th25 cells. It is considered a Th2-cytokine, and is also produced by mast cells and eosinophils. This cytokine is part of the epithelial Th2-response immune program with TSLP and IL-33 too. IL-17B is produced by chondrocytes, neurons, pancreas, gut, cartilage, and by embryonic limb buds. Finally, IL-17D is produced by nervous system, muscle, heart, and adipose tissue.

IL-17s are part of the Neutrostat feedback loop (IL-23-IL-17-CSF3/GCSF) which regulates the number of neutrophil populations.

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7. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins)- Known and Predicted Protein-Protein Interactions, collaboration between The Novo Nordisk Foundation Center for Protein Research (CPR), The European Molecular Biology Laboratory (EMBL), The University of Copenhagen (UCPH), The Swiss Institute of Bioinformatics (SIB), The Technische Universität Dresden, and The University of Zurich (UZH). http://string-db.org/
8. The Human Protein Atlas project is funded by the Knut & Alice Wallenberg foundation. http://www.proteinatlas.org/
9. Universal Protein Resource (UNIPROT), collaboration between European Bioinformatics Institute (EBI), the SIB Swiss Institute of Bioinformatics and the Protein Information Resource (PIR). http://www.uniprot.org/

10 MAJOR HISTOCOMPATIBILITY COMPLEX: ANTIGEN PROCESSING AND PRESENTATION

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INTRODUCTION

The Major Histocompatibility complex (MHC) system known as the human leukocyte antigen (HLA) in humans is located on the short arm of chromosome 6 (6p21.3) and contains the most polymorphic gene cluster of the entire human genome. Furthermore, the HLA consists of three regions which have been designated as class I, class II, and class III based on the structure and function of gene products. The main function of HLA class I gene products (HLA-A, -B, and -C) is to present endogenous peptides to responding $CD8^+$ T Cells while the class II coded molecules HLA-DR, -DP, and –DQ have restricted expression and process exogenous peptides for presentation to $CD4^+$ helper T Cells. The class III region, in turn, contains genes which encode for immune regulatory molecules, e.g., tumor necrosis factor (TNF), factors C3, C4, and C5 of complement and heat shock proteins. This chapter will discuss the genetic, structural and functional characteristics of HLA.

HISTORY

The major Histocompatibility Complex (MHC) was initially discovered as a genetic locus associated with the acceptance or rejection of transplanted organs in mice. In 1954, the same genetic system was described in humans by Jean Dausset and Jan van Rood and was called human leukocyte antigens (HLA). This discovery was made as the result of the presence of antibodies against antigens expressed on leukocytes from patients who had received multiple blood transfusions. Also, these antibodies were present on leukocytes from multiparous women and patients who had gone through kidney transplants. Subsequently, two distinct classes of HLA molecules were defined: HLA class I antigens and HLA class II antigens. HLA class I antigens are expressed on all nucleated cells and platelets (except those of the central nervous system) while the HLA class II antigens are expressed on antigen presenting cells (APC) such as B lymphocytes, dendritic cells, macrophages, monocytes, Langerhans cells, endothelial cells, and thymic epithelial cells. All this knowledge led to the practice of serological typification from different HLA variants in patients and donors before the performance of organ transplants. The HLA system revolutionized the transplant practice, and subsequent studies allowed the elucidation of its importance in the development of various diseases (e.g., autoimmune diseases), its application in genetic population studies and in genomic medicine (1,2).

GENETIC ORGANIZATION OF HLA

The HLA system is located on the short arm of chromosome 6 on band 6p21.3. This gene system is the largest cluster in the human genome, and it is divided into three main subregions: the genes of class I, class II, and class III, which are all involved in immune response and suppression (3) (Figure 1).

Relative to their chromosome position, the class I region is located nearest to the telomere, and the genes within this region encode for the class I molecule α-polypeptide chain. It is noteworthy that the gene encoding β2-microglobulin –the common light chain of the HLA class I molecules– is not located in the HLA complex but is on chromosome 15. Genes encoding HLA class I α-chain have a characteristic structure in which different domains of the protein are encoded by different exons. The leader peptide is encoded by exon 1, and the three extracellular domains (α1, α2, and α3) are encoded by exons 2, 3, and 4 respectively. Meanwhile the transmembrane anchor is encoded by exon 5, the cytoplasmic tail by exons 6 and 7, and the 3' untranslated region by exon 8 (Figure 2).

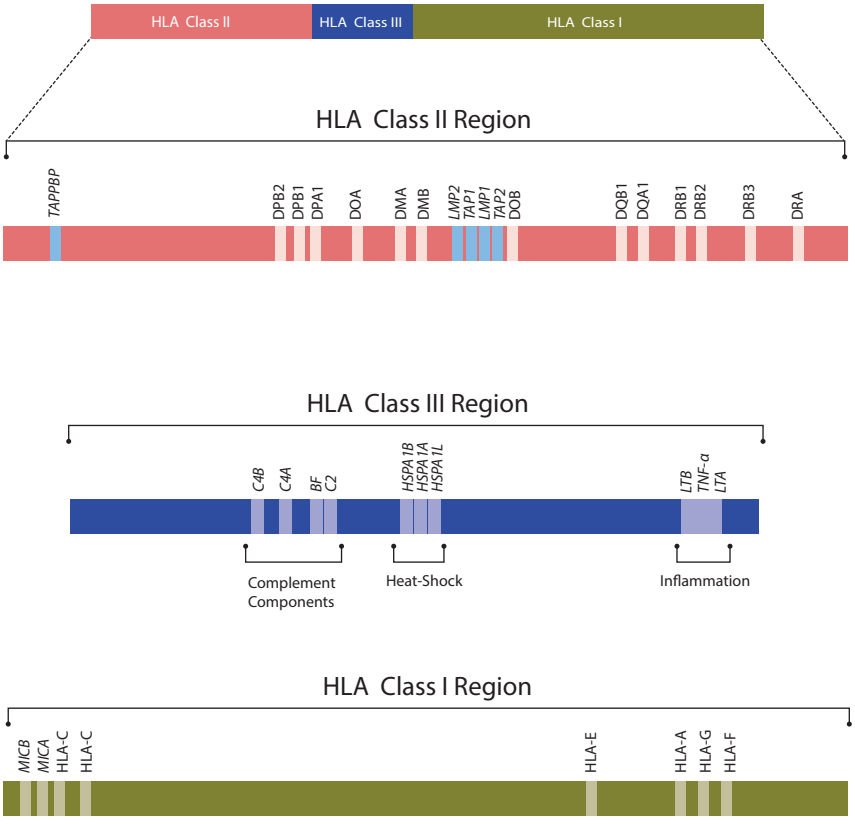

Figure 1. Map of the human HLA. The complex is conventionally divided into three regions: I, II, and III. Each region contains numerous loci (genes), only some of which are shown. Abbreviations: tapasin (*TAPBP*); large multifunctional proteases 1 and 2 (*LMP1* and *LMP2* respectively); transporter associated with antigen processing 1 and 2 (*TAP1* and *TAP2* respectively); complement components 2, 4A and 4B (*C2*, *C4A* and *C4B* respectively); complement factor B (*BF*); heat-shock protein 1A A-type, heat-shock protein 1A B-type, and heat-shock protein 1A–like (*HSPA1A*, *HSPA1B* and *HSPA1L* respectively); lymphotoxins A and B (*LTA* and *LTB* respectively); tumor necrosis factor α (*TNF-α*); major histocompatibility complex class I chain genes A and B (*MICA* and *MICB* respectively).

Overall, there are three types of class I genes in the HLA region*: HLA-A, -B*, and *-C*, the so-called classic. Furthermore, there are other loci in this region called *HLA-E, -L, -J, -K, -H,* and *-G*, which encode the non-classical HLA molecules (4).

Likewise, the HLA class II molecules are heterodimers composed of α and β chains. The exon-intron organization of the class II genes is like that of the class I genes because the different domains of the protein are encoded by different exons. The α- and β-chain genes have a similar structure in which exon 1 encodes for the leader peptide, and exons 2 and 3 encode the two extracellular domains. In the β-chain genes, exon 4 encodes for the transmembrane domain, and exon 5 encodes for the cytoplasmic tail. In contrast, in both α-chain genes, the transmembrane and the cytoplasmic tail are encoded by exon 4 (Figure 3).

As a whole, there are five isotypes of the class II HLA protein designated as HLA-DM, -DO, -DP, -DQ, -DR. To classify them into their respective loci on chromosome 6, there is a nomenclature of three letters: the first (D) indicates the class, the second (M, O, P, Q, or R) the family, and the third (A or B) the chain (α or β respectively) (4).

Meanwhile, the class III region (although less polymorphic) is also polygenic and more than 50 genes have been identified within it. Among the genes found, those encoding for factors C3, C4, and C5 of complement, heat shock proteins, and the TNF family including TNF-α are of interest (4).

REGULATION OF GENE EXPRESSION

Along with the CCAAT and TATA elements, which are involved in the binding and positioning of the basal transcription initiation complex, there are other relevant upstream DNA sequences in the HLA class I promoter region involved in the regulation of HLA class I gene expression.

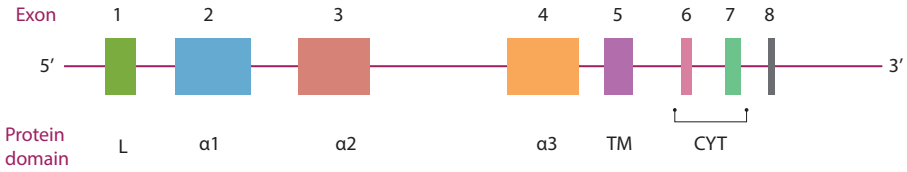

Figure 2. Exon-intron organization of an HLA class I gene. Within the class I heavy chain each protein domain is encoded by different exons. Abbreviations: leader sequence (L); transmembrane region (TM); cytoplasmic tail (CYT).

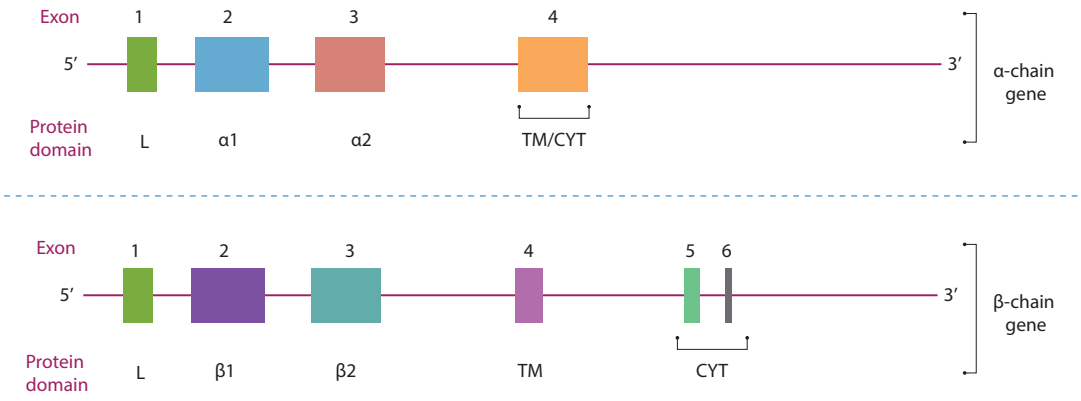

Figure 3. Exon-intron organization of the genes encoding α and β chains of HLA class II. Abbreviations: leader sequence (L); transmembrane region (TM); cytoplasmic tail (CYT).

These conserved DNA sequences include the enhancer A element, the interferon-stimulated response element (ISRE), site α, and enhancer B element (Figure 4).

The enhancer A element, which encompasses two putative NF-kB binding sites (kB1 and kB2), plays an important role in the constitutive and cytokine-induced expression of HLA class I genes (5). For instance, the kB1 and kB2 sites of the HLA-A locus bind members of the NF-kB/rel family of transcription factors, e.g., p50, p65, and c-Rel. In addition, kB elements are also potential target sequences for zinc finger proteins such as MZF1, which bind with high affinity to the NF-kB binding sites of the HLA class I promoter.

Alternatively, HLA class I expression can be induced by members of the interferon (IFN) family (6). For instance, interferon regulatory factor 1 (IRF-1) acts as an activator of HLA class I transcription, whereas IRF-2 and IFN consensus sequence-binding protein (ICSBP) act as repressors of HLA class I transcription. In addition, IFN-γ induces the expression of the IRF-1 gene through the JAK/STAT pathway of signal transduction. IRF-1 subsequently binds to the conserved ISRE element in the HLA class I promoter, which is adjacent to the enhancer A element, thereby mediating the induction of HLA class I expression by IFN-γ.

Expression of HLA class II molecules is regulated at the transcriptional level by a complex process involving highly conserved sequences, which recruit specific binding factors and generate the HLA class II enhanceosome. At the DNA level, this regulatory unit consists of four sequences (W/S, X, X2, and Y boxes), the SXY module, which has been found in genes encoding the three human HLA class II isotypes (HLA-DP, HLA-DQ, and HLA-DR) (7) (Figure 5). The SXY module is also found in the promoters of the genes encoding invariant chain (Ii) and the non-classical HLA class II molecules, HLA-DM and HLA-DO. These are accessory proteins that are required for intracellular trafficking and peptide loading of HLA class II molecules. Moreover, sequences that resemble SXY module also contribute to the function of HLA class I promoters.

The Y box is ubiquitous in the genome and binds the heterotrimeric transcription factor NF-Y, which consists of NF-YA, NF-YB, and NF-YC subunits. NF-YB and NF-YC contain histone fold domains that are thought to bend the DNA at promoters, a process that may facilitate the efficient assembly of multiple transcription factors and provide easy access to RNA polymerase. The X2 box was found to bind to the AMP response element binding protein (CREB) which is important for the stability and assembly of the X1 box factor, and the regulatory factor X (RFX) at specific promoter SXY (8).

The main transacting factors that interact with the SXY module were identified in patients with Bare Lymphocyte Syndrome (BLS), a severe hereditary immunodeficiency disease in which there is defective synthesis of class II molecules. Mutations associated with this syndrome were found in genes encoding for RFX, RFX-associated ankyrin-containing proteins (RFXB) (9), and also for class II transactivator (CIITA) (10).

CIITA has been implicated in promoting transcription by various mechanisms: first, recruiting components such as transcription factor IID (TFIID) and TFIIB from the general

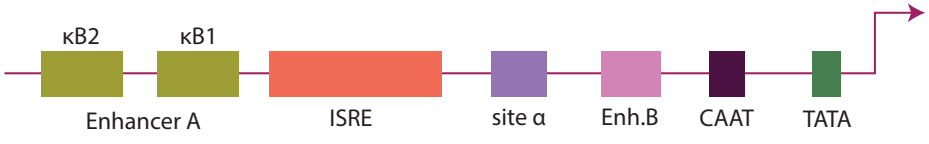

Figure 4. Schematic representation of the regulatory elements within the promoter of HLA class I genes. The Enhancer A (comprising the κB-binding sites), interferon-stimulated response element (ISRE), site α, Enhancer B, and CCAAT, and TATA elements are indicated.

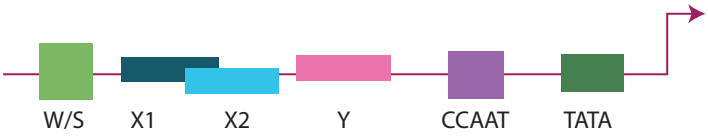

Figure 5. Schematic representation of the regulatory elements within the promoter of HLA class II genes. The W/S, X (comprising the X1 and X2 halves) and Y boxes together with the CCAAT, and TATA elements are indicated.

transcription-initiation machinery; second, inducing phosphorylation of RNA polymerase II; third, interacting with the positive transcription elongation factor b (P-TEFb); and last, recruiting co-activators that alter chromatin accessibility by inducing histone acetylation. Briefly, CIITA does not bind DNA directly, but instead assembles at SXY promoters through direct interactions with the RFX-CREB-NF-Y complex (10,11). When bound, CIITA activates transcription through a potent acidic activation domain located in its N-terminal region. Through this domain, and other sequences, CIITA interacts with components of the basal transcription machinery. CIITA also interacts with three co-activators, including the CREB binding protein (CBP), p300 and p300/CBP associated factor (PCAF) (8). As a consequence, CIITA is known as the master control factor or master regulator of HLA class II genes and related genes (12) (Figure 6).

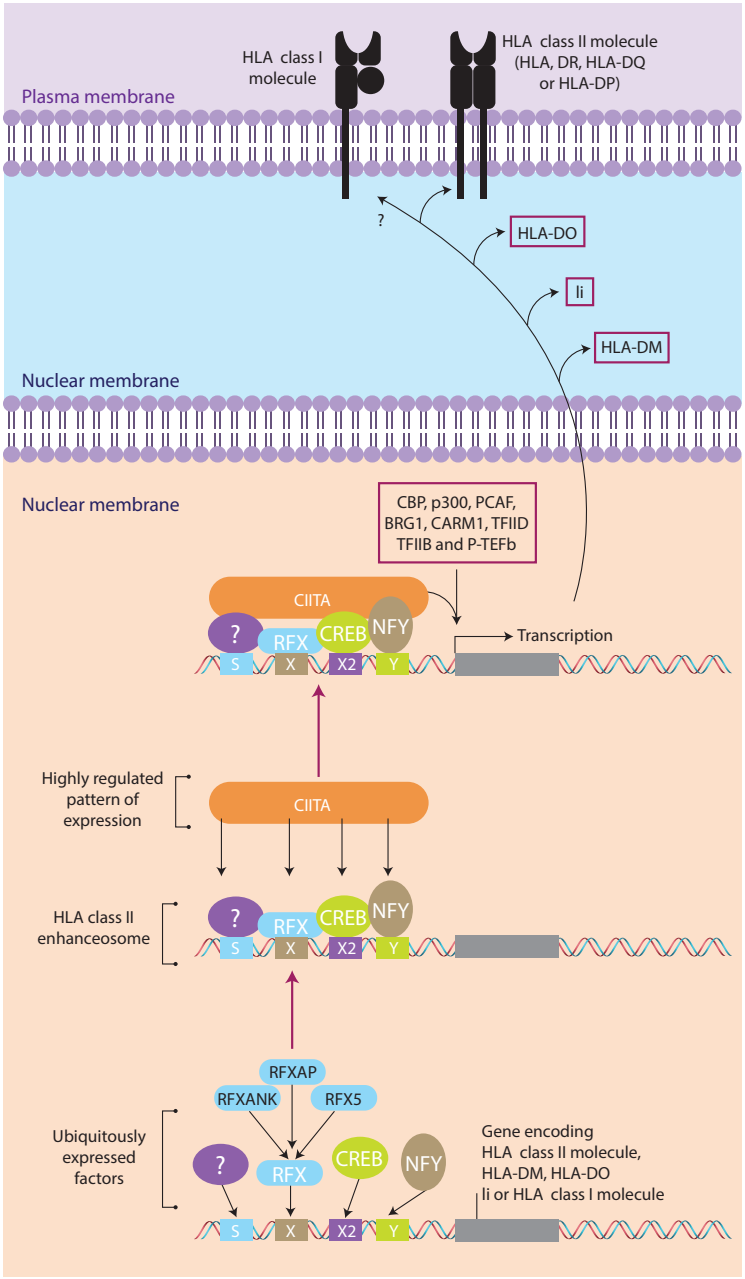

Figure 6. Regulation of the transcription of HLA class II genes. The SXY module that is mainly present in all classical HLA class II genes is bound cooperatively by four factors: the RFX factor, which is composed of RFX5, RFXAP, and RFXANK; the X2-box-binding factor is CREB; the Y-box-binding factor is NFY; and an unknown S-box-binding factor. This multiprotein complex (which is known as the HLA class II enhanceosome) is a platform to which the CIITA is recruited by multiple synergistic protein–protein interactions. CIITA coordinates the recruitment of additional factors that are involved in chromatin modification and remodeling: these are CBP, p300, PCAF, BRG1, and CARM1. CIITA also coordinates the recruitment of factors that are involved in transcription initiation (that is, TFIID and TFIIB) and in transcription elongation (that is, P-TEFb).

Abbreviations: Heterotrimeric X-box-binding factor regulatory factor X (RFX); RFX-associated protein (RFXAP); RFX-associated ankyrin-containing protein (RFXANK); cyclic-AMP-responsive-element-binding protein (CREB); nuclear transcription factor Y (NFY); class II transactivator (CIITA); CREB-binding protein (CBP); p300/CBP-associated factor (PCAF); brahma-related gene 1 (BRG1); co-activator-associated arginine methyltransferase 1 (CARM1); transcription factor IID and IIB (TFIID and TFIIB respectively); positive transcription elongation factor b (P-TEFb).

Adapted by permission from: Reith W, *et al*. Regulation of HLA class II gene expression by the class II transactivator. Nature Reviews Immunology. 5:793-806, copyright (2005)

ORIGIN AND EVOLUTION OF HLA

The acquired immunity and antigen presentation function of the HLA originates from ancestral vertebrates. Thus, the HLA is evolutionarily conserved, and this can be demonstrated not only at the functional level but also at the genetic level (13).

As was mentioned previously in the text, the HLA is an organized genetic system that encodes for a great variety of molecules that have key roles within biological pathways. Therefore, the expression and function of these molecules are strictly controlled. This control involves the action of chaperone molecules, which, despite their close relationship with the HLA function, are not located within the same gene complex. HLA generally contains genes which encode proteins whose function is closely related to each other. This clustering suggests that positive selection pressure occurred, and it would induce the binding of functionally related gene subsets. Moreover, this conservative selection pressure would prevent the separation of the loci within the system (13).

The HLA evolution has been explained by two successive duplications in the genome and their consecutive expansion. This hypothesis was proposed by Kasahara *et al*, after they discovered that the human genome contains at least three paralogous regions located on chromosomes 1, 9, and 19 of humans (14).

It is suggested that this evolutionary process took place by grouping the most basic functions and the most complex and specialized ones. Indeed, while the ancestral genes *C3, C4, C5*, and *TNF* (HLA III) acquired a role in both innate and acquired immunity, genes involved in antigen presentation such as *HLA-A, -B, -C, -DR, -DQ*, and-DP (HLA I and II) appear to have been generated more recently. For instance, molecules encoded in the HLA II became part of the acquired immunity system, which only appeared late in the evolution of the immune system with the advent of adaptive immunity. Hence, this late evolutionary progress shows the importance of higher immune system complexity and the requirement for an immune response that is not just more diverse but also more specific (15).

HLA CHARACTERISTICS

The HLA system is composed of three regions. The class I region corresponds to the genes coding for molecules HLA-A, -B, and -C. In addition, the class II region encodes HLA-DR, -DQ, and –DP. Finally, the class III region, in which genes are encoding for proteins of the complement system and TNF family genes. The function of HLA-encoded class I and class II molecules is to bind peptide antigens and display them for recognition by antigen-specific T lymphocytes. Peptide antigens associated with HLA class I molecules are recognized by $CD8^+$ T Cells while HLA class II molecules are recognized by $CD4^+$ T Cells (16).

HLA ALLELES	NUMBER
HLA Class I Alleles	7.089
HLA Class II Alleles	2.065
HLA Alleles	9.154
Other non-HLA Alleles	156
Number of Confidential Alleles	13

Table 1. Number of HLA Alleles.
From: http://hla.alleles.org/nomenclature/stats.html

POLYMORPHISM

The HLA system contains the most polymorphic gene cluster in the entire human genome. To date, the IMGT/HLA Database has reported 9,154 HLA alleles (17) (Table 1 and Figure 7). Comparison of the sequences of alleles of the polymorphic class I and class II loci shows that nucleotide substitutions are concentrated in the exons that encode the peptide-binding groove and the site of interaction with the T Cell receptor (TCR). It is possible that these features on HLA sequences can be explained by selection pressures exerted by epidemics of infectious diseases because these pressures lead to the selection of the HLA alleles that have distinctive peptide-binding properties. However, this is an unproven hypothesis. Polymorphisms in HLA class I and HLA class II molecules affect which amino acids are in the peptide-binding groove and thus their binding specificity, but the more open structure of the HLA class II peptide-binding groove and the greater length of the peptides bound in it allow greater flexibility in peptide binding (18).

The presence of multiple HLA alleles in the population will ensure that at least some individuals within a population will be able to recognize protein antigens produced by virtually any microbe, thus reducing the likelihood than a single pathogen can evade host defenses in all individuals in a given species.

NOMENCLATURE OF HLA ALLELES

The HLA system nomenclature is updated by an international committee (17). This nomenclature differs based on the detection method used. The HLA antigens, defined by serology, are designated by the denomination of the gene locus (e.g., *HLA-A*, *HLA-DR*) and followed by the numerical identification of the antigen (e.g., *HLA-A1*, *HLA-DR1)*. The nomenclature of the C locus incorporates the letter "w" (e.g., *HLA-Cw1*, *HLA-Cw2*) to differentiate it from the complement system.

In contrast, the nomenclature of HLA alleles defined by molecular biology varies based on their class. For class I, the denomination *HLA-A, HLA-B*, and *HLA-C* is used to designate antigens defined by serology. An asterisk is added to define the method as being one used in molecular biology (e.g., *HLA-A**), and two to eight digits are then added (e.g., *HLA-A*02:01*). The first two digits refer to antigen serological typing. The third and fourth are related to the denominations of specific alleles. The fifth and sixth describe allele variations, and the seventh and eighth represent variations at introns (5' or 3' gene regions).

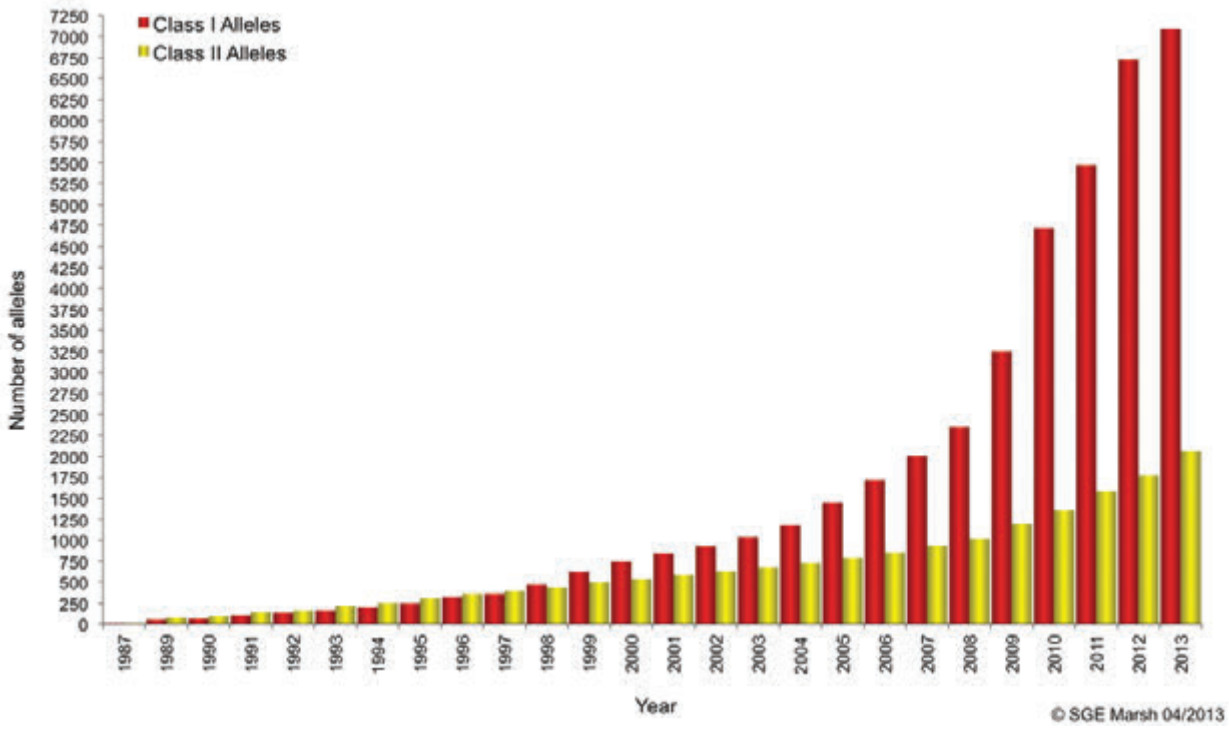

Figure 7. Number of HLA class I and II alleles officially recognized between 1987 and 2013. Reprinted by permission from: Robinson J, *et al*. The IMGT/HLA database. Nucleic acids research. 41:D1222-7, copyright (2013)

For HLA class II, the procedure is not exactly the same. After the designation of the HLA and its gene locus, the letter "A" or "B" is added to represent the polymorphic α and β chains of the HLA-DR and HLA-DQ and only the letter "B" to represent the polymorphic β chain of the HLA-DP (e.g., *HLA-DQA, HLA-DRB, HLA-DPB*). Since some regions have several genes for the α and β chains, each locus receives a corresponding number (e.g., *HLA-DQB*1). Next, as defined for HLA class I, four to eight digits are added after an asterisk (e.g., *HLA-DQB1*03:01*).

In addition to the specific allele number, there are additional optional suffixes that may be added to an allele to indicate its expression status. Those alleles which have been shown to be alternatively expressed are defined in Table 2.

INHERITANCE

Histocompatibility genes are inherited as a group (haplotype), one from each parent. Thus, HLA genes are co-dominantly expressed in each individual. A heterozygous human inherits one paternal and one maternal haplotype, each containing three class I (A, B, and C) and three class II (DP, DQ, and DR) loci. Each individual inherits a maximum of two alleles for each locus. The maximum number of class I HLA gene products expressed in an individual is six. Thus, as each chromosome is found twice (diploid) in each individual, a normal tissue type of an individual will involve 12 HLA antigens. Haplotypes are normally inherited intact, and hence, antigens encoded by different loci are inherited together. However, on occasion, there is crossing over between two parental chromosomes which results in new recombinant haplotypes (1,18).

LINKAGE DISEQUILIBRIUM (LD)

As was mentioned before, due to the proximity of the genes in the HLA complex, there are preferential associations between different loci. As a consequence, these loci are inherited as haplotypic blocks with minimal probability of genetic recombination. The result is that some combinations of alleles or genetic markers occur more or less often in a population than would be expected from a random formation of haplotypes. This is known as linkage disequilibrium (See chapter 16).

STRUCTURE OF HLA CLASS I AND CLASS II

STRUCTURE OF HLA CLASS I

HLA class I molecules consist of two non-covalently linked polypeptide chains, an HLA-encoded α chain or heavy chain (44 to 47 kD), and a non-HLA encoded subunit called β2microglobulin (12 kD) (19) (Figure 8). The α chain has three regions including a cytoplasmic region containing a peptide-binding groove made from the α1 and α2 domains, a transmembrane region containing hydrophobic amino acids by which the molecule is anchored in the cell

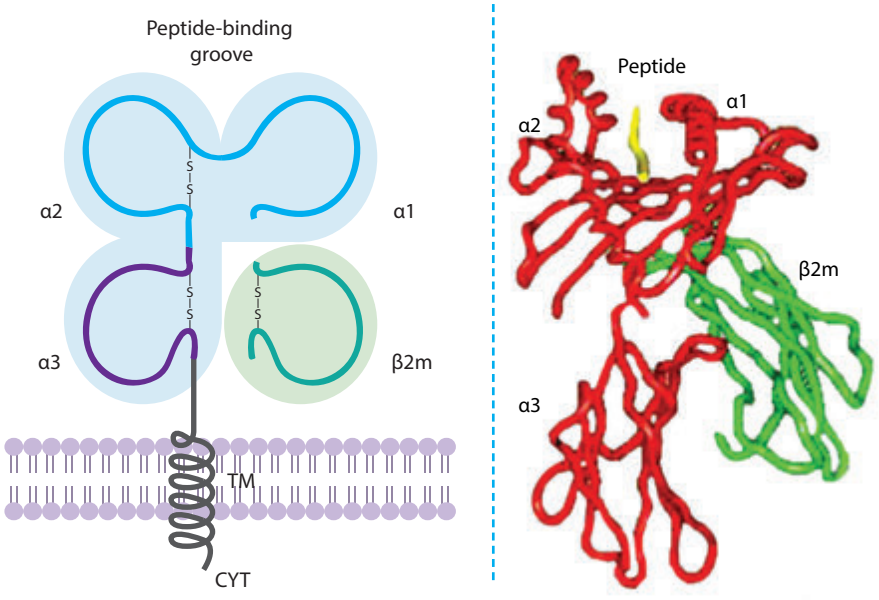

Figure 8. Structure of HLA class I molecules. The schematic diagram illustrates the different regions of the HLA-A2 class I molecule. β2-microglobulin (β2m) is the light chain of the class I molecule. In addition, the α chain of the class I molecule has two peptide-binding domains (α1 and α2), an immunoglobulin-like domain (α3), the transmembrane region (TM), and the cytoplasmic tail (CYT).

membrane, and a highly conserved α3 immunoglobulin-like domain to which CD8 binds (3).

A peptide-binding groove is formed between the α1 and α2 helices with a β-pleated sheet as its floor. Usually, the groove will accommodate peptides of approximately 8-10 amino acids in length. There, they are ligated by a network of H-bonds between conserved α chain residues and the free amino acid carboxyl-terminus of the peptide. This allows peptides with different sequences at their termini to be bound the same way by different class I molecules (Figure 9). Nevertheless, peptides longer than 10 amino acids can occasionally bind class I molecules although the termini generally remain tucked into the cleft such that the central part of the peptide bulges upwards to accommodate the extra residues (19).

The antigen-binding groove contains distinct pockets –A, B, C, D, E, and F– that vary in their chemical properties from one allele to another. There is a preferred sequence "motif" which generally contains two or three preferred primary anchor residues, and their amino acid side chains fit well into distinct pockets within the class I cleft where they provide significant energy binding (20). Dominant anchor sites usually occupy the B-pocket and F-pocket of class I molecules. The intervening residues between dominant anchor sites are much more varied in their side-chains. Moreover, the peptide often contains secondary anchor sites that

ALLELE	EXPLANATION
HLA	Refers to the HLA region and prefix for an HLA gene
HLA-DRB1	Refers to a particular HLA locus i.e. *DRB1*
*HLA-DRB1*13*	Refers to all alleles in the *DR13* serologic group
*HLA-DRB1*13:01*	Refers to a specific HLA allele
*HLA-DRB1*13:01:02*	Refers to an allele that differs by a synonymous mutation from *DRB1*13:01:01*
*HLA-DRB1*13:01:01:02*	Refers to an allele which contains a mutation outside the coding region from *DRB1*13:01:01:01*
*HLA-A*24:09N*	Refers to a 'Null' allele, an allele which is not expressed
*HLA-A*30:14L*	Refers to an allele encoding a protein with significantly reduced or 'Low' cell surface expression
*HLA-A*24:02:01:02L*	Refers to an allele encoding a protein with significantly reduced or 'Low' cell surface expression, where the mutation is found outside the coding region
*HLA-B*44:02:01:02S*	Refers to an allele encoding a protein which is expressed as a 'Secreted' molecule only
*HLA-A*32:11Q*	Refers to an allele which has a mutation that has previously been shown to have a significant effect on cell surface expression, but this has not been confirmed and its expression remains 'Questionable'

Table 2. **Basis for HLA nomenclature alleles (17).**

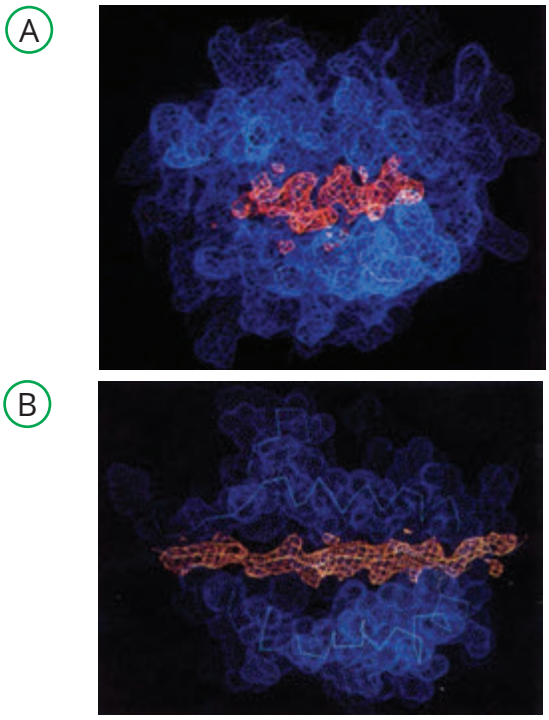

Figure 9. Peptide binding groove of HLA molecules. Crystal structures HLA molecules. **A.** Peptides bind to HLA class I molecules at each of their ends (upper panel). **B.** HLA class II molecules, the peptide extends beyond the peptide-binding groove and is held by interactions along its length (lower panel).

Upper panel. Reprinted by permission from Macmillan Publishers Ltd. From: Bjorkman PJ, *et al*. Structure of the human class I histocompatibility antigen, HLA-A2. Nature. 329: 506-512, copyright (1987).

Lower panel. Reprinted by permission from Macmillan Publishers Ltd. From: Brown JH, *et al*. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature. 364: 33-39, copyright (1993).

contribute to HLA-binding but are usually more degenerate in the side chains they will accommodate at these sites and still support strong binding (21).

Furthermore, the antigen-binding groove is the region responsible for the high polymorphism of HLA class I molecules. Consequently, there will be changes in the electrostatic charge, hydrophobicity, and shape of the cleft which will thereby alter the peptide binding properties of allelic class I molecules (22).

STRUCTURE OF HLA CLASS II

Class II HLA molecules are composed of two non-covalently associated polypeptide chains, a 32 to 34 kD α chain, and a 29 to 32 kD β chain (23) (Figure 10). The genes encoding both chains of class II molecules are polymorphic and present in the HLA locus. Both chains have three regions including a cytoplasmic region containing a peptide-binding groove made up of the α1 and β1 domains, a transmembrane region containing hydrophobic amino acids by which the molecule is anchored in the cell membrane, and the highly conserved α2 and β2 domains to which TCR binds (3).

A peptide binding groove is formed between the α1 and β1 domains with a β-pleated floor. Unlike class I molecules, the cleft of the class II molecules is open. As a result, class II molecules accommodate longer peptides than class I molecules do. Typically, the peptides bound to HLA class II molecules are 12 to 24 amino acids in length, but longer ones are not uncommon (20) (Figure 9). HLA class II molecules bind their peptides in an extended conformation with about a third of the peptide surface being accessible for interaction with the TCR. The termini of class II-bound peptides are not ligated by the same network of H-bonds that bind class I peptides so they may hang over the end of the cleft (23). The superantigens, in turn, bind class II as an interactive protein outside the conventional peptide antigen-binding site which explains their lack of restriction to any particular class II alleles (24).

FUNCTIONS OF HLA CLASS I AND CLASS II

Class I and class II molecules are essential for T Cell-mediated adaptive immunity. The foreign antigens recognized by the TCR are peptides produced by intracellular protein degradation which are bound to class I or class II molecules on the surface of human cells. While degradation of foreign proteins to produce peptides is called antigen processing, the binding of peptides by HLA molecules to produce ligands for TCR is called antigen presentation (1).

When TCR recognizes HLA-associated peptides on an APC, several T Cell surface proteins and intracellular signaling molecules are rapidly mobilized to the site of T Cell-APC contact. This set of molecules includes TCR complex which consists of αβ TCR non-covalently linked to the CD3 and ζ proteins, CD4 or CD8 co-receptors, co-stimulatory molecules (e.g., B7 molecules that are involved in signal transduction), and accessory molecules (e.g., ICAM-1 and CD58, which are important in strengthening the adhesion between the T Cells and APCs).

The effector functions of $CD8^+$ and $CD4^+$ T Cells are different. CD8+ T Cells have a cytotoxic function that enables them to kill cells infected with viruses and cancer cells. $CD4^+$ T Cells, in turn, have a wider range of effector functions, all

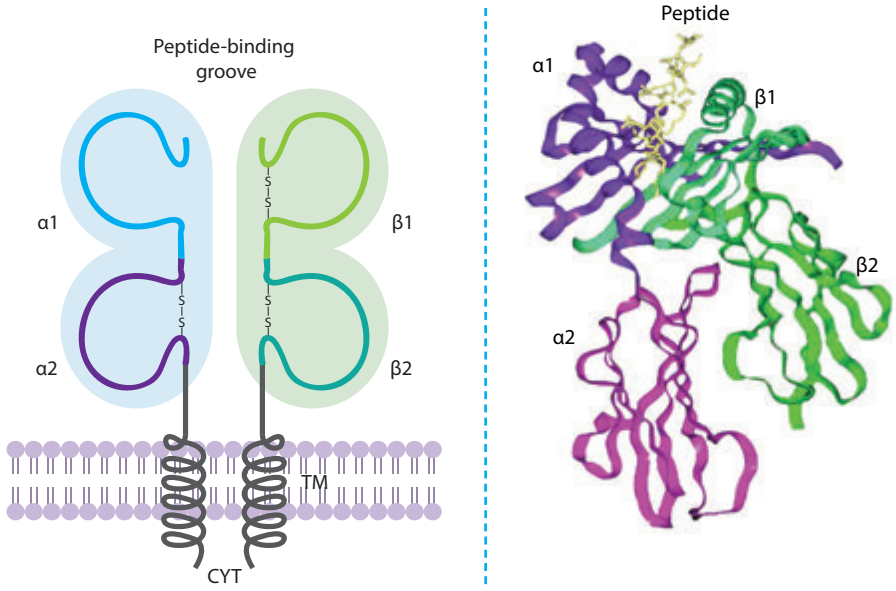

Figure 10. Structure of HLA class II molecules. The schematic diagram illustrates the different regions of the HLA-DR1 class II molecule. Each of the class II α and β chains has four domains: the peptide binding domain (α1 and β1), the immunoglobulin-like domain (α2 and β2), the transmembrane region (TM), and the cytoplasmic tail (CYT).

of which involve the targeted delivery of cytokines. $CD4^+$ T Cells are often called helper T Cells, and three distinct types of $CD4^+$ T Cells have been distinguished: T_H1 cells that secrete IFN-γ, which mediate defense against intracellular microbes; T_H2 cells that secrete IL-4 and IL-5, which favor IgE and eosinophil/mast T Cell-mediated immune reactions against helmints; and T_H17 cells, which promote inflammation and mediated defense against extracellular fungi and bacteria.

In general, while endogenous antigens associated with HLA class I are recognized by $CD8^+$ T Cells, exogenous peptides associate with HLA class II and are recognized by $CD4^+$ T Cells (Figure 11).

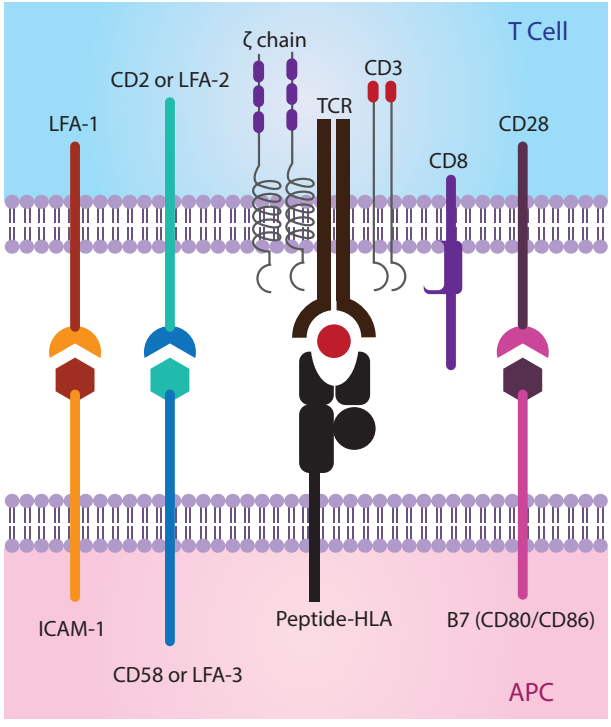

Figure 11. T cell and antigen presenting cell interaction. Schematic shows how the T cell co-receptors interact with HLA class I and HLA class II. T cell recognition of HLA-peptide complexes is aided by two surface glycoproteins called co-receptors: CD4 which binds HLA-II and CD8 which binds HLA-I.

Abbreviations: lymphocyte function-associated antigen (LFA); cluster of differentiation (CD); T cell receptor (TCR); antigen presenting cell (APC); intercellular adhesion molecule (ICAM).

ANTIGEN PROCESSING AND PRESENTATION

HLA CLASS I ANTIGEN PRESENTATION PATHWAY

In general, foreign antigens presented by class I molecules are derived from intracellular infections caused by viruses, or from proteins synthesized in the cytosol, mature proteins which have fulfilled their cell cycle, or defective ribosomal products that are thought to be derived from poorly folded nascent proteins including a high proportion of all newly translated products in living cells, called DRIPs (25).

Assembly of class I molecules with antigenic peptides requires the coordination of multiple processes to first create peptides, then to transport and load them into the cleft of nascent class I molecules in the endoplasmic reticulum (ER) (26,27). Many of these polypeptides are ubiquitinated and are thus tagged for degradation by the proteasome (25). The peptides are transported into the ER by the adenosine triphosphate-dependent transporters associated with antigen processing (TAP) where they associate with heterodimers of HLA class I heavy chain and β2-microglobulin. The TAP-associated glycoprotein called tapasin functions to facilitate peptide loading of class I molecules in the ER. Tapasin bridges HLA class I molecules to TAP in association with the chaperone molecules calreticulin and the thioloxidoreductase ERp57. Together, these molecules (TAP, calreticulin, ERp57, and tapasin) make up the peptide loading complex.

Likewise, Tapasin stabilizes the empty class I dimer by retaining it in the ER until peptide assembly by the peptide loading complex. When stably assembled, the HLA class I-peptide complex is transported to the cell surface via the ER and Golgi network to be recognized by the specific TCR on the $CD8^+$ T Cell (27) (Figure 12 and Table 3).

HLA CLASS II ANTIGEN PRESENTATION PATHWAY

In general, the foreign antigens presented by class II molecules are derived from pathogens present in the extracellular spaces. APCs use specialized receptors to bind and internalize microbes in vesicles called phagosomes which may fuse with lysosomes to produce phagolysosomes or secondary lysosomes. Though this occurs less often, cytoplasmic and membrane proteins may be processed and displayed by HLA class II molecules. In this case, cytoplasmic proteins are trapped within membrane bound vesicles called autophagosomes. These vesicles fuse with lysosomes, and the cytoplasmic proteins are degraded by proteolysis. In both cases, degraded proteins are then able to bind to HLA class II molecules (25).

HLA class II α and β chains assemble in the ER with a non-polymorphic protein called invariant chain (Ii). The interaction with the Ii has the effect of stabilizing the structure of the HLA class II molecule while preventing the binding of peptides within the ER. Ii is anchored in the ER membrane, and the cytosolic portion of the molecule directs intracellular sorting of class II molecules through the Golgi to the HLA class II compartment (MIIC). Within MIIC, the Ii is degraded and can be replaced by a peptide derived from degradation in the endosomes or lysosomes of endocytosed material.

Afterwards, proteolytic enzymes such as cathepsins, which generate peptides from internalized proteins also act on the Ii to degrade it and leave only a 24 amino acid remnant called class II-associated invariant peptide (CLIP), which sits in the peptide binding groove (28). Then, CLIP is removed by the action of the HLA-DM. This HLA-DM action can be modulated by a second class II-related molecule called HLA-DO which synergises with HLA-DM under specific conditions. Complexes of HLA II and peptide are then taken to the plasma membrane where they can be recognized by $CD4^+$ T Cells (29) (Figure 13 and Table 3).

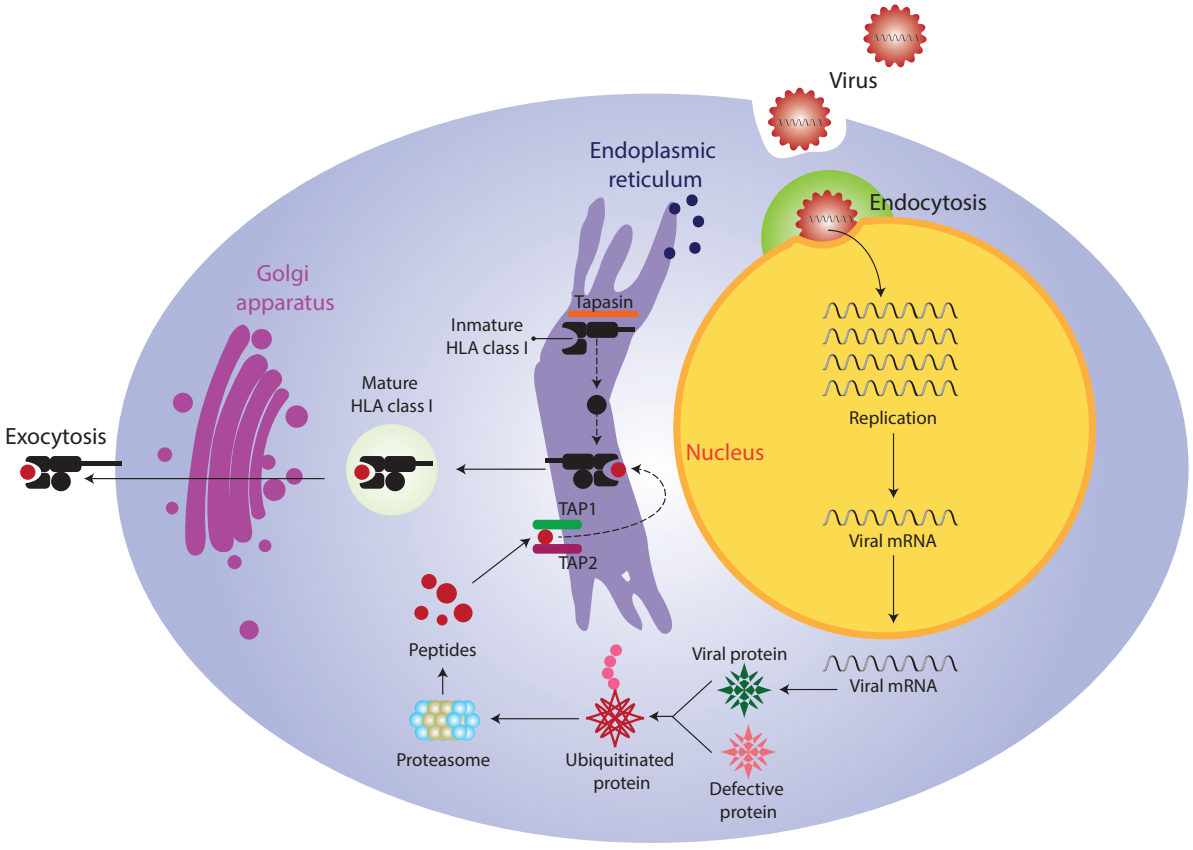

Figure 12. Pathway by which intracellular antigens are processed and presented by HLA class I molecules. Proteins in the cytosol are degraded by proteosome to make small peptides which are transported by TAP protein into the lumen of ER. HLA class I molecules are synthesized on ribosomes and translocated into the lumen of the ER where they are assembled and are bound to the peptide. Complexes of HLA class I and peptides leave the ER and move through the Golgi apparatus to the plasma membrane where they are recognized by CD8+ T cells. Abbreviations: Transporter associated with antigen processing 1 and 2 (TAP1 and TAP2, respectively).

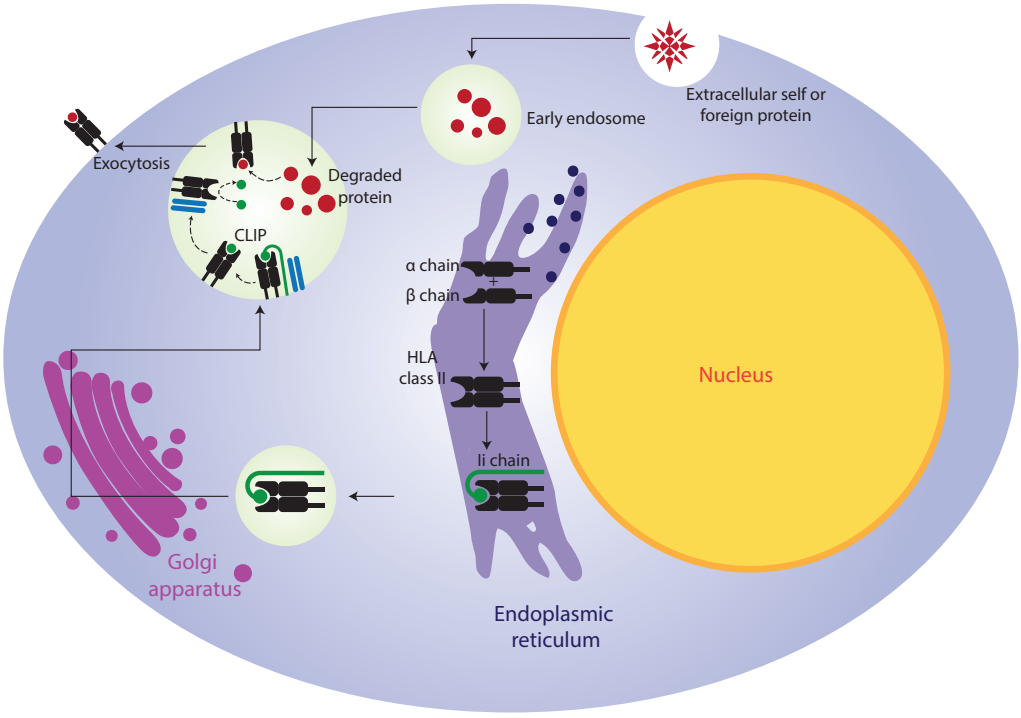

Figure 13. Pathway by which extracellular antigens are processed and presented by HLA class II molecules. Extracellular proteins are taken into the cell by phagocytosis and are then degraded to peptides in endosomes and lysosomes. The peptides are then sorted into vesicles where they can meet HLA class II molecules. HLA class II α and β chains, and Ii molecules are synthesized on ribosomes and translocated into the lumen of the ER where they are assembled into heterotrimers that cannot bind peptides because Ii occupies the peptide-binding site. Heterotrimers leave the ER and pass through Golgi apparatus to enter the vesicles. There the Ii is degraded and a peptide can be bound with the help of HLA-DM and HLA-DO. Complexes of HLA class II and peptides are then taken to the plasma membrane where they can be recognized by CD4+ T cells. Abbreviations: Class II-associated invariant chain peptide (CLIP), invariant chain (Ii).

HLA CLASS I CHAPERONES	
Calnexin	Calcium-binding, endoplasmic reticulum (ER)-associated protein that interacts transiently with newly synthesized N-linked glycoproteins, thus facilitating protein folding and HLA assembly. It may also play a central role in the quality control of protein folding by retaining incorrectly folded protein subunits within the ER for degradation
Calreticulin	Protein that binds to misfolded proteins and prevents them from being exported from the ER to the Golgi apparatus
Tapasin	Transmembrane glycoprotein which mediates interaction between newly assembled HLA class I molecules and TAP, which is required for the transportation of antigenic peptides across the ER membrane.
ERp57	Protein of the ER that interacts with lectin chaperones calreticulin and calnexin to modulate folding of newly synthesized glycoproteins.
TAP	Membrane-associated protein is a member of the superfamily of ATP-binding cassette (ABC) transporters. TAP is involved in the pumping of degraded cytosolic peptides across the ER into the membrane-bound compartment where HLA class I molecules assemble.
HLA CLASS II CHAPERONES	
Invariant chain (Ii)	Protein that regulates antigen presentation for immune response. It also serves as cell surface receptor for the cytokine macrophage migration inhibitory factor (MIF) which, when bound to the encoded protein, initiates survival pathways and cell proliferation.
CLIP	The part of the invariant chain (Ii) that binds HLA class II groove and remains there until the HLA receptor is fully assembled. The purpose of CLIP is to prevent the binding of self-peptide fragments prior to HLA class II location within the endo/lysosome.
HLA-DM	This protein plays a central role in the peptide loading of HLA class II molecules by helping to release the CLIP molecule from the peptide binding site.
HLA-DO	This protein is found in lysosomes of B cells and regulates HLA-DM-mediated peptide loading on HLA class II molecules.

Table 3. **Molecular chaperones involved in HLA class II antigen presentation.**

CROSS-PRESENTATION

In contrast to all other cells, APCs can present exogenous antigens to naïve CD8+ T lymphocytes. Two main intracellular pathways have been described for the cross-presentation of phagocytosed antigens (Figure 14):

- Cytosolic pathway: Internalized proteins access the cytosol where they are degraded by the proteasome. Proteasome-generated peptides are transported to ER TAP-dependently. Then, peptides are cut by ER-associated aminopeptidase 1 (ERAP1) and endosomal insulin responsive aminopeptidase (IRAP) and, finally, loaded on HLA class I molecules (30).

- Vacuolar pathway: This is TAP-independent and insensitive to proteasome inhibitors. Internalized antigens are degraded into peptides by cathepsin S in the phagosome, and peptides are then loaded onto HLA class I molecules. Finally, the resulting complexes are transported to the cell surface through vesicular recycling. Note that there is evidence that the cytosolic pathway can also occur apart from TAP, possibly through another, as yet unidentified, peptide transporter (31).

- The origin of the HLA class I molecules involved in cross-presentation is also a matter of debate. Initially, it was suggested that cross-presenting HLA class I molecules originated from the plasma membrane and recycled to endosomes. A conserved tyrosine residue in their cytosolic tail which is required for internalization from the cell surface was shown to be crucial for cross-presentation. However, it was recently shown that CD74 promotes the trafficking of newly synthesized HLA class I molecules from the ER to endocytic compartments in DCs and that this routing is required for the cross-presentation of cell-associated antigens (32,33).

CD1 MOLECULE AND PRESENTATION OF NON-PROTEIN ANTIGENS

Regarding genetic structure, human *CD1* is located on the long arm of chromosome 1 on band 1q23.1. The five *CD1* genes that have been identified are designated *CD1A*, *CD1B*, *CD1C*, *CD1D*, and *CD1E* and correspond to five CD1 proteins: CD1a, CD1b, CD1c, CD1d, and CD1e (34). On the basis of sequence analysis, the CD1 isoforms can be classified into three groups: group 1 consists of CD1a, CD1b, and CD1c; group 2 consists of CD1d; and group 3 consists of CD1e.

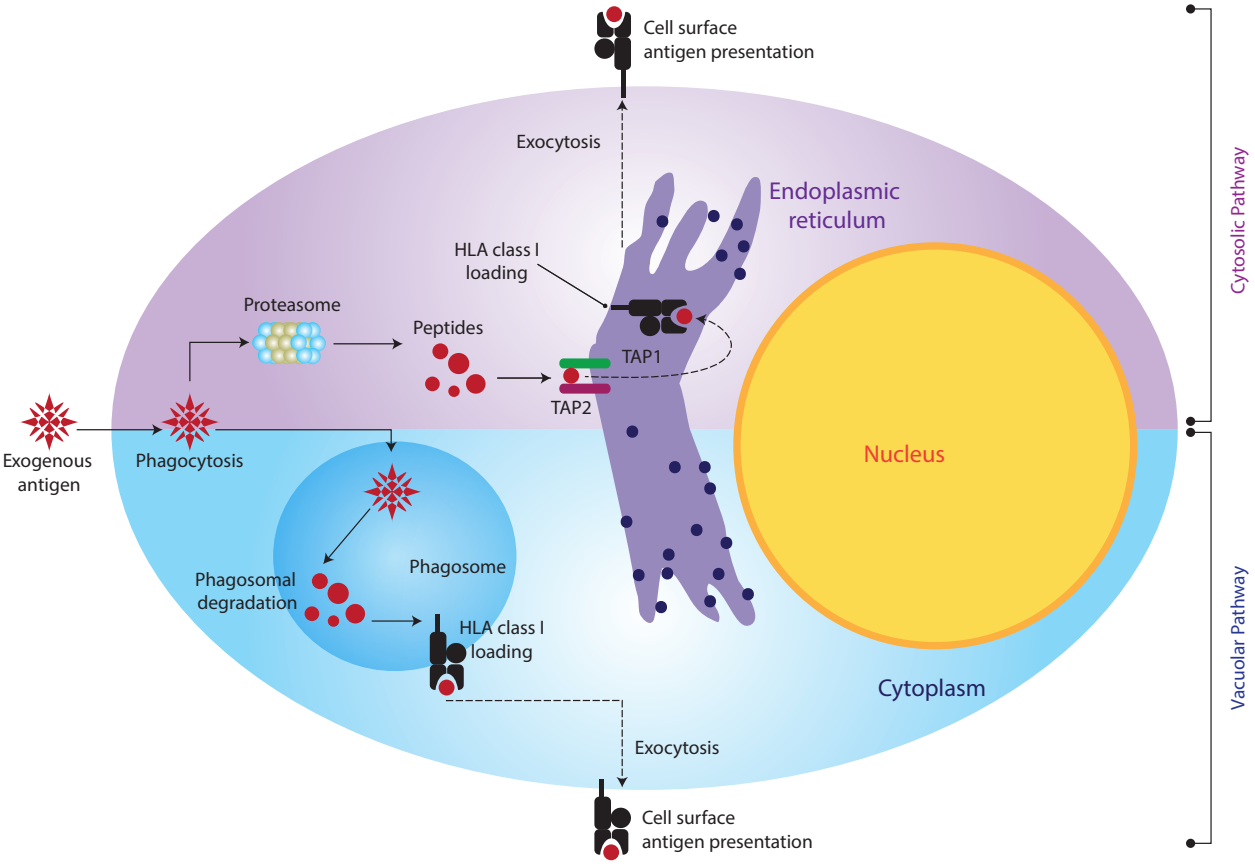

Figure 14. Cross-presentation of antigens to CD8+ cells. After phagocytosis, exogenous antigens can be exported into the cytosol, where they are processed by the proteasome. The processed antigens can then be loaded on HLA class I molecules in the ER (the cytosolic pathway with ER loading) or re-imported into the phagosome to be loaded on HLA class I molecules (the vacuolar with phagosomal loading).

All CD1 molecules produce heterodimers together with β2-microglobulin and are expressed on the cell surface (with the exception of CD1e which remains intracellular). Crystal analyses of CD1a, CD1b, and CD1d molecules have revealed a three dimensional structure resembling that of classical HLA class I molecules. CD1 proteins are organized into three extracellular domains (α1, α2, and α3), a transmembrane region, and a cytoplasmic tail. The membrane distal α1 and α2 domains adopt a conformation similar to that of other antigen-presenting molecules consisting of two antiparallel α-helices overlying a β-pleated sheet floor. These two distal domains are supported by the α3 domain that interacts with β2-microglobulin (35).

However, unlike HLA class I molecules, CD1 proteins bind alkyl chains in hydrophobic channels that reside beneath the surface of CD1 molecules. The hydrophilic head groups of the lipid antigens, in turn, protrude where the hydrophobic channels open to the membrane distal surface of the CD1 molecule. These head moieties are stabilized by hydrogen bonds, which also contribute to the correct positioning of the lipid antigens. Moreover, the different architecture of the antigen binding groove of different CD1 isoforms allows them to bind distinct lipid antigens (36) (Table 4).

CD1 trafficking in the endocytic pathway

Newly synthesized CD1 molecules have signal sequences for translocation into the lumen of the ER. Following synthesis, they rapidly become glycosylated to bind the ER chaperones calnexin and calreticulin (37). CD1d molecules, unlike CD1b, can move to the cell surface in the absence of β2-microglobulin. This difference is thought to be a result of the association of CD1d with the ERp57, which is important for the formation of disulfide bonds within the CD1 heavy chain prior to assembly with β2-microglobulin (38). Before leaving the ER, CD1 molecules are loaded with ER-resident lipids.

Following assembly in the ER, CD1b follows the secretory pathway through the Golgi directly to the plasma membrane (39). CD1d has also been observed to associate with HLA class II molecules and Ii chain, which can direct CD1 complexes with these proteins from the trans-Golgi network to endosomal compartments without first going to the plasma membrane (40).

After crossing the plasma membrane, CD1 molecules are internalized in endosomes. This clathrin-dependent pathway is common to proteins that contain well described tyrosine-based sorting motifs which bind adaptor protein complex 2 (AP2) and allow sorting of cargo proteins into clathrin-coated pits. In contrast to the other isoforms, CD1a does not contain any sorting motifs in its cytoplasmic tail, and yet, it is also internalized from the plasma membrane into endosomal compartments by an unknown mechanism (36).

After internalization by the early or sorting endosomes, the different CD1 isoforms follow different trafficking pathways. CD1a and CD1c head for the endocytic recycling compartment, whereas CD1b molecules move mainly through late endosomes and lysosomes. Thus, CD1b can bind both AP2, which mediates CD1 internalization from the plasma membrane, and AP3, which diverts these CD1 molecules from the early recycling pathway to late endosomes and lysosomes (41).

Lipid trafficking

Because of their hydrophobicity, lipids circulate in association with membranes or with lipid-transferproteins (LTP) (e.g., high density lipoproteins (HDL) and very low density lipoproteins (VLDL)). For instance, lipoproteins bound to Apolipoprotein E (ApoE) are internalized through specific receptors and reach the endosomal system. In acidic intracellular compartments, ApoE dissociates from VLDL and, then, associates with HDL and is secreted. During this exchange, intracellular lipids may become incorporated into membranes and remain in the cell or, instead, become associated with nascent HDL particles and thus be released into the extracellular space.

There are several ways exogenous lipids become internalized: through specific interaction of lipoproteins with lipoprotein-specific receptors, upon insertion into the plasma membrane, by binding of C-type lectins with mannose residues on glycolipids, or through internalization of apoptotic bodies (36).

The first mechanism involves lipoprotein-specific receptors which deliver lipids into clathrin coated pits allowing traffic through early recycling endosomes. Lipids such as sulfatide and sphingomyelin are internalized through clathrin-coated vesicles, then, reach late endosomes/lysosomes and are degraded. The second mechanism is related to internalization of extracellular lipids that associate with the plasma membrane. Glycosphingolipids such as gangliosides, globosides, and lactosylceramide are instead internalized clathrin-independently. The third mechanism involves the calcium-dependent binding of C-type lectins to carbohydrate moieties via their carbohydrate-recognition domains (CRDs).

Most C-type lectins bind mannosylated moieties which are present in several CD1 lipid antigens. Indeed, lipoarabinomannan has been shown to be internalized and delivered to late endosomal and lysosomal compartments by the macrophage mannose receptor. Finally, uptake of apoptotic cells is another important mechanism of lipid antigen internalization (42). During mycobacterial infection, phagocytic cells may die by apoptosis and apoptotic bodies are internalized by macrophages and dendritic cells which may simultaneously present both peptide and glycolipid antigens (43).

$CD1a^-$, $CD1b^-$, and CD1c-restricted T Cells were initially identified among the $CD4^-CD8^-$ double negative T Cell subset but have now been recognized to be even more common in the $CD4^+$ and $CD8^+$ αβ TCR T Cell pool. Conversely, CD1d-restricted iNKT Cells comprise a large pool of T Cells that respond rapidly and display the features of innate immune cells. Overall, the antigen presentation pathway by CD1 molecules is highly important in infectious, tumoral, and autoimmune processes (41).

SOURCE	ANTIGEN	CD1 ISOFORM
Mycobacterium tuberculosis and other mycobacteria	Mycolic acids Glucose monomycolate Sulpholipid (diacylatedsulphoglycolipid) Phosphatidylinositolmannosides Mannosylatedlipoarabinomannan Mannosyl-β1-phosphomycoketides Didehydroxymycobactin	CD1b CD1b CD1b CD1b, CD1d CD1b CD1c CD1a
Sphingomonas spp	α-Glucuronosylceramide	CD1d
Borrelia burgdorferi	α-Galactosyldiacylglycerol	CD1d
Leishmania donovani	Lipophosphoglycan	CD1d
Mammalian (self)	Phosphatidylinositol Phosphatidylglycerol Phosphatidylethanolamine GM1 GD3 Sulphatide Isoglobotrihexosylceramide	CD1d CD1d CD1d CD1b CD1d CD1a, CD1b, CD1c CD1d
Synthetic or marine sponge	α-Galactosylceramide	CD1d

Table 4. **CD1-restricted lipid antigens (36).**

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11 IMMUNOSENESCENCE

Gladis Montoya-Ortíz

INTRODUCTION

Senescence is a normal biological process that occurs in all living tissues which involves a decline in cell functions caused by molecular regulatory machinery alterations. In addition, this event is closely related to telomere erosion in chromosomes. This phenomenon in the context of the immune system is known as immunosenescence and refers to the immune function dysregulation or remodeling due to cell aging. Therefore, functions of several cells involved in the innate and adaptive immune response are severely compromised with age progression (e.g., changes in lymphocyte subsets, decreased proliferative responses, chronic inflammatory states, etc.). These alterations make elderly individuals prone to not only infectious diseases but also the phenomenon of malignancy and autoimmunity.

In this chapter, we will explore the molecular aspects of processes related to cell aging, their importance in the context of the immune system, and their participation in some autoimmune diseases (ADs).

AGING

Aging can be defined as the progressive decay of tissue functions which eventually results in organ dysfunction and death. This decline may be the result of the loss of post-mitotic cell function or lack of replacement of such cells due to a decreased stem cell ability to maintain cell division and replication (1). Note that aging is not a disease but rather a natural process occurring in all humans. However, its functional impairment can vary considerably between individuals. Although, at present, it is thought that aging is a process which occurs in parallel in all organs, recent evidence shows how some diseases, including infectious diseases, can accelerate the aging of specific organs (2). Moreover, when organisms suffer some operating ability mismatches or deterioration, the molecular machinery maintenance and repair is required in order to ensure the longevity of the organisms. If this damage is irreparable, the senescence or aging process will take place by limiting the cells' proliferative potential and the propagation of errors. Some control mechanisms include differential gene expression which may be detrimental (3). However, there is a cell renewal mechanism that ensures damaged cell replacement. This mechanism corresponds to a set of proliferating precursor cells that provide a source of cell replacement within the tissues. The immune system provides an interesting case of replacement: cells that die by apoptosis are replaced by new ones, a process which is essential for immune system longevity. This phenomenon is described in the immune system section of this chapter.

TELOMERES AND AGING

One of the most striking features of cell aging is its close relationship with telomere length (4). There is an inverse relationship between telomere length and cellular aging, e.g., very short telomeres force their cells to enter senescence.

Telomeres are located at the end of chromosomes and function to protect and prevent DNA cleavage by the cell's repair machinery. Furthermore, telomeres promote chromosome stability in each mitotic division and prevent chromosome binding and / or breaking during mitosis. This limits cell proliferation and protects cells from excessive telomere loss and cell death (5).

Human telomeres contain guanine-rich repetitive sequences (i.e., TTAGGG) which are associated with several specific proteins (this is known as the shelterin complex). Therefore, chromosomes gradually lose the TTAGGG repeats in each mitotic division given the fact that the DNA

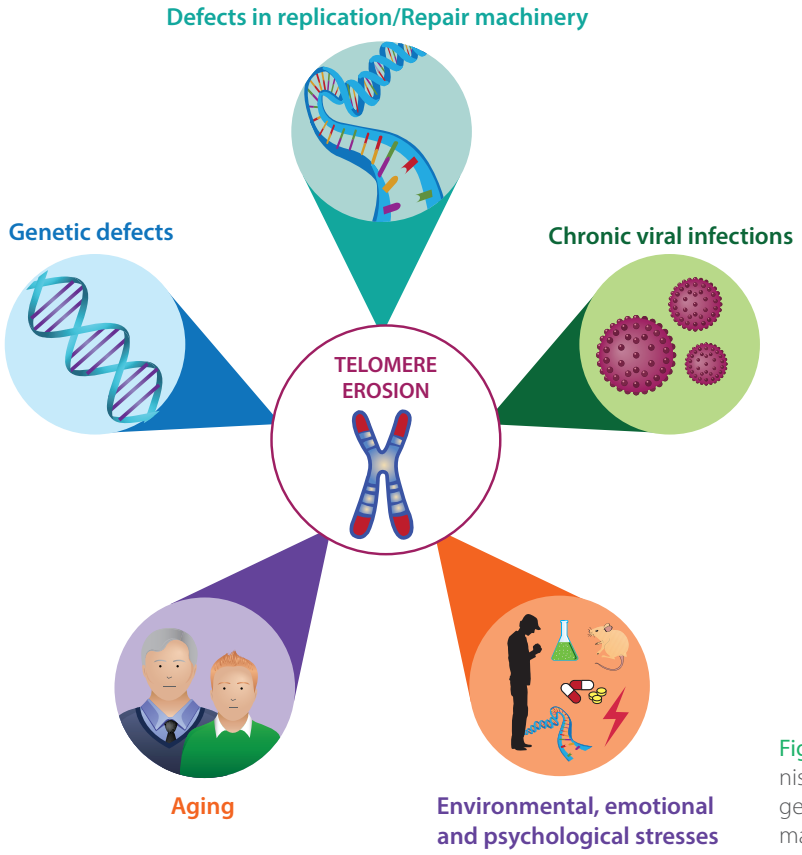


Figure 1. Factors related to telomere erosion. The mechanisms contributing to the loss of telomere length include genetic defects, chronic viral infections, defects in repair machinery, aging, and stress.

polymerase is unable to replicate linear chromosomes in a process known as telomere erosion (Figure 1). This process functions as a mitotic clock for which the length of the telomeres represents the number of cell divisions sustained by the cells (6). The major mechanisms contributing to the loss of telomere length include oxidative DNA damage, oxidative stress, lower telomerase activity, and replication errors (7–9).

Telomere length varies significantly between species and chromosomes. In humans, the average telomere length is between 0.5 and 15 kilobases (10). Moreover, telomerase (enzyme responsible for elongation of telomeres which consists of TERT enzymes and TERC) is crucial in each division cycle. Although this enzyme damps telomere loss (estimated between 50 to 200bp per cell cycle), it does not allow full recovery of the initial telomere length. Telomerase activity is differentially regulated in different cell types. For example, mature cells express low levels of this enzyme while it appears to rise in stem cells, germ cells, and lymphocytes (5). Protection of the telomeric regions is achieved by the shelterin complex proteins (TRF1, TRF2, POT1, TPP1, TIN2, and RAP1) evading the influence of the DNA repair machinery activity by several mechanisms including: secondary structure stabilization, replication error prevention, telomere length regulation, telomerase interaction, complex stability, and inhibition of fusion between chromosomes, etc. (1).

EPIGENETICS AND AGING

Aging affects all organisms, but their lifespan is species-specific. There is also a significant variability with respect to the speed of aging and quality between and within populations (11). This heterogeneity results from interaction between genetic, environmental, and stochastic factors. Therefore, numerous studies have shown changes in the expression profiles during aging which produce physiological and even pathological alterations in different tissues (12).

The epigenetic and autoimmunity chapter describes the main epigenetic mechanisms involved in gene expression changes. However, it is important to mention some of them just to show their involvement in the aging phenomenon. Several epigenetic alterations have been associated with aging and diseases caused by aging (e.g., DNA methylation state, histone modification, miRNA, etc.) (13).

DNA METHYLATION

Several studies have shown loss of methylcytosines with age, especially in CpG islands within Alu repetitive sequences and endogenous retroviral sequences. Recently, it has been demonstrated that hypomethylation occurs in all gene regions such as promoters, introns, and intergenic regions (14). Many target genes with alterations in their methylation status are age-related diseases; e.g., neurodegenerative (*ARC*)

and cardiovascular disorders (*ESR1* and *ESR2*), cancer *(*RB1, p16, VHL and CDKN2A), and immune suppression (*LFA*-1, *ITGAL*, CD5) (15–18). On top of this, a study of monozygotic twins showed that, as young people, they retain similar methylation profiles while other twins who were between 50 and 60 had different methylation profiles, a methylcytosine differential pattern distribution, and a H3 and H4 differential acetylation state (19). These results indicate that accumulative differences between twins possibly result from diverse environmental exposures. Another important study that included newborns, young people, and older individuals identified 29 genes which changed their methylation pattern. Most of these genes were methylated as the age rose although other genes presented a hypomethylated state. Hypomethylation can be explained as the consequence of DNA (cytosine-5-)-methyltransferase 1 (DNMT1) enzyme activity decreasing because of its low expression (20). Hypermethylated genes, in turn, might be the consequence of enzyme DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3b) action, which is paradoxically overexpressed in elderly individuals (20, 21). These results indicate that the cause of abnormal DNA methylation in aging cells is probably due to changes in the expression of these enzymes.

FoxP3, a hypermethylated gene, is a member of the forkhead transcription regulator family and is associated with the development of ADs (22). However, the gene coding for the CD11a chain of lymphocyte function-associated antigen 1 (LFA-1) –a protein which is associated with the development of development of ADs- is hypomethylated is hypomethylated with age and thus overexpressed in aging cells (23). Therefore, it is possible that the age-dependent hypomethylation of LFA-1 may play an important role in the development of autoimmunity in elderly people because of its over-expression. Additionally, there are other reports on elderly subjects indicating DNA hypomethylation states which could lead to an increase in immunogenicity (24).

HISTONE MODIFICATIONS

Another mechanism related to epigenetic changes in aging involves chromatin remodeling. This includes H3K9, H3K27, and H4K20 trimethylation, decreased H3K9 acetylation, and increased H3S10 phosphorylation (25). An example of this is the decrease in the H3K27 methylation state of insuline/IGF1 genes, which modulates lifespan through gene expression regulation of genes related to senescence. This mechanism operates especially in genes triggering senescence through p53 and retinoblastoma signal pathways. A decrease in H3K27 methylation together with an augmentation in H3S10 phosphorylation support the idea of a change in the heterochromatin and euchromatin dynamics in aging cells.

In addition, there are reports of the decline of histone chaperone levels during aging. This is important because chaperons are relevant in positioning the nucleosome. Finally, there are other chromatin-related molecule alterations within aging that include histone deacetylases (HDACs), sirtuin 1 (SIRT1) protein, and histone methyltransferases (26).

miRNA

In addition to modifications of DNA structure, several studies in both murine and humans have shown that miRNAs may influence aging and longevity. miRNAs are non-coding RNAs (ncRNA) which are important for gene expression and regulation (see epigenetics and autoimmunity chapter). Recently, multiple miRNAs related to aging have been described including lin-4, miR-1, miR-145, and miR-140. Characteristically, these miRNAs regulate lipid metabolism and insulin secretion, insulin/IGF-1, the ISR receptor substrate-1, and IGF-1R. In addition, there are other miRNAs which modulate cell senescence critical molecules such as class I HDAC, SIRT1, p21, p53, and pRb. Thus, the miRNAs implicated in regulation of these enzymes and pathways are miR-34a and miR-449th. Furthermore, miR181a is an important miRNA related to TCR signaling which has serious implications in elderly people and autoimmunity (this topic will be discussed later on in this chapter). Recently, Liu *et al* summarized the miRNAs involved in cell senescence (27).

IMMUNE AGING

Aging is associated with several changes in multiple systems including the immune system. In this regard, one characteristic of elder people is their inability to respond properly to vaccines and infections. This condition could be the result of their low immune system efficiency (28) and occurs because of thymic involution in which the thymus loses its ability to produce and replace naïve T cells on the periphery. As a result, thymic dysfunction produces a decrease in cell-mediated response to foreign antigens, self-tolerance, and the naïve T cell population. In turn, it could increase the auto-proliferation of T cells, and eventually, the induction of premature T cell maturation which would also conduce to tolerance reduction (29). These alterations lead to not only modifications in lymphocyte subsets but also functional changes in cell population subsets. For instance, longitudinal studies have demonstrated an association between immunosenescence and an increase in cytomegalovirus (CMV) anergic CD8 T cells (30), which indicates that aging is related to not only changes in the lymphocyte subsets but also functional changes of these cell subsets.

One of the main characteristics of the immune system is the constant renewal of its cells. At the same time, this renewal is highly dependent on the efficiency of telomere maintenance. Immune system cells are derived from hematopoietic progenitor cells that come from myeloid and lymphoid linage. These cells are constantly dividing and differentiating throughout their lifespan and that leads to changes in their telomere length. Note that a high rate of telomere loss in the first years of life has been observed, perhaps because of their high rate of mitotic division. However, this telomere loss is not a linear process over time since, in older people, it is possible to find significant telomere erosion. In fact, several studies have shown a decline in the length of telomeres with aging (31). A lot has been written regarding

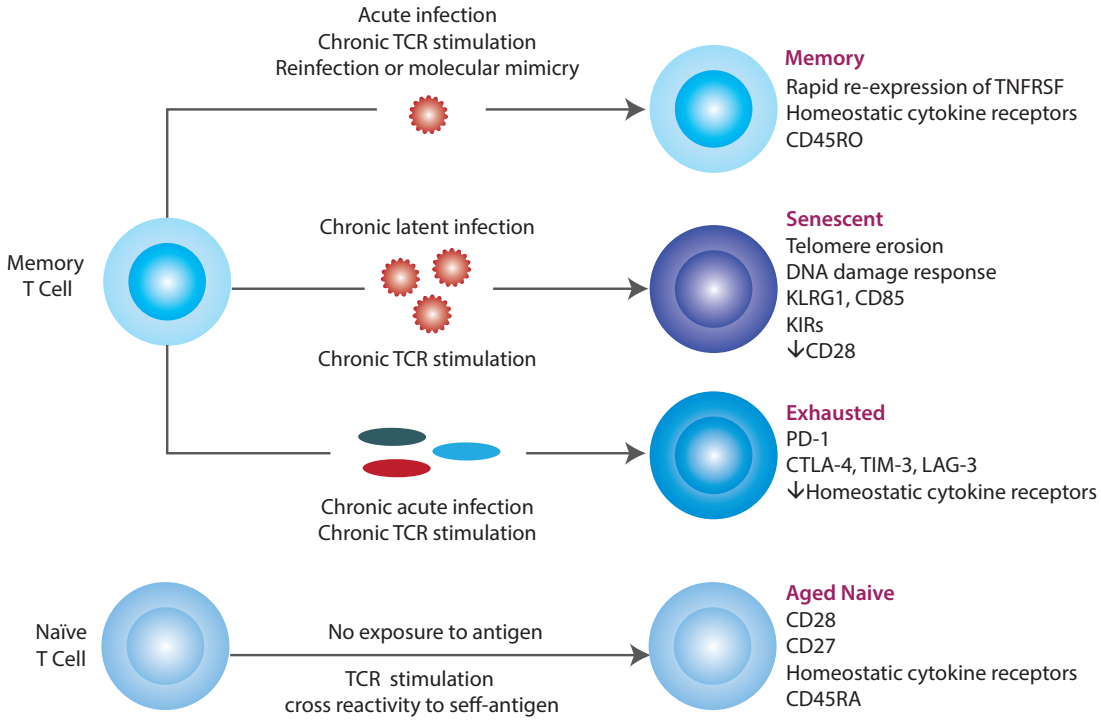


Figure 2. Changes in the T cell pool and individual cells during aging. Proportion of T subsets depends on individual infection antecedents and the environment. Memory T cell subset can change to senescence by a chronic latent infection and chronic TCR stimulation. Meanwhile, exhausted T cells are produced by the same type of stimulation and the chronic acute infection. Aged naïve cells are generated from naïve cells stimulated by self-antigen exposure. Adapted from (44). KIR, killer cell immunoglobulin-like receptor; *KLRG1*, killer cell lectin-like receptor subfamily G, member 1; *LAG-3*, lymphocyte activation gene-3; *PD-1*, Programmed Death-1; *TIM-3*, T Cell Ig- and mucin-domain-containing molecule-3; *TNFSRF*, tumor necrosis factor receptor superfamily.

the relationship between aging and thymic degeneration and changes in the bone marrow cells (32–34). However, in this chapter, we will focus only on immune senescence with an emphasis on circulating cell populations.

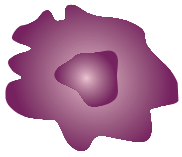
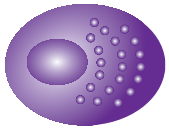
There are reports about age-related changes in peripheral blood cell populations: an increase in monocytes, decrease in lymphocytes, a decrease in naïve cells and an increase in memory cells (Table 1. (35)). Curiously, memory T cells ($CD4^+CD45RO^+$, and $CD8^+CD45RO^+$) increase with age and are preferentially located in tissue, whereas there is a similar proportion of $CD45RA^+$ and $CD45RO^+$ subsets in peripheral blood (36). Unlike somatic cells, lymphocytes have a robust capability to proliferate given their clonal expansion and present an overexpression in the telomerase. This process ensures no significant telomere shortening during each division. Interestingly, T cells possess several special features regarding their phenotype and their telomere length. T cell memory cells have shorter telomeres than naïve T cells and $CD28^+$ T cells have longer telomeres than $CD28^-$ T cells.

Immune aging or Immunosenescence not only affects adaptive response but also has implications in the innate response (Table 1) (37). It has been found that older individuals who exhibit a breakdown of their innate immune barriers such as epithelial skin barriers, lungs, and gastrointestinal tract could be vulnerable to a pathogen attack. Among the cell types involved in innate response, there are neutrophils, macrophages and Natural Killer (NK) cells, which also suffer functional alterations through aging (Table 1)

T CELLS

T Lymphocytes suffer alterations due to aging. Most of the observed changes are attributed to alterations during the initial activation step of the T Cell Receptor (TCR). There is evidence of alteration in the downstream signaling of the TCR in the case of elderly people. This includes a decrease in intracellular free Ca^{2+}, deficiencies in protein kinase C translocation, low Lck and ZAP70 activation, NFAT impairment, and NF-κB translocation, low ras-mitogen activated protein kinase (MAPK) pathways, and a decrease in proteasome activity, etc. (38). These alterations have been demonstrated in both naïve and memory T cells (39).

TCR has the function of discriminating between self-antigens and responding to foreign peptides. This is caused by its activation threshold level, and therefore, the loss of TCR sensitivity is closely related to aging. As mentioned before, there is an important microRNA–named miR-181a – implicated in this phenomenon which controls the expression of several phosphatases related to the negative regulation of proximal CD4 TCR signaling events. Indeed, in a murine model,

		CELL TYPE	CHARACTERISTICS	REFERENCES
Innate Immunity		Neutrophils 	↓ Phagocytic chemotaxis capability ↓ Superoxide anion production ↓ Ability to respond to soluble factors (GM-CSF) and bacteria (LPS, fMLP) ↓ Molecule recruitment into lipid raft, apoptosis and signal transduction	(37, 113–115)
		Dendritic cells 	↓ Cell number, antigen presentation, TLR-mediated signaling, IFN I/III production, chemotaxis and endocytosis ↓ Ability to stimulate lymphocytes in the frail elderly, ↑ function in healthy elderly	(37 ,82, 116)
		Macrophages 	↓ Phagocytic activity and chemotaxis ↑ Synthesis of pro-inflammatory cytokines (IL-6, IL-8, TNF-α, IL-1β) ↓ Apoptosis, superoxide production and signal transduction ↓ TLR expression and function ↑ PGE2 production ↓ MCH clas II production	(37, 115, 117)
		NK cells 	↑ CD56dimCD57+ population ↓ Function of cytotoxicity ↓ Secretion of IFN-γ induced by IL-2 ↓ HLA-DR, IFN-α;CD57 and CD95. Cell proliferation ↑ Production of IL-1, IL-4, IL- 6, IL-8 and TNF-α	(118–120)
Adaptive Immunity	**Cellular Response**	Thymus 	Involution from age of 9 months, thymic remnant after 50 years	(34, 121)
		T Cells 	Variable number (↓ proliferation to PHA, varying age and health status) - HLA B8/DR3 associated with high proliferative responses ↑ Proportion of memory cells ($CD45RO^+$), especially $CD8^+$ ↓ Proportion of naïve cells ($CD45RA^+$) ↓ Proliferative capacity ↓ Synthesis of IL-2 receptor and IL-2 in memory cells ↓ $CD28^+$ ↑ $CD28^-$T cells mainly $CD8^+$ $CD28^-$ (characterized by oligoclonal expansion, shortening of telomeres, potential decreased proliferation, resistance to apoptosis, and increased production of TNF-α and IL-6) ↓ CD4 T lymphocytes Change from Th1 response to Th2 response with ↓cell-mediated responses directed against intracellular bacteria (Th1 function) and relative preservation of humoral (Th2 function) ↓ Treg population ($CD4^+$ $CD25^+$) that plays a role in the manifestations of autoimmunity Impaired immunological synapse formation and signaling pathways (calcium response, phosphorylations) ↓ CD4/CD8 rate	(28, 121–123)

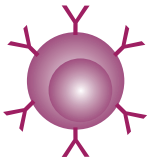
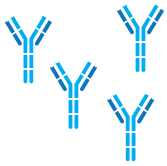
		CELL TYPE	CHARACTERISTICS	REFERENCES
Adaptive Immunity	**Humoral Response**	B Cells 	↓ pre-B lymphocytes with peripheral B lymphocyte count unchanged ↑ $CD5^+$ B cells ($CD19^+$ $CD5^+$ clones B) that produce low affinity antibodies without cooperation of T cell ↓ naïve B cells Accumulation of memory B cells with ↓ diversity and affinity of antibodies Reach primary humoral response (dependent T cell cooperation). Conserved secondary humoral response	(28, 60, 61, 64, 121, 124)
		Immunoglobulins 	↑ serum levels of IgA and IgG (IgG1, IgG2 and IgG4). Monoclonal immunoglobulin production by $CD19^+$ $CD5^+$ clones Secretion self-Ab non organ-specific (rheumatoid factor, antinuclear antibodies, antiphospholipid antithyroglobulines and parietal cells)	(62, 90)
		Interleukins 	↓ IL-2 production because ↓ cooperation of T cells with antibody producer B cells ↑ Production of IL-4, IL-6, IL-8, IL-10 and TNF-α ↓ Production of IL-1 and IFN-γ	

Table 1. Age-associated changes in immune cell populations and function. *fMLP*, N-formyl-methionyl-leucyl-phenylalanine; *GM*-CSF, Granulocyte Macrophage Colony-Stimulating Factor; *HLA*, Human Leukocyte Antigen; IFN-γ , Interferon gamma; *IL*, Interleukin; *LPS*, Lipopolysaccharide; *MHC*, Major Histocompatibility Complex; *PHA*, Phytohaemagglutinin; *PGE2*, Prostaglandin E2; *TLR*, Toll Like Receptor; *TNF-α*, Tumor Necrosis Factor alpha.

miR181a over expression lowers TCR activation threshold and restores TCR ability to respond to autoantigens (40). Note that miR-181a expression declines throughout life and shows an significant loss after the age of 70 (41).

Another interesting point is that there are reports indicating changes in gene expression of surface molecules on T cells. Changes in surface molecules may have a negative impact on T cell activation by increasing phosphatase expression such as DUSP family. Furthermore, a study evaluated the effect of aging on surface molecule gene expression and found that IL-6R, CD8, CD27, and CD28 show down-regulation while ILT2 (CD85j), KLRG, KIR, CD44, CD96, Klrf1, and CD94 exhibit up-regulation (42,43). Some of these molecules (ILT, KLRG, and KIR) function as negative regulators of TCR activation and proliferation (at least in murine models) or as specific molecules of particular T cells such as CMV peptide-specific T cells (KIR and ILT2). They also appear to be related to T cell exhaustion although the relationship between aging, senescent, and exhausted T cell gene expression seems to be different (Figure 2). An excellent review of this topic was done by Cavanagh *et al*. (44)

In addition to surface molecule gene expression alteration, there are other processes related to TCR signaling alteration and aging. For instance, it is well known that a cell-intrinsic environment (ROS species, DNA damage) and a cell-extrinsic environment (cytokines) both modulate TCR responses and that they are also altered in the elderly. Moreover, studies have shown that increased oxidative stress produces displacement of LAT from the cell membrane, thus inhibiting TCR signaling. Along with this, activated CD4 T cells from aging humans express increased levels of metallothoineins, which are an important redox system (45). Additionally, there are alterations in molecules that participate in nuclear and cytoplasmic signaling pathways such as DNA repair kinases (ATM, ATR, and DNA-PKCs), which are activated not only by DNA double-strand breaks (DSB) but also by telomere attrition (as we will see later). Both of them are related to activation of DNA repair kinases (ATM, ATR and DNA-PKcs) (46).

Host environment and specific cytokine profiles have enormous implications in the T cell signaling threshold. Some cytokines such as IL-7, IL-21, and IL15 have been studied in this regard. These cytokines signal through PI3K, STAT3, and STAT5 (47) and participate in ERK pathway activation. Note that IL-7 and IL-15 have profound implications in lymphopenia development in RA animal models. This has been shown through the fact that when animals are primed with IL-7 or IL-15, T cell response to autoantigens is enabled. In addition, another cytokine implicated in signaling alteration during aging is the IL-6. This cytokine has implications in the JAK-STAT pathway through activation of SOCS3 transcription. Note that STAT molecules are highly phosphorylated in elderly humans, a phenomenon that also occurs through type I interferon T cell activation (46).

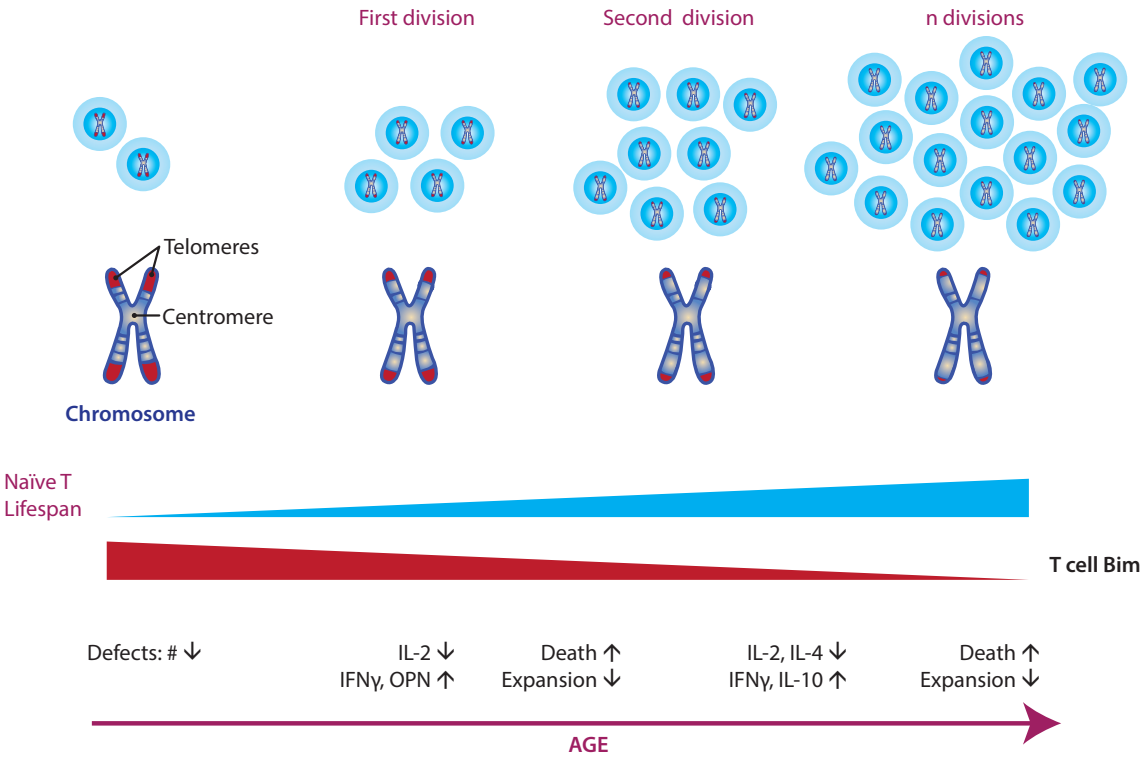


Figure 3. Schematic representation of T cell divisions and their implication in telomere erosion and aging. Constantly dividing cells are accompanied by a decrease in their telomere length, which is related to aging phenotype: Decreased Bim expression, increased naïve lifespan, and important functional changes. *IL*, Interleukin; *OPN*, Osteopontin.

As was discussed previously, telomere attrition is very frequent in elderly people and it is also known that T cell replication is important for maintaining lymphocyte function. This suggests that they employ the best mechanisms for telomere maintenance during clonal expansion. Indeed, there is evidence that telomerase activity is highly regulated during T lymphocyte development and differentiation (48). The resting $CD4^+$ and $CD8^+$ and naïve T cells recorded no telomerase activity on the periphery. However, the telomerase is activated by lymphocyte stimulation. The level of telomerase activity decreases during successive stimulations of the lymphocyte Figure 3 (49). The rate of telomere shortening seems to be different among $CD4^+$ and $CD8^+$ cells and it has been estimated to be 33bp/year for $CD4^+$ T cells and 26bp/year for $CD8^+$ T cells. One of the most outstanding features of aged $CD4^+$ naïve T cells is their inability to produce significant levels of interleukin 2 (IL-2) after stimulation of their TCR. This inability subsequently leads to poor Th1/Th2 polarization. However, these cells may retain their ability to suffer Th17 differentiation (50, 51), which, in turn, could favor an inflammatory and autoimmune phenotype development. On the other hand, the number of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) increases (2.4 fold) and they retain and gain functions during aging. Nonetheless, their ability to produce IL-10 is low. They may also contribute to Th17 bias (production of high levels of IL-17, IL-21, and IL-22) and show a decrease in antitumor responses too (51). Meanwhile, aging $CD8^+$ lymphocytes show a reduction in the diversity of the TCR repertoire, low antitumor response, and marked clonal expansion development but without the ability to replicate after stimulation (38). Note that Th2 inflammatory cytokines favor antibody production by B cells, and this condition could explain autoantibodies in the aged population.

One of the most important traits of immune aging is the loss of the CD28 surface marker. CD28 is one of the molecules expressed in T cells that provides co-stimulatory signals that are required for T cell activation, T cell proliferation, cytokine production, and T cell survival promotion. Loss of CD28 expression is a phenotypic change associated with senescence in T lymphocytes, and it has been associated with functional alterations such as: enhanced cytotoxicity, suppressive functions, and resistance of $CD4^+$ T cells to apoptosis. Loss of CD28 expression is characterized by telomere shortening and reduced proliferative ability, both *ex vivo* and *in vitro* (2, 52). At birth, virtually all T cells express CD28, but with age, the marker decreases about 40 to 50% for $CD8^+$ T cells and 85 to 90% for $CD4^+$. This reduction in the markers is attributed to repeated antigenic stimulation in peripheral blood (53). However, when the CD28 is lost, cells suffer reprograming and, as a consequence, they express new receptors such as KIR, CD70, and perforin. Moreover, phenotypic CD28- T cell characteristics include interferon gamma (IFNγ) production, potent cytotoxic capability as well as CD158, CD158b, CD158J, DAP12, CD94, and CD244

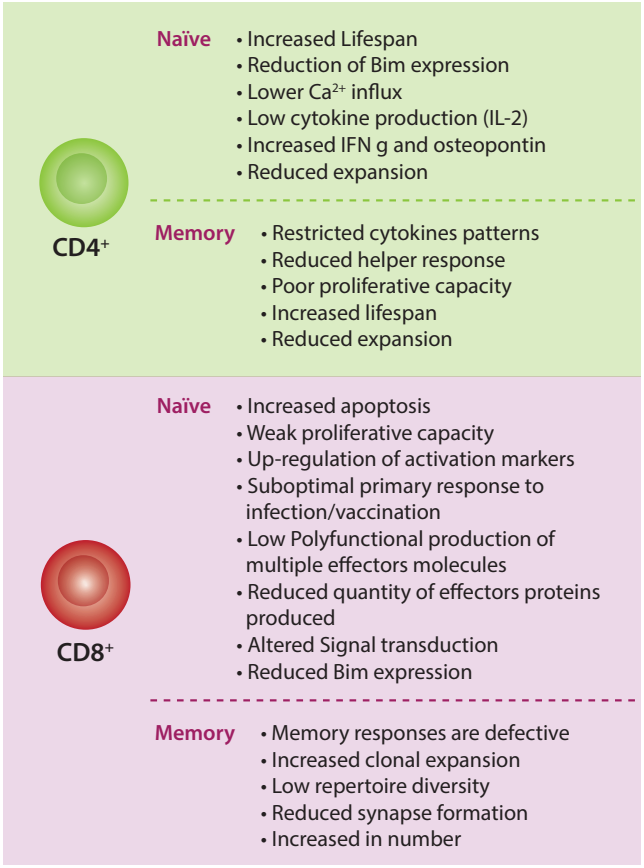


Figure 4. Aged-related functional changes in T cells subsets. Alterations are produced in both memory and naïve subsets. These alterations depend on the T cell micro-environmental history, exposures to stressor agents, and stochastic events. There are differences in changes between $CD4^+$ and $CD8^+$ concerning aging, but in both cases there is reduction of naïve subtype, increase in lifespan, and defective immune response.

receptor expression (similar to NK cell characteristics). These receptors give them the potential to interact with accessory cells such as mesenchymal origin cells, which include the fibroblasts of inflamed joints (54). Furthermore, in elderly individuals with chronic viral infections and autoimmune diseases (e.g., Multiple Sclerosis (MS), Rheumatoid Arthritis (RA), and Wegener's disease) an increase in the frequency of $CD28^-$ T cells has been detected (53). It has been suggested that autoantigens can lead to clonal expansion of these cells. Thus, there are reports (55, 56) indicating how they can, for instance, show reactivity to myelin basic protein (MBP). The presence of $CD4^+$ $CD28^-$ T cells in both elderly individuals and patients with ADs has supported the concept that ADs are closely related to the cell aging process. In this regard, the loss of CD28 molecule could favor CTLA-4 interaction with their ligands (CD80 and CD86), which are implicated in autoimmune phenotype too.

The main differential and functional alterations of $CD4^+$ and $CD8^+$ T cell subsets are summarized in Figure 4.

B CELLS

It is known that with aging, there is a decrease in not only the frequency and absolute number of pro-B cells but also their ability to differentiate into pre-B (between 60 and 90%). Nevertheless, in healthy individuals, mature peripheral B cell numbers do not change with age although the relationship between naïve and memory cells is altered, i.e., there is an increase in long-lived memory cells (homeostatic expansion of antigen-experienced or activated B cells) and a decrease in naïve cells (57). This condition seems to depend on different factors other than genetic ones. A study comparing old individuals with healthy centenarian offspring could determine that centenarian offspring have more IgD^+ $CD27^-$ naïve B cells than older people. Nevertheless, the double negative memory cells (IgD^- $CD27^-$ B cells) are only found in healthy elderly individuals, and there are no differences between groups (58). Recently, studies have reported a novel peripheral B cell subset in the elderly named aging–associated B cell (ABC) subset ($CD19^+CD11b^+CD11c^{+)}$ (59). *In vitro*, the ABC subset responds only to innate stimuli producing secretion of autoantibodies and cytokines, and this subset also has the ability to potentiate Th17 polarization, thus relating it to an autoimmune phenotype.

Another important fact is the presence of alterations in the repertoire of the B cell receptor (BCR), which exhibits a decreased affinity and diversity to the antibody response with aging (60). Indeed, elderly patients have impaired B cell proliferation and activation, possibly as a result of defects in their threshold of activation (38). Also, there is a loss of precision in distinguishing self from non-self antigens due to the oligoclonal expansion of the B lymphocyte subpopulation with a high proportion of antigen-experienced cells (61). This subpopulation expresses CD5 on their surface, thus giving them the ability to produce low affinity antibodies T cell-independently. In the context of autoantibody generation, this is important for triggering an autoimmune response.

Moreover, the germinal centers (GC) elderly people have are small and have few cells producing IgM. In these individuals, levels of immunoglobulins, especially IgA and IgG, are raised (62). Furthermore, it has been shown that IgG_+/IgA^+ B cell subsets (both $CD27^+$ and $CD27^-$) express Ig mutated genes in their variable regions and high levels of CD80 and CD86 on their surface, thus exhibiting a similar B cell memory phenotype (63). According to the reports, this subtype of cells declines with aging (62). Finally, aging B cells have been observed to produce antibodies with low avidity because of their somatic hypermutation deterioration which leads to a gradual decline in the humoral response. Nevertheless, repertory changes are not synchronous within aging, and decreased diversity has been related to poor health status (64).

An interesting study (65) done to evaluate naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) switch B cell subsets in elderly individuals showed a decrease in total B cells and, although the quantity of naïve cells rose as a percentage, they decreased or remained constant in number. Moreover, another striking result is that the B cell memory ($CD27^+$) rose as a percentage, but not significantly. In con-

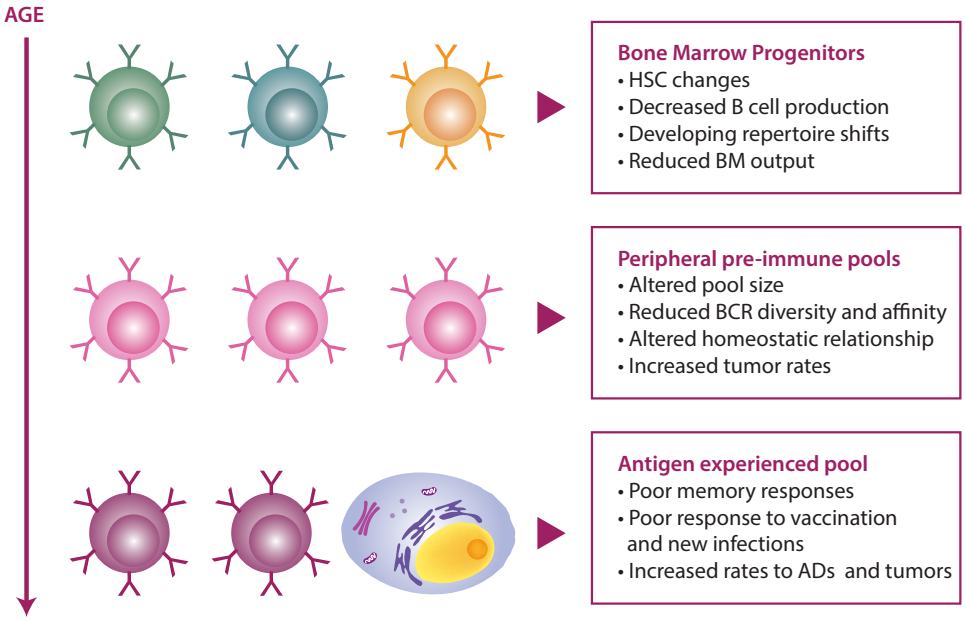


Figure 5. Age-related changes in the generation and function of B cells. There is a reduced output of B cells in the bone marrow, which induces accumulations in the periphery of antigen-experienced subsets with poor immune response and low diversity. *HSC*, hematopoietic stem cell; *BCR*, B cell receptor; *BM*, bone marrow. Adapted from (60)

trast, memory cells producing IgM subtype decreased in number but not in percentage. Finally, the memory switch cells decreased both in number and percentage with aging. An interesting point about memory B cells is that they have a hyporesponsive state to antigen-induced activation with less clonal expansion or less ability to differentiate into antibody secreting cells (66). This condition may be caused by the decreasing number of antibody-high affinity B cells that elderly people have.

Taken together, these evidences indicate that there is an accumulation of antigen-experienced B subsets in aging individuals. In these cases, overall B cell numbers are unchanged, but they vary in their functional abilities (Figure 5).

DENDRITIC CELLS

Dendritic cells (DCs) are important because they function as a checkpoint between immunity and tolerance. DCs from aging individuals display a basal level of activation, increased secretion of proinflammatory cytokines such as Tumor Necrosis Factor alpha (TNF-α) and IL-6 (67), and high levels of NF-κB activation. However, they do not exhibit CD86 and CD80 molecule upregulation on their surface which suggests that they are partially activated. Another important characteristic is that they are more reactive to self-antigens compared with their young counterparts and exhibit an impaired clearance of apoptotic cells and antigens (68). This could produce a higher production of self-antigens and, consequently, an activation of autoreactive lymphocytes. An interesting point is that these partially activated DCs have a greater ability to stimulate T cells, thus indicating that their ability to induce tolerance to self-antigens is affected.

Some explanations have been given regarding partially activated DCs: 1) there is an increased age-associated level of proinflammatory mediators; and 2) age-associated modifications in auto-antigens, which increase their immunogenicity (24).

Note that the functions of myeloid DCs (mDCs) such as IL-12 production, chemotaxis, and their ability to activate naïve CD4 T cells via antigen presentation appear to be altered in elderly individuals (68). This inability is due to decreased PI3k activation (69), which leads to activation of NF-κB, as was previously mentioned. This subtype of DCs also shows decreased capability in their antigen processing and increased expression of CD86.

Plasmacytoid DCs (pDCs) from elderly people, in turn, exhibit reduced type I IFN and type III IFN production after stimulation via TLR (70). Additionally, they have an impaired ability for antigen presentation to CD4 and CD8 T cells.

INFECTION AND IMMUNOSENESCENCE

To produce an adequate response to large numbers of pathogens throughout life, there are homeostatic mechanisms guaranteeing competent memory and a naïve cell pool for prolonging the survival of memory cells. However, under the conditions of advanced age, these mechanisms are seriously affected. As we have seen, during aging many changes occur in the immune system which means that immunosenescence becomes a factor which contributes significantly to a higher risk and severity of infections. The most important diseases in elderly are urinary tract infections, influenza and pneumonia, chronic viral infection reactivation (herpes virus, varicella-zoster virus) as well as bacterial (tuberculosis), fungal (candidiasis), or parasitic infections, and more rarely, opportunistic infections such as Clostridium and Staphylococcus (71, 72). Although the

immune response to antigens may be preserved in elderly individuals, their ability to be immunized against new antigens is reduced. This may be the result of an increase in the proportion of memory cells and progressive decrease in naïve cells from the thymus (73).

While it is true that aging is associated with the emergence of infectious diseases, it is also true that these infectious events will promote aging. It is well known that viral infections (particularly the herpes virus family) are strong stressors which alter the lymphocyte phenotype and functionality: altered cytokine profile, resistance to apoptosis, and shortened telomeres (74). These features are similar to those found in the elderly, thus it is possible that viral infections could represent an important extrinsic factor for aging by the repeated antigen stimulation characteristic of persistent latent infections (75). Furthermore, it has been suggested that latent herpes virus infections are primarily responsible for in vivo generation of senescent $CD8^+$ T cells, perhaps due to constant and prolonged virus-specific T cell proliferation (76). Additionally, the Epstein-Barr Virus (EBV) latent infection has also been associated with telomere shortening in antigen-specific $CD8^-$ T cells because EBV antigens cause a decrease in telomerase activity associated with T cell proliferation (77). In contrast to the above, (and related to telomere erosion and its relationship with CD28 molecule expression) the majority of T cells in a study done by Vescocini were $CD28^+$, unlike what was found for cytomegalovirus (CMV) which were mainly $CD28^-$ (78). During Human Immunodeficiency Virus (HIV) infection, in turn, it has been reported that early presence of $CD8^+$ $CD28^-$ T cells is a predictive characteristic of rapid disease progression (79).

These data indicate that chronic infections during aging produce significant changes in the $CD8^+$ cell subset. Additionally, this shows that expansion of $CD28^-$ T cells is age-dependent, and they have a positive correlation with proinflammatory cytokines. At the same time, these cytokines are heavily involved in the pathogenesis of immunological disorders which could favor the emergence of different pathologies including ADs.

AUTOIMMUNE DISEASE

Currently, it is clear that changes occurring in the immune system during aging affect the onset of ADs. This is due to the fact that aging is related to increased reactivity to self-antigens and loss of tolerance. The overall tendency supports this hypothesis because elderly people experience general systemic inflammation and, at the same time, they aggravate degenerative diseases, which, in turn, increase the risk of developing ADs (80, 81). In addition to this, it is important to remember (as we have seen previously) the epigenetic changes occurring in elderly people and how these may affect important genes involved in autoimmune disorder development (23). Another important aspect of aging that is closely related to ADs is the increase in inflammatory cytokines and chemokines such as TNF-α, C-reactive protein, IL-8, MCP1, and RANTES (82,83). There is a substantial amount of evidence of age-associated alterations in the T cell cytokine profile which could contribute to development of ADs. Studies have shown that there is a change from Th1 to Th2 (mainly IL-4 and IL-6) in the cytokine profile as age advances (84). IL-6 is a potent proinflammatory cytokine closely related to disability in patients with RA; therefore, it represents a therapeutic target for this disease (85). In addition, there are reports of an imbalance between Th17 and Treg cells. A considerable number of IL-17-secreting naïve $CD4^+$ T helper cells have been detected in the elderly in contrast to reduced IL-17-secreting memory $CD4^+$ T helper cells (86).

Some ADs are very frequent in younger patients and are not limited to elderly people although the occurrence or presence of autoantibodies is greater at advanced age (87, 88). Autoantibody production such as rheumatoid factor as well as antinuclear, antiphospholipid, and antithyroglobulin antibodies are present during aging (88, 89). Autoantibody production has been attributed to altered T and B cell function (90), especially to the decrease in antibody affinity maturation. This evidence supports the idea that autoantibody levels may be closely related to the clinical characteristics of elderly and to patients with ADs.

One of the important causes of dysfunctional immune responses is telomere abnormalities which may lead to autoimmunity. This observation is significant since numerous studies have shown an association between mean telomere length in peripheral blood mononuclear cells (PBMCs) and different diseases (74). This evidence suggests an increase in $CD8^+$ $CD28^-$ T cell proportions in several pathologies such as in the case of some ADs as shown below.

Reports of telomere length alteration in patients with ADs such as RA (91,92) scleroderma (SSc) (93), Systemic Lupus Erythematosus (SLE) (94), Wegener's disease (95), psoriasis, and atopic dermatitis (96), suggest an excessive cell replication with its corresponding telomere erosion. These findings have been interpreted as evidence of T cell accelerated proliferation in the autoimmune process.

At present, it is believed that there are differences among telomere abnormalities and various ADs. Some of these differences could be explained by the genetic background of the individuals studied. For example, a study done of patients with SSc and their family members reported short telomeres (93). The idea of a genetic predisposition to telomere shortening is also suggested in patients with RA, who exhibit telomere erosion in not only memory cells but also naïve cells. Moreover, this evidence shows acceleration in telomere erosion occurring at the precursor cell level (97). Another striking fact is that the genetic predisposition to short telomeres is strongly related to HLA-DR4 haplotype which is shared by RA and T1D in some individuals (98, 99).

Some reports concerning the relationship of immunosenescence with some ADs are considered below with telomere dysfunction receiving the most emphasis.

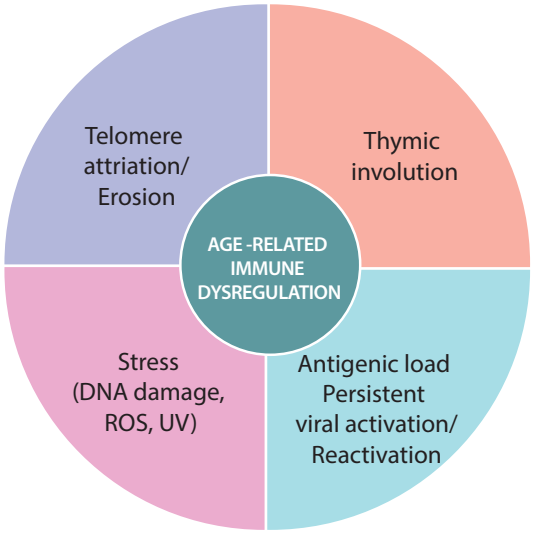


Figure 6. Main mechanisms contributing to immune dysfunction during aging. Age immune dysregulation is achieved by the combination of intrinsic (telomere erosion/attrition) and extrinsic (stress, thymic involution and viral infections) factors within the cell environment. *ROS*, Reactive Oxygen Species; *UV*, Ultraviolet.

RHEUMATOID ARTHRITIS

Lymphocytes, especially CD4 lymphocytes which are involved in the synovial tissue inflammatory infiltrates, play a major role in this condition (100). Also, it is well known that the above mentioned lymphocytes aid in the production of proinflammatory cytokines and autoantibodies. Memory T cells of RA patients are characterized by increased loss of CD28 expression and reduced TCR diversity (101). In fact, shorter telomeres in $CD4^+$ $CD45RA^+$ T cells from patients in comparison to control subjects has been reported (91). However, these cells reach appropriate clonal expansion. It is noteworthy, as we have mentioned previously, that individuals with the HLA-DR4 haplotype exhibit greater telomere loss;nonetheless, this loss does not induce cell cycle arrest or apoptosis (92). Furthermore, another important fact is the lack of telomerase activity in $CD4^+$ naïve T cells when they are stimulated through their TCR. This phenomenon applies to memory cells or hematopoietic progenitor $CD34^+$ cells although the latter have a significant loss of telomeres with aging (97). This is a key point because it highlights the possibility that telomere abnormalities may originate in these patients' bone marrow.

Telomere erosion in RA patients is not only attributable to the factors described above but may also be related to a defect in telomerase activity (97). Moreover, it could be attributed to an incorrect DNA break repair by the ataxia telangiectasia mutated kinase (ATM) enzyme or by induction of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) which eventually leads to a stress state and promots cellular DNA damage (102, 103).

Another point to keep in mind is the relationship between TCR alterations detected in aged-individuals and RA. TCR/CD3 assembly can be disrupted by chronic exposure to high TNF-α concentration, which contributes to not only reduced calcium influx response (104) but also down-regulation of CD28 expression. These changes could be responsible for low T cell response in RA patients.

SYSTEMIC LUPUS ERYTHEMATOUS

Currently, it is not clear if there is a relationship between telomere loss and SLE. Some studies have shown an increased telomere erosion in SLE patients (105,1 06) while others report normal telomere length when compared with healthy controls (107, 108). Nevertheless, it is clear that there is a reduction in telomerase activity in naïve $CD4^+$ T cells and an increase in this activity in B cells (106,108). In this regard, a recent study showed a differential expression of shelterin complex molecules in patients with lupus (109), but, unfortunately, it was not done cell-specifically.

Abnormalities in TCR signaling which have been documented in SLE patients are similar to those in RA patients. This may also be related to the elderly, for instance, TCR zeta chain expression is defective in these patients (110).

TYPE 1 DIABETES

Few studies have focused on aging and this disease. However (as has been reported for RA), studies have shown a significant telomere shortening in the PBMCs of T1D patients (111). A high susceptibility to T1D associated with the HLA-DR4*04 haplotype risk has been described and may be partly explained by a telomere loss similar to what has been found in RA patients. Curiously, T1D patients have been shown to have a high frequency of $CD4^+$ $CD28^-$ T cells (112), a phenotype that is highly associated with inappropriate immune aging.

CONCLUDING REMARKS

In a world where the elderly population is higher every day and, in which, its mortality and morbidity are due to infectious diseases, it is necessary to understand the changes in the immune response produced by immune system aging. Many of these changes also participate in the development of certain pathologies such as ADs. To date, the mechanisms and causes of these changes are not clear but there are three main processes that could explain this phenomenon: 1. thymic involution, 2. intrinsic damage, and 3. chronic antigenic stimulation (Figure 6). Thus, the microenvironments, soluble factors, surface and signal transduction molecules as well as processes such as telomere erosion and infection diseases can result from a decreased immunity through aging. Understanding these mechanisms will make it possible to establish appropriate treatment strategies, optimize responses to pathogens, and improve the quality of life for the elderly.

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12 THE ENDOCRINE SYSTEM AND AUTOIMMUNITY

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INTRODUCTION

The human immune system is a complex network of soluble factors and specialized cells that interact with genetic and environmental stimuli in order to maintain health and lifelong protection. Autoimmune diseases (ADs) are the result of the combination of genetic and environmental factors. ADs have a very strong female gender bias, and as a consequence, hormones have been implicated as environmental factors in driving the disease (1). A variety of evidence supports the existence of bidirectional communication between the central nervous (CNS), endocrine, and immune systems from the embryonic period until the end of life. This interaction is challenged by stressors such as infections, autoimmune diseases, or trauma that activate the immune neuroendocrine system. The main stress response systems are: 1. the hypothalamic-pituitary-adrenal axis (HPA), 2. the hypothalamic-pituitary-gonadal axis (HPG), 3. the hypothalamic-pituitary-thyroid axis (HPT), 4. prolactin /growth hormone (PRL/GH) system, and 5. the autonomic nervous system (ANS). Hormones, neuropeptides, and cytokines which are released by cells in these systems and autonomic nerves act through receptors to activate or inhibit the immune response (2-5). The rupture of this homeostatic-molecular balance participates in autoimmunity and in the pathogenesis of ADs (4,5).

There is increasing evidence that the immune system is regulated by circadian rhythms. Critical immune mediators, such as cytokines, undergo daily fluctuations. Circadian information reaches immune tissues mainly through diurnal patterns of autonomic and endocrine rhythms (6). The aim of this chapter is to analyze the immune-neuro-endocrine interaction in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) – the prototype of non-organ specific ADs, – and autoimmune uveitis, which is an example of organ-specific ADs in order to obtain a better understanding of these entities and to optimize treatment strategies based on new perspectives.

IMMUNE-NEURO-ENDOCRINE INTERACTION AND RISK OF DEVELOPING AN AUTOIMMUNE DISEASE

It has been established that the immune-neuro-endocrine system controls diverse physiological processes, e.g., growth and cell differentiation, immune response, metabolism, and human behavior. Hormones such as cortisol, corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), PRL, estrogens (E), progesterone (P), androgens (A), GH, insulin-like growth factor (IGF-1), thyroid stimulating hormone (TSH), etc., regulate a great variety of immunologic events. Moreover, cytokines released by immune system cells affect the neuro-endocrine system. Cytokines frequently act on the hormones and growth factors as a regulatory signal (Chapter 9). This homeostatic balance system is active even in healthy individuals and is mediated by specific receptors for cytokines, hormones, and neuropeptides, which are distributed around the immune-neuro-endocrine system and other tissues (7) (Figure 1).

During a local or systemic inflammatory/immune process, cells from the immune system release pro-inflammatory cytokines (interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6)) which cross the blood-brain barrier and reach the brain. Blood-brain-barrier disruption is mediated by lipopolysaccharides and cytokines (8, 9), which are potent activators of the CNS functions and produce behavioral changes, cognitive alterations, arthritis, and fever with consequent activation of stress response systems. The injection of IL-1 in mice produced an HPA axis activation and plasmatic release of

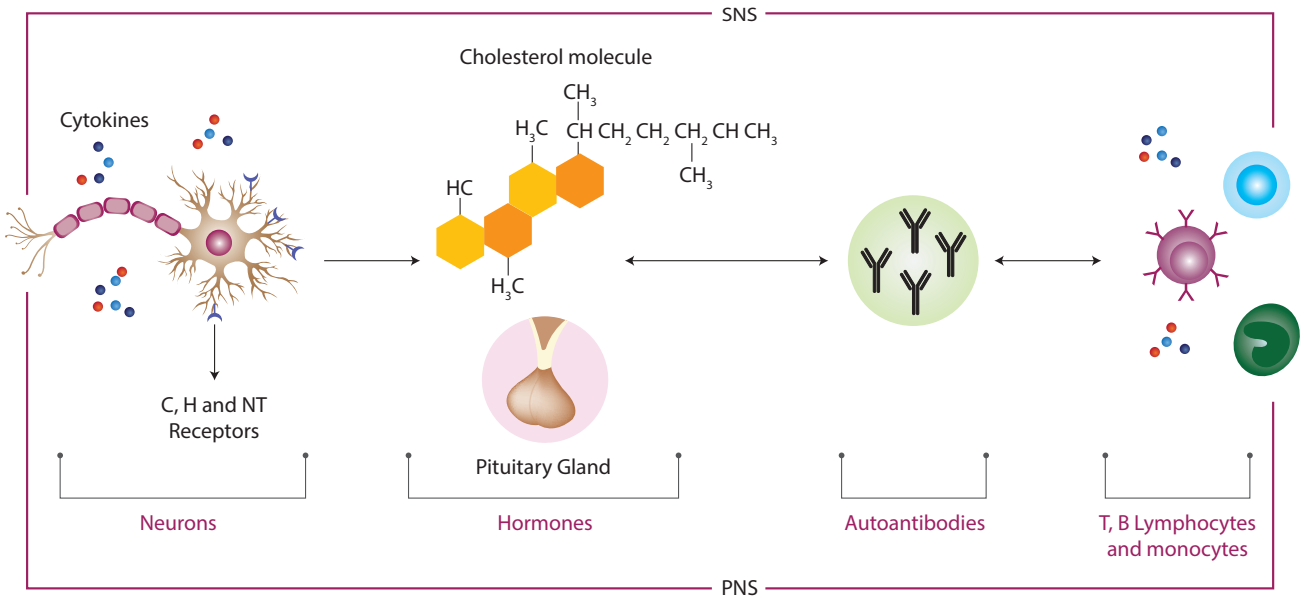

Figure 1. Relationship among neurons, hormones, autoantibodies, and immune cells. The interaction is mediated by cytokines (C), hormones (H), and neurotransmitters (NT) Receptors of these molecules are present in immune-neuro-endocrine system.

ACTH and cortisol (8). Besedovsky *et al*. (10) considered their results a direct effect of IL-1 over the HPA axis. This hypothesis was confirmed later with the demonstration of neuronal activation of the paraventricular nucleus of the hypothalamus which controls CRH and arginine-vasopressin release (8).

A characteristic of ADs is the loss of immune tolerance. It has been demonstrated that tolerance occurs when the antigen presenting cells process and present the antigen in the context of a diminished expression of the Class II Major Histocompatibility Complex (MHC-II) and co-stimulating molecules in a micro-environment that contains cytokines and anti-inflammatory hormones (i.e., IL-4, IL-10, cortisol, P, A). When the micro-environment is related to cytokines and pro-inflammatory hormones (i.e., IL-1, TNF-α, IL-6, E, and PRL), the immune response will be aggressive with a consequent loss of tolerance and the development of an autoimmune disease (11). During systemic infections, cancer or ADs, the immune signals to the brain can lead to an exacerbation of illness and the development of depression. Inflammation is, therefore, an important biological event that might increase the risk of depressive episodes (12). It is clear that an alteration of these processes of physiological regulation may evolve into a potential risk factor for the development of autoimmune rheumatic disease (ARD). In fact, an abnormal response of the HPA axis in patients with RA, SLE, Sjögren's syndrome (SS), and fibromyalgia has been found. However, these alterations may be the consequence of the inflammatory and immunological abnormalities observed in these entities (13). The evolution from acute inflammatory disease to chronic disease is an important process. In this regard, in ARD and other chronic inflammatory diseases, large amounts of energy for the activated immune system have to be provided by energy metabolism. Therefore, a new theory using insights from evolutionary biology and neuroendocrine immunology was developed describing the moment of transition from acute inflammatory disease to chronic inflammatory disease as a time in which energy stores become empty (complete energy consumption). Depending on the amount of stored energy, this point in time can be calculated to be 19-43 days (14).

Genetic, environmental, hormonal, and immunological factors are determiners for the development of ADs. In relation to environmental factors, retrospective studies have found that a high proportion of patients (80%) mention an unusually stressful situation before disease onset. Furthermore, illness by itself generates stress. Hormones released under stressful situations lead to an alteration of the immune response by increasing synthesis and release of cytokines. Therefore, AD treatment should include stress management in order to prevent immunological imbalance and reactivations (15).

Hormonal changes prior to the development of an ARD have been little analyzed. A group of pre-menopausal women who developed RA before the age of 50 had low serum levels of dehydroepiandrosterone (DHEA), cortisol, and testosterone (T) along with an increase in IL-1, TNF-α, and IL-6 12 years before the appearance of the disease. These findings suggest a disarrangement of the immune-neuroendocrine system in a pre-symptomatic phase of RA (16). Some patients have presented hyperprolactinemia (HPRL) before the development of Graves' disease, dermatomyositis, SS, and SLE. A group of SLE patients with prolactinoma has been described. A subset of these patients presented HPRL 5 years before SLE diagnosis, and they developed SLE manifestations when their PRL levels were between 20-40 ng/ml (3,17).

A recent study analyzed whether childhood traumatic stress increased the risk of developing AD in adult life. The conclusion was that childhood traumatic stress increases the likelihood of hospitalization with a diagnosed AD in adulthood (18). Another study investigated whether stress following major and minor life events might precede the onset of SS. A higher number of patients with SS reported the occurrence of negative stressful life events prior to disease onset compared with patients with lymphoma and healthy controls (19).

In summary, immune-neuro-endocrine alterations may precede the onset of ARD by several years. These alterations should be identified in individuals with genetic susceptibility to the development of ADs.

IMMUNE–NEURO-ENDOCRINE INTERACTION IN RHEUMATOID ARTHRITIS

Rheumatoid arthritis is an ARD characterized by a complex interaction between genetic, environmental, hormonal, and immunological factors. Women are at greater risk of developing RA than men which suggests the involvement of the hormones in the development of the disease. The molecular explanation for this clinical observation is based on experimental models of inflammation, which have shown a relationship between the H-P-A axis and cytokine production. The central and peripheral nervous systems and the endocrine system interact with the immune system in the development of experimental arthritis. Multiple clinical observations have shown the significant influence of the immune-neuro-endocrine system on RA, e.g., remission of the disease during pregnancy, protective effect of oral contraceptives, reduced risk with hormone replacement therapy, protective effect of hemiplegia, influence of psychological stress on the development of RA, and influence of the circadian rhythm of hormones on the inflammatory symptoms (20-23). The clinical and therapeutic importance of the multiple disturbances of the immune-neuro-endocrine system in RA is discussed below.

HYPOTHALAMUS-PITUITARY-ADRENAL AXIS (HPA)

The initial studies done at experimental level demonstrated that the susceptibility of Lewis rats (LEW/N) to an inflammatory/autoimmune disease similar to RA is due, at least in part, to an abnormal response in the HPA axis (CRH, ACTH, and glucocorticoids) (24).

Replacement of glucocorticoids in these rats reverses susceptibility to developing arthritis. HPA axis alteration in LEW/N rats is due to a decrease in the release of CRH which is genetically determined by the inhibition of its messenger RNA and alterations on pro-opiomelanocortin, globulin binding glucocorticoid, and its receptors (25). However, a normal response by HPA does not necessarily protect rats from the development of arthritis. As has been demonstrated in other experiments, rats exposed to high levels of stress develop arthritis despite high levels of cortisol (26).

In arthritis induced by immunization of rats with type II collagen, corticosterone levels rose only transiently, and adrenaline blood levels, hypothalamic IL-1beta and IL-6 overexpression rose during the induction phase of the disease. The increase in hypothalamic noradrenaline content during the symptomatic phase was paralleled by a gradual loss of sympathetic fibers in the joints. No correlation between hypothalamic IL-1beta expression and noradrenaline content was observed. The dissociation between hypothalamic cytokine gene expression and noradrenergic neuronal activity, the lack of sustained stimulation of the stress axes and the loss of sympathetic signals in the joints indicate that the immune-neuro-endocrine communication is disrupted during experimental arthritis, thus producing chronification of arthritis (27).

The alteration in immune-neuro-endocrine communication plays a role in osteo-articular damage. In an interesting study, LEW/N rats were selected as high-active (HA) and low-active (LA) animals according to neuro-endocrine and immune reactivity. During adjuvant-arthritis (AA), no differences in the severity of inflammation and corticosterone response were observed. However, LA rats had more osteoporosis, periostal new bone formation, and bone destruction than HA rats as determined by radiographs taken on day 30. Splenocytes of LA rats had lower production of IL-10 and IFN gamma. Histological examination revealed more intense factor VIII staining in arthritic joints of LA animals that indicates synovial angiogenesis with high plasma VEGF (an important angiogenic factor). Expression of RANKL, a crucial factor promoting bone resorption, was also higher in joints of LA animals. Lower production of bone-protective cytokines and a higher rate of angiogenesis leading to more synovial proliferation may be responsible for the severe joint destruction in LA animals (28)

In RA patients, cortisol levels similar to control subjects have been found, even in the presence of high pro-inflammatory cytokine levels, which represent an inefficient response in this pro-inflammatory environment from the early stages of the disease (29, 30). Subsequent studies showed that after experiencing physical or psychological stress, patients with RA have low cortisol levels due to defects in the HPA axis. Other studies showed that the circadian rhythm of cortisol is lost in patients with active RA, and the HPA axis response to surgical stress is impaired by defects in the expression of CRH (3).

These disruptions are mediated by hypothalamic and pituitary hormones and some pro-inflammatory cytokines such as IL-6 and TNF-α. In fact, it was demonstrated that anti-TNF- α therapy prevents abnormalities of the HPA axis in patients with RA and reduces fatigue, indicating that pro-inflammatory cytokines alter CNS function (31-33). A recent study showed that patients with good response to anti-TNF-α treatment also presented an increase in serum cortisol levels in contrast to the patients with poor response. This study demonstrated for the first time that, inflammation induced by TNF-α interferes with HPA axis integrity in humans and correlates with treatment response. Determination of plasmatic cortisol may be a sensitive marker to predict response to anti TNF-α treatment (34).

HYPOTHALAMUS-PITUITARY-GONADAL AXIS (HPG)

In patients with RA, an abnormal metabolism of sex hormones has been demonstrated systemically and locally (in synovial fluid) starting from the early stages of the disease. While low serum concentrations of T, dehydrotestosterone, DHEA, and dehydroepiandrosterone sulfate (DHEAS) have been found, E levels are normal or even high (35). From the start of treatment with disease modifying antirheumatic drugs, a recent study evaluated changes in the HPG axis in men with early RA over a 2-year period. Men with RA, including patients older than 50, had mean T levels that were lower than the controls had early in the course of the disease which suggested mild hypogonadotropic hypogonadism. In patients who responded to treatment, T levels increased significantly. A decrease in DAS28 during the 2 year follow-up significantly correlated with increased T levels (36). A subset of glucocorticoid-naïve premenopausal females with RA had a relative hypocompetence of adrenocortical function with low levels of basal cortisol and DHEAS as well as alterations in adrenal synthetic pathways or deficiencies in steroidogenesis (37).

Furthermore, an accelerated conversion of androgenic precursors to 17-β-estradiol has been suggested. This imbalance in E metabolism results in an elevation of 16α-hydroxiestrone/4-hydroxiestradiol metabolites (38). These hydroxilated E metabolites have the ability to stimulate monocyte proliferation and growth and to play a role in synovial hyperplasia (39). These disturbances may be attributed to the fact that some pro-inflammatory cytokines (i.e., TNF-α, IL-1, IL-6) stimulate aromatase activity. This partially explains the abnormal peripheral synthesis of E in RA and their higher availability in the synovial fluid (40). This imbalance leads to a decrease in the increased androgen to estrogen conversion in the synovial fluid of RA patients and thus indicates the existence of a proinflammatory hormonal environment (41,42).

In addition, synovial cells express E receptors that correlate positively with synovial secretion of IL-6 and IL-8 (43). Therapeutic blocking of TNF-α has demonstrated a benefit because it increases DHEAS levels, indicating that biological therapy may improve the HPG axis function (44).

AUTONOMIC NERVOUS SYSTEM (ANS)

The studies of the autonomic nervous system in patients with autoimmune diseases have shown conflicting results because symptoms of autonomic dysfunction are nonspecific and extremely varied. Parasympathetic and sympathetic dysfunction has been detected in 24% and 100% respectively of patients with AD. Cardiovascular autonomic dysfunction is the most common type of autonomic disturbance in patients with ARD (45).

Several abnormalities of ANS or the peripheral nervous system occur at different stages of RA (46). Activation of the sympathetic nervous system (SNS) and the parasympathetic nervous system in RA occurs parallel to HPA axis activation and to cytokine, hormone, and neuro-transmitter expression. This hypothesis is based on experimental models. In the arthritis model induced by type II collagen, it has been found that a transient increase in cortisol is followed by a rise in adrenaline levels and hypothalamic overexpression of IL-1β and IL-6 during the arthritis induction phase. The symptomatic phase showed an increase in hypothalamic noradrenaline followed by loss of noradrenergic fibers in joints. These findings indicate a disruption of immune-neuro-endocrine communication and noradrenergic activity during experimental arthritis (47).

In patients with juvenile idiopathic arthritis (JIA), an increase in SNS tone has been found. This data shows that the function of the ANS patients with JIA has been altered. The alteration is associated with a higher central noradrenergic outflow that presumably leads to increased vasoconstriction and results in a decreased response to an orthostatic stressor (48). Patients with early RA have high cardiac SNS activity while the parasympathetic activity is normal. These results suggest that inflammatory stress is responsible for these alterations in patients with RA (49). Tissue with inflammation from RA patients had less sympathetic nervous fiber in comparison with patients with osteoarthritis or trauma. Sensory nerve fibers contain two major neuropeptides, substance P (SP), and calcitonin gene-related peptide (CGRP). The pro-inflammatory role of SP is known, while CGRP has anti-inflammatory activities. In RA, there is an increase in sensory fibers and SP and a decrease in sensory fibers for the peptide related to the calcitonin gene (CGRP) in comparison with osteoarthritis (50,51). Some markers of SNS activity in patients with RA have been identified. Semaphorin 3C, a factor directed against sympathetic nerve fibers seems to be the main one responsible for reduction of tissue innervations in RA (52). Soluble neuropilin, a nerve repellent receptor, is another factor responsible for the disappearance of sympathetic nerve fibers soon after the beginning of inflammation (53). There are elevated levels of adrenal chromographin A in RA patients (54). Activation of SNS has pro- and anti-inflammatory effects, depending on the stage of the inflammatory process and the stimulated receptors. The acute phase of RA is characterized by β-adrenoreceptor stimulation with pro-inflammatory effects, whereas the chronic stage discloses stimulation of α-adrenoreceptors with anti-inflammatory effects (55).

The increased activation of ANS negatively influences the RA course by participating in the increase in cardiovascular risk observed in RA patients. Experimental evidence indicates that the SNS is critically influenced, at both the central and peripheral level, by the factors regulating vascular function such as nitric oxide, reactive oxygen species, endothelin, and the renin-angiotensin system. Additionally, there is indirect evidence of a reciprocal relationship between endothelial function and SNS activity (56).

Early detection of SNS hyperactivity in RA patients may be helpful in preventing these complications. In this regard, neuropeptide Y (NPY), an excellent indicator of sympathetic activity, is elevated in patients with RA and SLE and could be used as a sympathetic hyperactivity marker (57). However, another study found that NPY levels did not differ between RA and the

control group or in heart rate variability parameters considered to reflect sympathetic activity (58). In any case, the HPA axis and the SNS act synergistically in many ways.

An association between variants in the gene encoding the beta 2-adrenergic receptor (ADRB2) and RA have been demonstrated. In contrast, no association was found between JIA and alleles, genotypes, or haplotypes of ADRB2, especially in the case of the haplotype R16/Q27. These observations suggest that JIA and RA have a specific genetic association (59).

In summary, in patients with RA and other chronic inflammatory diseases, an ANS activity with increased sympathetic nervous tone is reported. The reason for this high sympathetic activity is probably the inefficient cortisol production in RA and other types of chronic arthritis (60).

PROLACTIN-GROWTH HORMONE SYSTEM (PRL-GH)

Initial studies in humans did not show abnormalities in PRL and GH levels in RA patients. However, PRL bioactivity, using NB2 cells from mice lymphoma was significantly lower in comparison with healthy controls matched by age and gender which suggests a PRL deficiency in RA (61). The first evidence of a PRL role in arthritis was found in children who had juvenile chronic arthritis with positive antinuclear antibodies, and who had a significant PRL increase in direct correlation with IL-6 serum levels (62,63). However, studies in RA patients have not shown a consistently higher PRL. A correlation among higher PRL levels, clinical activity, and macrophage activity indicators such as macrophage inflammatory protein-1-alpha (MIP-1α) (64) was found. An interesting study in males with RA found that high PRL levels were associated with long-term RA and a poor functional classification (65). These findings have been confirmed, and they are associated with RA activity as well (66). PRL levels in synovial fluid from RA patients are similar to those of patients with osteoarthritis, but GH and other gonadal hormones and neuropeptides were also elevated in the synovial fluid of both groups of patients suggesting that these hormones have a local pro-inflammatory role (67). In summary, one third of the RA patients have been found to present basal HPRL indicating that this hormone plays a role in the inflammatory process of RA (17).

Hormonal functional studies allow us to analyze a normal or abnormal pituitary response. In RA, the results of these studies are controversial. One of the first studies demonstrated that patients with RA developed HPRL and a dissociation of the ACTH response after stimulation with TRH and CRH respectively independently of the clinical activity (68). This alteration is more evident in HLA-DR4+ patients than in HLA-DR4- patients and this suggests an alteration of HPA and PRL release in post-menopausal women with RA (69). Another study demonstrated that RA patients secreted excessive amounts of PRL during a stressful situation, e.g., surgery in comparison with chronic osteomyelitis patients. High PRL levels may contribute to disease activity by increasing the inflammatory/immune process regardless of genetic factors (70). However, in patients with a recent RA diagnosis and without steroid treatment or remission induction drugs, there was no difference between patients and controls in ACTH, cortisol, PRL, and TSH serum concentrations before and after stimulation with their respective hypothalamic releasing hormones (71). These discrepancies may be due to multiple factors including the hour at which the study was done. Studies done in the afternoon are not suitable since pro-inflammatory cytokine release is lower and the rheumatoid factor in early RA is absent; hence, this can suggests a diagnosis other than RA. Other studies employed an insulin induced hypoglycemia stress test in order to measure cortisol, TRH, and PRL in active RA patients without steroids and demonstrate an HPA axis dysfunction and normal PRL release (72). Another investigation found that PRL and GH responses to hypoglycemia stress were similar in RA patients under treatment and controls (73) although the receiver operating characteristic (ROC) curve analysis of PRL secretion was minor in RA patients. Other authors using the same stimulus found a lower PRL response in patients with non-treated active RA which normalized after conventional RA treatment. These findings suggest that RA activity and/or conventional treatment affect the regulation of central secretion of PRL (74). An analysis of this controversy indicates that there are patients who are highly responsive to the stress test and others that are not. Moreover, RA activity with the release of pro-inflammatory cytokines that modulate PRL response seems to be more important for assessing the response to hypoglycemia than for treatment (75).

In this regard, a study was done to evaluate the relationship between the level of leptin, PRL, IL-4, and IL-5 and the activity of RA and SLE. The authors concluded that while leptin cannot be used to evaluate disease activity in these entities, PRL can be utilized to assess disease activity in RA and SLE (76).

Despite these controversies, PRL receptors were identified in synovial cells and lymphocytes which infiltrate the synovial membrane with experimental evidence that T cells and fibroblasts of patients with RA may produce PRL. PRL increases synovial proliferation and the production of pro-inflammatory cytokines and metalloproteases. Bromocriptine (BRC) reduces not only PRL production but also pro-inflammatory cytokines and collagenases by RA synovial cells (77).

In support of these findings, a recent study evaluated the levels of PRL in the serum and synovial fluid of patients with RA and OA. The PRL that was found in the serum and synovial fluid was significantly higher in RA than in OA. The PRL synovial fluid correlated significantly with the RA activity and serum PRL levels with the total Larsen score. The increase in PRL may play a role in disease severity and the joint damage of patients with RA (78).

Non-controlled studies suggest clinical improvement of the disease with the use of BRC (79). Treatment of RA with BRC induced a significant decrease in the immune function *in vitro*, and these changes correlated with an improvement

in activity parameters of RA (80). A case of RA refractory to conventional treatment improved with cabergolide, a PRL antagonist indicated for coincident HPRL treatment (81).

The PRL gene is located in close proximity to the HLA region on the short arm of chromosome 6. In this regard, it has been hypothesized that the associations between DR4 and reproductive risk factors in RA are due to linkage imbalance between DR4 and an abnormally regulated PRL gene polymorphism (82). In fact, there is linkage disequilibrium between HLA-DRB1 disease susceptibility alleles and microsatellite markers close to the PRL gene among women with RA and SLE (83). In contrast, none of the polymorphisms of neuroendocrine genes, including PRL gene, showed any statistically significant associations with JIA (84).

The PRL 1149 T (minor) allele decreases PRL expression and may be associated with AD. In order to determine the role of the PRL -1149 G/T polymorphism (rs1341239) in RA susceptibility, the association between PRL -1149 G/T and RA risk was examined in 3,405 RA cases and 4,111 controls. The results of this study indicate a possible association between the PRL -1149 T allele and decreased RA risk (85).

In conclusion, there is a growing body of evidence demonstrating an intriguing link between PRL and RA in experimental models and humans. New studies are necessary in order to find the PRL action mechanisms in RA and to test the efficacy of PRL antagonists in this disease (86).

IMMUNE-NEURO-ENDOCRINE INTERACTION IN SYSTEMIC LUPUS ERYTHEMATOSUS

SLE, the prototype of autoimmune-inflammatory disease affecting women, reveals that hormones act as an immuno-modulator. The main evidence about the role of hormones and their therapeutic consequences in SLE patients is discussed here.

HYPOTHALAMUS-PITUITARY-ADRENAL AXIS

The existence of a defect in the HPA axis in SLE has been shown in murine and human SLE. The MRL/lpr murine model presents a significant decrease in cortisol levels after stimulation with recombinant IL-1 (87). HPA axis studies are scarce and are influenced by treatment. Patients with SLE and moderate activity have decreased cortisol levels with normal ACTH both basal and after stimulation. Functional studies of responses to induced hypoglycemia demonstrated decreased cortisol in women with active SLE (88). In contrast, a recent study did not show alterations in the HPA axis in SLE patients without treatment. These contradictions may be due to the small sample size and the different methods employed (89). However the overall analysis of ACTH, androstenedione, cortisol, and DHEAS before and during a CRH test in patients with moderately active SLE undergoing low dose long-term glucocorticoid therapy showed marked adrenal insufficiency and a shift in steroidogenesis to cortisol in these patients but a completely normal pituitary function. This may depend in part on prior long-term glucocorticoid therapy and changes in steroidogenesis due to cytokines. Further longitudinal studies on untreated patients are necessary to investigate the endocrine-immune interplay and its consequences during the course of SLE (90).

HYPOTHALAMUS- PITUITARY-GONADAL AXIS

The major risk factor for developing SLE is being young and female. Menses and pregnancy may cause an SLE flare. These observations strongly suggest a role for E and A in gender bias and in immunological and clinical manifestations of human SLE. In fact, abnormalities in both E and A metabolism in SLE patients have been demonstrated. Increased hydroxylation of estradiol at c-16 (potent E metabolite) was found in both males and females with SLE when compared to normal subjects. The conversion of upstream A precursors to 17β-estradiol is accelerated in RA and SLE patients (91,92). Specific abnormalities of A metabolism associated with SLE may contribute in some way to morbidity and mortality. The reduction of serum concentrations of DHEAS, T, and P in both male and female RA and SLE patients strongly supports the existence of accelerated peripheral metabolic conversion of upstream A precursors to 17β-estradiol. These data may have implications for future therapeutic regimens based on male hormone replacement (93).

A recent study utilized gene profiles of activated T cells from females with SLE and healthy controls which showed that E up-regulated six pathways in SLE T cells including interferon-alpha signaling. These results indicate that E alters signaling pathways in activated SLE T cells and contributes to SLE onset and disease pathogenesis (94). The ovarian function in SLE patients with active disease before treatment with high doses of glucocorticoids and cytotoxic agents was studied. Menstrual cycle disorders with oligomenorrhea were observed in 54% of SLE patients. The hormonal studies showed decreased P level, reduced E2 concentration, and increased levels of LH, FSH, and PRL. Menstrual period disorders, decreased P levels, and HPRL were found to be significantly related to a high SLEDAI score. SLE women may be considered a group that is at risk of developing an altered ovarian function (95). Menstrual disturbances are frequent and may be associated with pituitary dysfunction, which would confirm a decrease in P production. The follicular reserve seems to be low regardless of intravenous cyclophosphamide treatment (96).

The origin of altered ovarian function in SLE is diverse: SLE activity, organ damage, and immunosuppressive treatment. Ovarian necrotizing vasculitis is a rare complication described in SLE which may be another cause of gonadal dysfunction (97).

Sex hormone-binding globulin (SHBG) regulates the bioavailability of sex hormones to target tissues. In this regard, the distribution of the SHBG functional polymorphism Asp327Asn (rs6259) was analyzed in SLE patients and controls in a Polish population. A higher risk SHBG327Asn vari-

ant was found to lead to the development of SLE. SHBG has a much higher affinity for T than E, and the SHBG327Asn variant displays lower E clearance. The opposing effects of E and T on the immune system and on the imbalance in the levels of these hormones in SLE patients can be enhanced by the SHBG327Asn protein variant (98).

There is a remarkably low number of male patients with SLE. However, male SLE patients have severe renal involvement and they need treatment with cyclophosphamide and other drugs that may affect testicular function. Recently, testicular function in SLE has been analyzed. This study identified a high frequency of testicular Sertoli cell dysfunction in male SLE associated with semen abnormalities determined by urological evaluation, testicular Doppler ultrasound, hormone profile, and anti-sperm antibodies. In addition, inhibin B levels, a heterodimeric glycoprotein hormone produced almost exclusively by testicular Sertoli cells, was lower in SLE patients (99). Note that male patients with untreated hypogonadism, including Klinefelter's syndrome, have a greater risk of developing autoimmune rheumatic diseases such as ankylosing spondylitis, SLE, RA, etc. (100).

In conclusion, female and male patients with SLE have alterations of the HPG axis as a consequence of clinically and immunologically active disease, immunosuppressive treatment, and genetic predisposition. The interactions between gonadal hormones and the immune system support the hypothesis that an abnormal immune neuroendocrine alteration may be the cause of clinical expression of AD.

PROLACTIN-GROWTH HORMONE SYSTEM (PRL-GH)

The relationship between PRL and the immune system has been demonstrated and created a new vision of immunoendocrinology. PRL is secreted not only by the anterior pituitary gland but also by many extrapituitary sites including the immune cells. The main function of PRL is to regulate the growth and differentiation of the mammary gland and the ovary and to act on the innate and adaptive immune response. HPRL has been described in both non-organ-specific ADs (e.g., SLE, RA, systemic sclerosis, psoriatic arthritis) and organ-specific ADs (e.g., celiac disease, type 1 diabetes mellitus, Addison's disease, and autoimmune thyroid diseases). PRL increases the synthesis of IFN-gamma and IL-2 by Th1 lymphocytes. Moreover, PRL activates Th2 lymphocytes with autoantibody production (101,102). Experimental models and a few controlled studies of dopamine agonist treatment in humans with SLE support the potential efficacy of such agents even during pregnancy and postpartum. PRL is an integral member of the immune neuroendocrinology network. The presence of PRL mRNA in human lymphoid tissue implies that locally synthesized PRL may play a critical role in immunocompetence. PRL-receptors (PRL-R) are distributed throughout the immune system and are included as members of the cytokine receptor superfamily. PRL-R signal transduction is mediated by a complex array of signaling molecules of which the JAK2, Stat1, and Stat5 pathways have been well studied. PRL is now considered a cytokine based on both molecular and functional evidence (103-105). Structurally, PRL-R is related to the cytokine/hematopoetin family which includes the growth hormone (GH), the granulocyte-macrophage colony stimulating factor (GM-CSF), the erythropoietin, and the interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-13, and IL-15 receptors. However, the effects of PRL depend on the local production by immunocompetent cells and its concentration as well as other hormonal factors and cytokines (106).

With respect to the different PRL actions, a recent study in mice susceptible to lupus found that HPRL accelerated the disease and increased the absolute numbers of T1 and T3 B cells but not of mature B cells. This suggested a primary effect of PRL on the early stages of B cell maturation in the spleen and a role for PRL in B cell differentiation, in which it contributed to SLE onset (107).

During the acute phase response (infection and various forms of injury), the adaptive immune response is suppressed and the innate immune function is amplified. The HPA axis stimulates natural immunity and suppressor/regulatory T cells, which down-regulate the adaptive immune system. Adaptive immunocompetence is maintained by vasopressin (VP), GH, and PRL. VP controls adaptive immune function and stimulates ACTH and PRL secretion (108).

Estrogens have long been regarded as major physiological activators of PRL synthesis and lactotroph proliferation and have been implicated in the pathophysiology of HPRL and prolactinomas. E2 stimulates PRL gene expression through binding E receptors. E2 and TNF-α have synergistic activation of the human PRL promoter. This effect is mediated by nuclear factor kappa B (NF-kappa β) thus suggesting a novel, promoter-specific signaling interaction between E and TNF-α for PRL regulation in vivo (109).

Early reports showed HPRL in SLE patients. The first study was done on male SLE patients without treatment. These patients had high levels of PRL both before and after intravenous administration of LH–RH stimulation associated with increased estrone and decreased T, and DHEA levels compared to healthy controls (110). Pregnant SLE patients also had HPRL as did healthy pregnant women and RA patients, which is associated with E and T decrease, especially in active SLE (111). An open study demonstrated significant HPRL in SLE patients in comparison to normal controls and other ARD with a direct correlation with clinical and serological activity suggesting that PRL plays a role in SLE pathogenesis (112). HPRL has been seen in SLE patients, but whether HPRL is associated with SLE activity is still controversial. These discrepancies may be explained by multiple factors including heterogeneous groups of patients, variability of the SLE activity index, treatments, circadian PRL rhythm, and different isoforms of PRL and anti-PRL antibodies in the sera of SLE patients (113). Despite this controversy, HPRL and elevated circulating IL-6 were reported in SLE patients with active lupus nephritis or neuropsychiatric involvement. These findings support the hypothesis that PRL and IL-6 are accumulated in the inflamed organs and

act as stimuli for T and B lymphocytes to infiltrate the tissue. It is noteworthy that PRL-R has been described in the choroid plexus, hypothalamus, and other areas of the central nervous system. In addition, at 7.5–14 weeks of gestation, PRL R is expressed chiefly in the human fetal kidney (113-116). The high concentrations of PRL and IL-6 in urine and cerebral spinal fluid may be the consequence of local synthesis of both mediators. A subset (30–40%) of SLE patients with HPRL has detectable anti-PRL antibodies with fewer clinical and serological manifestations. In contrast, SLE patients without macroprolactinemia (PRL-IgG complex) had significantly higher levels of disease activity compared to patients with macroprolactinemia. PRL-IgG complex was produced by 23-kd non glycosylated PRL that bound to IgG, and it was fully active *in vitro*. Therefore, the absence of symptoms of HPRL or lower levels of lupus activity in patients with anti-PRL antibodies is not explained by the lower bioactivity of the complex. Delayed clearance of the PRL-IgG complex may account for increased serum levels of PRL in SLE patients with anti-PRL autoantibodies (117).

The association of PRL with disease activity has been analyzed over the last 10 years. In 2001, three consecutive studies of independent centers demonstrated the association among PRL levels and disease activity, malar rash, CNS involvement, fatigue, fever, and renal involvement in 397 SLE patients. Additionally, anti-dsDNA, anti cardiolipin antibody titers, elevated erythrocyte sedimentation rate, anemia, low levels of C3 were associated with HPRL. Serum PRL concentrations were determined by immunoradiometric (IRMA) and biological assays which evaluate Nb2 lymphoma cell proliferation. Therefore, high serum PRL levels are an important independent factor related to lupus activity (118-120). Recent studies confirmed these findings, and a significant correlation between PRL levels, clinical disease, and serological activity of SLE was found. In addition, menstrual cycle disorders, decreased P level, and HPRL were found to be significantly associated with a high SLEDAI score before treatment with cyclophosphamide and high doses of GC (121, 95). According to meta-analysis of the role of PRL in SLE, a significant increase in PRL concentration in SLE patients has been demonstrated. Serum PRL levels have been positively associated with lupus disease activity. Abnormally high PRL levels during pregnancy in SLE also correlate with disease activity (122).

In vitro studies from human cells support the association of PRL and SLE. Lymphocyte derived PRL has been shown to be higher in patients with SLE compared to normal subjects (123,124). Jacobi *et al*. (125) found that physiological concentrations of PRL (20 ng/ml) induced IgG production more effectively than high concentrations of PRL (100 ng/ml) in lymphocytes from active SLE patients. As a result, PRL-induced IgG production was associated with SLE activity. Chavez-Rueda *et al*. (126) showed that early activation of T and B lymphocytes from SLE patients by PRL was associated with disease activity and PRL production by lymphocytes. The addition of anti-PRL antibodies resulted in a reduction of CD69 and CD154 expression. PRL impairs the negative selection of autoreactive B lymphocytes that occurs during B cell maturation into fully functional B cells. PRL has an anti-apoptotic effect and enhances proliferative response to antigens and mitogens and the production of immunoglobulins and autoantibodies (127). Controlled clinical trials demonstrated that treatment with BRC reduced the expected number of lupus flares and improved anxiety and distress and thus supported the role of PRL in disease activity. BRC is currently considered an unproven therapy for SLE, and its use is entirely experimental (128). HPRL in patients with lupus activity at onset and after 6 months of conventional treatment was associated with both SLE disease activity and remission induced by conventional treatment (129). Acute phase proteins, PRL, and disease activity in SLE patients have been evaluated during quinagolide therapy at onset and after 3 months of treatment. A significant decrease in the SLEDAI score, IL-6, and PRL levels was revealed after 3 months of therapy confirming the hypothesis that quinagolide may become a valuable and safe drug for SLE treatment (130). HPRL, hyperferritinemia, and hypovitaminosis D have different immunological implications in the pathogenesis of ADs, including SLE. It has been recently suggested that, in addition to therapy for HPRL with dopamine agonists, preventive treatment with vitamin D may be considered in certain cases (131). Due to deficiency of dendritic cell functions and abnormal PRL secretion in SLE, another possible participation of PRL in the pathogenesis of SLE is the interaction of PRL and dendritic cells. The interaction skews their function from antigen presentation to a pro-inflammatory phenotype with high INF-α production (132). There are patients with SLE in whom no secondary cause for HPRL can be found. Defects in peptidergic modulators and dopamine metabolism may, in part, explain the HPRL in SLE. Lymphocytes in active SLE have an increased production of PRL. Impaired hypothalamic function has been associated with HPRL in SLE (133,134). Moreover, STAT5 signaling is very important in the regulation of the immune response in SLE. Basal activation of STAT signaling and a reduced response to the cytokines IFNα and IL6 were observed in the peripheral blood of SLE patients. The phosphorylation of STAT5 is associated with cytokines such as PRL, IL2, G-CSF, and IFNγ (135).

In conclusion, experimental models, *in vitro* studies, and clinical observations have shown an intriguing link between PRL, autoimmunity, SLE, and other ADs. HPRL is associated with active disease and organ involvement in SLE patients. This knowledge might have important implications for treatment. Further studies are necessary to confirm the efficacy and safety of PRL lowering agents as therapy for SLE.

AUTONOMIC NERVOUS SYSTEM (ANS)

The manifestations of autonomic nervous system (ANS) dysfunction in ARD have been the subject of many studies. In one case, a study found that while RA and SLE patients had depressed heart rate variability compared to controls, the QTc interval was more prolonged in patients with SLE. In this

study, autonomic dysfunction in SLE was predominantly sympathetic while in RA vagal predominance was evident (136).

In patients with moderately active SLE, an altered ANS response which was more pronounced in comparison to healthy subjects was found during the hCRH stress test. The authors concluded that a hypersympathetic reaction may lead to a greater risk of cardiovascular diseases in these patients (137).

The autonomic neural control of cardiovascular and immune functions involves a number of autonomic neuropeptides such as neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP), which participates in the orchestration of cytokines and exerts modulatory effects on immune cells. In a study of adolescents with SLE and juvenile idiopathic arthritis (JIA) patients, both groups had significantly lower serum NPY and VIP with an association between cardiovascular autonomic neuropathy and disease manifestations including activity. This finding may denote hypofunction of sympathetic and parasympathetic divisions of ANS in lupus (138).

In contrast, another study found an increase in sympathetic outflow measured by NPY and a rise in the NPY/ACTH ratio in SLE patients which suggests that low levels of cortisol in relation to neurotransmitters may be pro-inflammatory because cooperative anti-inflammatory coupling of the two endogenous response axes is missing (57). Further studies are needed to elucidate the prognostic significance and clinical implications of impaired autonomic functions as well as the immune neuroendocrine network in patients with SLE (e.g., cardiovascular risk).

IMMUNE NEUROENDOCRINE INTERACTION IN AUTOIMMUNE UVEITIS

Uveitis is a group of diseases characterized by intraocular inflammation in which there are multiple related entities. They may be associated with infections, but there is an important relationship with immune-mediated diseases, e.g., RA, JIA, SLE, HLA-B27 associated spondyloarthritis, sarcoidosis, Vogt–Koyanagi–Harada disease, Behcet's disease, Granulomatosis with poliangitis, and other ANCA related vasculitis, etc. There is a subset of immune-mediated uveitis that affects only the eye such as: autoimmune idiopathic anterior uveitis, idiopathic intermediate uveitis (i.e. pars planitis), or retinal vasculitis. (139).

The factors that participate in the pathogenesis of uveitis are genetic, immunological, hormonal, and environmental. There are some studies related to emotional stress as an important factor related to onset and recurrence of uveitis. However, stress and the immune-neuroendocrine interactions have not been completely studied.

HYPOTHALAMUS-PITUITARY-ADRENAL AXIS

The use of corticosteroids as the cornerstone for the treatment of uveitis strongly suggests an abnormal immune-neuroendocrine interaction in these localized AD. CRH is a major regulator of the HPA and the main coordinator of the stress response. During inflammatory stress, the cytokines TNF α, IL-1, and IL-6 stimulate hypothalamic CRH and/or vasopressin secretion as a way of preventing an overreacting inflammation. This response has been observed in experimental models of inflammatory/immune diseases including uveitis induced by R16 peptide in Lewis rats (140).

In autoimmune uveitis, especially that associated with ADs, there are some studies that suggest an inhibition of the HPA axis characterized by significantly low levels of cortisol and HPRL as well as high levels of pro-inflammatory cytokines (i.e., TNF-α, IL-1, and IL-6) (141,142). The serum levels of cortisol and glycosaminoglycans (GAG) were studied in patients with uveitis in ARD. In acute uveitis, the mean serum cortisol level was significantly lower than that in infectious and idiopathic uveitis. In ARD, remission in uveitis was associated with an increase in the serum levels of cortisol. A poor course was observed when the low content of cortisol remained or decreased still further. GAG exchange in uveitis patients with RD changed: there was an inverse correlation between the level of hyaluronic acid and cortisol. The same correlation between GAG and chondroitin sulfate was found in males (143).

HYPOTHALAMUS- PITUITARY-GONADAL AXIS

Gender and sex hormones influence ocular diseases including uveitis and retinal disease as well as eye circulation, and optic nerve anatomy. Sex-based differences in ocular disease should be considered (144).

Pregnancy and postpartum period are associated with uveitis activity. In this regard, a prospective, observational case study was done. Four pregnant women in their first trimester with chronic non-infectious uveitis were followed monthly until 6 months after delivery. Serum female hormones (i.e., E, P, PRL) and cytokines (e.g., IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and TGF-β) were measured. Uveitis activity decreased after the first trimester but flared in the early postpartum period. Serum female hormones, after being highly elevated during pregnancy, drastically dropped in the postpartum. Cytokine levels, except TGF-β, were mostly undetectable suggesting that female hormones and TGF-β may contribute to the activity of uveitis during pregnancy and the postpartum period (145).

The relationship between hormones and the immune system in autoimmune uveitis is more evident in experimental autoimmune uveitis (EAU). The effect of E, P, and T in EAU was studied. In female rats, T decreased significantly, E was slightly enhanced, but P or E + P did not affect EAU. A correlation with the ocular levels of Th1 (IFN-γ) and Th2 (IL-10) cytokine messengers was found thereby suggesting that sex hormones may affect EAU by inducing changes in the cytokine balance. Sex hormone therapy could be considered an adjunct to anti-inflammatory agents used to treat autoimmune uveitis in humans (146). In this regard, the endotoxin-induced uveitis (EIU) and the effects of E were

studied in adult male, female, and ovariectomized female Lewis rats. The endotoxin injection produced cell infiltration, which was more marked in male than in female rats and in rats with ovariectomy. E receptor was found in the endothelium and iris-ciliary body, and E-selectin and IL-6 gene expressions were higher. Note that treatment with 17-beta-estradiol significantly reduced cell infiltration and gene expression in male and ovariectomized female rats. The down-modulation of these inflammatory genes by E may contribute to the reduction in cell infiltration in acute anterior uveitis (147).

PROLACTIN-GROWTH HORMONE SYSTEM (PRL-GH)

Prolactin (PRL) may play a role in the regulation of humoral and cell-mediated immune responses. On the basis of these observations, in 1991, PRL levels were measured in the serum and aqueous humor of 28 patients with cataract or anterior uveitis with concomitant cataract. Intraocular concentrations were significantly higher in uveitis patients. This was the first study measuring PRL concentrations in human aqueous humor (148). Previous studies suggested that experimental autoimmune uveitis as well as patients with uveitis, whether or not associated with spondyloarthritis, may respond to BRC treatment. (101,149). Serum levels of the hormone melatonin (MEL), PRL, and IL-2 were measured in 100 patients with uveitis and matched with healthy blood donors. MEL was reduced significantly in patients with iritis and iridocyclitis and significantly elevated in those with intermediate uveitis, chorioretinitis, and panuveitis. PRL was significantly reduced in patients with intermediate uveitis. IL-2 was reduced to about 50% of control values in all groups of patients. The results suggest a possible immune neuroendocrine interaction in uveitis patients (150). In contrast, a recent study evaluated basal serum PRL levels in patients with HLA-B27-associated uveitis in a prospective, nonrandomized comparative trial. Thirty-three patients with HLA-B27- associated uveitis and 30 age- and sex-matched healthy control subjects were included. PRL serum levels were significantly higher in patients vs. controls. However, no correlation was found between PRL levels, systemic treatment, and disease activity. These results suggest the role of serum prolactin levels in HLA-B27-associated uveitis pathogenesis. In a previous study, we found HPRL in patients with Reiter's syndrome. The frequency of conjunctivitis, urethritis, dysentery, and uveitis was higher in patients with HPRL than in normoprolactinemic patients with Reiter's syndrome (152).

In conclusion: uveitis is a group of inflammatory eye diseases with unspecified etiology. There is evidence that uveitis is an autoimmune disease mediated by T cells, but the triggering mechanism is unknown. A possible cause of such conditions is the disturbance at the immune neuroendocrine system level with alterations on the HPA axis, the HG axis, PRL hormone, and cytokine release. Finally, a variety of evidence suggests that uveitis is a systemic disease rather than simply an ocular disorder.

CIRCADIAN RHYTHM FROM HORMONES AND CYTOKINES IN HEALTH/DISEASE PROCESSES

Endogenous circadian rhythm is maintained by the central circadian timekeeper, the suprachiasmatic nuclei of the hypothalamus. The clock machinery is composed of heterodimeric transcription factors and transcription regulatory proteins. The expression of these factors oscillates rhythmically over 24-hour cycles. The rhythm of the central master clock is transferred to cells in the peripheral organs through hormonal and neuronal connections. The desynchronization of endogenous and geophysical time leads to fatigue as in jet lag. The molecular core of the circadian machinery is composed of transcription factors Aryl hydrocarbon Receptor Nuclear Translocator-Like (ARNTL/BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK), which heterodimerize and activate transcription from E-box elements located in the promoters of clock controlled genes (153).

There is increasing evidence that the immune system is regulated by circadian rhythms. The number of red blood cells and peripheral blood mononuclear cells as well as the level of cytokines undergoes daily fluctuations. Current experimental data indicate that circadian information reaches immune tissues mainly through diurnal patterns of autonomic and endocrine rhythms. In addition, cytokines can also influence the phase of the circadian clock and provide bidirectional communication of circadian information between immuno-neuroendocrine systems that function in synchrony in order to optimize immune response. Chronically stressed patients present blunted rhythmic characteristics that can disrupt this intrinsic orchestration as well as several endocrine signals (6).

RHEUMATIC DISEASES, CORTISOL, AND TH1 CYTOKINES

Cortisol, an adrenal steroid with anti-inflammatory functions, has a circadian rhythm with a maximum peak at 8 A.M. and nadir at midnight. In healthy subjects, it has been observed that bone resorption activity is higher between 5 to 7 A.M., because resorption is adjusted to cortisol circadian rhythm, TNF-α / IL-6, thus contributing to loss of bone mass (154). The circadian rhythm of cortisol in RA patients and low to moderate disease activity are not different from the rhythm of healthy subjects. However, as has been demonstrated, this rhythm may be altered in RA patients with high disease activity and thus lead to flattening of the hormone curve and the appearance of two peaks in the morning and afternoon (155). Although these patients have higher cortisol levels, they are insufficient in relation to the inflammation and, as a result, the altered circadian rhythm causes greater inflammation during the early hours of the morning (156).

Patients with apparently well-controlled RA may have debilitating symptoms such as morning stiffness, fatigue, and pain. The key to controlling these symptoms may be in understanding their pathophysiology. Nocturnal plasma levels of the pro-inflammatory cytokine IL-6 are elevated in patients with RA and correlate with levels of morning

stiffness. Endogenous cortisol secreted during the night may be insufficient to counter the actions of IL-6 in these patients. Consistent with this hypothesis, the beneficial effects of glucocorticoids on morning stiffness are enhanced by their administration at 02:00 h compared to conventional administration around breakfast time though it is inconvenient for patients to have to awaken to take therapy. Modified-release prednisone has been developed to allow treatment to be taken at a convenient time (≈ 22:00 h) with programmed delivery of the glucocorticoid 4-6 h later, which is a more appropriate time. Assessment of cytokine and cortisol levels over 24 h before and 2 weeks after treatment with modified-release prednisone 5 mg/day has confirmed the hypothesis. Clinical studies in patients with RA have shown that modified-release prednisone at the same dose significantly reduced the duration of morning stiffness without affecting tolerability. Furthermore, the administration of glucocorticoid in accordance with the natural circadian rhythm may improve hypothalamic-pituitary-adrenal axis function (157).

According to these clinical findings, the molecular machinery responsible for the circadian timekeeping is perturbed in RA. A recent study investigated the expression of the molecular circadian clock in RA. Gene expression of thirteen clock genes was analyzed in the synovial membrane of RA and control osteoarthritis (OA) patients. The effect of pro-inflammatory stimulus on clock gene expression in synovial fibroblasts was studied using IL-6 and TNF-α. Gene expression analysis disclosed disconcerted circadian timekeeping and immunohistochemistry revealed a strong cytoplasmic localization of BMAL1, a transcription factor of circadian rhythm, in RA patients. Perturbed circadian timekeeping is at least in part inflammation independent and cell autonomous, because RA synovial fibroblasts display altered circadian expression of several clock components and perturbed circadian production of IL-6 and IL-1β after clock resetting. Throughout the experiments, ARNTL2 and NPAS2 appeared to be the most affected clock genes in human immune-inflammatory conditions. This study demonstrated that the molecular machinery controlling the circadian rhythm is disturbed in RA patients (153).

PARTICIPATION OF OTHER HORMONES.

It was demonstrated that women with active RA showed a tendency towards GH insensitivity and a decrease in diurnal cortisol and DHEA in relation to their inflammatory state (158).

Melatonin, another hormone with a night circadian cycle produced by the pineal gland has the opposite effect of steroids. The elevated secretion in patients with RA seems to be an important factor for perpetuation and clinical circadian disease symptoms (159). In active RA, there is an imbalance favoring pro-inflammatory hormones (PRL and cytokines) which is responsible for the diurnal inflammatory rhythm of the disease (160). Furthermore, cytokines also have a circadian oscillation since IL-6, and TNF-α present maximum levels during the early morning hours. The IL-6 peak is at around 6 A.M. in healthy subjects with a decrease at 9 A.M. It is at 7 A.M. in RA patients and persistently rises until 11 A.M. thus influencing morning symptoms (161).

PERSPECTIVES OF TREATMENT

Reprogramming biological rhythms has recently gained a lot attention as a potent method to leverage homeostatic circadian controls to ultimately improve clinical outcomes. Elucidation of the intrinsic properties of such complex systems and optimization of intervention strategies require not only an accurate identification of the signaling pathways that mediate host responses but also a system-level description and evaluation (6).

Considering circadian rhythms and the impact of immunosuppressive schedule intake on efficacy, it would be of great interest to explore new therapeutic modalities such as those to neutralize cytokines, melatonin hormonal antagonists, new formulation of old drugs towards other release patterns and a major night inhibition of pro-inflammatory cytokines such as IL-6 and TNF-α, antagonists of IL-1 receptor. And so forth which of them are targeted at reducing RA symptoms during the early morning hours and improving quality of life for these patients. One of these new therapeutic agents, Tocilizumab, is a monoclonal antibody against IL-6 receptor. Clinical studies done of RA patients over the last few years have demonstrated that it is effective at improving symptoms and signs of RA. Moreover, inhibition of IL-6 may have positive effects on the functional status and radiological progression of the disease (161). Another interleukin expressed in the synovial membrane of RA patients is IL-17 that, with IL-1 and TNF-α, has a synergistic effect. IL-17 is a potent inductor of NF-kappaB. Anti-IL-17 therapy may be a new treatment option for bone destruction prevention along with anti-TNF-α and anti-IL-1 therapy (162). Regulation of transduction signals with small molecule inhibitors in rheumatic diseases, e.g., specific Jak3 inhibitors, spleen tyrosine-kinase (Syk) inhibitors, or mitogen activated kinase-like protein (MAPK) and endogen negative regulators of MAPK signals that have received recognition as inflammatory/immune response modulators with potential use in RA. Additional benefits seen in inflammatory disease treatment targeting CD80, IL-12/IL-23, AP-1 transcription factor, and cell activation receptor modulators such as cytokine receptors, toll-like receptors, and adenosine A3 receptors are being developed now (163). All of these potential treatments will probably modify the immune neuroendocrine response in order to stabilize the hormonal homeostasis.

The modulation of hormonal milieu has been a therapeutic option in ADs for several years. Clinical trials of DHEA at an oral dose of 200 mg/day for SLE patients have demonstrated an improvement in disease activity among female patients with mild to moderate lupus but to a lesser extent in cases of more severe disease. DHEA improves the ability to taper corticosteroids in patients with active lupus (164).

In relation to Danazol, a recent study reviewed the information on the use, effectiveness, and adverse effects

of danazol in patients with SLE. The study ran from January 1950 to July 2009 with a total of 153 patients, including 2 prospective trials of 7 and 16 patients respectively and 1 randomized controlled trial of 40 patients. Danazol has been used successfully in the treatment of hematological manifestations of SLE such as thrombocytopenia, Evan's syndrome, autoimmune hemolytic anemia, and a case of red cell aplasia. Thirteen patients responded to danazol after failing splenectomy. There is limited information on the use of danazol in nonhematological manifestations of SLE. Adverse effects were generally tolerable but high doses may produce undesirable side effects for female patients. Patients with primary antiphospholipid syndrome and RA were also treated with danazol with acceptable platelet counts within the first 4 weeks of danazol therapy that allowed the prednisone dosage to be tapered. (165-167).

Murine models of SLE demonstrate that PRL impairs the mechanisms of B cell tolerance induction (negative selection, receptor editing, and anergy), and therefore, it participates in the pathogenesis of autoimmunity. BRC, a drug that inhibits PRL secretion, restores the immune tolerance, and abrogates several immune effects of PRL (168). A double-blind, randomized, placebo-controlled study of BRC in SLE (mean follow-up 12.5 months) showed a decrease in the SLEDAI score and a significant reduction of flares/patient/month in the BRC group vs controls. Long term treatment with a low dose of BRC appears to be safe, and it is an effective means of decreasing SLE flares in these patients (169). Our pilot study suggests that BRC may play a role in the prevention of maternal-fetal complications such as premature rupture of membranes, preterm birth, and active disease (170).

CONCLUDING REMARKS

The immune neuroendocrine system is important for the maintenance of homeostasis and plays an important role in systemic and localized autoimmune disorders.

Multiple factors including stress are a risk factor for the development of autoimmune diseases because they produce the activation of the immune-neuro-endocrine system, abnormal systemic anti-inflammatory response, and energy consumption that leads to chronic disease.

A hyper- or hypoactive stress system associated with abnormalities of the systemic anti-inflammatory feedback and/or hyperactivity of the local pro-inflammatory factors may play a role in the pathogenesis of chronic inflammation and immune-related diseases (171).

The hyperactivity of the local pro-inflammatory factors may play a role in the pathogenesis of autoimmune diseases.

Therapeutic modification of the circadian rhythms of hormonal and pro-inflammatory cytokines opens a window of opportunity for new therapeutic regimens.

The modulation of hormonal milieu and stress are interesting therapeutic options in autoimmune diseases.

ABBREVIATION LIST

- **HPA:** hypothalamic-pituitary-adrenal axis
- **HPG:** hypothalamic pituitary-gonadal axis
- **HPT:** hypothalamic-pituitary-thyroid axis
- **PRL/GH:** prolactin/growth hormone
- **ANS:** autonomic nervous system
- **CRH:** corticotropin releasing hormone
- **ACTH:** adrenocorticotrophic hormone
- **E:** estrogens
- **P:** progesterone
- **A:** androgens
- **IGF-1:** insulin-like growth factor (IGF-1)
- **TSH:** thyroid stimulating hormone
- **DHEA:** dehydroepiandrosterone. DHEAS: dehydroepiandrosterone sulfate
- **T:** testosterone
- **HPRL:** hyperprolactinemia
- **SNS:** Sympathetic nervous system
- **SP:** Substance P
- **CGRP:** calcitonin-gene-related peptide
- **ADRB2:** beta 2-adrenergic receptor
- **BRC:** bromocriptine
- **LH:** luteinizing hormone
- **FSH:** follicle stimulating hormone
- **SHBG:** sex hormone-binding globulin
- **VP:** vasopressin
- **MEL:** melatonin

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13

CELL DEATH

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INTRODUCTION

Since 1973, several cell death modalities have been described. Recently, the Nomenclature Committee on Cell Death (NCCD) proposed a novel classification of the twelve subroutines based on biochemical features (Table 1) (1). These modalities or subroutines also differ in their morphological characteristics, the mechanisms of clearance of the death cell, and the molecular components involved in the process.

APOPTOSIS

In 1972 the term apoptosis was used to describe a form of cell death associated with specific morphological changes different from those observed in pathological conditions and necrosis (2). Apoptosis is a tightly regulated process that is essential for the development, establishment, and maintenance of tissue architecture (3). Apoptosis is characterized by biochemical (Table 1) and morphological chan- ges such as cellular shrinkage, DNA fragmentation, chromatin condensation, and cytoplasmic membrane blebbing (type of blisters formed by plasma membrane delamination) (Figure 1) (4,5).

Apoptosis is important in maintaining cell homeostasis, especially in, the immune system. The immune system generally produces more cells than necessary and the extra cells are eliminated by apoptosis (6).

Apoptosis can be induced by various intra or extra cellular signals such as environmental stress or binding of lethal ligands to their cognate death receptors on the cell membrane surface (3). Accordingly, the extrinsic pathway (extrinsic apoptosis by death receptors) is activated by extracellular signals via death receptors and the intrinsic pathway (caspase-dependent intrinsic apoptosis) depends largely on the mitochondrion (6,7).

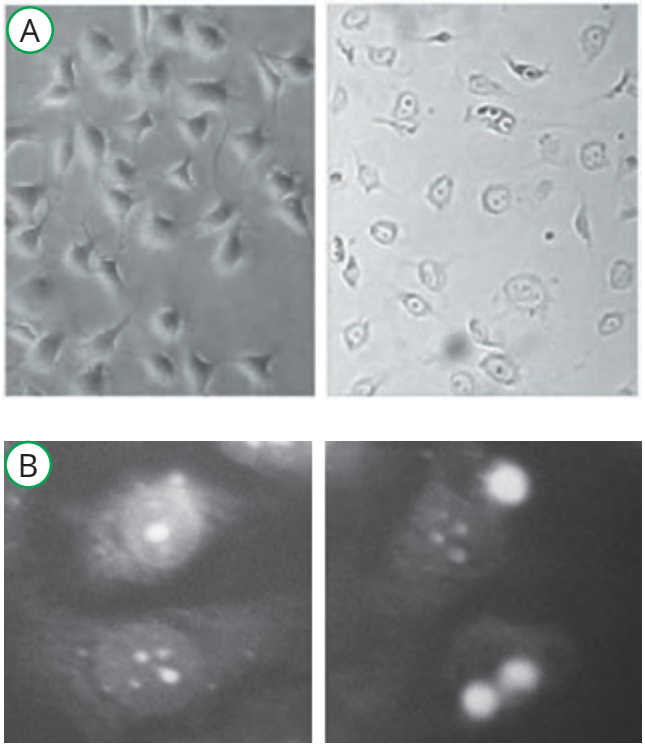

Figure 1. Morphological changes associated with apoptosis. A. Top left panel: Untreated endothelial cells (cell line EAhy.926) (5). Top right panel: Endothelial cells were treated with C2-ceramide (130 µM) for 6 hours. Morphology of cells was observed using a light microscope. 400X. Apoptotic cells presented detachment and shrinkage. B. Representative fluorescence microscopy of nuclei following ethidium bromide staining. 1000X. Bottom left panel: Untreated endothelial cells. Bottom right panel: Endothelial cells were treated with C2-ceramide (130 µM) for 6 hours. Normal cells displayed a diffuse chromatin while apoptotic cells showed condensed and fragmented chromatin.

TYPE OF CELL DEATH	CASPASE DEPENDANCE	INITIATION	BIOCHEMICAL CHARACTERISTICS
Extrinsic apoptosis by death receptors	Dependent	Binding of lethal ligand to their cognate death receptor	Lethal ligands FASL/CD95L, TNF, TRAIL Type I cells: activation of caspase-8 and 3 Type II cells: activation of caspase 8, cleavage of Bid and MOMP
Extrinsic apoptosis by dependence receptors	Dependent	Ligand deprivation-induced	Death receptor UNC5A-D and DCC Caspase-9 and 3 activation PP2A and DAPK1 activation
Caspase-dependent intrinsic apoptosis	Dependent	DNA damage, oxidative stress, calcium excess, mild excitotoxicity, UV, endoplasmic reticulum stress, ionizing radiation	Bioenergetic and metabolic catastrophe Irreversible MOMP Dissipation of mitochondrial transmembrane potential Respiratory chain inhibiton ROS generation Release of IMS proteins Inhibed by caspase inhibitors
Anoikis	Dependent	Loss of attachment to ECM	Lack of β1-integrin participation EGFR expression down regulation BIM overexpression Inhibition of ERK1 signaling pathway Activation of apoptotic executioners
Cornification	Dependent	Stress	Synthesis of enzymes and substrates of stratum corneum (transglutaminases) Caspase 14 activation Restricted to keratinocytes
Pyroptosis	Dependent	Microbial infection	Inflammasome or pyroptosome activation of caspase-1 Release of IL-1 β and IL-18 Activation of caspase-7
Caspase-independent intrinsic apoptosis	Independent	Intracellular stress	Translocation of AIF and ENDOG to nucleus Caspase independent DNA fragmentation HTRA2 fragmentation of cytoskeletal proteins
Regulated necrosis or necroptosis	Independent	Alkylating DNA damage, exocytotoxins, binding of lethal ligands to their cognate death receptor	Formation of necrosome and mitochondrial fragmentation Caspase inhibiton and activation of RIPK1 and/or RIPK3 Inhibited by necrostatin
Autophagic cell death	Independent	Amino acid withdrawal, glucose and growth factor deprivation, DNA damage, ER stress and development process	Massive cytoplasmatic vacuolization Regeneration of metabolic precursors and clearance of subcellular debris LC3/Atg8 lipidation and degradation of SQSTM1
Mitotic catastrophe	Independent	Chromosomal defects or perturbation of mitotic machinery	Caspase-2 activation Activation of TP53 tumor suppressor family proteins Mitotic arrest and activation of cell death or senescence
Entosis	Independent	Absence of cell interactions with ECM	"cell-in-cell" phenotype (engulfment)of cells of the same type Lisosomal degradation Contraction of host cytoskeleton by activation of Rho and ROCK Insensitive to intrinsic apoptosis inhibitors
Parthanatos	Independent	PARP1 over activation leading to PAR accumulation	Bioenergetic crisis (NAD+ and ATP depletion) Dissipation of mitochondrial transmembrane potential PAR and AIF interaction AIF nuclear translocation and DNA fragmentation Possibly represent a case of necroptotic cell death
Netosis	Independent	Bacterial, fungal and protozoal infection	Release of NET (in most cases) Massive cytoplasmatic vacuolization Histone citrullination and chromatin descondensation Dependant on ROS generation by NADPH oxidase Biochemical features shared with autophagic cell death and necroptosis

Table 1. Cell death modalities. Different types of cell death classified by Nomenclature Committee on Cell Death (NCCD). Abbreviations.AIF, apoptosis-inducing factor; ATP, adenosine triphosphate; BID, BH3-interacting domain death agonist; DAPK1, death-associated protein kinase 1; DCC, deleted in colorectal carcinoma; ECM, extracellular matrix; EGFR, epidermal growth factor; ENDOG, endonuclease G; ERK1, extracellular-signal-regulated kinases 1; HTRA2, high temperature requirement protein A2 IMS, mitochondrial intermembrane space; LC3/Atg8,microtubule-associated protein 1 light chain 3; MOMP, mitochondrial outer membrane permeabilization; NAD, Nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NET, neutrophil extracellular traps: PAR, poly(ADP-ribose); PARP1, PAR polymerase; PP2A, protein phosphatase 2A; RIPK1, receptor-interacting protein kinase- 1; RIPK3, receptor-interacting protein kinase- 3; ROCK, Rho-associated coiled-coil containing protein kinase; ROS, reactive oxygen species; SQSTM1, sequestosome 1; TNFα, tumor necrosis factor α; TP53, tumor suppressor TP53; TRAIL, TNF-related apoptosis inducing ligand. Adapted form reference (1).

In the extrinsic pathway, there is an interaction between the surface receptor and its cognate ligand after which, the signals are transmitted to the cytoplasm (Figure 2) (3). Several death receptors/ligands have been described: Tumor necrosis factor α (TNF α) and members of receptor super family of tumor necrosis factor (TNFRSF), Fas and Fas ligand (FasL) (also known as Apo-1, CD95, or TNFSF6), death receptors DR4 and DR5, TNF-related apoptosis inducing ligand (also known as TRAIL, ApoL2, or TNFSF10) (7). Extrinsic apoptosis can also be initiated by ligand deprivation-induced dependence receptor signaling (UNC5A-D and DCC netrin receptor in the absence of netrin-1) followed by activation of casase-9 and caspase-3 (1). The intrinsic pathway is activated by different forms of cellular stress (Table 1) (8). The integrity of the mitochondria relies on the rheostat between pro-apoptotic and anti-apoptotic protein members of the Bcl-2 family (Table 2) (9,10). When pro-apoptotic signals prevail, mitochondrial outer membrane permeabilization (MOMP) occurs leading to mitochondrial transmembrane potential dissipation, detention of ATP synthesis, uncoupling of the respiratory chain, ROS generation, and release of mitochondrial intermembrane space proteins (IMS) such as cytochrome c and Smac/DIABLO, apoptosis inhibitory factor (AIF), endonuclease G (ENDOG), and high temperature requirement protein A2 (HTRA2). The formation of the apoptosome followed by the activation of caspase-3 leads to cell death (Figure 2) (1,11,12).

Caspases are the main executers in the process of caspase-dependent apoptosis and are responsible for the morphological changes associated with this process. Caspases are serine proteases with a cysteine active site that cleaves the substrate at (Asp-X-X-X) motif. Caspases are synthesized as zymogens and are activated by cleavage of the zymogen between domains p20 and p10. This cleavage could be done by proximity to another caspase on the pathway, by proximity to the death ligand complex, or by holoenzyme formation (4).

Caspases have been classified into initiators (caspases 8, 9, 10) and executioners (caspases 3,6,7). These executioner caspases are involved in DNA fragmentation (by activation of CAD [caspase-activated DNase]), nuclear laminin degradation, alteration of the cytoskeleton (by disruption of actin microfilaments, gelsolin and fodrin), and the formation of apoptotic bodies (4,13,14) (Table 3) (15–19).

Apoptosis is regulated through the modulation of the proteins involved. The members of the Bcl-2 protein family are regulated by post-translational modifications that result in conformational changes that either release the protein from a complex or increase their affinity to their target. These modifications include proteolysis, N-myristylation, deamidation, and phosphorylation (mediated by PI3K/Akt survival pathway) (20–25).

Bcl-2, Bcl-XL, A1/Bfl1, PUMA,NOXA, and Bim are also transcriptionally regulated by several transcription factors such as p53, NFκB, and the forkhead transcription factor O family (FoxO) (25–27).

BCL-2 PROTEIN FAMILY (9-10)		
Anti-apoptotic	**Pro-apoptotic**	
	Multidomain proteins	BH3-only proteins
Bcl-2 Bcl-w Bcl-xL Mcl-1 A1/BFL-1 Bcl-B	Bax Bak Bok	Bad Bim Bid Noxa Puma Bik BMF HRK BNIP3

Table 2. Protein members of the Bcl-2 family. Pro-apoptotic and anti-apoptotic members of Bcl-2 family. Abbreviations.A1, Bcl-2-related protein A1; Bad, Bcl-2-associated death promoter; Bax, Bcl-2–associated X protein; Bak, Bcl-2 homologous antagonist/killer; Bok, Bcl-2 related ovarian killer; Bcl-xL, B cell lymphoma-extra large; Bcl-w, Bcl-2-like protein 2; Bid, BH3-interacting domain death agonist; Mcl-1, induced myeloid leukemia cell differentiation protein; BMF, Bcl-2-modifying factor; BNIP3, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3; Noxa, Phorbol-12-myristate-13-acetate-induced protein 1; Puma, p53 upregulated modulator of apoptosis.

Caspases are also post-translationally modified by proteolysis, phosphorylation, ubiquitylation, and proteasomal degradation (facilitated by IAPs proteins) (28).

APOPTOTIC CELL CLEARANCE

The corpses of the dying cells must be cleared out in order to avoid triggering secondary necrosis.This process is carried out by professional phagocytes namely macrophages and dendritic cells (DCs), and in some cases by "non-professional" neighbor cells (i.e., fibroblasts, endothelial cells, and mesothelial cells) (29). The process is divided into several phases: first, in the "Find-me" phase, the dying cell releases soluble signals to attract the phagocytic cell; then, in the "Eat-me"phase, cytoskeletal modifications in the phagocytic cell allow the engulfment of the corpses. After the dying cells are recognized and engulfed, the phagosome fuses with the lysosome which leads to the degradation of the apoptotic cell to nucleotides, fats, sterols, and peptides (Figure 3) (29,30).

During the clearance of apoptotic cells, macrophages downregulate secretion of pro-inflammatory cytokines (TNF, IL-8 and IL-1) and increase production of anti-inflammatory mediators (IL-10,TGF-β, and PGE2) while dendritic cells (DC) induce T cells to differentiate into regulatory cells which leads to the suppression of inflammation and the promotion of adaptive immune tolerance. However, when a failure to clear apoptotic cells occurs, the secondary necrotic cell releases alarmins or danger signals such as HMGB-1 that stimulate the release of pro-inflammatory cytokines. In this inflammatory context, DCs process, present, or cross-present ingested apoptotic proteins and express co-stimulatory molecules that trigger B and T cell activation leading to the production of autoantibodies (31).

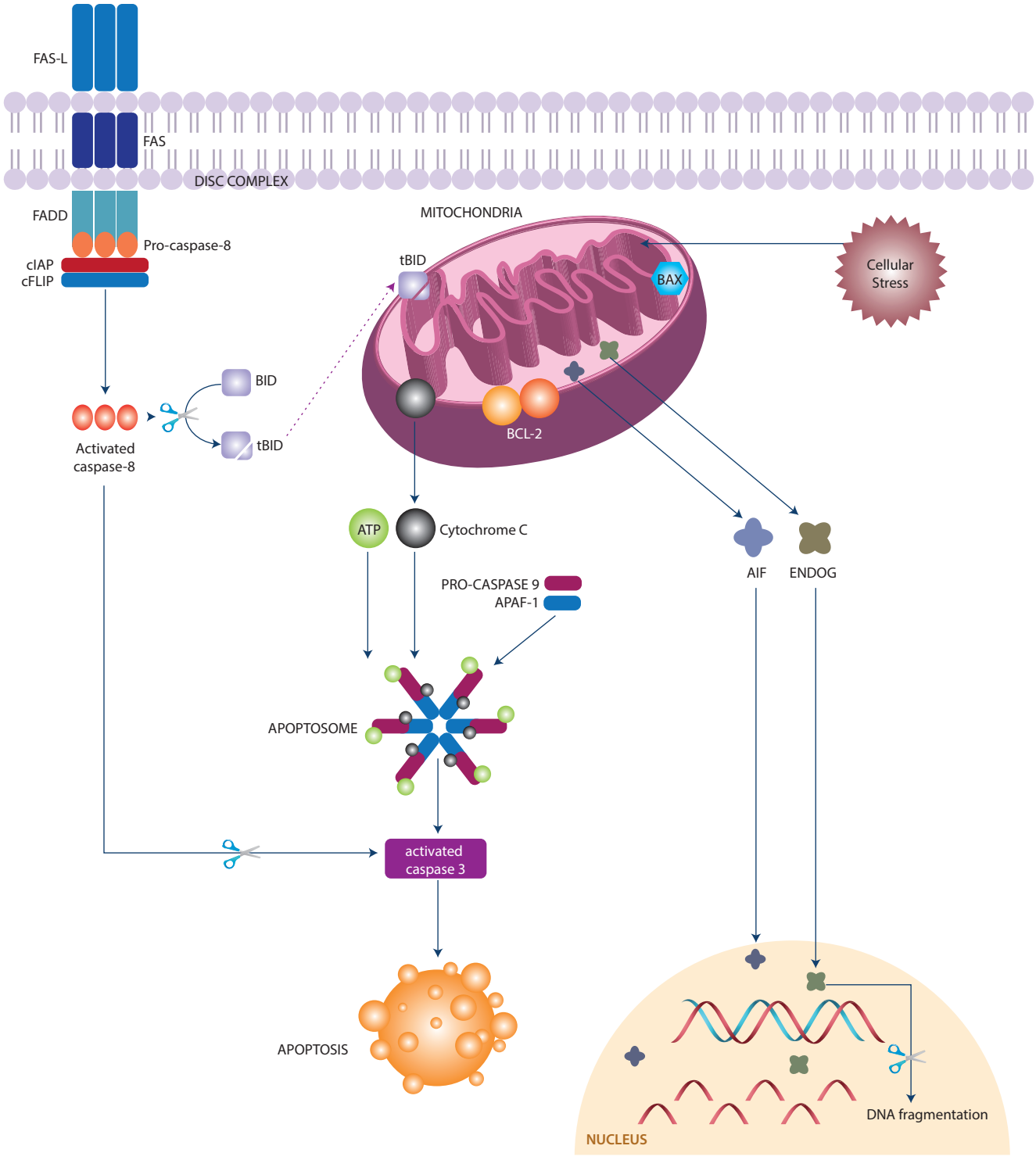

Figure 2. Apoptotic pathways. **Extrinsic pathway**. The activation of death receptors leads to the formation of DISC complex (death-inducing signaling complex) by the recruitment of FADD (FAS-associated protein with death domain), cIAPs (cell inhibitors of apoptotic proteins), c-FLIPs and pro-caspase 8 leading to activation of caspase-8 and / or 10 and the activation of caspase-3. In some cells such as lymphocytes, the cleavage of the cytosolic Bid protein produces truncated protein (tBid) which translocates to the mitochondrion and leads to pore formation in the mitochondrial membrane, release of cytochrome c, and formation of apoptosome and subsequent activation of the caspase-3. **Intrinsic pathway**. After the release of IMS proteins into the cytoplasm due to the intracellular stress conditions, cytochrome c with APAF1, dATP, and caspase-9 form a complex called apoptosome. The activation of caspase-9 triggers the activation of caspase-3 and ultimately cell death. Anti-apoptotic Bcl-2 proteins prevent mitochondrial outer membrane permeabilization (MOMP) by binding to BAK, BAX, and BH3- only proteins. **Caspase-independent intrinsic apoptosis**. Translocation of endonuclease G (ENDOG) and the apoptosis-inducing factor (AIF) to the nucleus produces DNA fragmentation independent of caspase activation. Adapted from references (1,11,12).

CASPASE	ALTERNATIVE NAME (15)	TYPE	FUNCTION (15-19)
1	Interleukin 1-beta converting enzyme (ICE), P45; IL1BC	Inflammatory-caspases	Cleaves pro-interleukin-1-beta (pro-IL-1-beta) and IL-18
2	ICH1; NEDD2; CASP-2; NEDD-2; PPP1R57	Initiator caspase	Two forms of caspase-2 exist that have antagonistic effects: caspase- 2L induces programed cell death and caspase-2S suppresses cell death. Caspase-2 is activated by caspase-3, or by a caspase- 3-like protease. Responsible for mitochondria-dependent apoptotic pathway by inducing the release of cytochrome C. *in vitro* caspase-2-released Cyt C is sufficient to activate the Apaf-caspase-9 apoptosome Cleavage of cytosolic Bid protein
3	CPP32; SCA-1; CPP32B	Effector/executioner caspase	Responsible for the proteolysis of the majority of cellular polypeptides, (e.g. poly (ADP-ribose) polymerase (PARP)), which leads to the apoptotic phenotype. Cleavage of Bid
4	TX; ICH-2; Mih1/TX; ICEREL-II; ICE (rel)II	Inflammatory-caspases	Contains a caspase-recruitment domain (CARD) in its N-terminal prodomain, which plays a role in procaspase activation. Able to cleave itself and the p30 caspase-1 precursor. Cleavage of pro-caspase-3
5	ICH-3; ICEREL-III; ICE (rel)III	Inflammatory-caspases	Cleaves itself and the p30 caspase-1 precursor. Cleavage of pro-caspase-3
6	MCH2	Effector/executioner caspase	Responsible for the proteolysis of the majority of cell polypeptides, (e.g. poly (ADP-ribose) polymerase (PARP)), which leads to the apoptotic phenotype. Directly activates caspase-8 and leads to cytochrome c released from the mitochondria; Cleavage and inactivation of laminin
7	MCH3; CMH-1; LICE2; CASP-7; ICE-LAP3	Effector/executioner caspase	Responsible for the proteolysis of the majority of cellular polypeptides, (e.g. poly (ADP-ribose) polymerase (PARP)), which leads to the apoptotic phenotype. Activated by the initiator caspases (caspase-8, caspase-9, and caspase-10. Direct substrate for caspase-1
8	CAP4; MACH; MCH5; FLICE; ALPS2B; Casp-8	Initiator caspase	Activator of the extrinsic apoptosis pathway by death receptor, triggered by death receptor ligation. Linked to cell surface death receptors such as Fas. Endogenous substrates for caspase-8 include procaspase-3, the pro- apoptotic Bcl-2 family member Bid, RIP, PAK2, and the caspase-8 activity modulator FLIP (L).
9	MCH6; APAF3; APAF-3; PPP1R56; ICE-LAP6; CASPASE-9c	Initiator caspase	Cleavage and activation of procaspase-3
10	MCH4; ALPS2; FLICE2	Initiator caspase	Bid, a Bcl2 protein, can be cleaved by caspase-10 The p15 fragment is N-myristoylated and enhances cytochrome c released from mitochondria

Table 3. Caspase classification and functions.

APOPTOSIS AND THE HOMEOSTASIS OF THE IMMUNE SYSTEM

During the several phases of immune response, both intrinsic and extrinsic apoptosis pathways play an important role: both pathways are involved in the elimination of activated T and B lymphocytes that are no longer functional or required, thus contributing to the maintenance of T- and B cell tolerance. After an acute immune response, levels of IL-7 and IL-15 cytokines decrease and Bim levels increase and lead to the activation of intrinsic apoptotic pathway. T cells, in turn, are eliminated through the extrinsic pathway activated by Fas- FasL under conditions of chronic infection or repetitive stimulation by autoantigens. Both pathways also cooperate in the selection of B lymphocytes expressing high-affinity BCRs during affinity maturation (32). Bim is also critical in the maintenance of tolerance and homeostasis of myeloid cells (33).

Apoptosis and other subroutines of cell death are also involved in the elimination of other cells of the immune system (Table 4) (32,34–37).

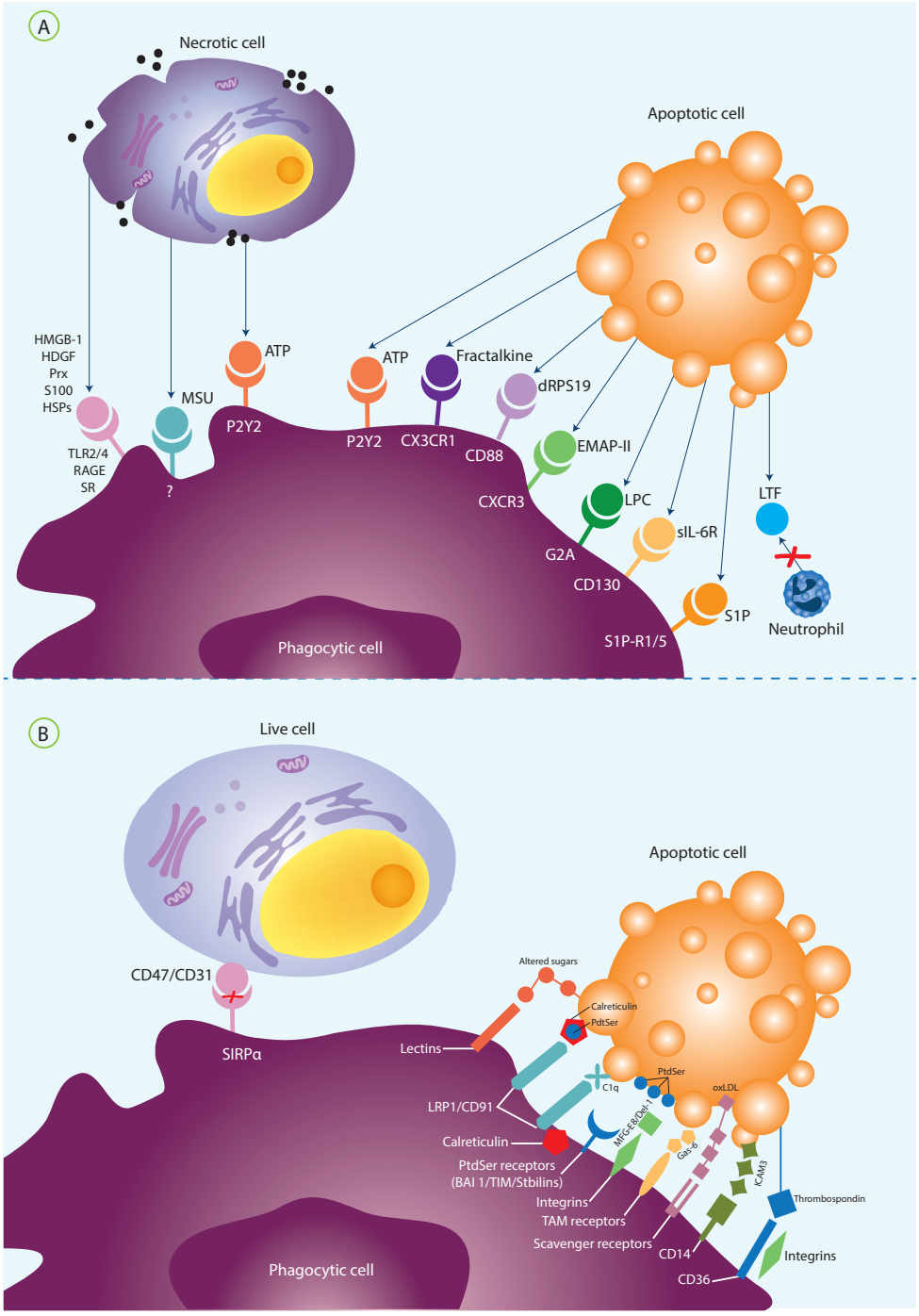

Figure 3. Dying cell clearance. A."Find me" phase. Dying cells release "find me" signals. These molecules bind the cognate receptors present on the phagocytic cell surface, which stimulates the phagocytic cell migration toward the dying target. In necrotic cells, the rupture of the plasma membrane releases the intracellular contents, and the released proteins (such as HMGB-1, HDGF, peroxiredoxins, S100, and heat shock proteins) act as danger signals (damage-associated molecular patterns, DAMP). These molecules activate TLRs on phagocytes thus facilitating antigen presentation and triggering the immune response. Apoptotic cells release lactoferrin (LTF) that acts as a "stay away" signal to inhibit neutrophil migration. B. "Eat me" phase. Dying cells Dying cells present "eat me" signals on their surfaces that are detected by the phagocytic cells. Sometimes the ligand- receptor binding is direct but, in many cases, intermediary molecules are used (such as Gas-6/TAM receptors, MFG-E8/αvβ3/5, and αvβ3/5 /CD36). Phosphatidylserine (PtdSer), the best known "eat me" signal, is recognized directly by BAI-1, TIM, and the stabilins 1 and 2 or indirectly through MFG-E8 and Del-1 that act as a bridge between PtdSer and integrins. The expression of CD47 (integrin-associated protein) and CD31 on the surface of live cells inhibit engulfment by phagocytes. Adapted from references (29, 30). Abbreviations: dRPS19, dimer of ribosomal protein S19; EMAP-II, endothelial monocyte activating polypeptide II; HDGF, hepatoma-derived growth factor; HMGB-1, high mobility group box 1 protein; ICAM3, intercellular adhesion molecule 3; LPC, Lysophosphatidylcholine or lysolecithin; sIL-6R, ectodomain of the IL-6 receptor; S1P, sphingosine-1-phosphate; RAGE, receptor for advanced glycation end products; TRL, toll-like receptor.

TYPE OF CELL	TYPE OF CELL DEATH (32, 34-37)
Lymphocytes	Apoptosis
Monocytes/ macrophages	Variable based on the pathogen infection: Pyroptosis, necrosis or apoptosis
Neutrophils	*In vivo*: Netosis or apoptosis *In vitro*: autophagic-like and autophagic-related necroptosis
Dendritic cells	Pyroptosis or necrosis
Mast cells	NETosis
Eosinophils	NETosis

Table 4. Subroutines of death involved in the elimination of immune cells.

APOPTOSIS AND AUTOIMMUNE DISEASES

Diseases produced as a consequence of unregulated apoptosis could be the result of increased cell survival due to inhibition of apoptosis, an augmentation in cell death due to hyperactive apoptosis, or the defective clearance of apoptotic cells. As previously mentioned, apoptosis plays a critical role in immune homeostasis and maintenance of immune tolerance. Although there are controversies regarding the role of cell death mechanisms in autoimmune disease pathogenesis, the available evidence suggests disruptions on the molecular mechanisms of death pathways (Table 5) (11,29,31–33,38–53).

TYPE OF CELL DEATH	CONSEQUENCE OF CLEARANCE	PHYSIOLOGICAL RELEVANCE	AUTOIMMUNE DISEASES PATHOGENESIS		OTHER DISEASES
Apoptosis (29,31-33,38-44)	Production of anti-inflammatory cytokines and tolerance induction	Organ and tissue remodeling during development, maintenance of homeostasis in adults	SLE	Defects in apoptotic cell clearance and accumulation of secondary necrotic debris (Defect on C1q and MFG-E8)	Cancer,Neurological disorders (Alzheimer, Parkinson, Huntington, stroke), Bronchial asthma; Inflammatory intestinal disease, Cardiovascular disorders (Ischemia, heart failure), Infectious disease (bacterial and viral)
				Deficiency in DNAse II leads to autoimmune phenotype	
				Over-expression of anti-apoptoic members of Bcl-2 family and TNF	
			ALPS	Reduced levels of Bim protein	
				Defects in FAS signaling pathway: germ line and somatic mutation on FAS or FAS ligand or caspase-10 or caspase-8	
			RA	Increase of joint cellularity due to decrease in apoptosis	
				Deregulation of Bim, increased expression of Bcl-2, increased expression of c-FLIP, over-expression of SUMO-1	
			SS	Apoptosis of epithelial cells through granule pathway (CD4+ and CD8+ T cells induced)	
Necroptosis (11,45)	Production of pro-inflammatory cytokines and chemokines, and stimulation of adaptive immunity	Stimulation of immune system in response to viral infection, ovulation, longitudinal growth of bones, intestine cell turnover	SLE	Necroptosis of T cells (instead of apoptosis)	Neuronal excitotoxicity, ischemia, myocardial infarction
Autophagy (11,46-47)	–	Regulation of innate and adaptive immunity, differentiation and development process	SLE	Mutation on ATG5 gene increases susceptibility to disease	Ischemia/reperfusion
Pyroptosis (11,48-49)	–	Defense against microbial infection	Vitiligo and vitiligo-associated Addison's disease	Mutation on NLRP1 gene increases incidence	Myocardial infarction, cerebral ischemia, neurodegenerative diseases, inflammatory bowel disease, endotoxic shock

TYPE OF CELL DEATH	CONSEQUENCE OF CLEARANCE	PHYSIOLOGICAL RELEVANCE	AUTOIMMUNE DISEASES PATHOGENESIS		OTHER DISEASES
NETosis (50-53)	Production of pro-inflammatory cytokines	Defense against bacteria, fungi, parasites, and virus infection	SLE	Deficiency in degradation of NETs	Table 7
			RA	Externalization of citrullinated autoantigens	
			Vasculitis	Presence of NETs proteases	
			Psoriasis	NETs are a source of IL-17	

Table 5. **Cell death subroutines and disease pathogenesis.**

GRANZYMES

Cytotoxic T lymphocytes and natural killer cells as well as CD4+ cells, mast cells, activated macrophages, neutrophils, basophils, dendritic cells, and T regulatory cells produce and deliver the serine proteases known as granzymes. In humans, five types have been described: GrA, GrB, GrH, GrK, and GrM. Although all of the five types have been associated with the induction of cell death, the GrB mechanism of action is the best described (Figure 4) (44,54).

Moreover, GrB (also known as CCP1 or cytotoxic T lymphocyte associated-1 (CTLA1)) cleaves both extracellular and intracellular substrates thereby uncovering cryptic epitopes of intracellular proteins which generate GrB unique autoantigenic fragments (Table 6) (44,55–61). In rheumatoid arthritis joints, GrB has also been implicated in cartilage destruction, apoptosis of resident cells, and extracellular matrix degradation (44). The expression of GrB has been described not only in the infiltrating immune cells (cytotoxic T cells, macrophages, NK cells, and T-helper cells) but in chondrocytes as well (62). GrA levels are also elevated in synovial fluid of rheumatoid arthritis patients (63).

AUTOIMMUNE DISEASES	CLEAVED AUTOANTIGEN
Systemic lupus erythematosus (55,56)	XRCC4
Rheumatoid arthritis (57)	Aggrecan
Sjogren's syndrome (58,59)	SS-B (La)
Myositis (60)	α-fodrin
	β-fodrin
	Type 3 muscarinic acetylcholine receptor
	PMS-1
	HisRS
Systemic sclerosis (61)	Topoisomerase I
	NOR-90
	Fibrillarin
	B23

Table 6. **Autoantigens generated by GrB protease activity.** Adapted from reference (44).

NETOSIS

Brinkmann et al. described a novel mechanism of neutrophil cell death that results in the externalization of antimicrobial peptides that are originated from nuclear granules, histones, and some cytoplasmic proteins (64). The extruded material makes traps that either capture and kill bacteria and fungi (64–66) or capture and inhibit bacteria and fungi dissemination (67). The NETs (neutrophil extracellular traps) consist of smooth filaments, each of which has a diameter of approximately 17 nm and is composed of stacked nucleosomes, and other filaments of approximately 50nm made of granular proteins. Using the high-resolution scanning electron microscopy, it was possible to clearly differentiate NETs from other fibrous structures such as fibrin (68).

This death mechanism has been associated with several pathologies and as has been discovered, it can be triggered by several factors such as IL-8, LPS (lipopolysaccharide), PMA (phorbolmyristate acetate), platelets (via TLR-4), INFα/γ and C5a, GM-CSF (granulocyte–macrophage colony-stimulating factor), etc. (69–71). Multiple microorganisms can induce NET production including gram-positive bacteria, gram-negative bacteria, fungi, parasites, and viruses (Table 7) (50–53, 64,65,70,72–98). Certain pathogens seem to have developed mechanisms to evade NET action by the production of DNAses, inhibition of proteins in the traps, or modification of cell wall structures (69,74,75,77,99).

During NET formation, there is loss of neutrophil intracellular and plasma membrane integrity, followed by cell flattering, chromatin descondensation, and granule disintegration. Afterward, the nuclear envelope disaggregates into vesicles and the nucleoplasm and cytoplasm form a homogenous mass. Finally, the cells round up and seem to contract until the cell membrane ruptures and the cell content is ejected into the extracellular space, producing the extracellular trap (68).

Approximately 20 components of neutrophils (granules, nucleus, cytoplasm, mitochondria, peroxisome) are present in the traps (83). The enzymes elastase (NE), myeloperoxidase (MPO), and peptidyl arginine deaminase 4 (PAD4) are essential in NET formation. These enzymes are involved in the cleavage of histones, chromatin descondensation, and the conversion of arginine to citrulline (100,101).

The production of these traps is not confined to neutrophils. Other immune cells such as eosinophil (EETs), mast

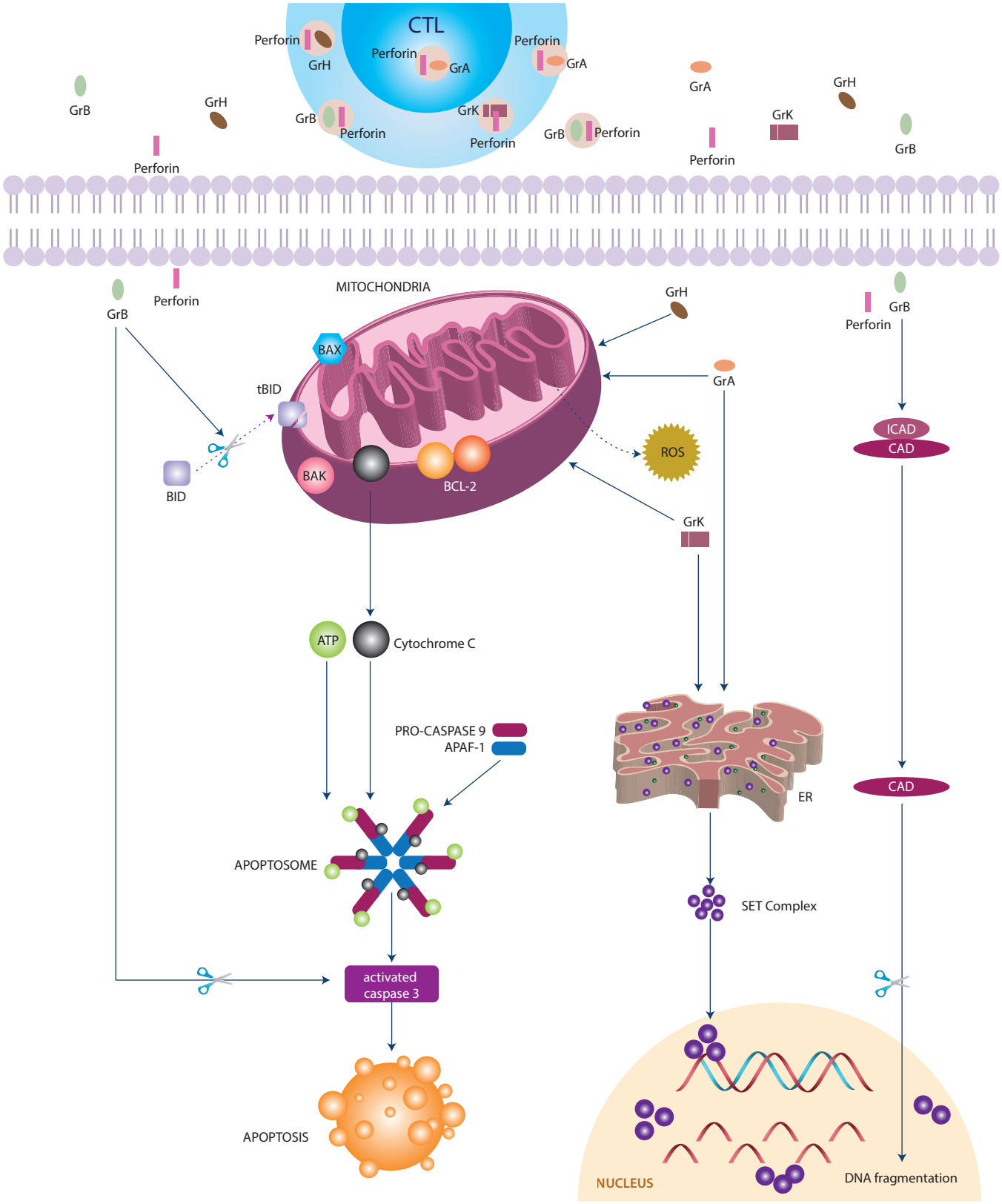

Figure 4. Granzyme induction of apoptosis. GrB activates caspase-3 and cleaves Bid, thus inducing inducing mitochondrial outer membrane permeabilization (MOMP). This enzyme also cleaves other pro-apoptotic proteins such as ICAD/DFF45, Mcl-1, laminin B and PARP. GrH also target the mitochondria. GrA and GrK induce ROS generation and SET complex translocation from ER to nucleus. Perforins are believed to facilitate GR penetration of the target cells. Adapted from references (44, 54).

PATHOGENS THAT INDUCE NETS	PATHOPHYSIOLOGICAL CONDITION
• *Staphylococcus aureus* (72-73) • *Streptococcus pyogenes* (74) • *Streptococcus pneumonia* (75) • *Escherichia coli* (76) • Group A *Streptococcus pyogenes* (77) • *Shigella flexneri* (64) • *Salmonella enteric* (64) • Nontypeable *Haemophilus influenzae* (78) • *Burkholderia pseudomallei* (79) • *Yerseni aenterocolitica* and *Yersenia pseudotuberculosis* (80) • *Mannheimia haemolytica* (81) • *Mycobacterium tuberculosis* (82) • *Mycobacterium canetti* (82) • *Lysteria monocytogenes* (82) • *Candida albicans* (65, 83) • *Aspergillu sfumigatus* (84) • *Schistosoma* (85) • *Lesihmania amazonensis* (86) • *Leishmania donovani* (87) • *Toxoplasma gondii* (88) • *Plasmodium falciparum* (89) • HIV-1 (90)	• Appendicitis (64) • Sepsis (70) • Fasciitis (74) • Pre-eclampsia (91-92) • Mastitis (93) • Periodontitis (94) • Cystic fibrosis (95) • Thrombosis (96) • Asthma (97) • Small vessel vasculitis (SVV) (50) • Crohn Disease (85) • Felty's s syndrome (98) • Systemic lupus erythematosus (52) • Rheumatoid Arthritis (53) • Psoriasis (51)

Table 7. Pathogens and pathophysiological condition associated with the formation of extracellular traps.

cells (MCETs), and monocytes (METs) have the ability to produce traps as well (69,85,97,102,103). Therefore, this novel cell death subroutine has been renamed "ETosis"(104).

NETOSIS AND AUTOIMMUNITY

Neutrophils play a major role in the autoimmune response and have been implicated in many autoimmune diseases such as vasculitis, systemic lupus erythematosus (SLE), dermatomyositis, rheumatoid arthritis (RA), and psoriasis. Moreover, the neutrophils are a major source of autoantigens involved in autoimmune diseases (Table 8) (34,98, 105–122).

The relationship between NETosis and autoimmunity has recently been pointed out. In 2009, Kessenbrock et al. were the first authors to point out the involvement of NETosis in the pathogenesis of small vessel vasculitis. They observed the presence of MPO and PR3 (proteinase 3) in samples from patients with small vessel vasculitis (50). The presence of NETs in patients with vasculitis could play an important role in the formation of thrombus (123).

Hakkim et al. were the first researchers to observe the relationship between NETs and SLE in 2010. They found that patients with lupus did not have the ability to degrade NETs by alterations in DNAse I activity (important for NET degradation) or by the presence of DNAse I inhibitors (52). Mutations on DNAse I gene had also been reported in patients with SLE (124).

Leffler et al. also observed deficiencies in NET degradation in a cohort of 94 patients with SLE. They found that C1q, together with DNAse I, are involved in the degradation process. They concluded that hypocomplementemia, previously observed in lupus patients, could be explained by the consumption of complement in the degradation of NETs (See chapter 4) (125). They also established that patients with poor degradation of NETs presented increased titers of double-stranded DNA antibody and that they had more risk of developing lupus nephritis (125).

In 2011, Lande et al. observed that LL-37 (intracellular peptide) from lupus patients created a complex with DNA and with anti-DNA autoantibodies which prevented the degradation of NETs. This complex also stimulates pDC (plasmocitc dendritic cells) via Toll-like receptor 9 (TLR9) to secrete IFN α (112).

In a SLE pediatric cohort study, Garcia-Romo et al. showed that SLE-derived neutrophils, primed *in vivo* with type I IFNs, died by NETosis upon exposure to SLE sera-derived anti-ribonucleoprotein antibodies (RNP). Such recognition of RNP

AUTOANTIGENS	AUTOIMMUNE DISEASES
Actin, cytoplasmic 1 and 2 (β/γ)	Rheumatoid arthritis (105)
α-Actin 1 and/or 4	Systemic lupus erythematosus (106)
Annexin AI	Systemic lupus erythematosus (107)
Azurocidin	Vasculitis (108)
C1q	Systemic lupus erythematosus (109)
Catalase	Systemic lupus erythematosus (110) Rheumatoid arthritis (110)
Cathepsin G	Vasculitis (111)
Defensins (HNP)	Systemic lupus erythematosus (112)
dsDNA/chromatin	Systemic lupus erythematosus (113)
Leukocyte elastase	Vasculitis (111)
α- Enolase	Rheumatoid arthritis (114)
Glyceraldehyde 3-phosphate dehydrogenase	Multiple sclerosis (115) Ovarian autoimmunity (116) Systemic lupus erythematosus (117)
Histones	Systemic lupus erythematosus (113) Drug-induced lupus (113) Felty's Syndrome (98) Rheumatoid arthritis (118)
Lactoferrin	Rheumatoid arthritis (119) Vasculitis (111) Autoimmune pancreatitis (120)
LL37	Systemic lupus erythematosus (112)
Lysozyme C	Vasculitis (111)
Myeloperoxidase	Vasculitis (111)
Myosin-9	Autoimmune polyendocrine syndrome type I (121)
Proteinase 3	Vasculitis (111)
Transketolase	Multiple Sclerosis (122)

Table 8. Autoantigens derived from proteins present in NETs and in autoimmune diseases. Adapted from reference (34).

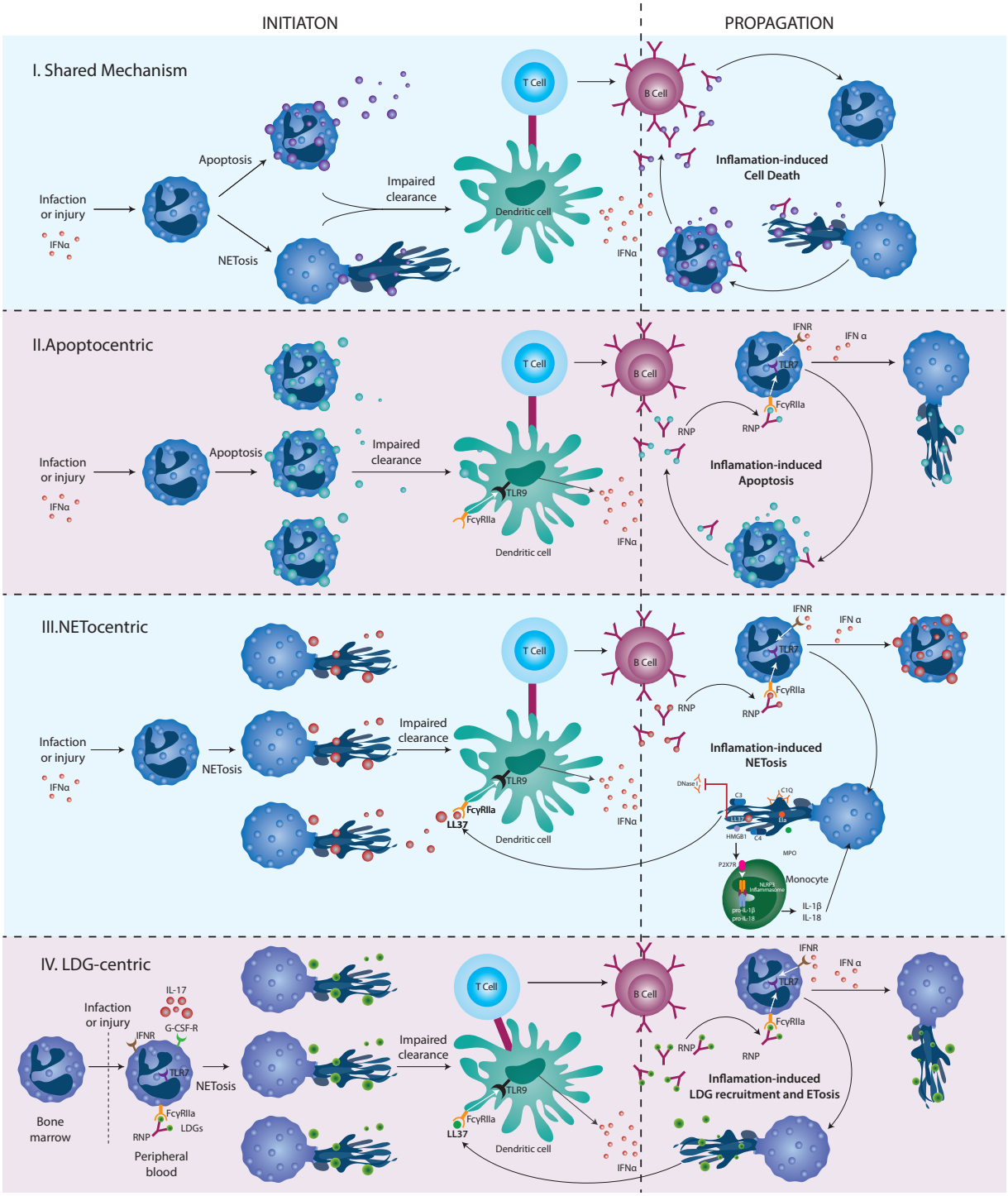

Figure 5. Theoretical models to explain the integration of apoptosis and NETosis into the paradigm of autoimmune diseases. **Model I.** Shared Mechanism. Impaired clearance pathways increase the presence of autoantibodies which triggers an autoimmune response. **Model II.** Apoptocentric. Neutrophils are actively dying by apoptosis and undergoing secondary necrosis. These apoptotic bodies are recognized by plasmacytoid dendritic cells (pDCs). Activation of pDCs leads to the production of IFNα and the presentation of autoantigens to T helper cells. T helper cells stimulate autoantibody production by B cells. The production of anti- RNP induced a prominent reduction in the apoptosis by changing the cell death program to NETosis. IFNα induce an augmentation in the expression of TLR7 in neutrophils and thus make them more susceptible to death by RNP-Ig-induced NETosis via FcγRIIa/TLR7 binding. **Model III.** NETocentric. Proteins present in the NETs are recognized by pDCs leading to the production of IFNα and presentation of autoantigens to T helper cells. C1q and LL37 present in the extracellular traps inhibit the action of DNase I. The monocytes recognize proteins present in NETs and increase the production of NLRP3, IL-1β, and IL-18, which then stimulates NETosis and leads to self-perpetuation of NET production. **Model IV.** LDG-centric. Low-density granulocytes (LDGs) are responsible for the generation of NETs. IL-17 is exposed in LDG-NETs, suggesting that this cytokine may be involved in tissue damage. Adapted from reference (34).

PROTEIN	FUNCTION
C1q	Active classical pathway of complement. Protects NET degradation from DNase I (125)
Elastase	Serine protease. Increases tissue damage (126,127)
Histones	Pattern of modification suggests transcriptional quiescence (127,129)
HMGB1	Alarmin; activates antigen-presenting cells (126)
HNP	May function similarly to LL37 (112)
IL-17	Proinflammatory cytokine (127)
LL37	Small cathelicidin peptide. Actives pDCs. DNase I protection (112,126,127)
MPO	Peroxidase activity. Increases tissue damage (112, 126,127)

Table 9. Proteins detected in extracellular traps of patients with SLE. Abbreviations. HMGB1: High-mobility group box 1; HNP: Human neutrophil peptides; pDCs: plasmocytoid dendritic cells; MPO: myeloperoxidase. Adapted from reference (128).

is dependent on FcγRIIa, NADPH-, and TLR7 (126). Moreover, Villanueva et al. revealed that low density granulocytes (LDGs) of patients with lupus have an enhanced ability to form NETs. These NETs (peripheral blood and skin) activate pDCs which increases IFN α production by externalization of IL-17 and contributes to tissue damage and immune deregulation. Biopsies of patients with lupus nephritis and skin lesion show high levels of neutrophil intracellular proteins such as LL37, double-stranded DNA, and IL-17. The authors suggest that the elevated immune response and uncontrolled development is associated with accelerated atherosclerosis (127) (See chapter 38).

Disruptions in the degradation of NETs support the hypothesis that neutrophil intracellular proteins expressed during the NETosis process become a source of antibody production and increased tissue damage. This, in turn, increases the autoimmune response and the production of IFN α. Several intracellular proteins from neutrophils have been detected in samples from lupus patients (Table 9) (112,125–129). According to a recent description, monocytes from patients with lupus, upon recognition of NETs and LL-37, can active caspase-1 via P2X7R. The subsequent activation of the inflammasome machinery and increased production of IL-1β and IL-18 stimulate NETosis, thus leading to a self-perpetuating cycle of NET production (130).

The role of the extracellular traps has also been reported in other autoimmune diseases such as psoriasis. These traps from neutrophils and mast cells are important sources of IL-17. Therefore, the production of IL-23 activates Th17 differentiation and triggers MCETs (51).

Recently, the role of NETosis in the pathogenesis of RA has been reported. Khandpur et al. observed that the production of NETs is enhanced in peripheral blood and synovial fluid of patients with RA. This correlates with a rise in the level of citrullinated protein antigens and systemic inflammation. These citrullinated autoantigens are externalized during NETosis. Moreover, in vitro experiments showed that stimulation of RA-derived neutrophils with IL-17A and TNF-α induce NETosis. In addition, in RA-derived synovial fibroblasts, NETs significantly augmented inflammatory responses including induction of IL-6, IL-8, chemokines, and adhesion molecules (53).

Based on several studies, four hypothetical models have been proposed in an attempt to integrate apoptosis and NETosis death subroutines and to establish the relationship with autoimmune disease (34) (Figure 5).

In conclusion, alterations in the removal of apoptotic bodies and proteins derived from NETs leads to the perpetuation of specific autoantibodies being generated, augmentation of secretion of proinflammatory cytokines, and tissue damage. Therefore, the inhibition of NETproduction has been proposed as an important therapeutic target in the treatment of autoimmune diseases.

CROSS-TALK BETWEEN THE SUBROUTINES

The existence of different types of death allows the cell to switch or combine the subroutine of death based on the nature and severity of the stimulus. For example, in the case of T cells, high levels of caspase activation induce apoptotic cell death while the presence of caspase inhibitors, genetic deficiency of FADD, or Caspase-8 leads to autophagy. Moreover, under different situations, apoptosis and autophagy can exert synergetic effects (131). Recently,Bcl-2, Bcl-xL,Bcl-w, Mcl-1, and c-FLIP have been shown to inhibit autophagy (55,132–135).

A similar situation occurs between apoptosis and ne- croptosis. Apoptosis shifts to necroptosis when ATP levels are low or caspases are inhibited. Both processes might also be induced simultaneously (136,137).

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Section II

From the mosaic of autoimmunity to the autoimmune tautology

14 FROM THE MOSAIC OF AUTOIMMUNITY TO THE AUTOIMMUNE TAUTOLOGY

Juan-Manuel Anaya
Adriana Rojas-Villarraga
Yehuda Shoenfeld

INTRODUCTION

Autoimmune diseases (ADs) are chronic conditions initiated by the loss of immunological tolerance to self-antigens and represent a heterogeneous group of disorders that affect specific target organs or multiple organ systems. The chronic nature of these diseases places a significant burden on the utilization of medical care, direct and indirect economic costs, and quality of life.

The mosaic of autoimmunity describes the multi-factorial origin and diversity of AD expression (1). This term implies that different combinations of many factors involved in autoimmunity produce several distinct clinical presentations that represent the wide spectrum of AD. The term "kaleidoscope of autoimmunity" portrays the possible change from one disease to another or the fact that more than one disease may coexist in the same individual or family (2). The fact that ADs share several clinical signs and symptoms (i.e. subphenotypes), physiopathological mechanisms, and genetic factors has been called the autoimmune tautology and indicates that they have several common mechanisms (3-7) (Table 1). Tautology (from Greek *tauto*, "the same" and *logos*, "word/idea") is an obvious statement. In logic, tautology is a formula, which is true in every possible interpretation. Thus, autoimmune tautology means that one AD is similar to a second one, to a third one, and so on. ADs cannot be equal because the target cell and organ are different in each case, and the trigger factors and age at onset vary among ADs. Ten shared characteristics supporting this logically valid propositional theory are discussed below (Table 1).

WOMEN ARE MORE PRONE TO AUTOIMMUNITY

Almost 5% of the world population develops an AD (8). Of this 5%, approximately 80% are women (9), and it is considered the fourth leading cause of disability for them (10). The more frequent the AD and the later it appears, the more women are affected. Women tend to have a different age at onset and different disease activity than men. Also, female gender appears to be a risk factor for polyautoimmunity (11). The most convincing explanation of female biased autoimmunity remains the hormonal theory followed by genetic factors (12).

SHARED SUBPHENOTYPES

Subphenotypes are shared by ADs including signs and symptoms such as arthralgia, arthritis, alopecia, fatigue, photosensitivity, Raynaud's phenomenon as are non-specific autoantibodies (e.g., antinuclear antibodies, rheumatoid factor, anti-Ro antibodies) and high levels of cytokines (e.g., TNF, IL-1, IL-6, IL-10, IL-17, etc.) which raises taxonomic concerns. ADs have a heterogeneous spectrum such that disease courses differ from patient to patient and, in addition, the disease goes through different phases within the same patient. Depending on the duration and activity of the disease, these subphenotypes might change. Mathematical approaches for precisely defining subphenotypes based on accurate clinical and immunological databases (13) combined with strengthening molecular genetic analyses have significant promise for a better understanding of ADs.

POLYAUTOIMMUNITY

Polyautoimmunity is defined as the presence of more than one AD in a single patient. When three or more ADs coexist, this condition is called multiple autoimmune syndrome (14,15). Polyautoimmunity represents the effect of a single genotype on diverse phenotypes. Polyautoimmunity was observed in 34.4% of 1,083 patients belonging to four AD cohorts (11) with autoimmune thyroid disease and SS being the most frequent diseases encountered (11). Factors signifi-

CHARACTERISTIC	COMMENT
Female predominance	The more frequent the AD and the later it appears, the more women are affected
Shared subphenotypes	Mathematical approaches for precisely defining subphenotypes based on accurate clinical and immunological databases combined with strengthening molecular genetics analyses have significant promise for a better understanding of ADs
Polyautoimmunity	Factors significantly associated with polyautoimmunity are female gender and familial autoimmunity
Familial aggregation	Unlike familial AD, which corresponds to the presence of one specific AD in various members of a nuclear family, familial autoimmunity uses the term "autoimmune disease" as a trait that encompasses all accepted pathologies for which evidence suggests an autoimmune origin
Age at onset influences severity	Early age at onset is a poor prognostic factor for some ADs
Similar pathophysiology	Damage induced by T or B cells, or both, plays a major pathogenic role in ADs. Although the autoimmune phenotype varies depending on the target cell and the affected organ, the local mechanisms for tissue injury are similar (e.g., activation of the Type I interferon pathway, decreased T and B regulatory functions)
Autoimmune ecology	Although a latitudinal gradient of infectious agents exists, Epstein-Barr virus and cytomegalovirus are notorious as they are consistently associated with multiple ADs. Some infections could be protective against AD development. Smoking and organic solvents have also been consistently associated with several ADs. Socioeconomic status influences ADs considerably
Ancestry	Amerindian ancestry influences the risk of acquiring ADs as well as its severity
Common genetic factors	The genetic risk factors for ADs consist of two forms: those common to many ADs and those specific to a given disorder. Combinations of common and disease-specific alleles in HLA and non-HLA genes in interaction with epigenetic and environmental factors over time will determine the final phenotype
Similar treatment	Similar biological and nonbiological therapies are used to treat diverse ADs

Table 1. Common mechanisms of autoimmune diseases (the autoimmune tautology). From references (3-7).

cantly associated with polyautoimmunity are female gender and familial autoimmunity (11).

Polyautoimmunity has been reported in most of the ADs including systemic lupus erythematosus (SLE) (41%) (16), systemic sclerosis (SSc) (26%) (17), primary biliary cirrhosis (32%) (18), vitiligo (27%) (19), myasthenia gravis (13%) (20), autoimmune thyroid disease (14%) (21), etc. Sardu *et al*. (8) confirmed a 5% prevalence of ADs in Sardinia. Among people with ADs, 95.6% were affected by one AD while the remaining 4.4% were affected by two ADs (8).

The main difference between polyautoimmunity and the overlapping syndromes lies in the fact that the former is the presence of two or more well-defined autoimmune conditions fulfilling validated classification criteria while the later is the partial presence of signs and symptoms of diverse ADs. Most of the cases of overlapping syndromes have been described in cross-sectional studies. As has been shown, there is a lag in the time interval between the first and the second AD. Just as in the mixed connective tissue disease (MCTD), the classical overlapping syndrome, in which some patients will develop SLE, SSc, or RA during the course of the disease, and some will presenting with a longstanding MCTD - incomplete sentence lacking main verb and the overlapping syndrome seems to have gotten lost! (22). In fact, long-term studies have shown that MCTD remains an overlapping syndrome in about 60% of the patients. The remaining 40% progress towards SSc (~20%), SLE (~10%), or rheumatoid arthritis (RA) (~5%) (23).For laccarino *et al* (24) polyautoimmunity is considered an "overlap syndrome" confined to "connective tissue diseases" [e.g., SLE, RA, SSc, polymyositis/dermatomyositis (PDM), and Sjögren's syndrome (SS)]. These authors reduce the spectrum of polyautoimmunity to just the rheumatic diseases and omitted several other systemic and organ specific ADs that are also associated with each other and observed in clusters (Figure 1) (11). However, they highlight that, in some cases, polyautoimmunity may be related to a specific autoantibody which supports the hypothesis that these syndromes are not a mere association of two or more ADs in the same patient, but a well-defined clinical entity with specific clinical characteristics (24). Two of such cases are: 1) Anti-t-RNA synthetase syndrome, characterized by the clinical features of SSc, RA, and myositis and the presence of antibodies against aminoacyl-t-RNA synthetase; and 2) Scleromyositis, characterized by features of both SSc and PDM and the presence of anti-PM–Scl antibodies (24).

FAMILIAL AGGREGATION

A primary characteristic of complex diseases is that affected individuals tend to cluster in families (familial aggregation, also referred to as recurrence risk or λ). The aggregation of a phenotype is observed when a disease occurs at a higher frequency in the relatives of an affected individual when compared to what is seen in the general population (14). Familial autoimmunity is defined as the presence of diverse ADs in multiple members of a nuclear family. Unlike familial autoimmune disease, which corresponds to the presence of one specific autoimmune disease in various members of a nuclear family, familial autoimmunity uses the term "auto-

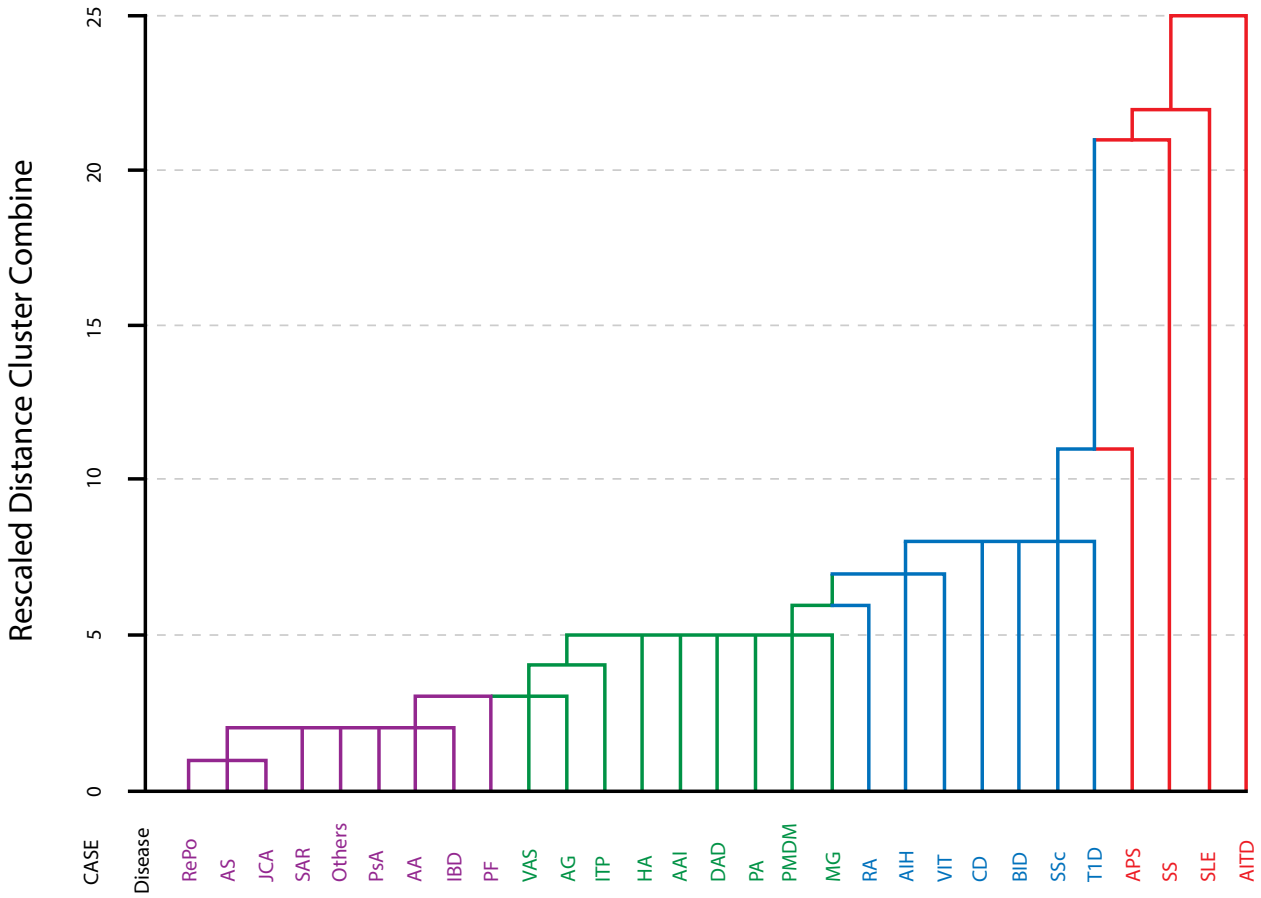

Figure 1. Cluster analysis dendogram of autoimmune diseases (ADs). Each node represents a stage in the clustering process. There were four clusters. The most hierarchical was composed of four ADs. *AITD*: autoimmune thyroid disease (including thyroiditis, Hashimoto disease, Graves disease); *SLE*: systemic lupus erythematosus; *SS*: Sjögren's syndrome; *APS*: antiphospholipid syndrome; *T1D*: type 1 diabetes mellitus; *SSc*: scleroderma (including localized, systemic, diffuse, limited); BID: billiary inflammatory disease (including primary biliary cirrhosis, primary sclerosing cholangitis); *CD*: celiac disease; *VIT*: vitiligo; *AIH*: autoimmune hepatitis; *RA*: rheumatoid arthritis; *MG*: myasthenia gravis; *PMDM*: polymyositis/dermatomyositis; *PA*: pernicious anemia; *DAD*: demyelinating autoimmune diseases (including multiple sclerosis, transverse myelitis, optic neuromyelitis); *AAI*: autoimmune adrenal insufficiency (Addison disease); *HA*: autoimmune anemia; *ITP*: idiopathic thrombocytopenic purpura; *AG*: autoimmune gastritis; *VAS*: vasculitis (including eosinophilic granulomatosis with polyangiitis, giant cell arteritis, microscopic polyangiitis, cryoglobulinemia, polyarteritis nodosa, granulomatosis with polyangiitis); *PF*: pemphigus (including vulgaris, bulloso, foliaceous); *IBD*: inflammatory bowel disease (including ulcerative colitis, Crohn's disease); *AA*: alopecia areata; *PsA*: psoriasis (including psoriatic arthritis); *SAR*: sarcoidosis; *JCA*: juvenile chronic arthritis; *AS*: ankylosing spondylitis; *RePo*: relapsing polychondritis. From Rojas-Villarraga *et al.* (11).

immune disease" as a trait that encompasses all accepted pathologies for which evidence suggests an autoimmune origin. Familial autoimmunity is more frequent than familial autoimmune disease (14,25) and represents the best model for the study of major autoimmunity genes (Figure 2).

AGE OF ONSET INFLUENCES SEVERITY

The age at onset refers to the time period in which an individual experiences the first symptoms of a disease. In ADs, these symptoms can be subtle but are very relevant for diagnosis. They can appear during childhood, adulthood, or late in life and may vary depending on the age at onset. Early age at onset is the worst prognostic factor for some ADs such as SLE and Type 1 Diabetes Mellitus. For others, either it does not have a significant influence on the course of disease such as in the case of SS, or no unanimous consensus exists, e.g., RA and multiple sclerosis (26). It is noteworthy that late-onset traits are more sensitive to environmental variation than genetic influence.

SIMILAR PATHOPHYSIOLOGY

Damage induced by T cells or B cells, or both, play a major pathogenic role in ADs. Similar immunopathological mechanisms lead to ADs (Figure 3). The autoimmune phenotype varies depending on the target cell and the affected organ. The predominant infiltrating cells include phagocytic mac-

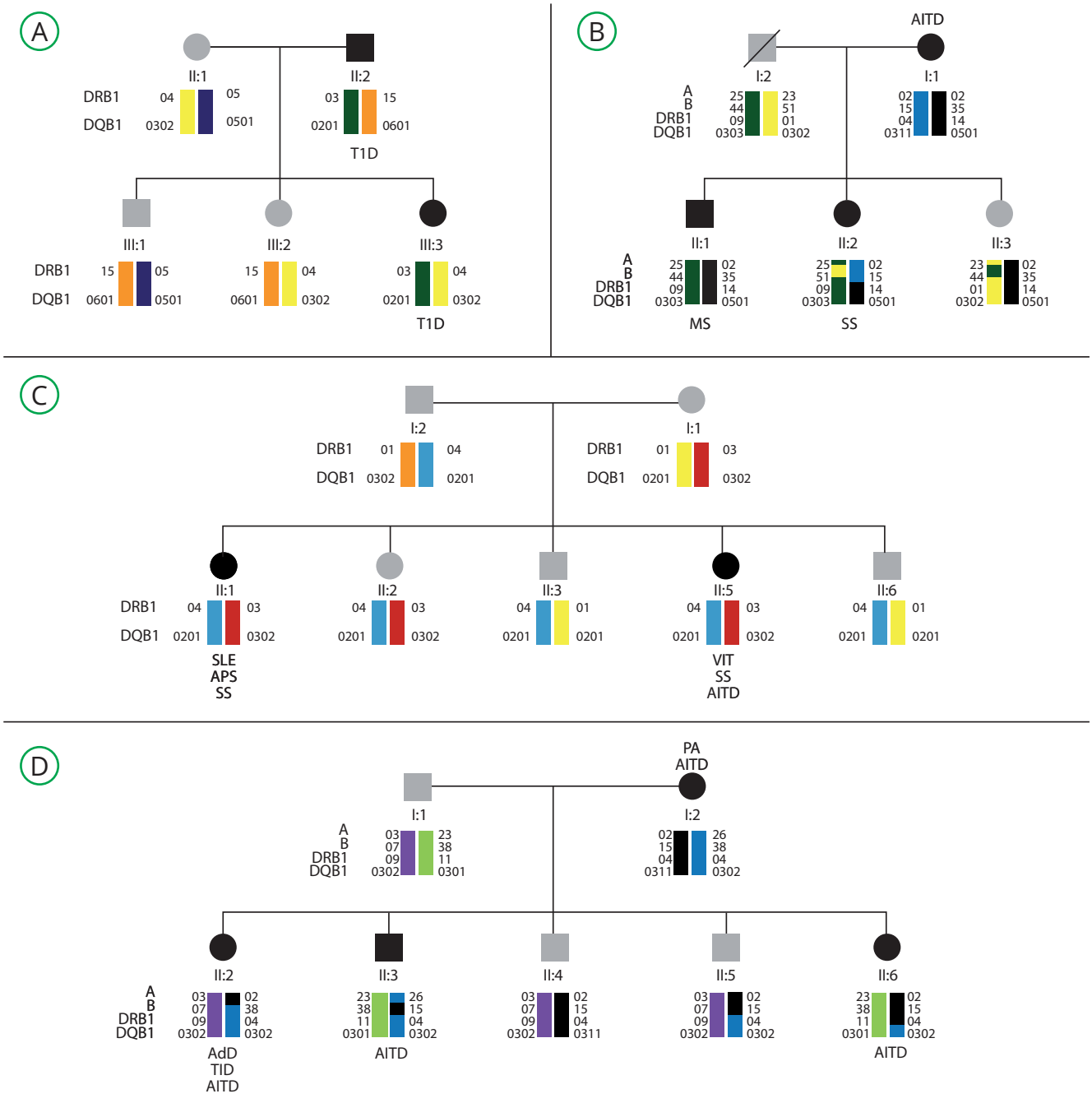

Figure 2. How do autoimmune diseases cluster in families? A) Familial autoimmune disease. This classical concept indicates the same AD in diverse FDRs. In this case, a proband and a FDR (that is, the father) present with T1D. B) Familial autoimmunity. This new concept corresponds to the presence of different ADs in a nuclear family. C) Multiple autoimmune syndrome. This condition refers to the presence of three or more autoimmune diseases in the same subject. In this case, two brothers met criteria for the syndrome. Moreover, this pedigree also meets criteria for familial autoimmunity. D) Polyglandular autoimmune syndrome type II. In this family, however, familial autoimmune disease and familial autoimmunity coexist. The results of HLA gene (i.e., A, B, DRB1, DQB1) typing are shown in colors (by reverse dot blot using InnoLipa Kit). A suggestive linkage among the HLA loci is observed. In these diagrams, people are represented by symbols: circles for female and squares for male, and the bottom line represents the offspring of the couple above. Solid symbols represent affected individuals. Symbols with a diagonal line indicates deceased individual. *AdD*, Addison's disease; *AITD*, autoimmune thyroid disease; *APS*, antiphospholipid syndrome; *FDRs*, first degree relative; *MS*, multiple sclerosis; *PA*, pernicious anemia; *SLE*, systemic lupus erythematosus; *SS*, Sjögren's syndrome; *T1D*, type 1 diabetes; *VIT*, vitiligo.

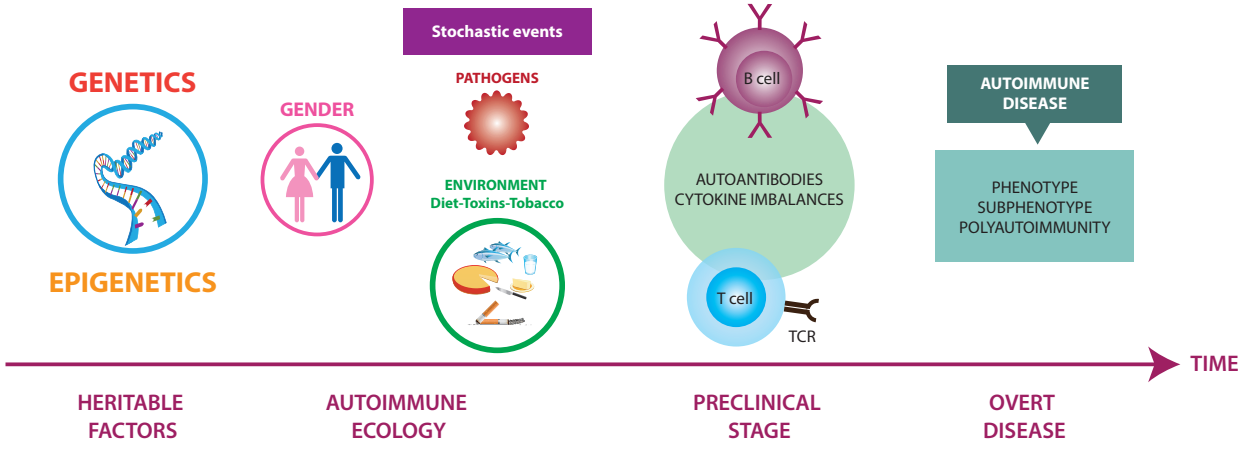

Figure 3. The fourth-stage-model for the pathophysiology of autoimmune diseases (ADs) (the autoimmune tautology). Outline showing the plausible stages for a multifactorial etiology to develop over time. Each stage shows the known phenomena that, when it accumulates, will be the causative scenario for the onset of ADs. First, heritable factors (genetics, epigenetics, and ancestry) have an impact over the life of the individuals. They converge and interact to create and increase or decrease the liability an individual would have to develop the phenotype. Second, the autoimmune ecology is characterized by the effect of environmental factors which, acting stochastically, will also influence the risk and course of disease. Once the autoimmune tolerance is lost by the interaction of heritable and environmental risk factors, a preclinical stage characterized by B and T cells dysregulation arises. This third phase may take years before the phenotype becomes clinically evident. Adapted from Anaya JM (4).

rophages, neutrophils, self-reactive $CD4^+$ T helper cells, and self-reactive $CD8^+$ cytolytic T cells along with smaller numbers of natural killer cells, mast cells, and dendritic cells. Among the T effector cells–Th1, Th17, and Th9 cells–contribute to pathogenesis of ADs (27).

Immune cells damage tissues directly by killing cells or, indirectly, by releasing cytotoxic cytokines, prostaglandins, reactive nitrogen, or oxygen intermediates. Tissue macrophages and monocytes can act as antigen-presenting cells to initiate an autoimmune response or, as effector cells once an immune response has been initiated. Macrophages act as killer cells through antibody-dependent, cell-mediated cytotoxicity and by secreting Th1 cytokines, which act as protein signals between cells. Macrophages and neutrophils damage tissues (and microorganisms) by releasing highly cytotoxic proteins, e.g., nitric oxide and hydrogen peroxide. Cytokines and other mediators released by macrophages recruit other inflammatory cells such as neutrophils and T cells to the site of inflammation (28).

Regulatory T cell populations, including activated CD25+CD4+ regulatory T cells, exist in peripheral tissues and are important in controlling inflammation and autoimmune responses by killing autoreactive cells. These regulatory cells also secrete anti-inflammatory cytokines that further inhibit Th1 and Th17 immune responses, thereby reducing inflammation and autoimmune disease. If regulation of self-reactive T-cells and autoantibody production by regulatory T-cell populations is disrupted by environmental agents, e.g., infections or toxins, then AD may result (28).

Defects in tolerance leading to AD occur in one or multiple tolerance mechanisms (Figure 3). For example, changes in the apoptotic cell death process, which result in inappropriate cell death or survival or disturbances in clearing apoptotic cells, are thought to be involved in the pathogenesis of a number of ADs (28). An additional mechanism common to several ADs is the activation of the type I interferon system (29).

Autoantibodies appear long before clinical symptoms thus providing a good predictive marker for the potential to develop a disease. In fact, the risk of developing an AD goes from about 10% if one autoantibody is present to around 60–80% if three autoantibodies are present for a particular AD (30).

SIMILAR ENVIRONMENTAL AGENTS (THE AUTOIMMUNE ECOLOGY)

Ecology (from Greek: *oikos*, "house"; *-logia*, "study of") is the scientific study of interactions between organisms and their environment. Therefore, the autoimmune ecology corresponds to the effects and relationships between all the environmental factors that may influence the risk and course of ADs (Table 2). Several environmental factors are common risk factors for ADs. Infectious agents are important in the pathogenesis of ADs since they are a major part of the environmental autoimmunity trigger. Although a latitudinal gradient of infectious agents exists (31), Epstein-Barr virus and cytomegalovirus are notorious as they are consistently associated with multiple ADs (32). On the other hand, some infections could be protective against AD development (33) (see Chapter 19). Smoking has also been consistently associated with several ADs including RA and SLE (34). This underlines the importance of smoking

1. Physical and chemical agents
2. Infections
3. Lifestyle. Diet, exercise
4. Social factors. Socioeconomic status, family, friends, health, employment
5. Psychological factors. Self-esteem, self-concept, relationships with family and friends, stress, cultural beliefs

Table 2. Factors involved into the autoimmune ecology.

prevention and eradication not only in respiratory disorders but also in autoimmune conditions as well. Organic solvents have also shown to increase the risk of acquiring ADs (35).

INFLUENCE OF ANCESTRY

Genetic ancestry contributes to the clinical heterogeneity and variation in disease outcomes among AD patients (36,37). Amerindian ancestry has been associated with an increased number of risk alleles for SLE (38). Genetic studies of AD subphenotypes will need to carefully address issues of population substructure based on genetic ancestry (36). In addition, latitudinal gradients in allele frequencies due to ancestry may influence the observed effect of genotype on phenotype across populations (39).

COMMON GENETIC FACTORS

The impact of genetic predisposition on susceptibility to ADs was first identified by the analysis of disease concordance rates between monozygotic twins (concordance rates ranged

BOX 1. Epistasis and Pleiotropy*

Epistasis is the interaction between two or more genes to control a phenotype. The term can be used interchangeably with gene–gene interaction, or genetic interactions.

Genetic interactions among major loci, disclosing odds ratio > 2, might explain a proportion of the missing heritability. Epistasis operates at direct interfaces between proteins, where alterations in the genetic code of either partner have the capability to alter the character and affinity of their physical interaction.

The term pleiotropy is derived from the Greek words *pleio*, which means "many," and *tropic*, which means "affecting." Genes that affect multiple, apparently unrelated, phenotypes are thus called pleiotropic genes.

Pleiotropy should not be confused with polygenic traits in which multiple genes converge to result in a single phenotype.

Cross-phenotype association is the association between a locus and more than one trait in a study regardless of the underlying cause for the observed association. In some cases, the same variants show association with multiple traits; in other cases, although the same overall region is implicated, distinct nearby markers show signals of association with different traits.

Differentiating the associations that represent genunely shared effects of single variants from those that represent the effects of colocalizing (i.e., different genetic variants in high linkage disequilibrium located in the same gene that affect different phenotypes) but independent variants are crucial as they imply different notions of pleiotropy and mechanistic models of shared function.

Biological pleiotropy refers to a genetic variant or gene that has a direct biological influence on more than one phenotypic trait. It can occur at the allelic level, where a single causal variant is related to multiple phenotypes, or at the gene (or region) level, in which multiple variants in the same gene (or region) are associated with different phenotypes. Biological pleiotropy is, in most of the cases, synergistic. However, it can also be antagonistic. Antagonistic pleiotropy occurs when a single gene causes competing effects, some of which are beneficial and some of which are detrimental to the fitness of an organism.

Mediated pleiotropy occurs when one phenotype is itself causally related to a second phenotype so that a variant associated with the first phenotype is indirectly associated with the second.

Spurious pleiotropy encompasses various sources of bias that cause a genetic variant to falsely appear to be associated with multiple phenotypes. Misclassification and ascertainment bias are the main causes of spurious pleiotropy.

Distinction between cross-phenotype association leading to spurious pleiotropy and true pleiotropy requires functional genetic studies and analysis of protein-protein interaction (see www.pathguide.org, or the Encyclopedia of DNA Elements at:

http://genome.ucsc.edu/ENCODE).

* From references 42-45.

from about 15% to 57%) (40). The decrease in the concordance rates of siblings compared to the rate for monozygotic twins supports the presence of multiple genes contributing to the autoimmune phenotype onset. Several reports have confirmed that autoimmune phenotypes represent pleiotropic outcomes of nonspecific disease genes (3,41). Epistasis and pleiotropy are crucial in the understanding of the common genetic pathways of complex traits including ADs (see Box 1).

However, not all ADs share the same genetic susceptibility. Therefore, the genetic risk factors for ADs consist of two forms: those common to many ADs and those specific to a given disorder. Combinations of common and disease-specific alleles in HLA and non-HLA genes in interaction with epigenetic and environmental factors over time will determine the final clinical autoimmune phenotype (3). Yet only around 10% to 15% of the inherited risk for ADs can be explained at present (46). Most of the common variants, individually or in combination, confer relatively small increments in risk (1.1- to 1.5-fold) and explain only a small proportion of heritability (i.e., the proportion of phenotypic variation in a population that is attributable to genetic variation among individuals). The amount of heritability depends on the population under study because variations in both additive and non-additive genetic factors and the environmental variance are specific to the population (47). As a corollary, genetic results should be confirmed in different populations.

Commonalities among ADs should be investigated based on real clinical associations (16) rather than arbitrary reviews (Figure 1). An inaccurate conclusion about a limited genetic overlap between SLE and 16 ADs was drawn because most ADs included in the analysis were either not ADs (i.e. ankylosing spondylitis) or were rarely associated with SLE (i.e. Kawasaki disease, sarcoidosis, Behcet's Disease, etc) (48). Some pitfalls and challenges of AD analysis as complex traits are summarized in Table 3.

SIMILAR TREATMENT

What we have learned about the etiopathogenesis of ADs, which supports the view that common features of different clinical entities outnumber their differences, makes it possible to use similar treatments for various ADs despite specific variations and regimen tailoring (50). Similar biological and non biological therapies are used to treat diverse ADs.

PITFALL AND CHALLENGE	PERSPECTIVE
Complex epistatic interactions	Better algorithms and control for phenotype and subphenotype studies. Data analysis is the next most expensive area to develop
Genetic heterogeneity	Larger size cohorts Familial studies to control for environmental and stochastic factors
Pleiotropy	
History of mutations. Difference in allele frequencies	Description and study of population genetic structure in light of information reported and publicly available Usage of newly reported algorithms for admixture analysis and pan-meta-analysis approaches
Population stratification	
Genetics in admixed populations	
Statistical power. Sample size	Correspondence in the use of specific clinical criteria or diagnostic biomarkers to define phenotypes in order to enhance prediction and diagnosis
Refining the phenotype. Subphenotypes	Development and application of bioinformatic approaches to classify diseases as quantitative and categorical entities
Family based studies vs. case-control studies	Application of genetic and classical epidemiological tools to describe new information available for other "omic" layers in the context of the genome from THE familial and population viewpoint
Gene-environment interaction	Further research on environmental factors, which, through exposure, might influence onset of disease (e.g., tobacco, coffee consumption, organic solvents, etc.)
Post-genomic era ("omics")	Use of the publicly available "omic" information already reported (e.g., ENCODE, GEO, HapMap, 1000 genome project) to explore, replicate, and hypothesize new experimental functional designs
Translational medicine	Use of genomic information that is applicable from the bench to the bedside and also from the bedside to the bench. Personalized medicine
Pharmacogenomics	Disentangle markers capable of predicting and diagnosing risk of disease even before symptom and sign onset

Table 3. Pitfalls and challenges of complex trait analysis. Adapted from Castiblanco *et al.* (49).

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15 EVOLUTION AND AUTOIMMUNITY

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INTRODUCTION

Evolution has allowed the development of myriads of species from very simple to highly complex ones. Initially unicelular microorganisms appeared without a nucleus (prokarytes) and later the organisms with a nucleus similar to amoebas. These ancestral amoebas developed in groups and have been called "social amoebas" (1). Some amoebaes specialized in receiving food and fending off other organisms, as a result of which, phagocytosis appeared. This may have been the origin of the immune system as we know it. These unicellular organism developed forms of signaling and adhesion molecules which allowed them to aggregate. Then, multicellular organisms (metazoans) appeared after unicellular organisms migrated from the sea (2). The vertebrate animals with their remarkable diversification appeared 500 million years ago. Both the immune system and its regulatory mechanisms evolved in parallel in a form of evolutionary accumulation (3) (Figure 1).

EVOLUTIONARY THEORIES THAT MAY EXPLAIN AUTOIMMUNE PHENOMENA DEVELOPMENT IN HUMANS

Life began when deoxyribonucleic acid (DNA) acquired the ability to replicate and mutate (4). This made the transmission of genetic information to offspring and the generation of biodiversity possible. Several theories have been formulated to try to explain the biological changes that, over time, determine the evolutionary phenomena of living. These theories may be applicable to the understanding of human biology, including that of diseases. Evolutionary theories can help us understand the origin of autommune diseases beginning with the concepts of genetic drift that refer to the change in the frequency of a gene variant (allele) in a population due to random sampling (5) to the concepts of classic Darwinian natural morphological selection and, concluding for now, with the most modern of molecular selection (6). The synthetic theory of evolution developed by Theodosius Dobzhansky (7) and Ernst Mayr (8), which initially combined Darwin's theory of evolution by natural selection and Mendelian genetics and, lately, has included modern genetic concepts, has also incorporated relevant knowledge. David Hull tries to cover these concepts in the phrase: "genes mutate, individuals are selected, and species evolve" (9). Recently, Richard Dawkins (10) showed us that the different forms of memory were the most relevant foundation for evolution. Information required for handling the present so as to survive into the future is necessarily obtained from the past. In fact, he proposed four levels of information gathering, which he called the "four memories," which would be the foundation of evolution. The "first memory" is DNA, the inherited database each species has and which is the result of non-random evolution. The "second memory" is the adaptive immune system, which is information accumulated during the life of an individual. The "third memory" is the nervous system. Our brain records past experiences and uses a trial-and-error process. The "fourth memory" is the collective memory of the social and cultural aspects of the human species. So, evolution, far from being a tautology as analyzed by the philosopher Karl Popper, is a complex process (11). However, what is clear is that evolution is a law of nature that has been scientifically proven in many ways.

Thus, based on the theories and thoughts presented here, we provide the key elements for understanding the biological significance of evolution in autoimmune phenomena.

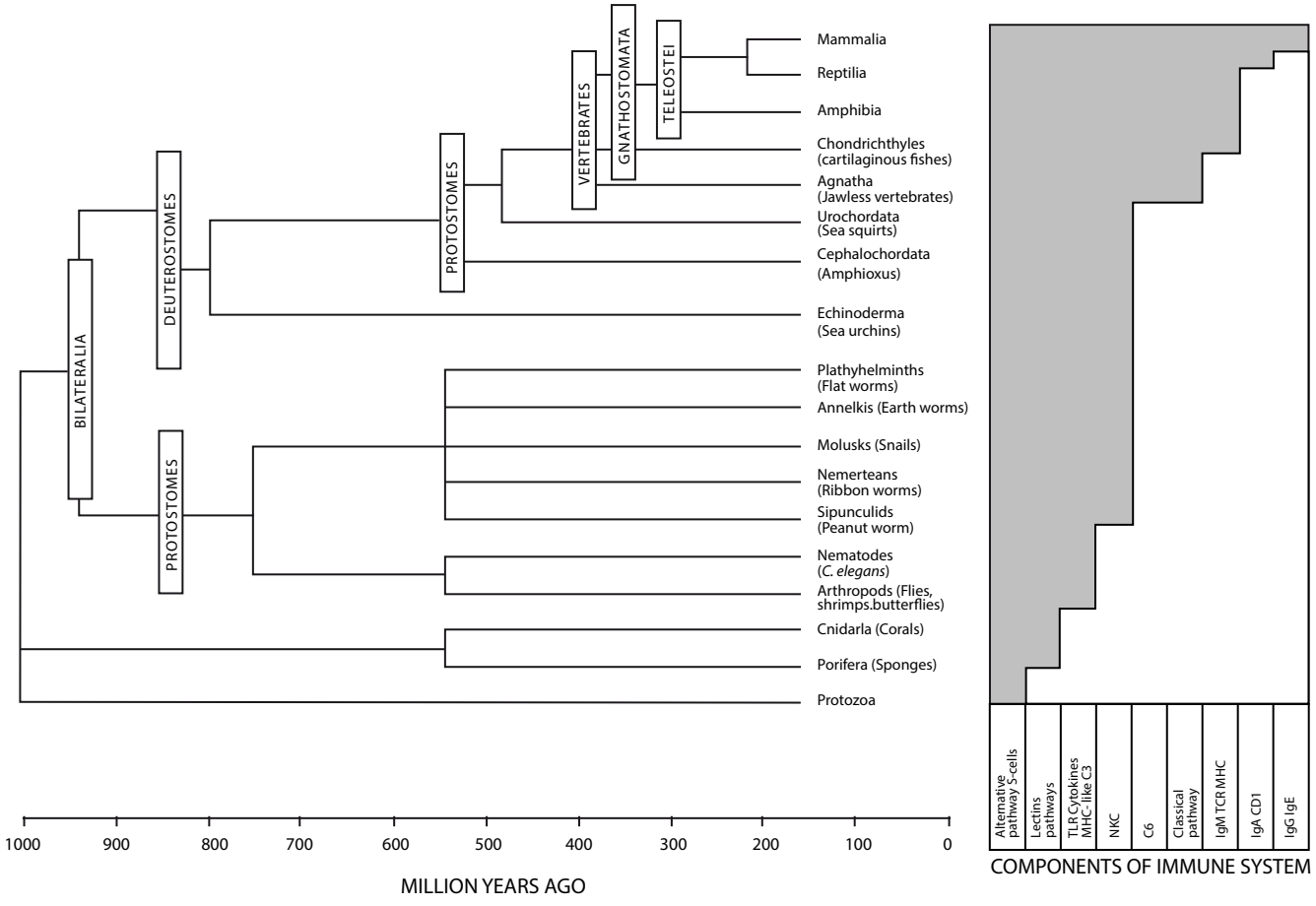

Figure 1. Phylogeny of animals and their immune system. Left: Phylogenic tree of animals. Right: component of immune system that develops an accumulative mechanism of defense. From Cañas & Cañas (3).

INFLUENCE OF THE FIRST FORM OF EVOLUTIONARY MEMORY ON THE AUTOIMMUNE PHENOMENA

Innate immunity is a system that does not create a new form of memory and should be included in the first memory of evolution. The innate immunity is natural, non-specific, non-anticipatory, and does not generate an accumulation of information. This system contains cells that resemble phagocytes which have generic receptors that recognize conserved patterns of pathogens and lectine-like soluble proteins, and they are essential in arthropods, nematodes, sipunculids, mollusks, annelids, platyhelminths, echinoderms, cephalochordates, and urochordates. Adaptive immunity, which has a highly diverse repertoire of lymphocytes, was added in agnathas (vertebrates without jaws) and gnathostomes (vertebrates with jaws). In fact, in the most complex species, e.g., mammals, the immune system consists of innate and adaptive immunities which include T and B lymphocytes and the production of cytokines and antibodies.

THE PATTERN RECOGNITION RECEPTORS (PRRS)

Human innate immunity shares similar cells, cell structures, and molecules with invertebrates. The PRRs are of special interest. These include members of nucleotide oligomerization domain proteins containing leucine-rich repeats (NLRs), retinoic acid inducing gene (RIG)-like helicases (RLHs), and toll-like receptors (TLRs). TLRs are highly conserved during evolution and were first identified in Drosophila melanogaster (12). The TLRs are necessary for defense from various microorganisms. Some TLRs in humans are present on cell surfaces and are able to recognize bacterial structures. Other groups of TLRs, including TLR3, TLR7, and TLR9, are located in the cell endosomes and recognize viral danger signals (double strand-dsRNA, single strand-ssRNA, and hypomethylated dsDNA respectively). This group of endosomal TLRs has been especially implicated in the pathogenesis of autoimmune diseases. Human-derived RNAs and DNAs that are targets of autoimmune responses in systemic lupus erythematosus (SLE) and related conditions have been found to induce activation of these receptors (13).

Altered expression and function of these receptors have been linked to clinical manifestations of lupus-like autoimmunity in animal models (14,15) and rheumatoid arthritis (RA) (16).

CYTOKINES

Proinflammatory cytokines and their receptors are present in early representatives of metazoans, e.g., such as cnidarians, and seem to be conserved in the entire animal kingdom. The family of the interleukin (IL)-1 is made up of IL-1α, IL-1β, and IL-18, which are proinflamatory cytokines, and the IL-1 receptor antagonist (IL-1ra) with pivotal roles in the regulation of acute inflammation. In humans, different forms of polymorphisms are implicated in the severity of a number of autoimmune diseases such as RA in which an adequate balance between IL-1 and IL-1ra is also required (17).

Tumor Necrosis Factor-alpha (TNF-α), mainly produced by monocytes/macrophages, regulates inflammation and cell immune responses. One of its functions is the modulation of the expression of IL-1, IL-6, and chemokines (18). Teleost fish have TNF-α and TNF-α receptors and the human recombinant TNF-α produces biological effects such as macrophage respiratory burst activity, neutrophil migration, and lymphocyte proliferation (19). A similar cross-reaction observed with IL-1 confirms an ancestral relationship with other species. Infliximab, a chimeric antibody in which the Fab portion has a mouse origin effectively blocks the human TNFα molecule and provides a clinical benefit for patients with active RA (20). TNF-α and their receptors have been implicated in the pathogenesis of diverse autoimmune diseases with their polymorphisms being of special interest (21).

A regulatory mechanism for reducing the inflammatory response in infections such as tuberculosis (TB) is presumably the development of polymorphism by natural selection. The −308 and −238 single nucleotide polymorphisms (SNP) of TNF-α may influence the presence of autoimmune diseases and TB. In fact, TNF −308G was both associated with TB and protective for autoimmunity. TNF −238A allele was protective for autoimmunity but represented a susceptibility factor for TB, and the haplotype−308A −238G was a protective factor against TB while, at the same time, it carried susceptibility for RA, SLE, and Sjogren's syndrome (SS) (22). These results support the hypothesis that autoimmune diseases are a consequence of natural selection for enhanced TB resistance.

Likewise, it is important to know that evolutionary mechanisms have been developed for the production of TNF-α regulatory mechanisms, and their disruption can lead to an increase in action and be associated with autoimmunity. Tristetraproline (TTP) is one of them. The TTP family of CCCH tandem zinc-finger proteins consists of three known members in mammals with a fourth member recently identified in frogs and fish. TTP is now known to bind to the so-called class II AU-rich elements within the mRNAs that encode TNF-α and the granulocyte/macrophage colony-stimulating factor (GM-CSF) (23). In both cases, this binding results in destabilization of the mRNA and decreased secretion of the protein (24). A TTP deficient mouse develops a deep inflammatory syndrome with erosive arthritis, autoimmunity, and myeloid hyperplasia (25). In patients with RA, a low TTP/TNF-α gene expression ratio could indicate failure to produce adequate amounts of TTP in response to increased TNF-α production (26).

CHEMOKINES AND THEIR RECEPTOR

Chemokines are involved in cell interactions and tropism in situations that are frequently associated with inflammation. Recently, the importance of chemokines and chemokine receptors in inflammation associated with autoimmunity has been highlighted. The molecular mechanisms that control these fundamental aspects of chemotaxis appear to be evolutionarily conserved, and studies in lower eukaryotic model systems have allowed us to form concepts, uncover molecular components, develop new techniques, and test models of chemotaxis. Some receptors of chemokines are also receptors for other molecules which include exogen agents such as microorganisms.

In the case of infections caused by P. vivax, the most widely studied evolutionary change that determines resistance is the genetic polymorphism of the parasite receptor known as Duffy Antigen Receptor for Chemokines (DARC) (27,28). In this receptor, the form lacking alleles does not allow the red blood cell to receive the parasite and let it enter. There are two kinds of alleles related to the DARC receptors: Fya and Fyb, which identify four possible phenotypical presentations: homozygous Fy (ab−) (absence of the receiver or null), homozygous Fy (a+ b+) and heterozygous Fy (a− b+) and Fy (a+ b−). These receptors belong to the family of seven transmembrane molecules, which were initially recognized as receptors for the P. vivax in human red blood cells and the simian P. Knowlesi. Later they were recognized as "promiscuous" receptors which were able to bind both CC and CXC chemokines and have a cleaning role in these molecules (29). Several chemokines related to the inflammatory process of RA are DARC ligands. These ligands include the CXC type chemokine, the interleukin-8 (IL-8) and neutrophil-activating protein derived from epithelial cells (ENA-78: epithelial cell-derived neutrophil-activating protein-78), the CC type, e.g., monocyte chemoattractant protein (MCP-1 monocyte chemoattractant protein-1), and the regulated T lymphocyte protein expressed and secreted in normal activation (RANTES: regulated on activation normal T-cell expressed and secreted). The DARC expressed on endothelial cells of the synovium is important for the recruitment of neutrophils in patients with RA (30).

However, the role of DARC in the pathogenesis of RA is unknown. As has already been mentioned, the DARC in this location plays a role in the clearance of circulating chemokines, a condition that could have an implication in the regulation of inflammatory processes. The DARC phenotypes have been studied in Caucasian patients from Italy with Behçet's disease, where the IL-8 and MCP-1 are pathogenics. In this study, there was no association between the genetic polymorphisms of these genes and the presence of the disease. It can be assumed that the absence or deficiency of DARC would be related to more severe forms of RA (31). Diverse mechanisms of resistence to malaria which may be implicated in the immune system regulation and RA pathogenesis are showed in Table 1.

COMPLEMENT SYSTEM

Serine proteinases appeared early in evolution. They have even been found in bacteria (32) and evolved to supply sev-

eral physiological needs in the immune system, etc. A serine proteinase cascade which shows similarities to the blood clotting system and the complement system of vertebrates is involved. There is even a functional link between immunity and haemostasis since coagulation factors activate immunological processes and various components of the complement also activate coagulation factors (33,34). The complement participates in the removal of immune complexes, aberrant and apoptotic cells, and cell debris and has important functions which, if they fail, are implicated in autoimmunity.

RECEPTORS FOR THE FC REGION OF IGG (FCGRS)

FcgR provides a type of link between the humoral and cell immune system. Inherited FcgR polymorphisms influence human phagocyte function. Single-aminoacid/SNP substitutions within the extracellular domains of FCgR alter the ability of the receptor to bind IgG and have been associated with the development of autoimmune and infectious diseases (35). FCgRII (CD32) has two isoforms, FCgRIa and FCgRIIb, which are expressed on mononuclear phagocytes, neutrophils, and platelets. FCgRIIa has 2 co-dominantly expressed alleles, H131 and R131, which differ at aminoacid position 131 in the extracellular domain (histidine or arginine) and differ substantially in their ability to bind human IgG2 (36,37). H131 is the high-binding allele, R131 the low-binding allele and heterozygotes have an intermediate function (38). FcgRIIa-H131 is essential for handling IgG2 immune complexes. These immune complexes are removed from circulation, primarily in the liver and spleen, by the mononuclear phagocyte system. Impaired removal of immune complexes is present in SLE which leads to an increase in the probability of tissue deposition of immune complexes, release of inflammatory mediators, influx of inflammatory cells, and damage to target-organs such as in the case of nephritis (39,40). In SS, there is a similar correlation with the presence of FCgRII (41) and FCgRIII (42) polymorphisms. Therefore, we postulate that primitive animals with an innate immune system may have been attacked by their own system at different times during evolution and, consequently, developed regulatory mechanisms such as RNAi, TTP, IL-1ra, regulator complement cascade proteins, and the FCgRIIb presented above. Another regulatory mechanism which is implicated in autoimmune phenomena when it fails is the one associated with the functions of suppressor T cells (43).

CLASS III MAJOR HISTOCOMPATIBILITY COMPLEX (CLASS III MHC)

This locus contains several genes that encode secreted proteins that have innate immune functions: components of the

FACTOR	EVOLUTIONARY MODIFICATION	RESISTANCE MECHANISM TO MALARIA	MODIFIED MMUNOLOGICAL PATHWAY	POSSIBLE ROLE IN RHEUMATOID ARTHRITIS PATHOGENESIS
Duffy Antigen	Duffy-negative allele	Lack of expression of chemokine receptor	Chemokine sink.	Amplification of immune response and lack of chemokine depuration
Beta-globin	Sickle hemoglobin	Suppression of parasite growth in red cells and enhanced splenic clearance of parasitized erythrocytes	Reduced parasite cytoadherence	Unknown Increased expression of VCAM-1, E-selectin, and ICAM-1? Decrease in immune complex clearance by asplenia.
FcgammaIIB	Substitution of threonine for isoleucine at position 232 in the transmembrane domain of FcγRIIB (T232)	Increased clearance of malarial parasites	Phagocytosis of Plasmodium falciparum-infected erythrocytes. Diferentiation of B lymphocytes	The abnormal function leads to an increase in immune reactivity mainly mediated by B lymphocytes
CR1	Polymorphisms of CR1 are involved in the amount of protein expression in the red cell membrane	Reduced ability of P. falciparum-infected CR1-deficient red blood cells to form rosettes	Reduced ability of P. falciparum-infected CR1-deficient red blood cells to form rosettes, and less severe disease	CR1 is a complement regulatory protein, responsible for removing immune complexes from circulation. Decrease in immune complex clearance
NK1.1(-) and NK1.1(+) subsets of TCR (int) cells	?	?	?	Autoantibody production?
GYPC	GYPC deficient erythrocytes	Protection from EBA-140-mediated invasion by P. falciparum parasites	Binding receptor-parasite protein	?

Table 1. Summary of the mechanisms of protection from malaria and their possible association with rheumatoid arthritis pathogenesis. Adapted from Bonilla-Abadía *et al.* (31).

complement system (e.g., C2, C4, and factor B) and inflammation-related molecules (cytokines such as TNF-α, LTA, and LTB) or HSP. Class-III has a function that is completely different from classes-I and II (described below in the text) but is between the other two on the short arm of human chromosome 6.

INFLUENCE OF THE SECOND FORM OF EVOLUTIONARY MEMORY IN AUTOIMMUNE PHENOMENA

Adaptive immunity is a form of second evolutionary memory and stores molecular information about micro-organisms in order to develop a faster and more effective defense against them in future exposures through cytokines and specific antibodies. The most important mechanism that nature has used to obtain and retain this type of information has been the immunoglobulin superfamily gene system. The adaptive immune system, as defined by rearranging antigen receptor genes in the immunoglobulin superfamily and by the major histocompatibility complex, has only been found in the jawed vertebrates (gnathostomes). The mechanism of recombination-activating gene (RAG)-mediated rearrangement exists in all jawed vertebrates, but the organization and structure of immunoglobulin (Ig) genes differ between species and reveal their capability for rapid evolution. (44). Other groups of proteins that belong to the immunoglobulin superfamily with a common ancestral origin are FcγR and the immunoglobulin-like receptor (KIR) which have an important function in the inflammatory response. The antibodies directed against their own structures (autoantibodies) play a primary role in autoimmunity being pathogenic in diseases caused by an attack on cell or tissue antigens (autoantigens) or in immune complex-mediated diseases. The autoantigens may originate from different sources.

Proteins that are hidden in some tissues and by external factors such as trauma begin to be recognized by the immune system as foreign. The immune system recognizes the new antigen and tries to eliminate it by innate immunity mechanisms, and later, by induction of acquired immunity mechanisms. An example of this condition is sympathetic ophthalmia (SO) where breaching of systemic ocular barriers compromises the relative immune privilege of the eye and causes sensitization to previously sequestered uveoretinal antigens (45). A similar mechanism is observed in relapsing polychondritis which begins with cartilage trauma and is triggered by an immune response to other cartilaginous or non-cartilaginous tissues (46,47).

Even Though There Are Proteins That Are Not Present in the Human Structure, the Genetic Information Is Present. These genes are ancestrally repressed and hidden. As a result of different causes, they start protein synthesis and this "foreign" protein is attacked by the immune system. One example of this is the endogenous retrovirus (ERV) which belongs to the large family of retrotransposable elements of human genoma (48). ERV may be activated by radiation, bacteria, chemicals, or recombination with an exogenous retrovirus and start the "autoantigen" protein synthesis that is the source for autoimmune processes implicated in the pathogenesis of SLE (49).

In vertebrates, allorecognition depends on proteins encoded by MHC genes. A MHC-like region is certainly very ancient and is believed to be present in the common ancestor of proto- and deuterostomes (50). The function of MHC is to present antigens to T-cell receptors. It has been suggested that the MHC region arose as a result of chromosomal duplications. In higher vertebrates, MHC is represented by two distinct classes, MHC I and MHC II. Class II MHC polymorphisms have been studied and their presence is a risk factor for various autoimmune diseases, for example, diverse HLA DRB1-04 alleles in RA, HLA DRB1-0301 in SLE, or HLA DR1-0301-DQB1-0201 in SS (51). Sometimes the risk is caused by the combination of polymorphisms (haplotypes) such as the 8.1 ancestral haplotype (52).

The HLA-DRB1 polymorphisms are ancient and, based on an estimate of the nucleotide substitution rate for MHC coding sequences, it has been argued that most of the alleles at the class II DRB1 loci predate the separation of hominoid species (53-55). However, the phylogenetic trees for primate exon-2 sequences are consistent with the notion that most of the alleles at some class II loci, e.g., DRB1 and DPB1, may have a more recent origin (56,57). Additionally, studies of human population groups with a defined degree and time of isolation have provided support for the view that new DRB1 alleles have been generated over the last 10,000 – 20,000 years (58,59). Thus, there are indications that the allelic variation at HLA loci may be much more recent than previously assumed (60). These alleles appear to be, on average, 250,000 years old thus implying that the vast majority (greater than 90%) of the more than 135 contemporary human HLA- DRB1 alleles were generated after the separation of Homo and Pan (61). This could be related to differences in these species in the prevalence of autoimmune diseases (62).

INFLUENCE OF THE THIRD FORM OF EVOLUTIONARY MEMORY IN THE AUTOIMMUNE PHENOMENA

Memory related to neuronal function in relation to autoimmune phenomena is a crucial factor for storing diverse experiences during life.

Neuroimmunoendocrine Network. An emotion such as stress or pain triggers endocrine responses which, in turn, affect the immune system and cause its activation and inappropriate response in the setting of autoimmune and infectious diseases. It is a vicious cycle because stress not only causes disease but the disease itself causes the patients considerable stress (63). Neuroendocrine hormones triggered during stress may lead to immune dysregulation and cytokine liberation resulting in autoimmflamatory/autoimmune disease risk. The stress response and induction of a disregulation in the cytokine balance can trigger the hypothalamic-pituitary-adrenal axis and sympathetic nervous system (64).

Neuron-Glial Relationship. A type of information stored in the brain which is related to the experience of pain. Somatic pain induces an immediate response that generates the reflex which moves the injured body part and prevents additional damage. A second form of pain is the subacute or chronic somatic pain that is caused by the rest of the affected area during the repair process. Another form of pain is the neuropathic pain which is due to the persistent response that is generated after an injury to peripheral nerve structures (65). In this condition, the microglia, which are the resident macrophages of the brain and spinal cord, plays an important role. It seems that the nerve injury activates the TLR4 which is only expressed on microglia in the central nervous system. Genetically altered mice lacking TLR4 showed markedly reduced microglial activation after nerve injury as well as reduced sensitivity to pain (66). Another candidate for microglial activation after nerve injury is fractalkine, a CX3C chemokine expressed on the surface of neurons (67). Ontogenetically, both microglia and other glial cells are important in the process of migration and location of neurons in the central nervous system and the nutrient supply (68). Not only glial cells but also neurons respond to the presence of several cytokines (69). The concept of integrated neuroendocrine-immune regulation has now been widely accepted although the physiological impacts on normal development and homeostasis or conditions of mental stress or physical disease are still poorly understood (70).

INFLUENCE OF THE FOURTH FORM OF EVOLUTIONARY MEMORY IN THE AUTOIMMUNE PHENOMENA

The fourth form of mankind's evolutionary memory is culture, and with this, there are many conditions that are related to the genesis of autoimmune phenomena. Dietary modifications for humans that led to protein-calorie malnutrition or otherwise to weight excess have been assessed and taken into account when seeking nutritional factors associated with development of autoimmunity (71,72). The lack of exposure to various antigens in early childhood, including the newly born and those growing up in an "aseptic" environment results in a very low exposure to antigens required for the development of cell-mediated immunity (leading to better and safer protection) and, at later stages, requires an antibody-mediated protection which can lead to allergy and autoimmunity phenomena (73). It is well known that industrialization decreases exposure to sunlight with the consequent development of vitamin D deficiency, now a well-studied factor related to autoimmunity (74). At the other extreme, the overexposure to sunlight due to working conditions or the cultural practice of changing the color of the skin (tanning) increases UV radiation. This is related to the development or exacerbation of cutaneous lupus and SLE by different mechanisms (75,76). Some jobs are related to risks of SLE. For example, school teachers are exposed to many viruses that could be the basis for an abnormal immune response in a genetically predisposed individual (77). Various habits such as smoking (78) or exposure to smog-related particles (79) are also associated with a similar outcome. With the evolution of human culture, pharmacology also developed and diverse drugs could induce autoimmune phenomena through various factors including epigenetic ones (Example: procainamide, hydralazine, chlorpromazine, isoniazid, phenytoin, and penicillamine) (80). Women have a greater predisposition to developing autoimmunity, in part, as a result of the effect of estrogen, which, when used for therapeutic or contraceptive reasons, increases the risk (81). It has been postulated that older factors such as migration and exposure for long periods of time to different forms of environment were determining factors for the development of different human races which have different risks for the development of autoimmune diseases (82). More recently there has been a genetic mixing of races due to migration and risk conditions for developing diseases have changed for each race. Race can also be modified without requiring migration; for instance, by religious or political influences that encourage the choice of partner for reproduction in order to ensure a race with certain characteristics which are more related to beauty and purity (83).

CONCLUSION

One way to understand autoimmunity is through knowledge of the biological significance of evolution. Since a specialized system for defense against microorganisms was set up, living things have theoretically been vulnerable to developing autoimmune phenomena. The existence of different regulatory mechanisms can only be explained as strategies that were developed to avoid these phenomena of self-destruction. The human species have a cumulative evolution of the most complex mechanisms of innate and acquired immunity which makes it very vulnerable to autoimmunity.

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16 INTRODUCTION TO GENETICS OF AUTOIMMUNE DISEASES

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INTRODUCTION

Epidemiology concerns the study of the demographic distribution of determinants and events related to health-status in populations. Genetic epidemiology is linked to traditional epidemiology since it focuses on the familial determinants of disease, mainly genetics, and their joint outcome with the environment. The later takes into account the underlying biology for the action of genes and genetic inheritance patterns. The genetic approach of a trait (e.g., Autoimmune diseases, ADs) based on genetic epidemiology is outlined in Table 1.

When DNA information was lacking, research tried to relate genetic variation to disease while relying on Mendelian inheritance (1), which required a biological model for the pattern of sharing genes between close relatives. This would make it possible to find a plausible pattern that would model how a putatively causative genetic variant might lead to disease and give rise to etiological inferences drawn from the distribution of disease and phenotypical aggregation within large families or across groups of families.

Disease characteristics = *Descriptive epidemiology*
Familial clustering = *Aggregation studies*
Genetic or environmental? = *Studies of adoption-migration*
Mode of inheritance = *Segregation analysis*
Susceptibility loci = *Linkage analysis*
Markers of susceptibility = *Association studies*
Causality = *Epistasis, pleiotropy and functional genetic studies*

Table 1. Genetic approaches to studying complex traits.

Once genetic markers were available and with them deeper knowledge of the human genome, there was a starting point for correlating disease although not necessarily finding complete determinants of health or disease. Incorporation of gamete formation biology and chromosomal recombination into a mathematical model to examine to what extent a given marker is transmitted through a family in conjunction with disease permitted causative genetic variants to be untangled close to markers defining disease. This approach summarizes the basis of genetic linkage analysis, which has achieved many of the breakthroughs in the genetics of disease causation.

A plethora of genome and "omics" information can now be included in genetic studies. Once a potentially causative gene is identified, looking for a correlation between variants in that gene and the disease of interest is fundamentally no different from an exploration of a disease-exposure association in traditional epidemiology. Knowledge about the underlying biology, coupled with the inferential tools of epidemiology, biostatistics, and data mining, allows important questions to be pursued in ways that are more rigorous, and often more powerful, than approaches that fail to make best use of epidemiology, genetics, and biology (2).

Many of the most important genetic correlations for monogenic disorders, where familial recurrence seems to follow the laws of Mendelian inheritance, have been reported (3). Currently, research is focused on complex common diseases (e.g., ADs) known to characteristically be caused by sets of interacting genetic and environmental determinants (4). This chapter aims to provide a framework for and illustrate the challenges of exploring the role of genetic variation in ADs. A workflow broken down into key steps represents a template around which this chapter has been structured (Figure 1). Although the workflow is

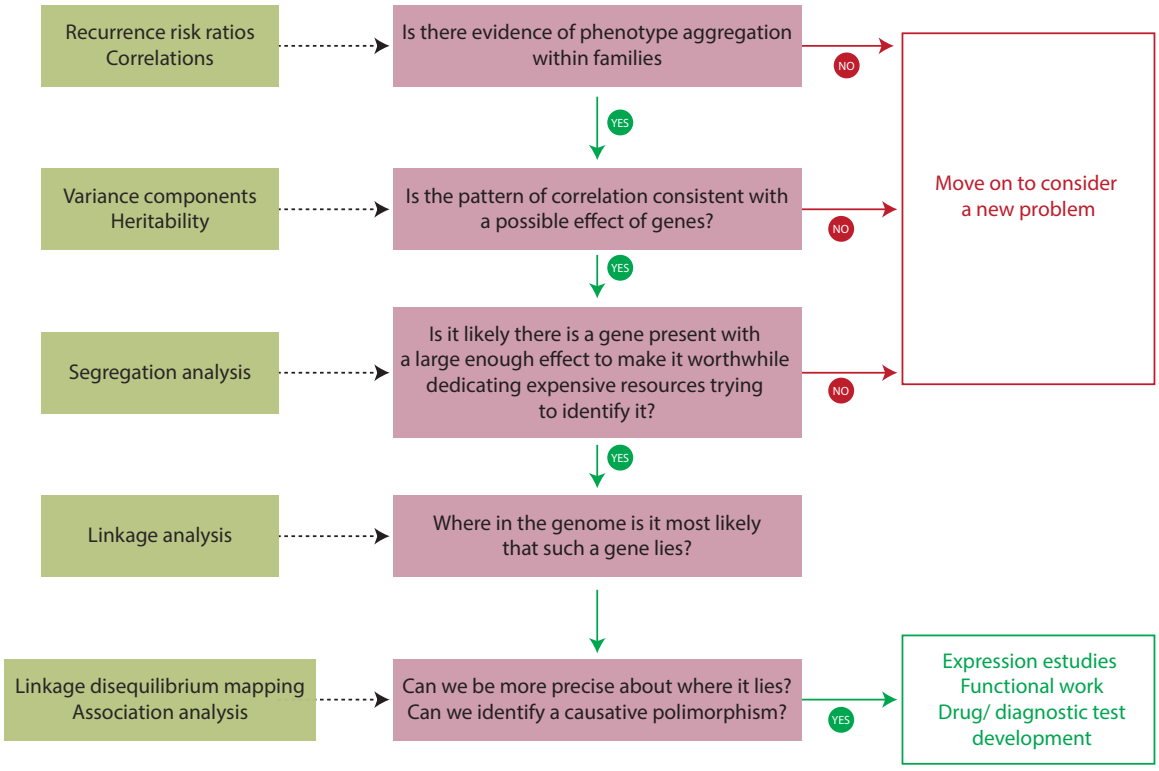

Figure 1. Algorithm outlining the approach for the identification and characterization of genetic determinants of complex disease.

meant to be a convention about how such research should be done, it is also meant to function as a reference point in order to have a better experimental structure when approaching questions regarding the study of disease genetics in populations.

GENETIC EPIDEMIOLOGY

Currently, lack of clear diagnostic tools and defined disease criteria leaves patients in a bureaucratic limbo and soaring through the system in search of a complete and accurate diagnosis, so that they can receive appropriate treatment. Clinical pathologies tempt us to envisage disease as either an independent entity or a diverse set of traits governed by common physiopathological mechanisms prompted by environmental assaults throughout life (5). ADs are not an exception to this premise given that they represent a diverse collection of diseases in terms of their demographic profile and primary clinical manifestations (6). The National Institute of Health (NIH) estimates that up to 23.5 million Americans suffer from an AD with more than 80 known forms of disease and at least 40 more having an autoimmune basis. This puts ADs among the top ten leading causes of death in Americans. The commonality between ADs is the damage to tissues and organs arising from the loss of tolerance and, in most cases, a gender imbalance (6). Research generally focuses on a single disease although autoimmune phenotypes could represent pleiotropic outcomes of non-specific disease genes underlying similar immunogenetic mechanisms (7). While it is apparent that multiple cases of a single disease cluster within families (8), what is more striking are the individuals in those families afflicted with multiple ADs (9).

Autoimmune disorder epidemiology varies based on individual conditions. Collectively, the prevalence of ADs in the general population is at least 5%, and they are one of the major causes of premature mortality in young and middle aged women (10) (Table 2). ADs can be categorized into two types of disorders: systemic (i.e., loss of immune tolerance is directed towards systemic antigens and disease manifestations can occur at a variety of different sites in the body) and organ-specific (i.e., predominantly or exclusively directed towards tissue-specific elements).

Though ADs encompass a broad range of phenotypical manifestations and severity, their pathogenesis is considered to be multifactorial and several of their features suggest they share common etiological factors. These shared disease features, in conjunction with epidemiological evidence that demonstrates the clustering of multiple ADs within individuals and families, strongly implicate shared etiological factors including shared genetic loci. Reasons for the diverse manifestations exhibited by different ADs remain unclear, but recent progress in elucidating genetic susceptibility loci for this group of disorders promises to shed light on this important issue (11). Most ADs are,

AD	SEX (F/M)	PREVALENCE (RATE PER 100,000)	MONOZYGOTIC CONCORDANCE RATES	DIZYGOTIC CONCORDANCE RATES
AITD	18:1	792	55% TPO Ab (64–65%)	0-40% TPO Ab (13–35%)
SS	(9-15):1	14–1600	N/A	N/A
SSc	12:1	4	4–5%	4–5%
AdD	12:1	5	N/A	N/A
SLE	9:1	2–7.6	20–39%	0–3.2%
MS	3:1	58	25%, 30–35%	0–5%
RA	3:1	860	22-26.7%	6.7-6.9%
CD	2:1	39	83-91%	20%
PSO	1:1	79	70%	20%
IBD	1:1	2	17-60%	0-8%
T1D	1:1	192	23–64.3%	3–20.8%
VIT	1:1	400	23%	0%
AS	1:2	129	40%	4.3%

Table 2. Prevalence, monozygotic and dizygotic twin concordance, and genetic basis of ADs. Adapted from (43,105). *N/A*: Not available.

however, multifactorial in nature with susceptibility controlled by multiple genetic and environmental factors.

Diverse populations present different allelic structures depending on the natural and epidemiological history of the population (12). In addition, the effects of genotype on phenotype in any given population may depend upon environment and length of exposure to an undefined etiological insult. Consequently, there is a need to explore genetic associations in diverse populations.

It is important to distinguish between the clinical sense of familial clustering (extended families that happen to have multiple cases of a disease or syndrome of interest) and the epidemiological sense of familial aggregation (there is, on average, a greater frequency of disease in close relatives of individuals with the disease than in relatives of individuals without the disease). Simple analyses of familial aggregation treat the family like any other unit of clustering. In addressing whether there is phenotypical aggregation within families, no attempt is made to determine the cause of any aggregation (13). Nevertheless, the observation and portrayal of familial autoimmunity and the outline of the multiple auto immune syndrome (MAS) has put aside environmental aggregation and given greater value to the common/rare genetic component for diverse autoimmune phenotypes with a generally common background (8).

FAMILIAL AGGREGATION

Familial aggregation is often assessed by the recurrence risk ratio. The pattern of recurrence risk ratios across different types of relatives can provide valuable information about the origin of a binary trait and can add to the statistical power of linkage studies (14). The recurrence risk ratio (λ_R) in relatives of type R relatives is the prevalence of the disease in relatives of type R of affected cases (P_R) divided by the prevalence in the general population (P). If the relatives are siblings, λ_S and P_S would be used. Families are often recruited because they have affected members. This outcome-based sampling is often more informative and increases power. Furthermore, it has obvious benefits for a study aimed at estimating λ_R, the prevalence of disease in a particular subgroup of relatives. However, because the familial determinants of the trait of interest are usually unobserved in a study of familial aggregation, this sampling method can lead to severe ascertainment bias (13).

For many complex diseases, the average λ_R in first-degree relatives is around 2. It tends to be greater the younger the age at onset in the affected individual, to fall as the familial relationship becomes more distant, and to increase as the number of affected relatives of the at-risk individual rises. Although a λ_R of 2 might appear modest, it does suggest that uncovering all sources of familial aggregation might well be worthwhile. A moderate λ_R generally implies the presence of underlying familial risk factors (genetic or non-genetic) that are, at least, an order of magnitude stronger than λ_R itself. Because a simple assessment of familial aggregation takes no account of the underlying biology, one should not assume that evidence of familial aggregation implies genetic effects. For many complex diseases, the non-genetic risk factors identified to date have a modest effect and are weakly correlated in relatives. They, therefore, seem to explain a little familial aggregation (13).

ADs show a tendency towards familial aggregation but the incidence in close relatives of affected individuals is usually low compared to the much higher figures that would be expected if these conditions were Mendelian-like (9). Recurrent associations of ADs in family members of patients have been reported and comprise autoimmune thyroid disease (AITD),

systemic sclerosis (SSc), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) as the most common ADs among relatives (15-22). Multiple sclerosis (MS), primary biliary cirrhosis (PBC), celiac disease (CD), and antiphospolipid syndrome (APS) have also been registered although less frequently (16,20,22-24). Shared immunopathological mechanisms among Sjögren's syndrome (SS), SLE, SSc, and AITD as well as a possible familial aggregation of these diseases has been also reported (25). In fact, SS may coexist with other ADs such as RA (26), SLE (27), AITD (28), and SSc (29). In addition, AITD has been the most common disease encountered among first-degree relatives as has also been reported in familial studies of MS (30), vitiligo (VIT) (31), juvenile RA (32), SLE (33), SS (22,28), and type 1 diabetes mellitus (T1D) (34,35) patients. T1D, SS, and SLE share similar susceptibility gene polymorphisms including HLA and non-HLA variants (36-39), which may account for aggregation.

Many disorders have demonstrated a familial aggregation that does not conform to any recognized pattern of Mendelian inheritance. These conditions show a definitive familial tendency but the incidence in close relatives of affected individuals is usually around the lower instead of the much higher figures that would be seen if these conditions were caused by mutations on single genes (40). The impact of genetic predisposition on susceptibility to AD was first identified by the analysis of disease concordance rates in monozygotic twins (ranging from concordance rate ranges from about 10% to 85%) (41,42) (Table 2). The decrease in the concordance rate of siblings compared to the rate for monozygotic twins supports the presence of multiple genes contributing to the autoimmunity phenotype onset (43). In fact, ADs are not inherited in a classical Mendelian pattern, but instead have a complex, yet to be defined mode of inheritance (44-46).

Twin studies are an invaluable source for researchers attempting to distinguish whether genetic or environmental factors (or varying degrees of both) contribute to the development of disease and set the basis for composite disease heritability estimates (43,47). Concordance rates are based on a comparison of disease status between monozygotic (MZ, i.e., developing from a single fertilized egg and, therefore, sharing all of their alleles, MZ), and dizygotic (i.e., developing from two fertilized eggs and, therefore, sharing on average 50% of their polymorphic alleles, the same level of genetic similarity as found in non-twin siblings, DZ) twins. The presumption is that as MZ twins share 100% of their genomic sequence, a phenotype concordance significantly higher than that for DZ twins would be suggestive of predominantly genetic influences while low concordance rates would indicate stronger environmental factors. Concordance rates for most conditions generally support both genetic and environmental influences with varying degrees of each. A combination of environmental and genetic influences has formed the basis of a complex multifactorial model of disease, where a genetically predisposed individual encounters several environmental exposures over a lifetime which culminates in disease development after stochastic exposures (Figure 2). This model has been proposed for several multifactorial diseases, including AD, and the limited applicability of the most robust genomic associations from genome-wide association studies (GWAS) has supported this notion.

Moreover, twin studies in ADs highlight the complexity and obstacles that may be faced from one disease to the next such as the variability of MZ and DZ concordance rates between reports of the same disease (Table 2). Several ADs, e.g., CD, have a strong genetic component with high concordance rates for MZ twins (43,47). Likewise, for example, SSc has been shown to have a less prominent genetic component with, therefore, more room for environmental factors. T1D, which is perhaps the most studied, discloses a concordance rate for MZ twins ranging from 23 to 64.3% (43). However, due to great variability in concordance rates, the interpretation of the results of these reports needs to be done cautiously. Sex differences have also been observed in twin studies on T1D. The proband and pairwise concordance rate for MZ male twins was 42.4% and 27% respectively, and 7.8% and 4.1% for DZ male twins respectively (48). Female MZ twins had a concordance rate of 43.5% probandwise and 27.8% pairwise. DZ females had a 4.4% and 2.3% probandwise and pairwise concordance respectively (48). A higher female concordance and greater time interval of discordance was noted in a study by Redondo *et al*. (49). A North American study found that the risk for male DZ twins was as high as that for the MZ male twins and significantly higher than DZ female twins (50).

AGE AND AUTOIMMUNITY

Consideration of age as a factor responsible for the onset of ADs at midlife (age, 40-60) has been proposed. Two specific circumstances should be considered. Firstly, detection bias, given that some ADs depict slow sign and symptom progression, making the age of onset not perceptible. Secondly, alteration of the biological functions, which may alter the development of the disease (i.e., decreased apoptosis and increased clonal activation of T cells, or decreased ability to respond to antigenic or mitogenic stimulation, etc.) (51). Nevertheless, age remains an important topic in autoimmunity not only because of the biological implications of aging on the immune system but also because of the setback it constitutes for epidemiological studies which has the common origin of ADs as its goal. The problem with age in epidemiologic studies lies in the fact that many ADs have different ages of onset. For children for example, the most common diseases are T1D, CD, and VIT (52). For young adults, MS, myasthenia gravis (MG), VIT, and SLE are the most frequent (53). Mid-age patients are more likely to have SS (54), SSc (55), and RA (56). Finally, the elderly are more prone to have SS (54), AITD (57), and MG (58). This mosaic of ages constitutes one of the biggest problems in aggregation and co-occurrence studies and can be summarized in two types of setbacks (59). The first is the reduced probability of

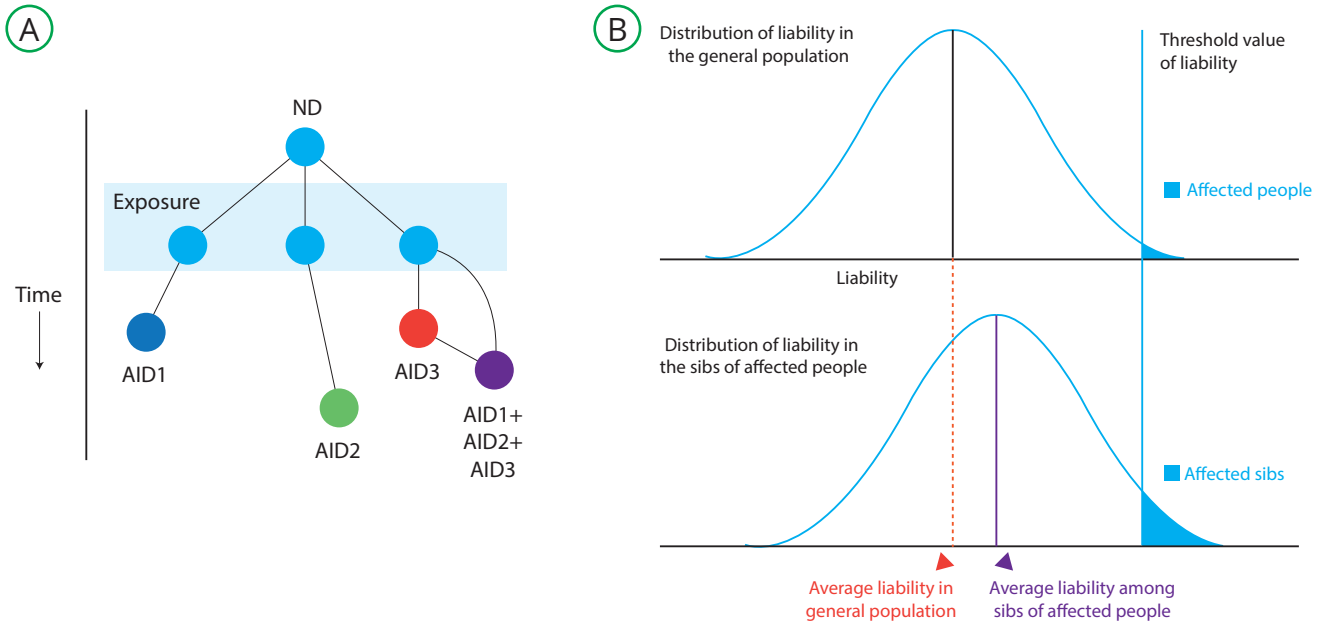

Figure 2. **A. Phenotype progression.** The exposures of an individual to environmental risk factors throughout his lifetime will define the vector of disease progression toward an autoimmune disease state depending on his population genetic background. Polyautoimmunity (AD1 + AD2 + AD3). **B. Familial and individual liability.** The curve represents the liability threshold model for the development of an autoimmune disease phenotype given by the frequency of the overlapped variants, within and among the constituents of families in a defined population. When a condition is polygenic and normally distributed (upper curve), people whose liability is above a certain threshold value are affected. Their sibs (lower curve) have an average liability that is higher than the population mean and a greater proportion of them have liability exceeding the threshold. Therefore the condition tends to run in families. *AD*: autoimmune disease; *ND*: no-disease.

finding aggregation of ADs for affected patients when age differences are considerable. For example, there is the case of a young child affected with T1D whose parents are young and restricted to a small group of ADs. The opposite scene would be the elderly patient whose parents are already deceased and whose children are too young to present many of these kinds of pathologies. The second type of setback is the one arising when doing co-occurrence studies. The limitation arises when two diseases are so far apart with respect to their time of diagnosis that a necessary and rigorous follow-up will be required in order to find co-occurrence in one patient (59).

GENDER, INHERITANCE, AND AUTOIMMUNITY

The vast majority of patients with ADs are female. The reason for this prevalence is poorly understood (Table 2). The more frequent the AD and the later it appears, the more women are affected. Many ideas that are mainly based on hormonal and genetic factors that influence the autoimmune systems of females and males differently have been proposed to explain this predominance. These hypotheses have gained credence mostly because many of these diseases appear or fluctuate when there are hormonal changes such as in late adolescence and pregnancy (60). The proportion of females with AD varies depending on the disease: from 18:1 in AITD to 1:1 in PSO to 1:2 in ankylosing spondylitis (AS) (Table 2). Even for specific populations, reports have described differences in gender and clinical presentation pertaining to sex and RA (61). ADs that are more prevalent in men are characterized by acute inflammation, appearance of autoantibodies, and a proinflammatory Th1 immune response whereas ADs that are more prevalent in women have known antibody-mediated pathologies. Moreover, ADs that are more prevalent in women and that clinically appear in women over age 50 are associated with chronic Th2-mediated pathology (62).

Sex hormones are involved in the susceptibility factors for AD through modulation of Th1/Th2 response. Impact of hormonal changes on the disease course in females is documented in pregnancy: severity of MS and RA has been reported to decrease during pregnancy whereas severity in SLE is either exacerbated or unaffected during pregnancy. This may be explained by high levels of hormones during pregnancy which enhance Th2 response and suppress RA and MS which are driven by Th1 response. In contrast, SLE is Th2 driven and may not be suppressed by the hormones (63).

Theoretically, X-chromosome inactivation and the resultant tissue chimerism might explain the female predisposition to systemic autoimmunity (64,65). In females, half their somatic cells express antigens derived from the paternal X and half from the maternal X. The Burnet-Jerne theory of somatic generation of antibody diversity and forbidden clone elimination states that lymphocytes under maturation

in the thymus are killed or suppressed if they present high or no affinity towards a histocompatibility antigen. If this were to hold for self-antigens as well, females would escape expressing one of her parental X chromosomes, which would still be able to react to self. Then lymphocytes happening to pass the selection in the thymus would meet only cells expressing one of the parental X chromosomes and it would be easier to predispose for a dysregulation of self-tolerance in females than in males. This is known as the Kast conjecture (66). Even though specific responses to immunization do not appear to account for the high sex ratio seen on ADs (67), there is still a chance that chimerism among immunological cells could represent a starting point for perpetuating or acquiring an unbalanced self-tolerance. Ultimately, both X monosomy as a resource to produce chromosome instability and haploinsufficiency for X-linked genes have been put forward as possibly playing critical roles in the predominance of ADs in females (68). None of the suggestions presented have a completely proven experimental background, and they are still part of a sex-connotation discussion.

A preferential inheritance of the autoimmunity trait from mothers has been observed in patients with primary SS (19,22), SLE (33), and T1D (34), thus indicating a preferential transmission of susceptibility genes from mothers to their offspring. Maternal transmission of autoimmunity could be influenced by the high preponderance of ADs in females compared to the general population given their greater intrinsic susceptibility to develop these diseases that can potentially arise from sex related physiological factors (69). Nonetheless, other reports have postulated a preferential transmission of the autoimmune trait through the father, specifically T1D (70) and MS (24). Given women's inherent excess of susceptibility to developing an AD, men would require an augmented risk to overcome the resistance towards autoimmunity relative to women. Thus, men would need a greater content of susceptibility variants that would trigger their phenotype and would also guarantee more frequent transmission of the autoimmunity trait to their offspring. This has been reported previously for MS by Kantarci *et al*. (24) under the Carter effect, which is defined by the observed higher incidence of the trait in relatives when the index case is the least commonly affected sex (71). Consequently, the incidence of the trait would be expected to be the highest in daughters of affected men and lowest in sons of affected women, a trend that has not been convincingly confirmed (24). Although the Carter effect for pyloric stenosis has been explained by a multifactorial threshold model (72), further research on familial autoimmunity must be done given the possibility of an ascertainment bias of the studied families through the affected probands that would generate a higher proportion of autoimmunity in the affected fathers than in mothers (70).

SEGREGATION OF AUTOIMMUNITY

The purpose of segregation analysis is to use familial phenotype data to resolve major loci predisposing to a trait and estimate the most likely parameters of the mode of inheritance. Although computationally demanding, it is now possible to set up models that include more than one mode of inheritance providing the family structures have sufficient information. Classical segregation analysis has no requirement for observed genotypes. It can be viewed as a special case of investigation of familial aggregation which often focuses on the pattern of aggregation within individual families rather than averaging across the population. The results of a segregation analysis can be very sensitive to inappropriate adjustment for ascertainment (73).

Segregation analysis for ADs alone and together with other ADs has been implemented. A single major gene has been hypothesized to confer susceptibility for autoimmunity indicating that a postulated autoimmune gene is expressed as an autosomal dominant trait with penetrance of approximately 92% in females and 49% in males (44). Furthermore, others have demonstrated the presence of a dominant major gene and strong environmental effects as the most parsimonious model of segregation for VIT (45). However, when analyzing RA together with other ADs, a mixed model fit the data significantly better than the major gene or polygenic models. (46) The above-presented models are initial approaches towards unraveling the dynamics of the polygenic component among families presenting the autoimmunity trait. Still, analyses combining the presence of autoimmune family history that take autoimmunity as a trait remain to be carried out.

GENETICS OF AUTOIMMUNITY

As multifactorial etiologies, ADs develop from the cumulative effect of diverse events on the immune system (59). It is now clear that they do not begin at the time of the clinical appearance but rather many years before (Figure 2). This implies the possibility of predicting autoimmunity (74). A common origin for diverse ADs is sustained by three levels of evidence (9). The first comes from clinical observations indicating the possible shift from one disease to another and the fact that more than one AD may coexist in a single patient (i.e., polyautoimmunity) (8,75-77), or in the same family (i.e., familial autoimmunity) (Figure 3) (78). The second refers to known pathophysiological mechanisms shared between ADs, and the third corresponds to the evidence implying common genetic factors (77). The importance of this concept focuses on the probability of having multiple ADs simultaneously in one patient, which goes beyond epidemiological inferences. Therefore, family history of ADs should be considered when doing genetic analysis as this new approach incorporates all accepted pathologies for which evidence suggests an autoimmune origin.

The human genome is distributed among 46 chromosomes, 22 homologous pairs of autosomes, and one pair of sex chromosomes. The complete set is the diploid complement, while one set is the haploid (i.e., gametic) genome. One chromosome in each of the 22 homologous pairs is derived from the mother and one from the father. The two

homologues will have the same sequence of genes in the same positions, but they will usually exhibit sequence variations at several loci and can therefore be distinguished. The human haploid genome is about 3·3 billion bp. Some 3% of the genome consists of coding sequences, and there are 30,000–40,000 protein-coding genes. Any two genomes typically differ by 0.1%, but the DNA sequence may vary between the two versions of the same chromosome several ways (79).

Many different types of DNA sequence variants exist, and they can be classified different ways (e.g., by the physical nature of the sequence variation, by the effect on protein formation, and by the associated susceptibility to a disease). The two most important structural classes are microsatellites and single nucleotide polymorphisms (SNPs). Microsatellites are highly variable, and most people are heterozygous at any given locus. Coding regions tend to not contain microsatellite sequences. SNPs, in contrast, represent variations in a single nucleotide that occurs with high frequency in the human genome (13).

The modern unit of genetic variation is the SNP, typically used as markers of a genomic region with the large majority of them having a minimal impact on biological systems. SNPs in protein-coding regions can be non-synonymous (i.e., missense) or synonymous (i.e., sense), depending on whether they do or do not modify the amino acid sequence in the gene product. Non-synonymous SNPs can also be called coding SNPs. Intronic and intergenic SNPs lie in the non-coding regions. A non-synonymous SNP in a coding sequence is generally more likely than other classes of SNP to affect the function or availability of a protein. The true distribution of disease-associated variants between non-coding and coding sequences is unknown; however, they are by far the most abundant form of genetic variation in the human genome (80).

Common variants (frequency >1%) in the population account for most of the genetic variation observed in the genome (79,81). Rare variants, each found in <1% of the population, make up the remainder of genetic polymorphism but are far more numerous than common variants in total counts given the large size of the human population. In all, 10–15 million common variants and billions of rare sequence variants constitute the human genetic diversity which makes the identification of those variants relevant to particular ADs challenging (82).

MODELS FOR COMPLEX DISEASES

Currently, three general models have been proposed to explain the genetic component of complex disease (83): the common disease/common variant (CD/CV) model, the rare alleles of major effect (RAME, Multilocus/multiallele hypothesis) model, and the infinitesimal model (Figure 4). These are neither mutually exclusive nor sufficiently precisely defined to allow them to be distinguished in any particular case, but they provide an essential conceptual background. However, it is also debatable whether they are collectively sufficient. The evidence for CD/CV is underwhelming, RAME is difficult to reconcile as a general explanation for familial risk distributions, and infinitesimal liability does not lend itself to reductionist genetic dissection.

The evidence now suggests that genetic susceptibility to common disease is probably due to hundreds or even thousands of alleles that may or may not be rare, that are at least as often ancestral as derived, and that can vary in frequency among human populations. Evidence for epistatic interaction between risk alleles and their role in ADs has started to gain more focus (84). Finally, some diseases yield to GWAS studies more readily than others, which suggests different levels of genetic buffering (85). Below is a brief description of the models that have been proposed to explain the genetic contribution to disease.

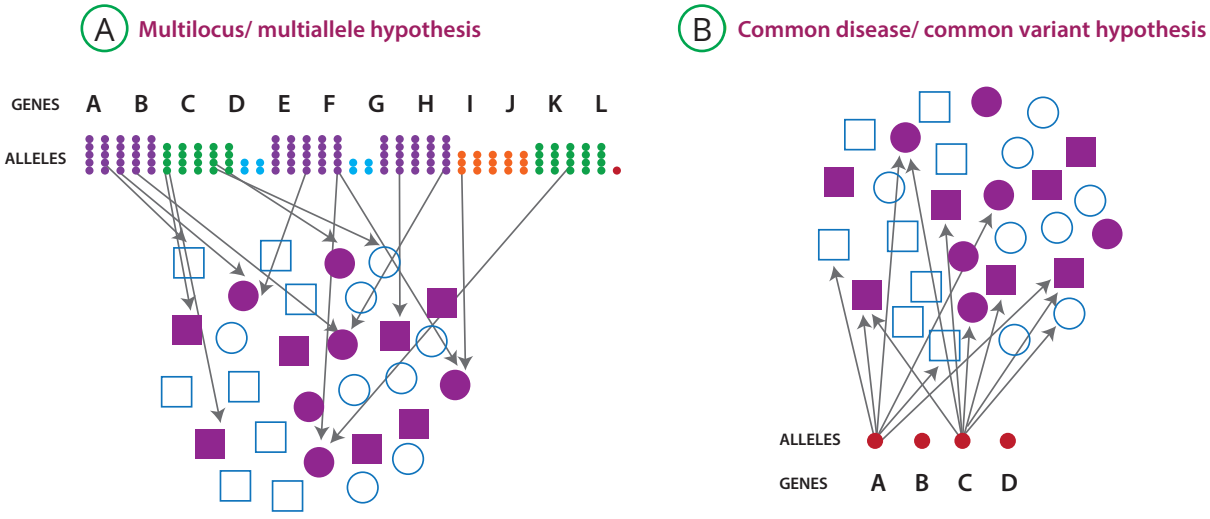

Figure 3. General models proposed to explain the genetic component of complex disease. Square: Male; Circle: Female; filled circle and square depicts affected. Adapted from Wright *et al.* (106).

1. Common disease/common variant (CD/CV) model (Figure 4) According to the CD/CV model, common disease susceptibility is caused by common polymorphisms, each of which makes a moderate contribution but generally explains no more than five percent of disease susceptibility. By analogy with QTL mapping, it is assumed that up to 20 or so common variants (each with a minor-allele frequency greater than 0.05) are associated with any particular disease, and that affected individuals carry an excess of the risk alleles (see chapters 17 and 18). Note that it is not clear whether the SNPs detected in GWAS studies typically capture common variants or a set of rare major-effect variants that are enriched on the susceptibility haplotype (83).

2. The rare alleles of major effect (RAME) model: This model postulates that some common diseases are actually highly heterogeneous with respect to their etiology (Figure 4). That is, rare variants with frequencies of less than 0.01 can promote disease. Hundreds of such polymorphisms, with homozygous individuals for each polymorphism occurring at a frequency of just 1 in 10,000, could add up to an appreciable incidence in the general population. Double heterozygous interactions could further increase the susceptible population, possibly to common disease status (83). Despite this expectancy, the characterization of causal rare variants has not, so far, been able to explain the observed effects as being the result of common tag variants mapping close to the discovered rare variants. This gives further support to the CD/CV model attributed to common variants with weak effects as disease defining factors (86).

3. The infinitesimal model: The infinitesimal model is gaining in popularity as GWAS reveal that most genetic variation for complex diseases must be due to variants that have relative risks that are less than 1.2 and explain a fraction of a percent of the liability. As disease is often regarded as a discrete state, it is often assumed that a threshold of liability separates cases from controls. However, the need for a threshold assumption has been questioned, at least in relation to autoimmune disorders. If disease liability follows the same distribution of effects as the best-characterized continuous trait, height, then hundreds if not thousands of variants covering a wide range of frequencies are likely to contribute to common diseases (83). It should also be noted that some subphenotypes have stronger genetic factors than the phenotype (i.e., the disease) itself. The infinitesimal model does not immediately explain how susceptibility becomes enriched in certain pedigrees, or why the prevalence of disease has increased so markedly in recent decades.

GENETIC CORRELATIONS

After obtaining evidence of a likely genetic component in the cause of a complex disease (without genotyping genes), the next step is to locate and identify any causative genes (Figure 1). However, for most complex diseases, there are so many candidates and so many genes whose usual effects are completely unknown that candidate gene work is often preceded or accompanied by an attempt to locate regions of the genome that are etiologically relevant. Regardless of assumptions about the genetic model of a trait, or the technology used to assess genetic variation, no genet-

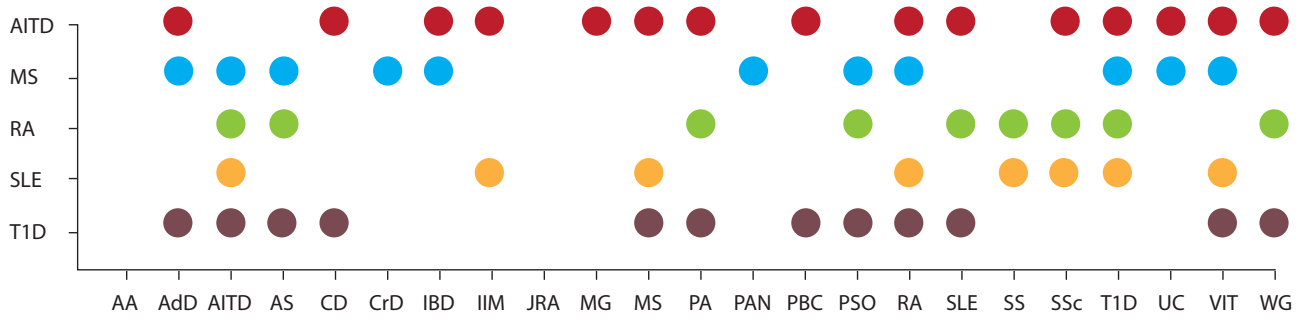

Figure 4. Familial autoimmunity. The vertical axis corresponds to the proband's disease and each disease individually. On the horizontal axis, diseases present in first degree relatives are shown. Each color belongs to the proband's disease. The figure only includes significant results and may serve as a guide for clinical practice in order to search for ADs in FDRs of probands. Note that familial autoimmune disease is excluded. *AA*, alopecia areata; *AdD*, Addison's disease; *AS*, ankylosing spondylitis; *AITD*, autoimmune thyroid disease; *CD*, celiac disease; *CrD*, Crohn's disease; *FDR*, first degree relative; *IBD*, inflammatory bowel disease; *IIM*, idiopathic inflammatory myositis; *JDM*, juvenile dermatomyositis; *JRA*, juvenile rheumatoid arthritis; *JSLE*, juvenile systemic lupus erythematosus; *MAS*, multiple autoimmune syndrome; MG, myasthenia gravis; *MS*, multiple sclerosis; *PA*, pernicious anemia; *PAN*, polyarteritis nodosa; *PBC*, primary biliary cirrhosis; *PSO*, psoriasis; RA, rheumatoid arthritis; *SLE*, systemic lupus erythematosus; *SS*, Sjögren's syndrome; *SSc*, systemic sclerosis; *T1D*, type 1 diabetes; *UC*, ulcerative colitis; *VIT*, vitiligo; *WG*, Wegener's granulomatosis. AS is considered an autoinflammatory more than AD. From Cardenas-Roldán *et al.* (78).

ic study will have meaningful results without a thoughtful approach to characterize the phenotype of interest (87). When embarking on a genetic study, the initial focus should be on identifying precisely what quantity or trait genetic variation influences. The commonly used strategies to find variants that influence disease are linkage and association studies. They are described below.

Genetic linkage Analysis. Major genes for monogenic conditions have been located by linkage analysis (3), but there have been far fewer successes with complex diseases. Genetic linkage analysis is perhaps the best example of a common investigative approach that derives almost entirely from a consideration of the underlying genetics since it relies entirely on the tendency for shorter haplotypes to be passed on to the next generation intact, without recombination events at meiosis. If a marker can be identified that is passed down through a family such that it consistently accompanies the disease of interest, this suggests a gene with a functional effect that is located close to that marker (88).

It is noteworthy that the modified use of traditional linkage approaches remains a useful tool for the study of polygenic diseases, especially if a major locus that contributes to the phenotype is known. It has been observed that, in some cases genetic loci overlap or co-localize between related disorders. Becker *et al*., based on previous AD linkage studies, first reported eighteen common non-major histocompatibility complex (MHC) loci clusters in 1998 and also hypothesized a shared and common genetic basis for the autoimmune trait (36). Tomer et. al., working with both Hashimoto's thyroiditis and Grave's disease in the same analyses reported linkage for AITD (89). Other studies which considered the presence of linkage for specific diseases have found shared autoimmunity loci (9,90). Limitations of genome-wide scans when applied to complex ADs, involve heterogeneity in disease phenotypes, population and ethnic differences, and lack of statistical and analytical models (9).

Association analysis. This approach can be seen as a traditional epidemiology approach applied to genotypes or alleles across a population. Thus, many of the analytical approaches used in epidemiology and biostatistics can be applied directly to the evaluation of associations. Among these are univariate methods and regression analysis. Furthermore, analysis can be extended to deal with data that have a complex correlation structure including: family data, longitudinal data, data naturally subject to geographical or temporal clustering, and/or data collected under a multistage sampling plan and applied to phenotypes in various classes, including binary traits, continuous normally distributed traits, and time to event (survival time) (13). A test of association can be informative even when based on genetic variants that are not functional. It can also be useful to detect linkage disequilibrium (LD) (BOX 1) between disease and a non-functional marker. An association analysis based on a putative functional genetic variant can be called direct, and one based on linkage disequilibrium with a marker indirect. Indirect association analysis allows finer mapping than conventional linkage analysis.

Despite the enormous diversity of genetic variants associated with susceptibility/protection from individual to individual with the same AD, a high degree of similarity is observed between ADs in the pool of genetic variants associated with disease (see chapters 17 and 18). These genetic associations can be dissected into three biologically distinct classes.

1. HLA associations: for most ADs, the strongest genetic associations are with the human leukocyte antigen (HLA) locus. Variations in the HLA region were the first polymorphisms investigated in association studies and turned out to play a major role in most ADs even though precise understanding of the effects is still under investigation owing to the highly polymorphic nature, exceptional LD, and high gene density of this region. Although some linked HLA haplotypes are shared between ADs, most HLA associations seem to be specific for a disease (82). For a complete description of HLA association the reader is directed to chapter 17.

2. Common autoimmune risk variants: Many of the strong loci that have been associated with one AD are also found

BOX 1. Linkage disequilibrium (LD) vs. Linkage

LD is a property of genetic markers on a contiguous stretch of a genomic sequence which describes the degree to which an allele of one genetic marker is inherited or correlates with an allele within a population. Population geneticists coined the term LD in an attempt to mathematically describe changes in genetic variation within a population over time. In turn, the term is related to the concept of chromosomal linkage, where two markers on a chromosome remain physically joined on the chromosome. Recombination events from generation to generation break apart chromosomal segments. This effect is amplified over generations, and in a population of fixed size undergoing random mating, repeated random recombination events will break apart segments of contiguous chromosome (containing linked alleles) until eventually all alleles in the population are in linkage equilibrium or are independent (91).

to be involved in multiple other ADs (See chapter 18). Typically, the allele of these shared variants that causes risk in one AD also causes risk in other ADs, indicating that the alleles participate in a shared immunological process common to the development of multiple, clinically distinct ADs. A degree of complexity exits in which precise mechanisms may differ between subgroups of ADs (92,93).

3. Disease-Specific risk variants: A smaller number of associations are observed that are specific to a single AD with no measured impact on other ADs. This suggests that they might drive a target organ-specific pathway toward disease.

GENOME-WIDE ASSOCIATIONS STUDIES (GWAS)

Debate has recently erupted in the field of genetics between the CD/CV and RAME variant hypotheses. GWAS are designed to study common variants typically present at an allele frequency of more than 5% and have been exceedingly successful in doing this for ADs (see chapter 18). GWAS exploit LD between SNPs and make it possible to assay a manageable number of variants while still capturing the majority of variation in a given population's genome. GWAS have been very successful in the identification of novel loci and pathways contributing to AD (See chapter 18). However, the effect sizes reported are usually rather modest, with odds ratios (OR) typically between 1.1 and 1.8. Note that most of the genes identified to date affect more than one autoimmune condition. One interesting observation has been that many genetic loci appear to harbor variants that are associated with multiple, sometimes seemingly distinct traits, and such associations are termed cross-phenotype associations (94). Cross-phenotype association differs from pleiotropy. A cross-phenotype association occurs when a genetic locus is associated with more than one trait in a study, regardless of the underlying cause for the observed association. Pleiotropy occurs when a genetic locus truly affects more than one trait and is one possible underlying cause for an observed cross-phenotype association [for complete review see Solovieff *et al*. (93)].

Genetic studies have led to important conclusions on the genetic architecture of ADs. A plausible disease trait scenario –multiple environmental variants interacting with several genes to conferred susceptibility on individuals in a population– has been projected (83). According to this theory, individuals would express the disease trait if they were located on the wrong side of the normal distribution. This concept has been widely accepted, but additivity must be assumed for all genetic variants accompanied by an equal effect on the trait. Autoimmunity might involve a genetic distribution that is not as straightforward as a normal distribution but rather an unknown one in which many loci would not add to but complement the individual's risk of developing the autoimmune phenotype (Figure 3b).

For many and perhaps most traits, the interaction with other genes and environmental factors might be genetically programmed or may be purely stochastic. Most common diseases probably contain major subsets that fall into this sort of causation. Even when the phenotypical manifestation is usually considered to be monogenic, it could be the result of gene-environment interaction (95). This does not mean impossibility in the modeling of a complex trait but a warrant towards generalization.

Mathematical models which assume a continuous distribution of liability to disease in the general population have been employed to explain observed "non-Mendelian" patterns of familial occurrence (96,97). Polygenic determination refers to the mathematical model in which a number of genes with small additive effects provide an underlying genetic predisposition to disease development. Likewise, the term multifactorial describes models in which environmental factors interact with genetic predisposition. This multifactorial model was adapted to account for discontinuous traits by the addition of a threshold, the point of risk distribution beyond which individuals are affected (Figure 3). Moreover, a number of observations in human population and experimental animals have shown consistency with the model of multifactorial inheritance (24,95). The relative merits of this hypothetical model (polygenic multifactorial-threshold and major single gene with incomplete penetrance) have been debated vigorously (95).

Families with multiple affected relatives appear to share common risk alleles with sporadic patients but may have a higher genetic load. A consequence of the polygenic model for complex diseases is that patients are inevitably highly heterogeneous in terms of the particular set of risk alleles they carry. It has been suggested that this may translate into different genetically determined disease mechanisms in subgroups of patients or a common disease mechanism that is complemented by additional pathways that are more or less predominant in different subgroups (82).

PERSPECTIVES

Much progress has been made in the area of genetics of complex diseases including ADs. Understanding the genetic basis of ADs is an important goal, since the pathways that affect the risk of disease in patients are also potentially good drug targets. However, many of the factors that define the onset and outcome of AD and other complex diseases remain to be identified. By far the biggest impacts in the long run will be from the newly adopted technologies including next-generation sequencing (NGS). New information from studies like the 1000 Genome Project, which is searching for novel variants in healthy subjects, would allow common and rare variations to be captured in hopes of better and more specific mapping (98).

Parallel to this, current studies on many phenotypes are presently using resequencing in regions found through GWAS to ensure that the majority of variation has been identified before embarking on detailed functional studies.

Other genomic features, e.g., chromatin marks, microRNA, epigenetic status, transcription factor binding regions, and expression need to be evaluated in conjunction with the associated regions (99,100). Studies must also carefully evaluate the impact of environmental influences in combination with genetic predisposition to disease to better understand the pathophysiological mechanisms underpinning autoimmune phenotypes.

Collectively, studies mapping genetic variation that contribute to transcriptional variation are referred to as expression quantitative trait loci (eQTL) mapping studies also known as genetical genomics (101,102). Their general design consists of genome-wide genotyping of subjects and capturing a transcriptome-wide mRNA profile using microarrays, or more recently, high-throughput RNA-sequencing (i.e., RNAseq). An eQTL analysis itself consists of applying regression-based or nonparametric models to test millions of genetic variants for regulatory effects on the expression of nearby and distant genes. cis-eQTL analyses are focused on assessing the role of genetic variants with respect to the expression of genes in their vicinity and, empirically, have been demonstrated to be able to detect regulatory effects that are replicated (103,104).

Finally, association, rather than causality, results from the combined effect of many variants each exerting a small effect on risk. Many different combinations of risk alleles are able to independently generate a high level of disease risk, without individual loci being necessary or sufficient for the development of disease. Thus, a long road lies ahead for the effort to disentangle and develop complete understanding of AD genetic architecture. However, multidisciplinary approaches involving autoimmunologists, geneticists, epidemiologists and statisticians together with new technology will nurture this landscape.

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17

HLA ASSOCIATION WITH AUTOIMMUNE DISEASES

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INTRODUCTION

Autoimmune diseases (ADs) are chronic complex inflammatory diseases. They are considered to be either specific or systemic and characterized by inducing immune humoral (B cell) or cell (T cells) responses. Although their etiology is unknown, they are well known to have environmental and polygenic components that are involved in defining susceptibility or protection. Thus, the sum of the genes involved in ADs makes up the genetic component that defines them. ADs are presented with a multifactorial genetic inheritance pattern which does not completely follow a classical Mendelian model.

Furthermore, studies in monozygotic and dizygotic twins have estimated the relative contribution of genetic effects as seen in Table 1 (1). Among the most relevant and studied genetic factors for ADs are genes located in the Major Histocompatibility Complex (MHC) and, in particular, loci from Human Leukocyte Antigen (HLA) class I and class II. An extensive list of ADs has been associated with different variants of the HLA genes, particularly, class II genes (2). Note that the set of alleles associated with various ADs may vary from one population to another, and within the same population, different alleles might be associated with different ADs. Moreover, the genetic effect of HLA might also be involved in changing and defining the relationship between the environmental factors associated with ADs.

Genetic mapping of the MHC has identified multiple genes as shown in chapter 10. Advances in molecular techniques, based on the genetic sequence and conformation of the different epitopes of HLA molecules and genes that encode them, have enabled the development of molecular and functional analysis. Together with genetic and molecular epidemiology, they have made it possible to establish and hypothesize the plausible mechanisms of association between HLA genetic variants and the pathophysiology of ADs, e.g., rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), type 1 diabetes mellitus (T1D), multiple sclerosis (MS), celiac disease (CD), Sjögren's syndrome (SS), etc., or HLA and autoinflammatory diseases such as ankylosing spondylitis (AS). However, the molecular mechanisms associated with these diseases are not fully resolved. The struggle in identifying genes presenting a primary and secondary association as well as the high linkage disequilibrium (LD) observed throughout the whole MHC region coupled with the interaction of these molecules with infectious agents and/or allergens and other environmental factors has hampered the definition of these mechanisms.

DISEASE	MONOZYGOTIC PAIRWISE CONCORDANCE	DIZYGOTIC PAIRWISE CONCORDANCE
SLE	11 – 40	0 - 4
RA	0 – 21	0 - 8.8
T1D	13 - 47.4	3.8 - 11.6
MS	0 – 50	0 - 16.7
AITD	17 - 22.2	0 - 1.9
CD	60 – 75	9.1 - 11
PSO	35 – 64	10 - 14
IBD	20 – 50	0 - 6.5

Table 1. Concordance rates in monozygotic and dizygotic twins in various ADs. Twin studies have been done on ADs with varying degrees of robustness and different cohort sizes, particularly since solid population-based studies cannot be done except in rare conditions. Adapted from Bogdanos *et al.* (1). Abbreviations: Systemic lupus erythematosus (*SLE*), rheumatoid arthritis (*RA*), type 1 diabetes (*T1D*), multiple sclerosis (*MS*), autoimmune thyroid disease (*AITD*), celiac disease (*CD*), psoriasis (*PSO*), inflammatory bowel disease (*IBD*).

The association hypothesis has been substantiated in: 1) the susceptibility or protection that may result from the combination of several molecules encoded by several HLA (class I and/or class II) or non-HLA (*TAP1* and *TAP2*) loci in the MHC region (3), 2) the different functions of the immune response and the processing and presentation of antigens (4), and 3) the distinction between self and non-self, and the activation of autoreactive T cells (3,4). Likewise, it has been thought that some peptide sequences that originated from infectious organisms (i.e., viral or bacterial) might present high similarity to peptides generated from self-proteins. The former would engender the chance of an immune response (i.e., molecular mimicry) leading to the presentation of these self-proteins in the context of HLA molecules despite their different origins (5).

EPIDEMIOLOGICAL ANALYSIS

Evaluation of the role of the HLA molecules and alleles in susceptibility or protection against any disease, particularly ADs, is a key component in the definition of the population at risk. Therefore, conventional epidemiological analysis of age, sex, ethnicity, and geographical origin might elucidate multiple environmental factors associated with these diseases. Furthermore, these variables should go hand in hand with molecular epidemiology which seeks to understand the genetic distribution in a population and its correlation with the distribution of the disease and associated alleles.

Molecular epidemiology is based on the study of homogeneous populations. The preferred groups are those that are genetically and geographically isolated such as indigenous communities, which make it possible to identify polymorphisms, new alleles, new sequences, extended haplotypes and determine the genetic and functional expression of HLA molecules in these populations. In addition, molecular epidemiology allows us to evaluate the natural dynamic of the population and frame it in a system which generates data such as the Hardy Weinberg proportions, genetic drift, inbreeding, and positive/negative selection for certain alleles (Box 1) (6). All these factors can lead to different patterns of association with the disease or the functional behavior of antigen presentation and immune response affected in pathologies such as ADs.

BOX 1.

Complotype
Describes haplotypical combinations of genetic variants of the MHC-linked complement genes.

Genetic drift
Corresponds to changes in allele frequencies due to the genes of the offspring that are not a random sample of the genes seen in the parents. It can be caused by a small population size.

Hardy-Weinberg (HW) proportions
Indicate a stabilized/expected distribution of the genotype frequencies in the values p2, 2pq, and q2, where p and q are allele frequencies. The HW proportion is important for two reasons. First, the proportions of genotypes in a population may diverge from the expected ones for many reasons, including natural selection, division of populations into different subgroups, or mating that is not entirely random. Comparing the expected and observed proportions of genotypes allows biologists to determine whether these evolutionary forces may be contributing to a population. The heterozygosity of a population is the expected proportions of heterozygotes, given the allele frequencies in the population.
Second, the proportions lead to a natural definition of genetic variation in a population: the heterozygosity. A population's heterozygosity is the expected proportion of heterozygotes from the HW formula, 2pq. Two populations may be compared by their heterozygosities: the one with the higher heterozygosity has a higher chance that any single individual will have two different alleles, which means the population is genetically more variable. Variation is a consequence of evolutionary history, including the patterns of selection and genetic drift, and the amount that individuals have moved from one population to another in the past. Thus, the HW proportions give an important way to study the evolution of populations over time.

Linkage disequilibrium (LD)
Occurrence of some combinations of alleles or genetic markers in a population more or less often than would be expected from a random formation of haplotypes from alleles based on their frequencies.

Relative Risk (RR)
Estimate of the strength of the association of a given gene relative to disease. Indicates how many times an individual has a risk of developing the disease by being a carrier of that gene. RR> 1 indicates susceptibility, RR <1 indicates protection/resistance.

Selection
Process by which an individual with a particular genotype has an advantage over another with a different genotype

MOLECULAR INTERACTION: MHC-PEPTIDES AND AUTOIMMUNE DISEASES

Given the characteristics of the interaction of peptides with the MHC molecules reviewed in chapter 10, it is clear that many efforts to explain the molecular association of these alleles with ADs are focused on the identification of autoantigens and HLA molecules that present them as will be reviewed below.

RHEUMATOID ARTHRITIS (RA)

Relevant HLA genes involved in susceptibility to RA are *HLA-DRB1*04:01*, **04:04*, and **04:08* in Caucasians; *HLA-DRB1*04:05* in Spaniards and Japanese; *HLA-DRB1*01:01* and **01:02* in Israelis; *HLA-DRB1*14:02* in some Native Americans such as Pima and Yakima Indians; *HLA-DRB1*10:01* in Greeks (7); and *HLA-DRB1*01:01*, **04:01*, **04:04*, and **04:05* in Latin Americans (8). Although the pathogenic mechanisms of these alleles in RA are still unresolved, different hypotheses have been postulated as follows: first, presentation of arthritogenic antigens; second, alterations of peptide affinity during T cell repertoire selection; and third, molecular mimicry with microorganism peptide residues. Another approach is the classical shared epitope (SE) hypothesis, proposed by Gregersen, *et al*. (9), who by comparing aminoacid sequences encoded by the disease-associated *HLA-DRB1* alleles listed above, demonstrated a conserved motif (L-LE-[Q/R]-[R/K]-R-A-A) including residues 70–74 in the third hypervariable region of the DRβ1 chain.

In addition, the *HLA-DRB1* locus also harbors some protective alleles known as the DERAA sequence at the same position in the third hypervariable region of the DRB1 chain residues 70-74, specifically, aspartic acid (D) at position 70. The DERAA alleles are *HLA-DRB1*01:03*, **04:02*, **11:02*, **11:03*, **13:01*, **13:02*, and **13:04*. People carrying *HLA-DRB1* alleles that express this DERAA sequence display a lower susceptibility to RA and have less severe disease than people with SE-negative and DERAA-negative *HLA-DRB1* alleles (10). Alleles associated with SE and DERAA sequence are listed in Table 2 (11).

It has been suggested that alleles carrying the SE induce the activation of autoreactive T cells. This is complemented by crystallographic studies in which it has been observed that a glutamine (Q) at position 70 and an arginine or lysine residue (R/K) at position 71 of the β chain establish a direct interaction with the T cell receptor (TCR) by selecting a specific population of T lymphocytes "SE recognizers."

Other studies have shown that changes at residues 70 to 74 of the β-chain of the SE alleles can generate a total change in the range of peptides that are initially presented by these molecules through directed mutagenesis (12). In the SE, both its residues defined by specific genotypes and the LD that these alleles may have with other loci — either as HLA and non-HLA — are important. This type of genetic pattern also affects the risk depending on the genotypic conformation. For example, if the individual has one or both alleles for SE, the risk effect for the SE homozygous individual is 50% less and for the heterozygous one, 30% adducing a penetrance variability dependent on genotype status. Therefore, it can be concluded that the relative risk (RR) can be high with respect to the presence of SE but not enough for RA to be present in 100% of individuals who carry it (7).

Further, when the relationship between anti-cyclic citrullinated peptide antibodies (ACPA) and *HLA-DRB1* have been studied, they have revealed that the association between *DRB1* SE alleles and RA was restricted to ACPA-positive RA but not ACPA-negative RA patients in different populations (13). Just 12.7% of the phenotypic variance can be explained by susceptibility loci within the MHC region compared to ~4% for non-MHC loci. This leaves most of the MHC association to be explained by *HLA-DRB1* in RA (14). Furthermore, there is evidence that four amino acids at positions 11, 13, 71, and 74 in the HLA-DRB1 molecule are important

AMINO ACID SEQUENCE	SE MOTIF	CODING *HLA-DRB1* ALLELES
QKRAA	+	**04:01;*04:09;*04:13;*04:16;*04:21;*14:19;*14:21*
DERAA	-	**04:02;*04:14;*01:03;*11:02;*11:16;*11:20;*11:21;*13:01;*13:02;*13:04;*13:08;*13:15;*13:15;*13:17;*13:19;*13:22;*14:16*
QRRAE	-	**04:03;*04:06;*04:07;*04:17;*04:20*
QRRAA	+	**01:01;*01:02;*01:05;*04:04;*04:05;*04:08;*04:10;*04:19;*14:02;*14:06;*14:09;*14:13;*14:17;*14:20*
RRRAA	+	**10:01*
RRRAE	-	**09;*14:01;*14:04;*14:05;*14:07;*14:08;*14:10;*14:11;*14:14;*14:18*
DRRAA	-	**04:15;*08:05;*11:01:1;*11:01:2;*11:04:1;*11:04:2;*11:05;*11:06;*11:08:1;*11:08:2; *11:09;*11:10;*11:12;*11:15;*11:18;*11:19;*11:22;*12:01;*12:02:1;*12:02:2;*12:03:1;*12:03:2;*13:05;*13:06;*13:07;*13:11;*13:12;*13:14;*13:21;*16:01;*16:02;*16:05*
QARAA	-	**15;*13:09*
QKRGR	-	**03; *04:22;*11:07*

Table 2. SE and SE-negative *HLA-DRB1* alleles. Adapted from Holoshitz (11).

for the susceptibility to RA in ACPA-positive patients (Figure 1). These amino acids have side-chains pointing into the groove and are thus important for and possibly define the peptide binding properties of the HLA molecule. Moreover, amino acids 71 and 74 are part of the SE and, as mentioned above, are implicated in conferring risk to RA. The association at these four positions with ACPA-positive RA patients is different and depends on the *HLA-DRB1* polymorphisms. Thus, *HLA-DRB1*04:01*, **04:08, *04:05,* and **04:04* are the risk alleles most significantly associated with ACPA-positive RA in European individuals. Additional associations for *HLA-B*08* and for a group of *HLA-DP1* alleles with ACPA-positive RA have also been reported. For both molecules encoded by these HLA genes, there is a substitution in position 9 which may allow a functional impact on antigenic peptide presentation to T Cells (15) (Figure 1).

According to new insights into the functional role of the SE in RA pathogenesis, this is transduced not by means of an adaptative response but by an innate response transduction. De Almeida *et al*., suggested that in dendritic cells (DC), a SE-triggered signaling is transduced via cell surface calreticulin (CRT), a molecule involved in clearance of apoptotic cells. Signaling by CRT ligation would occur with the SE through the P-domain of CRT. Following an initial binding, activation of nitric oxide (NO) and reactive oxygen species (ROS) production is initiated, leading to two different outputs. In CD11c(+)CD8(+) DCs, the SE inhibits the enzymatic activity of indoleamine 2,3 dioxygenase (IDO), a key enzyme in immune tolerance and T cell regulation, whereas in CD11c(+)CD8(-) DCs, the ligand activates the production of IL-6 and IL-17 (16). Likewise, the same group demonstrated that the SE ligand interacts with cell-surface CRT on osteoclasts (OC) and activates NO and ROS production. The former is supported by the fact that SE activates Th17-dependent osteoclastogenesis by enhancing the differentiation of RANKL-expressing IL-17–producing T cells (17) (Figure 2).

MULTIPLE SCLEROSIS (MS)

This condition corresponds to an autoimmune pathology with a predominant immune cell response characterized by the presence of autoreactive T cells that react against the myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and the proteolipid protein (PLP). The *HLA-DRB1*15:01* and *HLA-DQB1*06:02* alleles, which are in LD, are the main alleles associated with risk for MS in Caucasians and Latin Americans (18). Recently, in a large combined multinational cohort in the International Multiple Sclerosis Genetics Consortium (IMSGC) GWAS study, the *HLA-DRB1*13:03* allele was also identified as being associated with MS (OR=2.43). Furthermore, *HLA-DRB1*01:08* (OR=1.18) and *HLA-DRB1*03:01* (which is strongly linked to *HLA-DQB1*02:01*; OR=1.26) showed significant associations. Evidence of an additive effect for each additional allele was also described (19). In the Sardinia region of Italy, where MS prevalence is high, *HLA-DRB1*04*, *HLA-DRB1*03:01*, and *HLA-DRB1*13:01* (in addition to *HLA-DRB1*15:01*) positive associations with MS have been reported (20).

Several studies have explored phenotype-genotype correlation for associated HLA alleles in MS and reported that *HLA-DRB1*15* has been associated with younger age at onset and a worse Expanded Disability Status Scale (EDSS) score as well as severe morbidity in patients with primary progressive MS (21). Both the carriage of *HLA-DRB1*15* and the presence of oligoclonal bands in the cerebrospinal fluid have been reported to hasten disease progression (22).

MS studies in animal models [i.e., experimental allergic encephalomyelitis (EAE)] and human models have focused on the 84-102 MBP peptide, known as immunodominant epitope ENPVVHFFKNIVTPR, based on which the crystallographically complex *HLA-DRB1*15:01* - MBP peptide has been disclosed. The most prominent feature of this peptide is the capability of the P4 pocket DRB1*15:01 molecule to

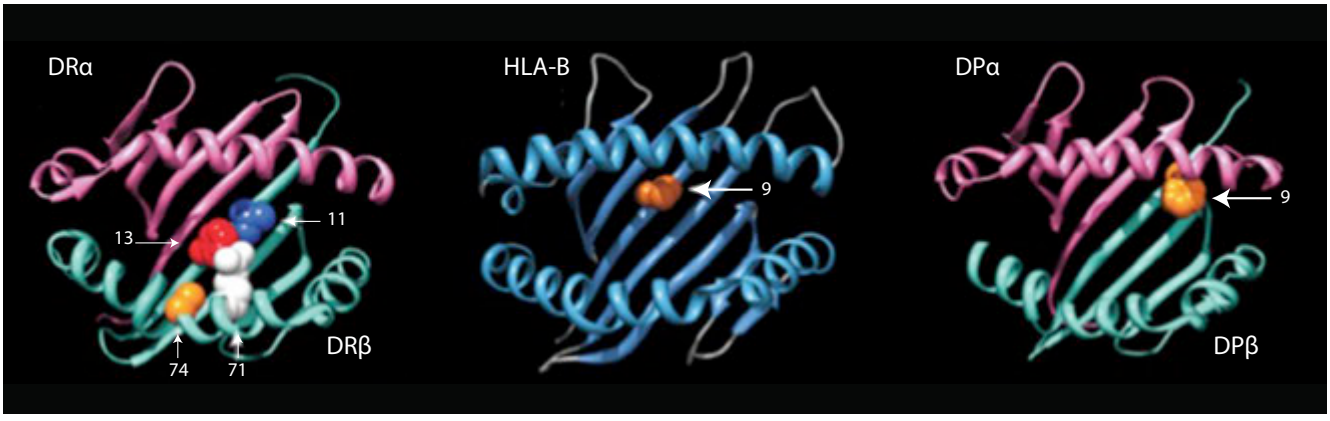

Figure 1. Three-dimensional ribbon models for the HLA-DR, HLA-B, and HLA-DP proteins. These structures are based on the Protein Data Bank (PDB) with a direct view of the peptide-binding groove. Key amino acid positions identified by the association analysis are highlighted.

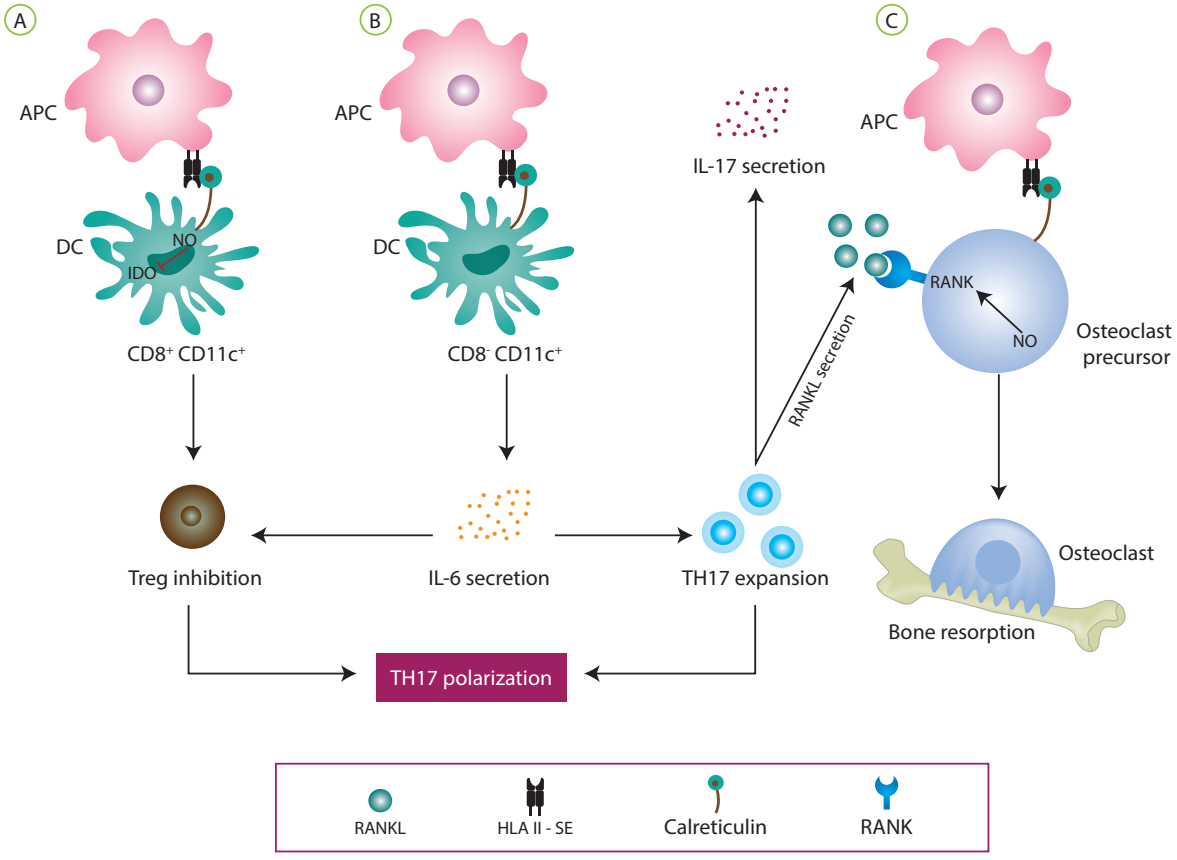

Figure 2. New insights into the functional role of the SE in RA pathogenesis: A proposed model. The SE ligand, expressed on APC interacts with cell surface CRT. In RA, the affinity of SE-CRT interaction could increase leading to an aberrant activation of the SE pathway, excessive Th17 polarization, and disease development. A) In $CD8^+CD11c^+$ DC, the SE inhibits the activity of IDO, an enzyme known to play an important role in regulatory T cell activation. B) In $CD8\text{-}CD11c^+$ DC, the SE triggers production of IL-6 leading to the activation and expansion of Th17 cells. C) SE ligand enhanced production of proosteoclastogenic factors and facilitated OC differentiation. As a consequence, increased bone destruction was observed. Abbreviations: Calreticulin (*CRT*); shared epitope (*SE*); cluster of differentiation (*CD*); Indoleamine-pyrrole 2, 3-dioxygenase (*IDO*); osteoclasts (*OC*).

bind hydrophobic residues due to the presence of an alanine (Ala), which receives the phenylalanine residue (Phe) peptide of MBP (2,23). The P4 not only accommodates the Phe, but because of its size, it can also incorporate residues, e.g., Ala (aromatic) and Lys, which interact perfectly with the negative charge of the pocket. Still, the role of the P4 anchor residues is not a prerequisite for developing the disease, so it is assumed that there are additional factors that may or may not be associated with HLA that can trigger the disease.

The potential of peptide-based therapy for treatment of MS and its relationship with the MHC molecules has been explored. Copaxone, is a Food and Drug Administration (FDA) approved drug for treatment of MS. The synthetic random amino acid copolymer, Copolymer 1 (Cop 1, Copaxone, glatiramer acetate) was the first drug based on four amino acids (L-alanine, L-lysine, L-glutamic acid, and L lysine) from MBP. Cop-1 suppresses EAE, slows the progression of disability, and reduces the relapse rate in MS. Cop 1 binds to various class II MHC molecules including *HLA-DRB1*15:01*, inhibits the T cell responses to several myelin antigens, shifts Th1 response to Th2, and upregulates T regulatory cell expression. Later, it was proved that Cop-1 causes demyelination arrest and induces remyelination when given to EAE mice (24).

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

The HLA, as per almost all the ADs, has been shown to exert the strongest genetic association and effect on SLE to date. The top association was found at *HLA-DRB1*. Studies examining HLA class II have consistently replicated the *HLA-DR2 (DRB1*15:01)*, *HLA-DR3 (DRB1*03:01)*, *HLA-DRB1*08:01*, and *HLA-DQA1*01:02* alleles associated with the disease in American and European populations with a two fold RR conferred by each allele (25–28). The extended *HLA 8.1* AH (ancestral haplotype) is considered a common European haplotype implicated in SLE susceptibility (See section below). GWAS in both European and Asian populations has shown that the strongest contribution to risk for SLE resides in the

HLA region and consists of multiple genetic effects (29). The long-range LD within the HLA region has made assessing the relative contribution of each component gene to disease susceptibility difficult. However, the available evidence suggests that genetic variants such as *HLA-DR2* and *HLA-DR3*, *HLA-DPB1*, *HLA-G*, and class III (such as *MSH5* and *SKIV2L*) genes, in particular, predispose an individual to SLE (30).

Moreover, the role of SLE-associated HLA class II alleles in initiating SLE-relevant autoantibody responses has been demonstrated in humanized mice expressing the *HLA-DR3* transgene but no other *DR* or *DQ* alleles (31). Microarray studies done on SLE patients have revealed that the MHC class I genes are under expressed when compared with controls (32). The MHC class I region is required for the detection of intracellular pathogens by $CD8^+$ T cells, and its absence seems to lead to a failure to defend against such pathogens. A certain gene transcription signature in $CD8^+$ T cells has been linked to SLE disease prognosis (33).

TYPE 1 DIABETES MELLITUS (T1D)

Several alleles have been associated with and linked to susceptibility to T1D including *HLA-DQB1*03:02* and *DQB1*02:01* (34,35). It is known that individuals with both alleles have a higher RR of developing the disease when compared to the general population. Similarly, many *DRB1*04* alleles, which are in LD with the *DQB1*03:02* allele may modify the RR for the disease. In summary, the HLA association with T1D is one of the most complex. It was initially suggested that *HLA-B8*, *HLA-B18*, and *HLA-B15* (B62) were higher in patients with this disease (36). Then, as the molecular tests were developed, the association expanded to *HLA-DR3* alleles (i.e., *HLA-DRB1*03:01*), *HLA-DR4 (HLA-DRB1*04)*, *HLA DQB1*02:01*, and *HLA-DQB1*03:02*. Although the quantity of alleles found associated with T1D in the *DR* and *DQ* loci is high, the role of the locus or loci conferring susceptibility/protection is unclear given that they are presented with low effects when compared to the identified susceptibility haplotypes of T1D (Table 3) (37). This can be explained by the variety of existing alleles in the HLA, population changes, and the pattern of inheritance for both susceptibility and protection alleles such as *HLA-DRB1*15:01* and/or *HLA-DQB1*06:02* (36,38).

Studies of human cell lines of T lymphocytes restricted by *DR* and *DQ* alleles have reported autoantigens associated with the disease including insulin, glutamate decarboxylase (GAD65), and tyrosine phosphatase pancreatic islet antigens known as IA-2. In addition, transgenic mice experiments for *HLA-DQ*03:02* helped identify the presence of GAD specific T lymphocytes (39) while others suggest that the peptide Ins B9.23 could be an immunodominant autoantigen restricted to HLA-DQ (40).

Initial reports indicate that *HLA-DQ*03:02* is the preferred molecule for peptides with a negative charge in the anchor residue which binds to the P9 pocket (41). These observations are supported by binding experiments between Insulin B chain (residues 9–23, SHLVEALYLVCGERG) and the HLA molecule *DQ*03:02* (42). Negatively charged peptides in the same position could make a bridge between the MHC molecule and Arg, which would help to stabilize the complex. This would increase its half-life in contrast to what was observed with peptides that had no negative charge. Furthermore, the molecules of *HLA-DQ*03:02* are larger at P4, which allows them to accommodate hydrophobic peptide residues such as phenylalanine (Phe) and tyrosine (Tyr) while unable to accept positively charged residues. The P1 of the *DQB1*03:02* molecules is highly polar. Therefore, it may contain positively charged residues such as histidine (His) and arginine (Arg). It has also been possible to establish a molecular characteristic for the molecule corresponding to the protective allele which is that, unlike *DQ*03:02, DQ*06:02* prefers aliphatic residues attached to P9 (43). Similarly, the 57 residue of the β chain of the DQ molecules also contributes to the pattern of peptides bound by these molecules. However, studies suggest that P9 is what determines the selection of autoreactive peptides involved in the development of T1D.

SJÖGREN'S SYNDROME (SS)

SS is an autoimmune exocrinopathy characterized by a lymphocytic and plasma cell infiltration of the salivary and lach-

HLA-A	RR	*HLA-B*	RR	COMPLOTYPE	RR	*HLA-DR*	RR	*HLA-DRB1-DQB1*	RR
*A*1*	0.99	*B*7*	0.52	*SC31*	0.53	*DR1*	0.90	*DRB1*03:01DQB1*02:01*	3.55
*A*2*	1.36	*B*8*	2.09	*SC01*	2.03	*DR2*	0.16	*DRB1*04 DQB1*03:01*	0.62
*A*3*	1.05	*B*18*	2.22	*F1C30*	8.49	*DR3*	3.55	*DRB1*04 DQB1*03:02*	3.95
*A*11*	0.63	*B*50*	1.0	*SC33*	3.10	*DR4*	2.91	*DRB1*01:01 DQB1*05:01*	0.90
*A*24*	1.58	*B*57*	0.15	*SB42*	3.20	*DR5*	0.24	*DQB1*03:01*	0.36
*A*26*	1.53	*B*60*	2.06	*SC2*	0.63	*DR6*	0.46	*DQB1*03:02*	3.95
*A*28*	0.28	*B*62*	2.01	*FC*	0.82	*DR7*	0.28	*DQB1*06:02*	0.10
*A*30*	2.17	*B*65*	0.68	*S1C2*	2.97	*DR8*	0.76	*DQB1*03:03*	0.23
*A*33*	0.43			*SC61*	0.08	*DR9*	0.72		

Table 3. MHC markers associated with autoimmune type 1 diabetes mellitus and their relative risks (RR). Adapted from Larsen *et al*. (37).

rymal glands. This is accompanied by *de novo* production of autoantibodies leading to keratoconjuntivitis sicca and xerostomia. A recent meta-analysis of association studies from around the world identified associations between HLA Class II and SS. A total of 1,166 cases and 6,470 controls from 23 studies were analyzed including 16 different populations. At the allelic level, *DQA1*05:01*, *DQB1*02:01*, and *DRB1*03:01* alleles were found to be risk factors for the disease. Conversely, the *DQA1*02:01*, *DQA1*03:01*, and *DQB1*05:01* alleles were protective factors (44). However, there are other risk alleles/haplotypes specific for each population such as *DRB3*01:01* in Norwegians (45), *DRB3*01:01* and *DQB1*06:02* in Danes (46), *DRB1*04:05-DQB1*04:01* in Japanese (47), *DRB1*08:03-DQB1*06:01 i*n Chinese (47), and *DRB1*11:01, DRB1*11:04, DQB1*03:01* in Israeli, Jews, and Greek (48).

Particular HLA class II alleles may play an important role in the regulation of the immune responses against the Ro and La ribonucleoproteins. The generation of these autoantibodies has been correlated with the alleles *DRB1*03*, *DQA1*05:01*, and *DQB1*02:01* in SS patients (49–52). Likewise, while *HLA-DR3* and *HLA-DR8* were correlated with anti-Ro and anti-La responses in patients with SS and SLE, HLA-DR2 is associated with anti-Ro responses in the absence of anti-La (53). Furthermore, an induction of strong T and B cell responses by a human recombinant Ro60 protein was observed in transgenic mice carrying *DR2*, *DR3*, or *DQ8* HLA genes but not in mice carrying *DQ6* genes (54). Thereafter, using two different artificial neural networks (NetMHCIIpan and the Immune Epitope Database Analysis Resource), five La peptides ($La_{18–32}$, $La_{49–63}$, $La_{101–115}$, $La1_{53–167}$, $La_{241–255}$), and three Ro peptides ($Ro_{125–139}$, $Ro_{244–258}$, $Ro_{523–537}$) with the ability to bind strongly to *HLA-DRB1*03:01* risk allele were identified (44). Thus, differences in the biochemical characteristics of critical amino acids are directly related to either the risk or protection conferred by HLA Class II alleles associated with SS.

CELIAC DISEASE (CD)

CD is a complex disorder of the small intestine caused by an inappropriate immune response to ingested wheat gluten (See chapter 33). CD has a strong genetic component as illustrated by a monozygotic twin concordance of nearly 90% compared to 10% in first-degree relatives (55). A significant proportion of the genetic predisposition comes from HLA genes. HLA-DQ2 (encoded by *HLA-DQA1*05:01-DQB1*02:01*) or HLA-DQ8 (encoded by *DQA1*03:01-DQB1*03:02*) is expressed in 30%–35% of the populations where CD is prevalent with only 2%–5% of gene carriers developing CD. This implicates other genetic as well as environmental factors as contributors to the manifestation of CD (56).

The principal disease triggering component of wheat gluten belongs to a family of closely related proline-rich and glutamine-rich proteins called gliadins. When genetically predisposed individuals who express HLA-DQ2 or DQ8 are exposed to certain gliadin epitopes, these epitopes are presented on the surface of antigen presenting cells (APC) in the lamina propria. These, in turn stimulate proliferation of gliadin-specific CD4 T cells in the mucosa. A 33-mer peptide of α2-gliadin, in particular, which is extremely resistant to gastrointestinal digestion because of its rich proline content, is the most powerful immunodominantgliadin peptide. In CD patients, undigested gliadin fragments present in the intestinal lumen can be transported and released intact in the mucosa thereby triggering an immune response and perpetuating intestinal inflammation (57).

DQ2 and DQ8 molecules can only bind gliadin peptides if they have been enzymatically modified by tissue transglutaminase (TG2). This pleiotropic enzyme, which is present in many organs including the small intestine, catalyzes a deamination of certain glutamine residues, the most abundant amino acid in gluten, by converting them into glutamate residues. When deamidated, most of the resultant negatively charged gluten peptides bind more strongly to HLA-DQ2 (or HLA-DQ8), which leads to a more rigorous gluten-specific $CD4^+$ Th1 T Cell activation (58).

Although DQ2- and DQ8-restricted T cells can recognize the same gliadin peptides in exactly the same registers (for instance peptides that share the core sequence QQPQQPFPQ), these peptides have been deamidated at different positions: deamidation at position P4 or P6 is mandatory for recognition by DQ2-restricted T cells, whereas deamidation at position P1 and/or P9 is critical for DQ8-restricted recognition. In addition, most of the characterized DQ2-restricted gliadin T Cell epitopes have proline residues at P1, whereas DQ8 is unlikely to tolerate a proline at P1 and so selects other sequences that are more likely to be sensitive to proteases, in particular, aminopeptidases (59).

Altogether, these data are significant for clinical practice because HLA-DQ2 and DQ8 are such strong disease risk factors that their absence has a negative predictive value for CD that is close to 100%. In addition, this knowledge will allow the design of new therapeutic approaches (60).

ANKYLOSING SPONDYLITIS (AS)

AD association with class I HLA alleles is rare, except in (AS), considered an autoinflammatory disease rather than an AD. *HLA-B*27* has been observed in 96% of patients suffering from AS. It is clear that not all of the *B*27* alleles are associated with the pathology. This is the main reason why the conformational differences in the α-chains have been analyzed. Lys at position 70 on the α-chain is a common residue of the *B*27* allele group. Yet, specific combinations of polymorphic residues correspond to His 9, Glu 45, Cys 67, and Ala 71 and all are grouped to form pocket P2 of the molecule. The Glu 45 and Cys 67 are located in the deepest part of P2 and receive higher affinity anchor residues of peptides containing Arg. Furthermore, the Lys 70 which is common to all *B*27* interacts with Asp 74 but does not seem to be critical in the associa-

tion with AS in contrast to what is observed when the position has a Tyr 74 belonging to the *B*27:01* allele which associates with MS. Although *B*27* associations appear to be strictly dependent on the conformation of their residues, this leads us to think that autoantigenic peptides should be an important component in the susceptibility and pathophysiology of AS. So far these autoantigens have not been identified (61,62).

HLA COMMONALITIES AMONG AUTOIMMUNE DISEASES

ANCESTRAL 8.1. HAPLOTYPE

Conserved DNA sequences that act as extended haplotypes or ancestral haplotypes (AHs) are a typical feature of the MHC because of a high LD phenomena observed between loci and alleles throughout the region. It is believed that about 30% of the MHC haplotypes are AH in populations such as Caucasian (63). The AH made by the MHC is identified as having identical allelic variants in the regions that are mapped between *HLA-B* and *HLA-DRB1*. Some of these AHs are accepted as susceptibility markers for ADs (64). The existence of these AHs has allowed alleles such as HLA-*DRB1*03:01* and **04:01* to be established (65,66).

The association of AHs with ADs has been studied more in the Caucasian population, and it has been established that the 8.1 AH is made up of *HLA-A*01,-C*07,-B*08* in the class I region and *DRB1*03:01-DRB3*01:01-DQA1*05:011-DQB1*02:01-DPA1*01-DPB1*03:01-TAP1*01:01 TAP2*02:01* in the class II region including genes composed of *C2-C4A-TNF2* in the class III region. This haplotype is associated with SLE, SS, and T1D. Functionally, it can be seen that HLA class II alleles that are part of this AH are the same as those susceptibility alleles discussed for each aforementioned AD. Furthermore, these alleles that constitute the extended haplotype possess well defined immunological characteristics (Table 4) that can be associated with the pathophysiology of ADs (64).

Another AH associated with ADs is the AH 7.2, which consists of the *HLA-A*03:01-C*07-B*07:02* in class I and *DRB1*15:01-DRB5*01:01-DQA1*01:02-DRB6*02:01-DQB1*06:02-DPA1*01-DPB1*04:01* for class II and includes the *TNF1* gene in the class III region. This AH is associated with susceptibility to SLE and MS although its importance lies in the association with infectious diseases such as leprosy and tuberculosis, and it also behaves as protective for T1D (67). Although their molecular function is not clear, this AH seems to behave like a slow stimulator of the innate and acquired immune response since there is a diminished synthesis of complement factors C4A and C4B. In addition, the synthesis of cytokines and chemotactic factors is less effective which may partially explain the susceptibility to infectious diseases.

Many studies have been done to establish the role of these AH. Therefore, after obtaining the complete sequence draft for the human genome, eight well characterized AHs for the MHC were reported as part of the human MHC Haplotype Project (Table 5). Further characterization of these AH in different populations and deciphering of their different functions (68) including the gene mapping in the class III region is crucial for a better understanding of the association between MHC and ADs.

CHARACTERISTIC	EFFECT
T cell Response	
Mitogen Proliferation	Decrease
IFNα, IL2, and IL2R Production	Decrease
CD69 Expression	Decrease
CD71 Expression	Normal
T Lymphocytes and NK Cell Numbers	Normal
NKT and NK Activity	Decrease
IL4 and IL6 Production	Normal
IL5 Production	Decrease
Acute phase Cytokines and Macrophages Function	
C3 and Fc Immune Complex Formation	Decrease
TNFα Leukocyte Production	Increase
IL1 Culture Bioactivity	Decrease
Spontaneous Fas Expression and Apoptosis	Increase
Fas Expression and Fas-mediated Apoptosis	Decrease
Antibody Production	
Antibody Titres	Increase
IgG Response	Increase

Table 4. Ancestral haplotype 8.1 immunological characteristics.

SHARED HLA ALLELES IN ADs

Through a meta-analysis, the genetic commonalities in ADs were analyzed by examining the contributions from HLA-II alleles which confer associated risk or protection on six ADs: RA, SLE, autoimmune hepatitis (AIH), MS, SS, and T1D in the Latin American (LA) population. A total of 3,727 cases and 8,465 controls were analyzed and different types of association between alleles and ADs were found (Table 6). These included three risk alleles for two or more ADs, four opposite associations (the same allele is a risk factor for one AD but a protective factor for another AD), thirteen risk alleles for a particular AD, and eight protective alleles that are disease-specific (8). The associations were grouped in the network in Figure 3.

Some HLA class II alleles for ADs in LA are similar to those reported for other population groups regardless of latitudinal gradient and admixture. For instance, *DRB1*03:01*, *DRB1*04:05*, *DRB1*04:01*, and *DQB1*02:01* risk alleles for T1D in LA also confer susceptibility in Caucasians and Asians (69). *DRB1*03:01* allele, which has been described as a risk factor for SS in the Colombian population, was also associated with the disease at the global level (44). However, there

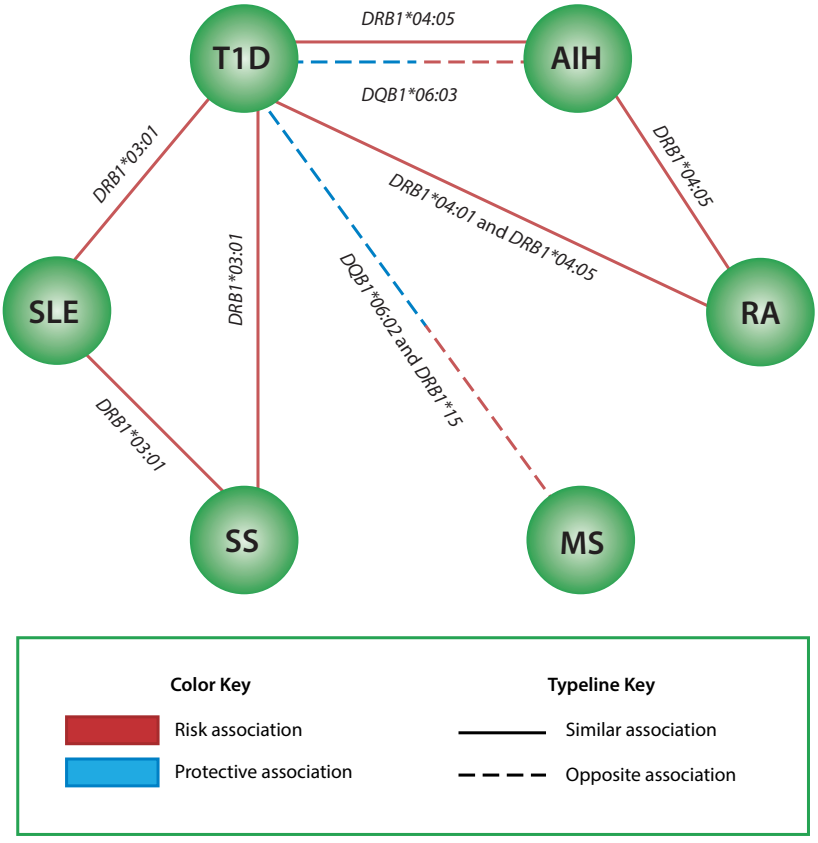

Figure 3. The complex interplay of HLA in six autoimmune diseases in Latin Americans.

are other genes that influence the development of ADs in a particular population which are not replicated in another one (i.e., *PADI4* and *SLC22A4* genes) (70).

Two alleles were found to influence the risk of developing three different diseases. The *DRB1*03:01* allele was found to be a risk for SLE, SS, and T1D while *DRB1*04:05* allele was associated with AIH, T1D, and RA. In addition to sharing HLA alleles, these ADs share other characteristics which reinforce the common origin of ADs theory (Table 7). At the genetic level, AD association with non-HLA genes has been observed. For instance, *PTPN22* 1858T/C (71) and *TNF-α* -308G/A (72–74) are associated with SLE, SS, and T1D. Likewise, the *CTLA4* gene has been reported to be a risk factor for AIH, T1D, and RA (14,75,76). Another consideration concerning genetic findings is the familial aggregation. Relatives of patients with ADs have a higher risk than general population of developing the same or other ADs (77). At the clinical level, shared autoantibodies in ADs have also been described. Antinuclear antibodies (ANAs) are present in multiple ADs such as SLE, SS, RA, T1D, AIH, and MS (8).

Regarding opposite associations, *DQB1*06:02* and *DRB1*15* alleles were found to be risk factors for MS but protective factors for T1D. These results are similar to those from other studies reporting that other MHC genes such as *CDSN* and *HLA-DMB* (rs3130981-A and rs151719-G respectively) are risk factors for MS but protective ones for T1D. However, there is also evidence of the inverse relationship. For instance, *TAP2* (rs10484565-T), *VARS2* (rs1264303-G), *NOTCH4* (rs2071286-A), *BTNL2* (rs2076530-G), and *TRIM40* (rs757262-T) were found to be risk factors for T1D but protective factors for MS (78). Despite the presence of these genetically opposite associations, it is important to mention that clinical evidence supporting the coexistence of MS and T1D has been reported (79). Thus, these pleiotropic effects may be explained by the combined action of different alleles of several genes and environmental factors that change the biological context of the SNPs in different individuals and populations (Table 7).

In summary, the results of this meta-analysis validate the common origin of the AD paradigm. The finding of significant risk and protective alleles in LA and the fact that they are shared with other populations around the world highlights the primary role of some HLA regions in genetic susceptibility to ADs regardless of latitudinal gradient and ethnicity.

HAPLOTYPE	*HLA-A*	*HLA-B*	*HLA-C*	*HLA-DRB1*	*HLA-DQB1*	*HLA-DPB1*
A3-B7-DR15	*03:01:01:01*	*07:02:01*	*07:02:01:03*	*15:01:01:01*	*06:02:01*	*04:01*
A1-B8-DR3	*01:01:01:01*	*08:01:01*	*07:01:01*	*03:01:01:01*	*02:01*	*03:01*
A1-B60-DR13	*01:01:01:01*	*40:01:01*	*06:02:01:01*	*13:01:01*	*06:03:01*	*04:02*
A2-B57-DR7	*02:01:01:01*	*57:01:01*	*06:02*	*07:01:01*	*03:03:02*	*04:01:01*
A29-B44-DR7	*29:02:01*	*44:03:01*	*16:01*	*07:01:01:01*	*02:02*	*02:01:02*
A32-B44-DR4	*32:01:01*	*44:02:01:01*	*05:01:01:02*	*04:03:01*	*03:05:01*	*04:01:01*
A26-B18-DR3	*26:01:01*	*18:01:01*	*05:01:01:01*	*03:01:01:02*	*02:01:01*	*02:02*
A2-B62-DR4	*02:01*	*15:01:01:01*	*03:04:01:01*	*04:01*	*03:01*	*04:02*

Table 5. Different HLA-homozygous typing haplotypes provided by The MHC Haplotype Project.
Adapted from: http://www.ucl.ac.uk/cancer/medical-genomics/mhc

ASSOCIATION	ALLELE	AD	OR AND 95% CONFIDENCE INTERVAL (CI)	P-VALUE
Risk (For only one AD)	*DQA1*03:01*	T1D	(OR: 2.65; 95%CI: 1.23-5.72)	0.013
	*DQA1*05:01*	T1D	(OR: 2.43; 95%CI: 1.34-4.38)	0.003
	*DQB1*02:01*	T1D	(OR: 2.97; 95%CI: 2.05-4.3)	<0.001
	*DQB1*03:02*	T1D	(OR: 4.45; 95%CI: 3.29-6.02)	<0.001
	*DRB1*03*	T1D	(OR: 2.69; 95%CI: 1.41-5.15)	0.003
	*DRB1*04*	T1D	(OR: 3.83; 95%CI: 2.02-7.27)	<0.001
	*DRB1*04:02*	T1D	(OR: 3.23; 95%CI: 1.63-6.39)	0.001
	*DQB1*06*	MS	(OR: 2.18; 95%CI: 1.55-3.08)	<0.001
	*DRB1*15:01*	MS	(OR: 2.59; 95%CI: 1.68-4.02)	<0.001
	*DRB1*15:03*	MS	(OR: 2.24; 95%CI: 1.39-3.62)	0.001
	*DRB1*01:01*	RA	(OR: 1.71; 95%CI: 1.23-2.39)	0.002
	*DRB1*04:04*	RA	(OR: 3.42; 95%CI: 1.54-7.63)	0.003
	*DRB1*13:01*	AIH	(OR: 4.84; 95%CI: 2.83-8.26)	<0.001
Risk (For more than one AD)	*DRB1*04:01*	T1D and RA	(OR: 3.86; 95%CI: 2.32-6.42)	<0.001
	*DRB1*03:01*	SLE, SS, and T1D	(OR: 3.56; 95%CI: 1.42-11.54)	0.009
	*DRB1*04:05*	AIH, T1D, and RA	(OR: 4.64; 95%CI: 2.14-10.05)	<0.001
Protection (For only one AD)	*DQB1*05*	T1D	(OR: 0.31; 95%CI: 0.19-0.51)	<0.001
	*DQB1*05:01*	T1D	(OR: 0.41; 95%CI: 0.24-0.68)	<0.001
	*DRB1*11*	T1D	(OR: 0.27; 95%CI: 0.17-0.42)	<0.001
	*DRB1*13*	T1D	(OR: 0.37; 95%CI: 0.24-0.58)	<0.001
	*DRB1*14*	T1D	(OR: 0.18; 95%CI: 0.06-0.55)	0.002
	*DQB1*03:01*	AIH	(OR: 0.33; 95%CI: 0.19-0.56)	<0.001
	*DRB1*13:02*	AIH	(OR: 0.16; 95%CI: 0.05-0.45)	0.001
	*DRB1*11:01*	SLE	(OR: 0.21; 95%CI: 0.006-0.72)	<0.001
Opposite associations	*DQB1*06:02*	MS risk	(OR: 2.49; 95%CI: 1.67-3.71)	<0.001
		T1D protection	(OR: 0.17; 95%CI: 0.09-0.29)	<0.001
	*DQB1*06:03*	AIH risk	(OR: 4.48; 95%CI: 1.28-15.73)	<0.001
		T1D protection	(OR: 0.29; 95%CI: 0.18-0.87)	<0.001
	*DRB1*15*	MS risk	(OR: 2.28; 95%CI: 1.69-3.07)	<0.001
		T1D protection	(OR: 0.38; 95%CI: 0.22-0.65)	<0.001

Table 6. Associations between HLA class II and six ADs: SLE, RA, T1D, AIH, SS, and MS. Each OR and its CI show the effect size and precision for individual studies and for the combined effect calculated by the random model. Adapted from Cruz-Tapias *et al.* (8)

ASSOCIATIONS	GENETIC ASSOCIATIONS	CLINICAL ASSOCIATIONS
SLE, SS, T1D	Shared risk genes *HLA-DRB1*03:01*, *IL2–IL21* (rs6822844), *PTPN22* (1858T/C), 8.1 Ancestral Haplotype, *TNF-α* (-308G/A)	Common clinical characteristics Human Endogenous Retroviruses (HERV) are associated with multiple ADs including SLE, SS, and T1D Presence of poliautoimmunity (presence of more than one AD in a single patient) has been reported Hepatitis C Virus has been related to ADs such as RA, AIH, T1D, SLE, SS, etc. High prevalence of ADs in siblings of probands affected with AITD, MS, RA, T1D, SLE, and other ADs ANAs have been detected in patients with SLE, SS, T1D, RA, and other ADs Familial aggregation
AIH, RA, T1D	Shared risk genes H*LA-DRB1*04:05*, *CTLA4*	
MS, T1D	Shared risk genes *CD226*(rs763361), *CLEC16A* (rs12708716), *SH2B3* (rs3184504), *ZSCAN23* (rs11752919) *KIF5A* (rs1678542), *SH2B3* (rs3184504), *CD226* (rs763361) Shared protective genes: *HLA-DRB1*01*, *HLA-DRB1*10*, *HLA-DRB1*11* and *HLA-DRB1*14* Opposite gene associations: Risk for T1D but protection for MS: *TAP2* (rs10484565), *VARS2* (rs1264303), *CDSN* (rs1265048), *NOTCH4* (rs2071286), *BTNL2* (rs2076530), *TRIM40* (rs757262) Risk for MS but protection for T1D: *CDSN* (rs3130981), *HLA-DMB* (rs151719) *IL2RA* (rs35285258), *IL2RA* (rs7090530)	Common clinical characteristics A latitudinal gradient characterizes both diseases. MS and T1D each become increasingly common as distance from the Equator increases Protective effect of vitamin D levels Association with Epstein–Barr virus infection Both MS and T1D are characterized by T cell mediated autoimmunity. The targets of T Cells are pancreatic islet and central nervous system antigens in both diseases Familial aggregation
AIH, T1D	Shared protective alleles: *DQB1*03:01* Controversial genetic and clinical characteristics: In children with AIH, the frequency of high risk *HLA-DQB1*03:02* or *DQB1*02* alleles was low and similar to control frequencies, indicating low-risk for T1D despite the presence of T1D-related autoimmunity markers	Controversial characteristics: One case report with Grave's disease, AIH, and T1D One cohort of 278 patients with AIH presented only two cases of T1D One study reported that the prevalence of ICA and IAA antibodies in children with AIH was 60.7 and 18.5% respectively. However, only one patient developed T1D

Table 7. Relationship between genetic and clinical features with HLA-ADs associations. Adapted from Cruz-Tapias *et al.* (8).

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18 NON-HLA GENES AND AUTOIMMUNE DISEASES

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INTRODUCTION

Autoimmune diseases (ADs) are responsible for a substantial amount of disability and morbidity worldwide. Although the epidemiology varies according to individual conditions, collectively autoimmune prevalence is at least 5% in the general population and is one of the major causes of premature mortality in young and middle-aged women (1).

ADs encompass a broad range of phenotypic manifestations and severity. The pathogenesis is considered to be multifactorial, and several of the features suggest shared etiologic factors (see Chapter 14). Most ADs are characterized by female predominance, and many are associated with the production of autoantibodies. A variety of pathogenic mechanisms are ultimately triggered during the progression of ADs, and dysregulations involving major cell signaling pathways and inflammatory responses are consistent features in most ADs (2,3). ADs can be categorized into two types of disorders. First, systemic ADs, such as systemic lupus erythematosus (SLE), in which the loss of immune tolerance is directed towards systemic antigens and disease manifestations can occur at a variety of different sites in the body. Second, organ-specific ADs, in which the immune response is predominantly or exclusively directed towards tissue-specific elements [e.g., type 1 diabetes (T1D) targets the pancreas, autoimmune thyroid disease (AITD) attacks the thyroid gland].

The inheritance pattern of ADs is polygenic; this indicates that multiple genes are involved in defining their development and outcome. Although the exact number of risk loci is not known for many ADs, the numbers are likely in the 100s for each. The human leukocyte antigen (HLA) region typically confers the strongest association with ADs discovered to date. However, other genes, both located within and outside of the HLA region, are risk factors for these diseases.

Recent advances in genomics have led to increased understanding of the molecular underpinnings of complex diseases. For instance, the technological and manufacturing advances made in the early 2000s led to the use of microarrays to conduct large-scale, mostly unbiased surveys of the human genome such as genome-wide association studies (GWAS) (BOX 1) to determine which genetic factors influence the onset and development disease. However, due to their multifactorial and polygenic nature and accompanied by a differential penetrance, genetic heterogeneity among populations, and the influence of environmental factors (4,5), untangling the genetic determinants defining their outcome and onset has proven to be extremely challenging. Data showing the existence of different ADs within a single family or within the same individual suggest a combination of genetic defects that may predispose individuals to different ADs sharing common pathogenic pathways (6,7). This chapter reviews the reported non-HLA shared genes, based on the plausibility of their functional or biological mechanisms, that affect the susceptibility to the most common ADs.

SHARED GENETIC VARIANTS AMONG ADs

GWAS has opened up a new horizon on the genetics of complex diseases by defining the genomic regions harboring disease risk alleles that display association at convincing levels of statistical significance (BOX 1) (8,9). In this regard, cohort studies have identified associations with common genetic markers across the entire human genome that satisfy stringent statistical criteria and have been replicated across multiple cohorts (Figure 1).

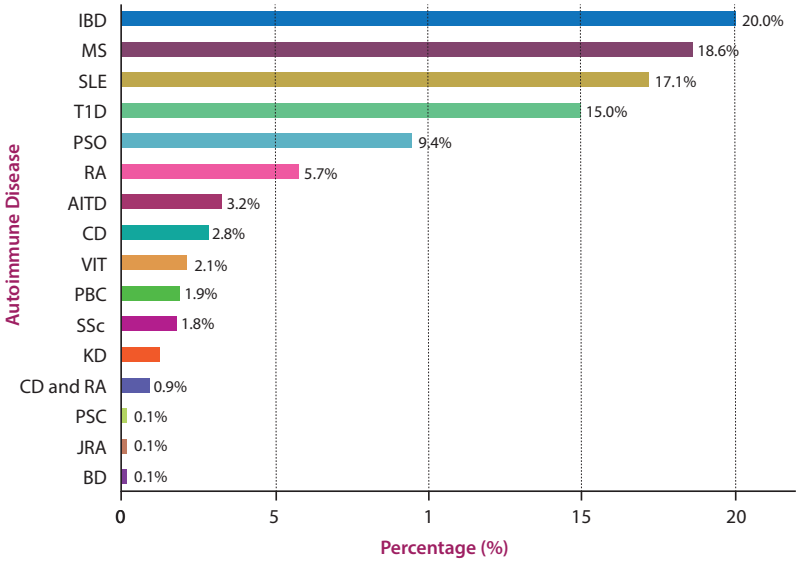

Figure 1. Frequency of autoimmune diseases with significant reported genetic variants (p<1x10-5) in genome-wide association studies (GWAS) curated from the (NHGRI, http://www.genome.gov/gwastudies/) and the database of genotypes and phenotypes (dbGAP, http://www.ncbi.nlm.nih.gov/gap), both accessed in September of 2012. For the NHGRI, a total of 8964 genetic variants were encountered, out of which 917 were significant variants associated with autoimmune disease susceptibility; while in dbGAP out of 31246 reported variants, 690 were mutually exclusive from the NHGRI for a grand total of 1607 genetic variants related to genes associated in a GWAS for any population. Autoimmune thyroid disease (*AITD*), Behcet's disease (*BD*), celiac disease (*CD*), rheumatoid arthritis (RA), inflammatory bowel disease (*IBD*), juvenile RA (JRA), Kawasaki disease (*KD*), multiple sclerosis (*MS*), primary biliary cirrhosis (*PBC*), primary sclerosing cholangitis (*PSC*), psoriasis (*PSO*), systemic sclerosis (*SSc*), systemic lupus erythematosus (*SLE*), type 1 diabetes (*T1D*), and vitiligo (*VIT*).

Perhaps nowhere have such studies been more fruitful than in ADs: more than 150 genetic loci have been shown to be associated with one or more autoimmune disorders (Figure 2). Although in most cases, the precise causal alleles or genes driving these associations have not been identified, some associated loci can be implicated with particular functional pathways including the intracellular signaling networks that drive the activation of T and B cells, signaling by cytokines and cytokine receptors, and pathways that mediate innate immunity and microbial responses (Table 1).

The results of several GWAS show that genetic variations in multiple genes are associated with each AD and that the associations are modest. The majority of GWAS have focused on case series of European ancestry, but studies of other populations show that some associations are observed across populations, and such associations point to pathways that may be particularly important in disease pathogenesis. Multiple ADs appear to have overlapping genetic associations and risk alleles, which suggested that common pathogenic mechanisms underpinning the diseases must be present (5,10-13). In addition, there is evidence that loci predisposing to one disease can have effects on the risk of a second disease (14) although the risk allele for one disease may not be the same for the second (15).

Each new genetic finding can suggest multiple hypotheses that need to be incorporated into an overall scheme of pathogenesis. Ongoing research points to some expected shared biology (16). However, the relative risk of each locus may differ between diseases. Before we can fully understand the relationships between ADs, we must first identify all risk loci for each disease, an effort which is still underway. Compelling and interesting observations have emerged implicating several genes with shared etiology that comprise the well-described examples for the major histocompatibility complex (MHC) (17) and non-MHC genes such as, *PTPN22,* which is associated with T1D, RA, SLE and AITD but not multiple sclerosis (MS) (18). Other examples include *CTLA4*, *STAT4*, *TNFAIP3,* and *SH2B3*, etc.

Many of the recently identified AD loci are involved in pathways related to B cell or T cell activation and differentiation, innate immunity, and regulation of cytokine signaling (7,19). This expected commonality has motivated several meta-analyses across pairs of diseases to establish their shared genetic basis. Approaches in such diseases as celiac disease (CD) and rheumatoid arthritis (RA) (20), T1D (14), and inflammatory bowel disease (IBD) (21) have revealed overlapping loci. However, these observations could potentially underestimate the actual extent of commonality as the lack of statistical power would inflate type 1 error for the associations found to genome-wide significance [i.e., $5x10^{-8}$]. In addition, modeling and simulation analyses estimate that, in several diseases where GWAS have been successful, further loci with low effect sizes remain to be discovered (22). This problem is exacerbated when considering independent discoveries across diseases since power would be multiplicative across studies. Therefore, estimates of the true extent of genetic sharing are probably underestimated by either simple overlaps or pairwise meta-analysis.

The main objective in genetic mapping studies is to identify the genetic variants and/or haplotypes affecting genetic susceptibility. Any associated marker in a locus may simply be genetically tagging the real causal variant, thus, statistical significance is not enough evidence to infer causality of variants. Another goal is to identify specific causal genes in regions when the associated region for a given trait encompasses multiple ADs. This requires additional refinement before inferences on genetic mechanisms and etiological causes of disease can be made (23). As an approach to be able to underpin these goals, part of the AD community de-

Figure 2. Weighted list created from the reported significant mapped genes in the current genome-wide association studies (GWAS) curated from the (NHGRI, http://www.genome.gov/gwastudies/) and the database of genotypes and phenotypes (dbGAP, http://www.ncbi.nlm.nih.gov/gap), both accessed in September of 2012. The word cloud shows the frequency of genes and their associated variants relative to their font size using the freely available java applet at http://www.wordle.net. Both databases were queried for the autoimmune disease (AD) reported of genetic variants with significant p-values ($p<1x10-5$). For the NHGRI, a total of 8964 genetic variants were encountered, out of which 917 were significant variants associated with autoimmune disease susceptibility, while in the dbGAP out of 31246 reported variants, 690 were mutually exclusive from the NHGRI, for a grand total of 1607 genetic variants related to a genes associated in a GWAS of any population. The autoimmune diseases queried were autoimmune thyroid disease, Behcet's disease, celiac disease, rheumatoid arthritis, inflammatory bowel disease, juvenile RA, Kawasaki disease, multiple sclerosis, primary biliary cirrhosis, primary sclerosing cholangitis, psoriasis, systemic sclerosis, systemic lupus erythematosus, type 1 diabetes, and vitiligo.

veloped shared resources, e.g., the ImmunoChip, a common platform for fine mapping AD-associated loci (24,25) as well as computational approaches to select the likeliest candidate genes from regions of association (26-28). Moreover, across the studied ADs, the application of a range of bioinformatics algorithms has generated plausible hypotheses about the causal genes and tissues underlying disease pathogenesis, motivated and functional experiments designed to test these hypotheses (29-31). In order to confront this critical aim of identifying pathways either shared across diseases or unique to specific ones, it is essential to develop network models for commonalities and to consider how genetic association data might be used to distinguish such models.

Currently, genomic technologies have been particularly attractive for acquiring, defining, and building network maps as they are amenable to automation. A limiting factor in annotating these networks is the careful acquisition of samples (i.e., rigorous standards in statistical experimental design, cell isolation, flow sorting, and sample preparation) required to gain cell-specific annotation. Parallel high-throughput gene expression studies are currently under way. Other types of information regarding the state of the DNA [e.g., DNase I hypersensitivity (32), nucleotide methylation status by sequencing (33), chromatin immunoprecipitation and sequencing (34), etc] are equally valuable and helpful are for capturing different types of information that could be used for deciphering the pathogenicity of the associated disease pathways.

Along with this extensive amount of information, the issue of interpretation comes into play. For example, several correlated alleles appear to confer risk of some diseases but are protective in others (15,35) and it remains unclear how this evidence should be incorporated into a pathway view of disease. Under a shared pathway architecture causal variants in each disease perturb a limited number of cellular processes. Thus pathway enrichment analysis (36,37)

CHR.	REPORTED GENE (S)	STRONGEST SNP-RISK ALLELE	DISEASE
1p13.2	*PTPN22*	rs6679677-A RA T1D, rs2476601-T T1D, rs2476601-G IBD	RA, T1D, SLE, IBD
1p36.23	*PARK7, TNFRSF9*	rs12727642-A	CD
1q25.1	*TNFSF4*	rs2205960-A SLE	SLE, CD
2p14	*SPRED2*	rs934734-G	RA
2p22.3	*RASGRP3*	rs13385731-A	SLE
2q33.2	*CTLA4/ICOS/CD28*	rs4675374-A CD, rs231735-T RA	CD, T1D, IBD, RA, MS, SLE
3q13.11	*CBLB*	rs9657904-T	MS
3q13.33	*CD80, KTELC1*	rs11712165-C	CD
4p15.2	*RBPJ*	rs874040-C	RA
4q24	*BANK1*	rs10516487-G	SLE
6p21.32	*HLA-DQ*	rs6457617-T RA, rs9272346-G T1D	RA, T1D
6p21.32	*HLA-DQA1*	rs477515 IBD, rs2187668-A CD	IBD, CD
6p21.32	*HLA-DRA*	rs9268923-C IBD, rs9268877-T IBD, rs3135388-A MS, rs3135338-A MS	IBD, MS
6p21.32	*HLA-DRB,HLA-DQB1*	rs2040406-G	MS
6p21.32	*HLA-DRB1*	rs660895 RA, rs2647044-A T1D, rs9271366-G MS	RA, T1D, MS, SLE
6p21.33	*HLA-C*	rs10484554-T	PSO
6p21.33	*HLA-C, CCHCR1*	rs9263739-T IBD	IBD, MS, SLE
6p21.33	*HLA-C, MSH5*	rs3131379-A SLE	SLE
6p22.1	*HLA-B*	rs2523393-A	MS
6q25.3	*TAGAP*	rs1738074-A CD	CD, T1D, RA
7p12.2	*IKZF1*	rs4917014-A	SLE
8p23.1	*BLK*	rs13277113-A SLE, rs7812879-G SLE, rs2736340-A RA	SLE, RA, MS
10p15.1 11p17	*PRKCQ* *CD44*	rs947474-G T1D rs2732552	T1D, RA, CD SLE
11q24.3	*ETS1*	rs6590330-A SLE, rs11221332-A CD	SLE, CD
12p13.31	*CD69*	rs4763879-A	T1D
12q13.3	*KIF5A,PIP4K2C*	rs1678542-C RA	RA, T1D, MS
12q24.12	*SH2B3, ATXN2*	rs653178-G	CD
12q24.13	*SH2B3/LNK*	rs17696736-G T1D	T1D, CD
18p11.21	*PTPN2*	rs2542151-G T1D IBD, rs1893217-G CD	T1D, IBD, CD, IBD, RA
18q22.2	*CD226*	rs763361-A T1D	T1D, MS, RA, SLE
20q13.12	*CD40*	rs4810485-T RA	RA, MS, IBD
21q22.3	*ICOSLG*	rs762421-G IBD, rs4819388 CD	IBD, IBD, CD, T1D

Table 1A. AD susceptibility loci involved in immune signaling (including the MHC region). Common SNPs are considered with a minor allele frequency > 0.01 and a significant association ($P<5x10^{-8}$) in GWAS. Adapted from Rai and Wakeland, 2011 (19) and Lessard *et al.* (179) and Lessard *et al.* (214). Abbreviations: celiac disease (*CD*), rheumatoid arthritis (*RA*), inflammatory bowel disease (*IBD*), multiple sclerosis (*MS*), psoriasis (*PSO*), systemic lupus erythematosus (*SLE*), type 1 diabetes (*T1D*), Systemic sclerosis (*SSC*), and single nucleotide polymorphism (*SNP*).

CHR.	REPORTED GENE (S)	STRONGEST SNP-RISK ALLELE	DISEASE
1p13.1	*CD58*	rs2300747-A	MS
1p31.3	*IL23R*	rs11209026-G IBD, rs11209026 IBD IBD, rs2201841-G PSO	IBD, PSO, RA, IBD
1p36.11	*RUNX3*	rs10903122	CD
1q32.1	*IL10*	rs3024505 T1D, rs3024505-T IBD	T1D, IBD, SLE
2q12.1	*IL18RAP*	rs917997-A CD	CD, IBD, T1D
3q25.33	*IL12A*	rs17810546-G	CD
4q27	*IL2, IL21*	rs13151961	CD
4q27	*KIAA1109,TENR,IL2,IL21*	rs6822844-G CD	CD, T1D, RA, IBD, PSO, SLE
5q31.1	*IL13*	rs20541-G	PSO
5q33.3	*IL12B*	rs10045431-IBD, rs2082412-G PSO	IBD, PSO, TD, MS
6q22.33	*PTPRK, THEMIS*	rs802734-G	CD
10p15.1	*IL2RA*	rs706778-T RA, rs12722489-C MS	RA, MS, T1D
11q12.2	*CD6*	rs17824933-G	MS
12q13.2	*IL23A, STAT2*	rs2066808-A	PSO
12q15	*IFNG, IL26, IL22*	rs1558744-A	IBD
16p11.2	*IL27*	rs4788084-G T1D, rs8049439-G IBD	T1D, IBD
17q21.2	*STAT3*	rs744166-A IBD, rs744166-G MS	IBD, IBD, MS

Table 1B. AD susceptibility loci involved in T cell differentiation. Adapted from Rai and Wakeland, 2011 (19) and Lessard *et al.* (179) and Lessard *et al.* (214). Abbreviations: celiac disease (*CD*), rheumatoid arthritis (*RA*), inflammatory bowel disease (*IBD*), multiple sclerosis (*MS*), psoriasis (*PSO*), systemic lupus erythematosus (*SLE*), type 1 diabetes (*T1D*), systemic sclerosis (*SSC*), and single nucleotide polymorphism (*SNP*).

and functional genomics datasets can be queried to reveal pathway components that are preferentially encoded in associated loci. These approaches have shown that this cumulative burden hypothesis is true (28), which substantiates the argument that susceptibility alleles accumulate and perturb pathways to influence risk (38). These approaches can be expanded to incorporate genetic data from multiple diseases in much the same fashion (39).

Available genetic information points out that nearly half of the loci of an individual disease identified through GWAS have also been identified as influencing the risk of more than one additional disease. The argues for a genetic basis to co-morbidity. Moreover, there are examples of several variants with differing risk profiles depending on the disease. Support for the idea of common patterns of association and shared biological processes is obtained by loci clustered over a pattern of diseases, as they affect and harbor genes encoding for interacting proteins at a much higher rate than by chance. These results suggest that multi-phenotype mapping will identify the molecular mechanisms underlying co-morbid, immune-mediated inflammatory and autoimmune diseases.

SHARED GENETIC FACTORS

Extensive clinical and epidemiologic observations have shown that multiple ADs can occur in the same individual or in closely related family members. This clustering of multiple diseases appears more frequently than would be expected if the phenotypes were independent. Familial approaches have documented the clustering of certain ADs among the relatives of individuals who have RA, MS, SLE, T1D and other diseases (42-48) (see chapter 17).

The role of common variants versus rare variants in mediating susceptibility to common diseases has reemerged as a controversial topic (49,50). The controversy arises predominantly from the fact that the sum of the risk attributable to all of the loci identified can only account for a small fraction of the genetic heritability exhibited by many common diseases. This is often referred to as "missing heritability." This has led to the possibility that rare variants with strong functional effects actually contribute significantly to the overall heritability of common diseases, which still needs to be validated and completely supported. In this regard, until recently rare variants were not regularly included in GWAS arrays, and several studies using re-sequencing of only the exons of each protein coding gene, called the exome, have identified a few rare mutations or copy number variations that contribute to the pathogenesis of ADs (51-57).

Recent reviews have summarized emerging work that identifies both genetic loci that are shared across the spectrum of ADs and the biological pathways whose involvement is implicated by these shared loci (3,19,51,58). The extent to which immune-related signaling and/or other pathways are implicated for each of these disorders varies and suggests

CHR.	REPORTED GENE (S)	STRONGEST SNP-RISK ALLELE	DISEASE
1p36.13	*OTUD3, PLA2G2E*	rs4654925-G IBD, rs6426833-G IBD	IBD
1p36.32	*TNFRSF14, MMEL1*	rs3748816 CD	CD, RA
1q23.3	*FCGR2A*	rs1801274	IBD, SLE
2p16.1	*REL*	rs13017599-A RA, rs13003464-G CD	RA, CD
2q24.2	*IFIH1*	rs1990760-A T1D	T1D, SLE
2q32.3	*STAT4*	rs3821236 RA	RA, SLE, T1D, IBD, IBD, PSO
2q37.1 3q13.33	*ATG16L1* *TMEM39A*	rs2241880-G IBD rs1132200	IBD, IBD SLE
5q11.2	*ANKRD55,IL6ST*	rs6859219-C	RA
5q33.1	*IRGM*	rs13361189	IBD
5q33.1	*TNIP1*	rs10036748-A SLE, rs17728338-A PSO	SLE, PSO
6p25.3	*IRF4*	rs1033180-A	CD
6q21	*PRDM1, ATG5*	rs548234-G	SLE
6q23.3	*TNFAIP3*	rs610604-G PSO, rs2327832-G CD, rs10499194-C RA	PSO, CD, RA, SLE, IBD
7q32.1	*IRF5, TNPO3*	rs4728142-A SLE, rs12537284-A SLE, rs10488631-C RA	SLE, RA, IBD, MS
9p24.1	*JAK2*	rs10758669-C	IBD
9q32	*TNFSF15*	rs6478109 IBD, rs4263839-G IBD	IBD, IBD
9q34	*TRAF1-C5*	rs3761847-G RA	RA, SLE
11p15.5	*KIAA1542, IRF7*	rs4963128-C, rs1131665-G, rs 1061501-T	SLE
12p13.31	*TNFRSF1A*	rs1800693-C	MS
12q24.32	*SLC15A4*	rs1385374-A	SLE
16p11.2	*ITGAM, ITGAX*	rs9888739-T SLE, rs11574637-C SLE	SLE
16p13.13	*KIAA0350*	rs2903692-G T1D	T1D, MS
16q12.1	*NOD2*	rs5743289-T IBD, rs2076756 IBD, rs17221417-G IBD	IBD, IBD
16q24.1	*IRF8*	rs17445836-G MS, rs11644034 SLE, rs2280381-A SLE, SSc, rs11642873-A SSc	MS, SLE, SSc
17q21	*IKZF3*	rs1453560	SLE
19p13.2	*TYK2*	rs2304256-C T1D	T1D, SLE, MS
20q13.33	*TNFRSF6B*	rs2315008-G	IBD
22q11.21	*HIC2, UBE2L3*	rs131654-A SLE	SLE, CD, RA, IBD
22q13.1	*C1QTNF6*	rs229541-T	T1D
Xp22.2	*TLR7, TLR8*	rs5979785 CD, rs3853839-G SLE	CD, SLE
Xq28	*IRAK1*	rs2239673, rs763737, rs5945174, rs7061789 haplotype GGGG	SLE

Table 1C. AD susceptibility loci involved in innate immunity. Adapted from Rai and Wakeland, 2011 (19) and Lessard *et al*. (179) and Lessard *et al*. (214). Abbreviations: celiac disease (*CD*), rheumatoid arthritis (*RA*), inflammatory bowel disease (*IBD*), multiple sclerosis (*MS*), psoriasis (*PSO*), systemic lupus erythematosus (*SLE*), type 1 diabetes (*T1D*), Systemic sclerosis (*SSC*), and single nucleotide polymorphism (SNP).

CHR.	REPORTED GENE (S)	STRONGEST SNP-RISK ALLELE	DISEASE
2q24.2	*IFIH1*	rs35667974-A, rs35337543-G, rs35744605-G, rs35732034-G	T1D
3p21.31	*TREX1*	1 3'UTR, 11 NS coding	SLE
6p21.33	*HLA-C*	rs2395029-C	PSO
6q23.3	*TNFAIP3*	rs2230926-C, rs5029939	SLE
11q24	*SIAE*	12 defective NS coding	RA, MS, SLE, T1D, IBD, IBD
12q12	*LRRK2, MUC19*	rs11175593-T	IBD

Table 1D. Rare or low frequency alleles associated with AD susceptibility. Adapted from Rai and Wakeland, 2011 (19) and Lessard *et al*. (179) and Lessard *et al*. (214). Abbreviations: rheumatoid arthritis (*RA*), inflammatory bowel disease (*IBD*), multiple sclerosis (*MS*), psoriasis (*PSO*), systemic lupus erythematosus (*SLE*) and, type 1 diabetes (*T1D*), and single nucleotide polymorphism (*SNP*).

BOX 1. Genome wide association studies (GWAS)

GWAS have emerged as the method of choice for genetic analyses of susceptibility to complex diseases. GWAS typically are conducted with single nucleotide polymorphism (SNP) typing platforms that can genotype large numbers of genetic variants (i.e., >100,000 SNPs to current >5,000,000 SNPs/assay) distributed throughout the genome in a single experiment. In general, GWAS are performed by large consortiums of investigators and involve the analysis of thousands of patients and controls. Many GWAS utilize a collection of tagging SNPs, which are selected based on their capacity to capture information for 65–70% of the common variations throughout the human genome (40). GWAS datasets are viewed as providing a largely unbiased, genome-wide survey for risk loci that contribute to susceptibility to the disease of interest. These studies have several analytical advantages: (1) the dense datasets produced allow for the identification of duplicate samples or samples from genetically related individuals; (2) the datasets are sufficient to allow for the application of strategies to correct for case–control stratification; and (3) the datasets are sufficient to impute SNP genotypes for untyped sites when performing genome-wide meta-analyses across multiple studies.

Although GWAS represent a potent strategy for the detection of novel and unbiased genetic associations with susceptibility to complex diseases, their interpretation is subject to many important limitations. First, GWAS focus on common variants (>1%) in the population, and these SNPs generally do not occur in genomic regions anticipated to impact gene function. However, the extent and nature of any functional variations is poorly defined; thus, the functional and biological significance of the association of a specific SNP haplotype with disease susceptibility is unknown. Secondly, GWAS guidelines established extremely stringent thresholds for a significant association with disease susceptibility (i.e., *p-value* < $5x10^{-8}$) and, as a result, many genome scans provide a plethora of suggestive associations (SNPs having p-value < 0.05 and >$5x10^{-8}$) (8). Thirdly, the common SNPs that are assessed in GWAS, particularly in European populations, represent only a subset of all the variability that is associated with complex diseases. That is, genetic predisposition to a common disease is likely to involve complex interactions between genetic variations exhibiting a broad spectrum of population frequencies, including a subset of rare genetic variants (i.e., frequency lower than 1%) that have a major effect on disease susceptibility in some individuals (41). Therefore, at the GWAS level, when associations are taken individually, they each can only account for a fraction (i.e., 1-5%) of the total genetic heritability associated with a complex disease. Finally, the functional variation causing the association of a specific SNP with disease susceptibility may or may not be associated with a gene in close proximity to the tagging SNP. Although the causal lesion should be located within the linkage disequilibrium (LD) block that is tagged by the associated SNP, the functional variation could potentially impact the expression or functional properties of a gene or genes that are more distal, or possibly interacting epistatically with the associated allele to cause the disease (40). Thus, the precise location and identity of the specific genes mediating the functional variations associated with a specific SNP association can be difficult to predict.

that most of these pathways contribute or play a role to a variable degree in most of these disorders. The latter underscores the fact that most of these loci can be mapped to a few shared biological pathways.

Table 1 illustrates some of the susceptibility loci/alleles identified by genetic studies in ADs thus far, either by candidate gene and/or GWAS approaches. These associated loci have been organized on the functional role that their top positional candidate plays in the immune system. Although the adaptive immune system has long been a focus of attention, innate immune mechanisms are now viewed as main players in the pathogenesis of autoimmune-related disorders. Even further, the concept of quantitative thresholds for immune-cell signaling has emerged in the past decade as a potential way of understanding how multiple genetic factors of relatively small effect sizes may combine to create a state of susceptibility to autoimmune activation. The new genetic findings also emphasize that the identification of the environmental components that interact with host genetic factors will be critical in developing a deeper understanding of autoimmunity as well as new approaches to prevention, diagnosis, and treatment.

The immune system is in a constant struggle to maintain a balance between immunity (i.e., elimination of foreign pathogens) and tolerance (i.e., lack of response to self-tissue). When these regulatory immune response mechanisms fail, inflammatory destruction might occur. The following section describes evidence for genes associated with and between ADs (Table 1). Putative immune functions for each gene are given, and each is classified below into a few broad categories for clarity.

GENES MODULATING ADAPTIVE IMMUNITY

Variations in the properties of the antigen receptor pathways of T and B cells have long been postulated to be key elements in genetic predisposition to ADs. The activation of autoreactive T and B lymphocyte clones is the essence of autoimmunity. As shown in Table 1, several AD susceptibility genes that impact the adaptive immune system have been identified thus far. These susceptibility alleles are anticipated to modulate the development, activation, and regulation of a variety of immune cell lineages.

Genes impacting T cell activation and signaling

The differentiation of T cells into functional subsets is a major element in the diversification and regulation of adaptive immune responses. Defects in this process or imbalances between the populations have been linked to the development of ADs. The differentiation of naïve T cells into functional subsets is controlled by a variety of cell intrinsic (i.e., receptor specificity) and extrinsic (i.e., cytokines and cell–cell interactions) factors. The activation signals generated by the innate immune system significantly impact T cell differentiation and, as discussed above, variations in signals from the innate immune system can significantly impact T cell function. Additionally, T cell intrinsic molecular pathways that interpret signals from the innate immune system can also impact T cell differentiation.

PTPN22 (Protein tyrosine phosphatase, non-receptor type 22) encodes the protein tyrosine phosphatase, LYP, that functions as a negative regulator of T and B cell responses by dephosphorylating key downstream signaling molecules [e.g., lymphocyte-specific protein tyrosine kinase (LCK), protein-tyrosine kinase fyn (FYN) and ζ-chain (TCR)-associated protein kinase 70 kDa (ZAP70)] (59). A missense mutation at rs2476601 results in a change from arginine to tryptophan at position 620 (i.e., R620W). The functional consequences of this substitution appear to be a decreased ability to bind C-terminal Src tyrosine kinase, an important negative regulator of lymphocyte-specific protein tyrosine kinase (60,61). The consequences of this substitution are controversial, with decreased activation through both the BCR and TCR reported by some (62-64) and increased TCR signaling reported by others (65). The *620W* allele has been associated with risk for SLE, T1D (60), and RA (61), whereas in IBD it is protective (66). In addition, there is evidence that this allele is not associated with other ADs, such as CD (67) and MS (18). Likewise, a rare missense substitution (R263Q) in *PTPN22* was shown to reduce phosphatase activity and was associated with protection from SLE (68).

CTLA4 (cytotoxic T-lymphocyte-associated protein 4) is a key negative regulatory molecule that impacts antigen-driven activation of T cells. *CTLA4* polymorphisms are reported to be associated with T1D (69), IBD (70), RA (71), CD (72), MS (73), and SLE (74). This gene maps to a region in strong LD with *CD28* and *ICOS,* both of which also have important regulatory roles in adaptive immunity. A recent study reported the association of both genes with RA (75). However, the strong LD in the *CD28/CTLA4/ICOS* gene cluster makes it difficult to identify the true causal locus. In many respects, it is probably most accurate to interpret the association of SNPs from this LD block as representing the functional consequences of carrying a *CD28/CTLA4/ICOS* haplotype rather than focusing completely on one member of the cluster until more data becomes available to further dissect this association.

SH2B3 (SH2B adaptor protein 3), which encodes for the negative regulator of T cell receptor signaling, LNK (lymphocyte adaptor protein), harbors a non-synonymous variant, particularly associated with T1D (76) and CD (77) but also SLE. Mice genetically deficient for *SH2B3* are hypersensitive to stimulation with multiple cytokines (78). SH2B3 also functions as a regulator of T cell signaling as overexpression of SH2B3 inhibits the activation of nuclear factor of activated T cells (NFAT) following TCR stimulation *in vitro* (79). Variants that affect *SH2B3* function could, therefore, alter the signaling thresholds through many different receptors on cells of both the lymphoid and myeloid lineage. A non-synonymous SNP that leads to a R262W substitution in *SH2B3* is associated with SLE, T1D, and CD (14,80) and occurs in the pleckstrin homology domain, which is known to be important for targeting the protein to the plasma membrane (14,81,82).

Similarly, *TAGAP* (a T cell activation GTPase-activating protein) is associated with CD (80), T1D (14), and RA (83). *ICOSLG*, which encodes a B7-related peptide involved in T cell activation and differentiation and binds to ICOS expressed on the surface of T cells, has been associated with IBD, CD, and T1D (84-86). All of these genes modulate antigen-driven T cell activation in a manner that potentiates susceptibility to multiple ADs.

CD226 is a type I transmembrane receptor of the immunoglobulin superfamily, mainly expressed on the surface of lymphocytes (87). *CD226* induces protein kinase C (PKC) and Src family kinase Fyn upon binding to its ligands (88) and is involved in T cell activation and differentiation (89). A missense variant in the gene (Gly307Ser) is associated with T1D, MS, AITD, RA, Wegener's granulomatosis, and SLE (82,90-93). The risk allele reduces the expression of *CD226* in T and NK T cells (93).

The *TNFSF4* [tumor necrosis factor (ligand) superfamily, member 4] binds to the surface of antigen-presenting cells by its receptor (*TNFRSF4*). TNFSF4-mediated signals inhibit IL-10-driven expression of regulatory T cell functions and *in vitro* production of IL-17 (94). Genetic variants in the promoter of *TNFSF4* define a haplotype associated with SLE (95). In addition, the risk haplotype led to the increased expression of the TNFSF4 transcript as well as cell surface protein expression of TNFSF4 when compared to non-risk alleles in lymphoblastoid cell lines (96). Moreover, *TNFSF4* and *TNFRSF9* are involved in T cell activation and are implicated in CD (86).

Susceptibility genes impacting B cell activation

Human genetic diversity approaches have identified a variety of polymorphisms in genes potentially impacting B cell activation. Among these, *BANK1* (B cell scaffold protein with ankyrin repeats 1) encodes a gene that is active in the transmission of BCR signaling. BANK1 promotes the protein tyrosine kinase (LYN)-mediated tyrosine phosphorylation of IP(3) R (inositol 1,4,5-trisphosphate receptor), thus affecting B cell receptor-induced calcium mobilization from intracellular calcium reservoirs (97). Potentially functional *BANK1* variations are reported to be associated with SLE in Scandinavian (98), European, American, and Chinese populations (99-101).

Multiple members of the Src family of tyrosine kinases have polymorphisms associated with susceptibility to AD. *LYN* is associated with antigen receptor signaling in B cells and is associated with SLE (102,103). *BLK* (B lymphoid tyrosine kinase) encodes another Src family tyrosine kinase that also plays a role in B cell signal transduction. The variants rs2248932 and/or rs13277113 associate *BLK* with SLE (103,104), RA (105), ankylosing spondylitis (AS) (106), and scleroderma (SSc) (107). The risk allele of the SNP, rs13277113, is associated with low mRNA expression levels of *BLK* and high mRNA expression levels of *FAM167A* (104). Interestingly, BLK expression levels are downregulated by epigenetic factors like exposure to high amounts of interferon and infection with Epstein-Barr virus (108).

CD40 (CD40 molecule, TNF receptor superfamily member 5) plays a crucial role in the activation and differentiation of B lymphocytes. *CD40* is associated with susceptibility to RA (109), MS, and IBD (110). *KIF5A* and *PIP4K2C* are in tight LD and are located on chr.12q13. *PIP4K2C* encodes a phosphatidylinositol-5-phosphate 4-kinase that is expressed in B cells and is predicted to be involved in phosphatidylinositol signaling. *KIF5A* encodes a member of the kinesin family with no obvious role in the immune system. The *KIF5A/PIP4K2C* LD block is a susceptibility locus for RA (109), T1D (11), and MS (111), although the functional mechanisms involved are unclear. ETS1 is a transcription factor in the ETS family that regulates differentiation of terminal B cells and Th17 cells (112). Distinct common variants within *ETS1* have been associated with SLE and CD (86,113).

Finally, *IL10* (interleukin-10) is an important immunoregulatory cytokine that downregulates the immune response by reducing T cell function and MHC Class II expression in antigen-presenting cells. IL-10 levels were found to be dysregulated in SLE patients (114). Specific variations in *IL10* are inconsistently associated with SLE (115). However, this locus has been identified as a risk factor for IBD (116) and T1D (117).

Genes affecting Th1 and Th2 cells

The contribution of different subsets of T-helper cells to the pathogenesis of inflammatory diseases is the subject of much research and debate (118). $CD4^+$ Th1 cells drive the cell-mediated immune responses that lead to tissue damage and particular IgG responses thought to play a role in many inflammatory diseases, whereas $CD4^+$ Th2 cells drive the production of certain antibodies (i.e., IgE) that predominantly underlie allergic responses (119,120).

IL18RAP, STAT1-STAT4, STAT3, and *IL12A* impact the differentiation of Th1 and Th2 cells, and all have polymorphisms associated with ADs. rs917997 is correlated with alterations in expression of *IL18RAP* and is associated with susceptibility to IBD. Interestingly, the same SNP is associated with T1D, yet the same risk allele is protective for IBD (35). rs7574865 from *STAT4* is associated with RA (121), SLE (103), T1D (122), and IBD (123). *STAT1,* which is adjacent to and in moderate LD with *STAT4*, is associated with MS (124) and SLE (125). Likewise, *STAT3* is associated with IBD (126) and MS (127).

IL12B encodes the p40 subunit of the heterodimeric cytokines IL-12 and IL-23. IL-12 is composed of a bundle of four alpha helices. It is a heterodimeric cytokine encoded by two separate genes, IL-12A (p35) and IL-12B (p40). IL12B variants are associated with T1D (128), MS (129), psoriasis (PSO) (130), and IBD (131). IL-13 and IL-4 are located directly adjacent to each other in a common LD block. IL-13 is involved in Th2 effector functions and can inhibit Th17 differentiation, while IL-4 is critical for Th2 differentiation. An IL-13 promoter variant is associated with PSO, and the protective allele results in enhanced IL-13 expression in Th2 cells (132).

PRKCQ (protein kinase C, theta) is predominantly expressed in hematopoietic cells and is involved in TCR regu-

lation (133). Mice deficient in *PRKCQ* show impaired differentiation of T-helper subsets, particularly in Th2- and Th17-mediated inflammatory responses (134). rs4750316 is located in an intergenic region on chr.10p15 proximal to *PRKCQ* and is associated with RA (135). On the other hand, rs947474, in the same gene is reported to be independently associated with T1D (136). *CD58* and *CD6* encode co-stimulatory molecules involved in T cell receptor signaling and differentiation. These molecules are associated with MS and RA (83).

Genes affecting Th17 cells

In recent years, the characterization of novel subsets of T-helper cells, most notably regulator T cells (Treg; see below) and Th17 cells, has led to a major paradigm shift in T cell biology, and these T-helper cell subsets have been shown to play an important role in the pathogenesis of ADs. Elevated IL-17 levels are reported in the serum of patients with active SLE (137), MS (138), and RA (139). Moreover, IL-17-producing T Cells are observed in inflammatory infiltrates of SLE, MS, and RA patients. In PSO, activated IFN-γ-producing T cells and IL-17 are detected in the skin and play an important role in disease pathogenesis. A key factor in the survival and expansion of Th17 cells is stimulation by IL-23 (140). A missense variant, rs11209026 (R381Q), in the receptor for *IL-23* strongly associates with IBD (141), PSO (142), AS (143), and AITD (144). Lastly, IL-17-producing T cells express CCR6, which encodes a member of the G protein-coupled chemokine receptor family and has emerged as a predisposing genetic factor for IBD in a recent meta-analysis of GWAS (126). Similarly, a functionally disruptive 32bp indel in *CCR5* associates with protection from T1D, RA, and CD (126). Polymorphisms in the *CCR3* and *CCR4* chemokine receptors are also associated with CD (86).

Genes affecting regulatory T cells (Treg cells)

Treg cells are involved in the maintenance of immunological tolerance and lymphoid homeostasis (145). Their deficiency can result from decreased production of the IL-2 (146). IL-2 is often absent in RA synovium (147), and severe autoimmunity develops in knockout mice deficient for IL-2, IL-2RA, and IL-2RB, presumably due to defective regulatory T cell production (148). Genetic variants within the LD block encompassing the *KIAA1109/Tenr/IL-2/IL-21* genes have been associated with CD (149), T1D (82), RA (150), IBD (151), PSO (152), and SLE (153). Given the strong LD, it is difficult to define a true candidate gene; however, *IL-2* and *IL-21* appear to be the plausible casual genes based on their functions in adaptive immunity (154).

IL2RA variants are associated with RA (155), T1D (117) and MS (156), and IBD (157), presenting allelic heterogeneity among diseases. Likewise, *IL2RB* is associated with RA (158) and T1D (136). Finally, *IL7RA* variations mediate reduced splicing of the transmembrane domain, decreasing soluble forms of IL-7RA, and are associated with IBD (159) and MS (160). IL7RA along with IL7 and IL2, helps maintain a healthy T effector cell:Treg cell balance (161). All of these variations are postulated to modulate AD susceptibility through their impact on Treg cell differentiation and maintenance.

TNF receptor superfamily genes associated with ADs

GWAS approaches have led to the association of several TNF superfamily members with ADs. *TNFSF15*, expressed predominantly on endothelial cells, has been shown to be upregulated on macrophages and $CD4^+$/$CD8^+$ lymphocytes of the intestinal lamina propria of IBD patients (162), with a SNP haplotype associated with in multiple populations (163). TNFRSF14, another member of TNF-receptor superfamily, interacts with TRAF proteins (164) and may indirectly affect NF-kB-mediated inflammation. This gene is associated with RA and CD (165). Moreover, *TNFRSF6B,* encoding a decoy receptor that prevents FasL-induced apoptosis, is associated with IBD (85). Increases in TNFRSF6B protein are observed in IBD (166) and RA (167), suggesting an impact in susceptibility. *TNFRSF1A* harbors a variant leading to an amino acid substitution, R92Q, which has been identified as a susceptibility locus for MS that correlates strongly with episodic multi systemic inflammation (168,169).

TNFAIP3 encodes the protein A20, an ubiquitin-modifying protein that is strongly induced in cells following TNF stimulation. This molecule is an inhibitor of NF-κB and plays a key role in limiting the severity of inflammatory processes (170). Several *TNFAIP3* variations are associated with RA, IBD, SLE, PSO, and CD (13,165,171). A recent meta-analysis confirmed the strongest association with severity of SLE (172,173).

INNATE IMMUNITY GENES

The innate immune system plays a crucial role in driving the activation of the immune response. Functions of the innate immune system include: (a) detection of infectious agents; (b) initiation of both local and systemic inflammatory responses by the production of cytokines; (c) attraction of effector cells into infected tissues via the production of chemokines; and (d) initiation of the adaptive immune response. There exists the possibility that exposure of a dysregulated innate immune system to environmental stimuli might initiate the development of autoimmune phenomena. Several studies have implicated viral or microbial infections with the initiation of ADs, although these studies are inconsistent (174). Studies in mice have established an important role for commensal gut flora in the development of arthritis (175) and IBD (176), and, more recently, in modulating susceptibility to T1D in the NOD mouse (177). The precise interactions involved are still unclear, although signaling through toll-like receptor (TLR) pathways appears to be crucial. Further analysis of the genes and molecular pathways within the innate immune system identified by GWAS studies may provide important insights into the events that trigger autoimmunity in genetically predisposed individuals.

The shared autoimmune loci in innate immunity are: *TNIP1* [(associated with psoriasis (178)], *IRF8* [(associated with MS (168) and SLE (179)], TYK2 [associated with MS (181) and T1D (182)] and *TNFAIP3* discussed elsewhere in this chapter. A missense allele in *IFIH1* is associated with risk for T1D (182), AITD (183), and SLE (115). *IFIH1* (also called *MDA5*) is a cytoplasmic RNA sensor that promotes IFN-I production when activated by viruses. As the innate immune response to viral infection is hypothesized to play a role in the pathogenesis of multiple autoimmune diseases, the inappropriate activation of nucleic acid sensors such as TLRs and IFIH1 may contribute to a general predisposition towards autoimmunity (184). *IFIH1* is associated with T1D, SLE, and AITD (56). A missense allele (*R32Q*) of *CFB*, an activator of the alternative complement pathway, is associated with protection from age-related macular degeneration (AMD) and SLE (185). *MIF* is an immunoregulatory cytokine that functions in both innate and adaptive immunity and is expressed by many immune cells such as monocytes, macrophages, and T and B lymphocytes. Polymorphisms in *MIF* have been associated with multiple autoimmune and inflammatory diseases [(reviewed in (186)].

Genes impacting the pathogen recognition receptor (PRR) pathways

The detection of microbial infections is predominantly mediated by the recognition of pathogen-associated molecular patterns (PAMPs) by the TLR and the NOD-like receptor (NLR) PRR families. Signaling through either the TLR or NLR pathways results in the secretion of pro-inflammatory cytokines such as IL-6, TNFα, IL1, and the Type I (interferon alpha/beta) and Type II (interferon gamma) interferons (IFN). Levels of these cytokines (and many others) are commonly increased in the active stages of ADs and are generally considered to play an important role in a variety of elements of disease pathogenesis.

Genetic polymorphisms throughout the TLR and NLR pathways are associated with susceptibility to ADs. Variations in *TLR7/8*, *TLR4*, and *NOD2* are associated with ADs (85,86,187). Polymorphisms in *TLR7/8* are associated with SLE. Genetic studies of IBD have associated a missense variant in *TLR4* with early onset of disease (85). Multiple variations in the *NOD2* gene are associated with susceptibility to IBD (85). Finally, multiple variants of the *CLEC16A* (C-type lectin domain family 16 member A) gene, which is predicted to play a role in sugar binding, are associated with T1D (188) and MS (189). All of these variations occur in PRR family receptors and presumably impact susceptibility to AID by modulating the ability of the innate immune system to identify environmental stimuli.

Innate system transcription factors associated with autoimmune susceptibility

Signaling through PRR pathways leads to the activation of multiple cellular functions and the secretion of a variety of cytokines and chemokines. These receptors are expressed in a variety of immune cell lineages, and the consequences of their signaling are dependent in part on the specific cells involved. The nine members of the interferon regulatory factor (IRF) family of transcription factors play crucial roles in mediating transcriptional activation following signaling through the TLR pathway. Genetic variations in *IRF4, IRF5, IRF7,* and *IRF8* are associated with susceptibility to various ADs (179). *IRF5* is strongly associated with SLE (190), RA (191), Sjögren's syndrome (SS) (192), IBD (193), MS (194), and primary biliary cirrhosis (PBC) (195).

As noted above, interferon pathways mediate both autoimmunity and viral defense (196). Genetic associations exist that lead to the increased expression of IRF5 in SLE (197) and RA (155). IRF5 broadly regulates innate immune responses through toll-like receptors (198) and type 1 interferons.

NF-κB, a central regulator of immune responses and inflammation, is a key player in innate immune responses. Though variations in NF-κB have not been associated with ADs, a variety of NF-κB-interacting and regulatory molecules do affect susceptibility. *c-Rel,* involved in T and B Cell activation, is associated with CD (86) and RA (115). *UBE2L3* (ubiquitin-conjugating enzyme E2L 3 isoform1) encodes a protein involved in ubiquitination, and its overexpression leads to quicker degradation of the NF-κB precursor and a diminished innate immune response. This gene is associated with SLE (199), CD (86), RA (155), and IBD (200).

Apoptosis/autophagy/immune-complex clearance

Further implicating a primary role for innate immunity, variants of autophagy genes (e.g., *ATG16L1*) that target intracellular components, including microbes, to lysosomes have also been associated with IBD (201). Another autophagy gene, *LRRK2* (leucine-rich repeat kinase 2), has been identified as an IBD susceptibility locus (126). The associated variant (G2019S) is known to alter neurite length and autophagic vacuole size (202). GWAS reported an association between autophagy *ATG5* (APG5 autophagy 5-like) gene and SLE. The associated SNP (rs6568431) lies in an intergenic region between *PRDM1* (PR domain containing 1, also known as BLIMP1) and *ATG5*. Since both genes are viable candidate genes for modulating SLE, the causative gene for this disease association remains to be elucidated (103). *MST1* encodes macrophage-stimulating protein (MSP) that regulates macrophage chemotaxis and activation and affects innate immune responses to several bacterial ligands. This gene is associated with IBD (203) along with AITD, RA, SLE, and T1D. Finally, CRP (pentraxin C-reactive protein) is a complement activator and an important innate immune modulator involved in the clearance of cellular debris and apoptotic bodies. Variants in the promoter of *CRP* have been associated with SLE and CRP levels in SLE patients (204).

Increases in the prevalence of IBD during the past century may well reflect corresponding changes in the compo-

sition of the intestinal microbiota resulting from changes in hygiene, such as eradication of intestinal parasites (205). However, the role of the intestinal microbiome is probably not limited to the intestinal immune system. Mouse models of autoimmune diabetes suggest that host differences in the capacity to sense intestinal microbes (177), and the specific composition of the intestinal microbiota modulate susceptibility to diabetes. The dynamic cross-talk between the host immune response and the intestinal microbiota will be an important focus of future research.

RARE VARIANTS ASSOCIATED WITH AD SUSCEPTIBILITY

A current listing of rare alleles with a major effect on ADs obtained both in classic studies and more recently via targeted resequencing studies is provided in Table 1. Classic prototypes of rare alleles with major effects on autoimmunity are the deficiencies in components of the complement system. Deficiencies in *C2*, *C4A*, *C4B,* and *C1q* result in highly penetrant susceptibility to SLE (206). The mechanisms by which these deficiencies mediate a dramatic increase in AD susceptibility are unknown.

Copy number variations (CNV) have also been associated with strong relative risk effects in ADs. CNV of both *C4* and *FCGR3B*, coding for FcγRIIIb, are associated with susceptibility to SLE (207). Other examples of CNV effects associated with AD susceptibility are the beta-defensins. These are small, secreted, antimicrobial peptides, that are encoded by *DEFB* genes, which show association between higher genomic copy number and the risk of PSO (53).

Next-generation sequencing technologies are currently being used to disentangle rare variants (i.e., frequency <1%). For this purpose, recent analyses in 480 T1D patients and 480 controls identified four rare variants in *IFIH1* associated with the disease (56). Likewise, *TREX1* encodes for a major 3' - 5' DNA exonuclease that may be responsible for a proofreading function during genome replication. *TREX1* deficiency has been associated with Aicardi-Goutières syndrome (208), characterized by high type 1 IFN levels. Several rare variants are associated with SLE and SS. The precise mechanisms by which these deficiencies increase disease risk remain unknown.

TRANSLATIONAL AUTOIMMUNITY

Drastic advances on human "-omics" are giving rise to new possibilities in medicine and terms such as clinical bioinformatics (209) and translational bioinformatics (210). All these options lead to one common premise: ways of mining meaningful information from the vast amount of -omics data being generated. In this sense, application of comprehensive molecular information to clinical settings is been referred to as "genomic medicine" (211) with the ultimate goal to nurture, improve, and develop personalized medicine (PM) approaches. A genomic medicine approach will always require participation at a multidisciplinary research expertise level. These valuable advances will strengthen the overall approach to population and individual genetic susceptibility (212) and promise the ability to manage each patient as a biological individual. This will lead to changing paradigms and increasing efficiency and will foster a PM approach as an added plug-in capable of assisting in better prevention, detection, and classification of disease.

Last but not least, the protection of human participants, whether patients, unaffected family members, or unrelated population controls, has to be ensured. These individuals are the key component of genomic research, and their legal rights need to be protected if we wish to continue on with genomic science and to eventually apply genomic-based medicine for the good of humankind. More importantly, we shall not forget the sometimes understated premise that, "... we should not only be interested in the human genome but also in the human beings that carry it" (213).

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19 INFECTION AND AUTOIMMUNE DISEASES

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INTRODUCTION

Autoimmune diseases (ADs) are chronic pathologies triggered by the loss of immunological tolerance to self-antigens, which can cause systemic or organ specific damage. ADs are common, with an estimated prevalence of 3,225/100,000. They are also a frequent cause of morbidity and mortality (1,2). Genetic and environmental factors are the main ones involved in the pathogenesis of ADs. Infections and exposure to pathogens or opportunistic organisms are among the environmental factors and may induce the initiation or exacerbation of ADs (3-5). Many types of infection may influence one or more of these diseases, and a single organism may be able to trigger more than one AD (1,6). In this chapter, the evidence indicating a causal role of infections in the development of ADs is reviewed.

INFECTIOUS AGENTS AND ADs

Autoimmune response is mediated by autoreactive T and B lymphocytes responsible for the production of soluble mediators (e.g., cytokines, nitric oxide, etc.) and autoantibodies. These will lead to tissue damage that may be systemic in the case of systemic lupus erythematosus (SLE) or organ specific such as in type I diabetes (T1D) (7).

Infections can be triggers of ADs as has been shown in animal models (1,3,6,7). Moreover, infections can participate in the activation and later clonal proliferation of autoreactive T and B lymphocytes that are crucial for the development of an AD. Almost all ADs have been associated with at least one infection (1). In addition, patients diagnosed with ADs have a higher risk of infections as a consequence of their treatment (1,4). For example, SLE patients under treatment who have a low grade vaccination against varicella zoster virus have an increased frequency of reactivation of the virus (8). According to the "hygiene hypothesis," infections may act as a protective mechanism for autoimmune development. However, it is well known that they also trigger autoimmune manifestations (9). Moreover, one or more microorganisms can be associated with the same AD (1,6). Note that, this association may depend on the population under study given the evolutionary effect exerted by some infectious agents to induce resistance in the host (see Chapter 15), which in turn, influences the risk of ADs (Table 1) (9–40). Autoantibodies may also be seen in infectious diseases in patients without ADs (41,42), indicating that the presence of a pathogen may lead to the occurrence of autoimmune phenomena.

Antiphospholipid syndrome (APS). High levels of IgM antibodies against Rubella, Toxoplasma *gondii*, Cytomegalovirus (CMV), and hepatitis C virus have been found. Moreover, cross-reactivity has been demonstrated between proteins of bacteria such as *Haemophilus influenzae*, *Neisseria gonorrhoeae*, tetanus toxoid, and CMV with anti β2 glycoprotein 1 (β2-GPI) antibodies, which is one of the typical antibodies of this AD (1,22,43).

SLE. Studies in animal models of SLE have shown that Epstein-Barr virus (EBV) can trigger the production of autoreactive antibodies with subsequent development of manifestations similar to those presented in the human disease (1,19,44). In patients, a high EBV seroprevalence has been observed as compared to healthy controls (1,19,45). Additionally, it has been suggested that Rubella (1) and CMV may induce the production of autoantibodies in patients with SLE (10,12,13). *Toxoplasma gondii* and *Helicobacter pylori* are also risk factors for ADs (46).

Rheumatoid arthritis (RA). It has been reported that the IgM response to some bacterial infections, e.g., *Escherichia coli,*

AD	PATHOGEN	MECHANISMS	OBSERVATIONS	REFERENCE
SLE	CMV	Molecular mimicry	SLE diagnostic after CMV acute infection Association with high levels of antibodies against CMV and Anti Sm autoantibodies.	(10–12) (13)
	EBV	Molecular mimicry, bystander activation, clearence deficiency	Higher prevalence in patients (99%) than in healty controls (70%). Higher viral load in blood cells from patients than in controls. B and T cell reactivity to Sm related to antibodies against EBNA and HLA-DR3.	(14) (15–17) (18,19)
	T. gondii	Molecular mimicry	Higher antibody titers to T. *gondii* in patients than in healty controls.	(20)
APS	EBV	Molecular mimicry	Higher prevalence in patients than in healty controls.	(21)
	C. tetani	Molecular mimicry	Relationship between tetanus toxoid and the presence of anti β2-GPI antibodies.	(22,23)
T1D	CMV		Relationship of presence of antibodies to islet cells and CMV infection Ealry infection during childhood cuases elevated risk of DT1	(24) (25)
	Saccharomyces cerevisiae		Higher prevalence of ANCA autoantibodies in patients than in controls	(26)
AR	*E. coli,* *K. pneumoniae* *P. miriabilis*	Molecular mimicry	Relationship of presence of IgM to infection and rheumatoid factor	(27)
	P. miriabilis	Molecular mimicry	Recurrent *P. miriabilis* infection in patients with RA.	(28–30)
	EBV	Molecular mimicry	Higher prevalence of infection in patients than in healthy controls.	(31)
	Endogenous retrovirus	Molecular mimicry	Evidence of induction of autantibodies	(32)
MS	EBV	Molecular mimicry	Higher frequency of EBV infection before MS onset in patients (95%).	(33–36)
	Acinetobacter sp *P. aeruginosa*	Molecular mimicry	Presence of autoantibodies that recognize autoantigens in patients but not in healthy controls.	(37)
SS	Coxsackie virus	Non especific activation	Possible presence of the virus in the cells of salivary glands in SS patients but not in helathy controls.	(38)
	EBV		Presence of previous infection or IgM to the virus before disease onset.	(39)

Table 1. Pathogens associated with most common autoimmune diseases. Adapted from Arango & Anaya (40). *AD*: Autoimmune disease, *SLE*: systemic lupus erythematosus, *APS*: antiphospholipid syndrome, *T1D*: type 1 diabetes, *RA*: rheumatoid arthritis, *MS*: multiple sclerosis, *SS*: Sjögren's syndrome, *CMV*: cytomegalovirus, *EBV*: Epstein-Barr virus.

Klebsiella pneumonia, and *Proteus mirabilis* is associated with rheumatoid factor (1,27). In the case *of P. Miriabilis*, a relationship with RA was established through structural similarities between self-epitopes and bacterial molecules, e.g., the shared epitope alleles (mainly *HLA-DRB1*01:01, *04:01, *04:04, *04:05* – See Chapter 17) and the bacterial haemolysin as well as the human type XI collagen and the urease from *P. Miriabilis* (28–30). Additionally, RA has also been associated with the presence of Hepatitis B virus which is higher in RA patients than in healthy controls (47). Similar findings have been reported for EBV in RA as well as for Sjögren's syndrome (1,31,48).

T1D. CMV infection may trigger the clinical manifestations associated with this disease. However, there are conflicting results concerning this matter (24,25). Associations between Coxsackie B4 virus, Rubella, and T1D have been reported (49). Furthermore, a homology has been shown between sequences of glutamic acid decarboxylase (GAD), a self antigen recognized by autoantibodies in T1D, and some protein sequences of several viruses, e.g., adenovirus, CMV, EBV, and rotavirus (50). There is, in addtion, some evidence of the association between islet autoantibodies and *H. pylori* (51).

Multiple sclerosis (MS). While some animal models suggest a protective effect of viral infections against myelin loss, other evidence suggests that murine CMV and EBV may favor the development of the disease (52). In humans, there is no agreement about the risk of developing MS confered

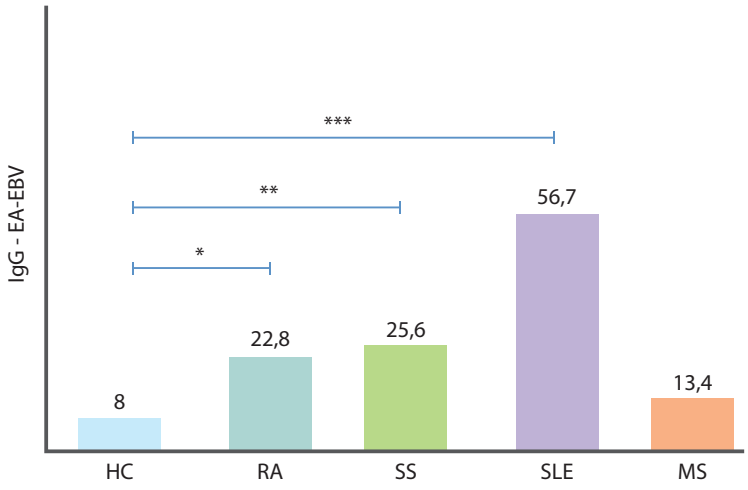

Figure 1. EBV infection in ADs. The percentage of individuals with IgG against EA-EBV is shown. (* OR=3.35 95%CI: 1.63-6.92, *p<0.001*, ** OR= 4, 95%CI: 1.82 - 8.83, *p<0.001*, *** OR= 15.22, 95%CI: 7.45-31.1, *p<0.001)*. EA-EBV, early antigen of Epstein Barr virus, *AD*: autoimmune disease, *HC*: healthy controls, *RA*: rheumatoid arthritis, *SS*: Sjögren's syndrome, *SLE*: systemic lupus erythematosus, *MS*: multiple sclerosis. *OR*: odds ratio, *CI*: confidence interval.

by infection (53,54). There are different types of viruses and bacteria associated with the induction of autoantibodies to myelin basic protein (MBP) (55) (e.g., *Acinetobacter sp*. and *P. aeruginosa*). These infections are associated with the occurrence of autoantibodies against MBP and myelin glycoprotein oligodendrocyte due to their similarity with some bacterial molecules (37). In a series of 593 Colombian individuals including 143 healthy controls, 99 MS patients, 152 RA patients, 120 SLE patients, and 82 SS patients, an evaluation for antibodies to EBV infection was done (Figure 1). An association between ADs and anti IgG antibodies wih the early antigen of EBV was observed in all ADs except for MS.

INFECTIONS: A TRIGGER MECHANISM FOR ADs

The AD definition has been framed by various direct and indirect criteria as well as circumstantial evidence about the disease (56–58). Infectious agents can trigger some ADs through different mechanisms (Table 2). Reconciling the criteria for the AD definition with Koch's postulates provides a better understanding of the relationship between ADs and infections. Koch's postulates are the criteria for establishing a causal link between a microbe and a disease. However, these four criteria are based on a simplistic view of ADs because they do not take into account the multifactorial

EVIDENCE			POSSIBLE INFLUENCE OF INFECTION AGENTS
Direct	Mediated by ABs	Circulating ABs which alter the function	+
		Localized Abs	+
		Immune Complexes located at lesion	+
		Passive transference	+
	Mediated by Cells	In vitro T-cell proliferation in response to autoantigen	+
		In vitro T-cell transference to immune-deficient mice	+
		In vitro T-cell cytotoxicity against target organ cells	+
Indirect		Disease reproduction by experimental immunization	+
		Disease reproduction by idiotypes	+
		Spontaneous animal models	-
		Animal models produced by immune system deregulation	+
Circumstantial		Auto-Abs	+
		Other AD association	+
		HLA association	+
		Lymphocytic infiltration in target organ	+
		Good response to immune suppression	-

Table 2. Classification criteria for autoimmune diseases in humans and role of infection. Adapted from Shoenfeld & Anaya (57). *Abs*: Antibodies, *AD*: Autoimmune diseases.

KOCH'S POSTULATES	OBSERVATIONS REGARDING ADs
The microbe or pathogen must be present in all cases of the disease	As a consequence of individual characteristics (i.e., genetic background), not all the patients with an AD are infected with the pathogens previously associated with the disease.
The pathogen can be isolated from the diseased host and grown in pure culture	The pathogen cannot be present in the patients. However, if they had a previous infection, which induced auto-reactive B or T cells, these cells will remain in the host, and they will perpetuate the disease. Therefore, the transplant of these cells can cause the disease in other individuals without the presence of the same pathogen.
The pathogen from the pure culture must cause the disease when inoculated into a healthy individual	Induction of the disease by inoculation of the pathogen is possible if the environmental and genetic background conditions are under a control setting (i.e., animal models).
The pathogen must be re-isolated from the new host and shown to be the same as the originally inoculated pathogen	

Table 3. Koch's postulates in autoimmune diseases. *AD*: Autoimmune diseases.

nature of autoimmunity (Table 3). Since environmental, epigenetic, and genetic factors also influence ADs, these factors should be taken into consideration. In other words, the inner characteristics of a population may influence the development of an AD when they are exposed to an infection. This explains why, despite the association of certain pathogens with a specific disease, there is still a considerable group of healthy individuals who, after exposure to a microorganism, does not develop the disease [e.g., EBV and SLE (14)].

There are different mechanisms by which infection may trigger ADs (Figure 2). First, there is **molecular mimicry** in which B and T cells are activated as a result of an infection, but they are able to recognize self-molecules or proteins that are similar to those from infectious agents. This cross reaction may generate an autoimmune response which has been reported in several ADs (1,3,19,52,59–61). The second is **epitope spreading**, which is also known as antigenic determinant. In this case, the autoimmune response is due to the spatial proximity and similarities between several self epitopes and epitopes from the microorganism (1,61). A third mechanism is **bystander activation**. This makes reference to the activation and clonal expansion of auto reactive T cells by the cytokine profile generated during the inflammatory process and swelling in response to an infection. This inflammatory cocktail activates cells with different specificity and which may be auto reactive. Finally, all these circumstances favor the inflammatory process and the development of an aggressive response leading to exacerbations in patients with ADs (1,3,52,61). In contrast with molecular mimicry and epitope spreading where cells are activated by specific antigens, the bystander activation mechanism assumes that pathogens may break immune tolerance by the induction of a non-specific polyclonal response. This mechanism is achieved through several pathways including the release of intracellular antigens after cell death known as cryptic antigens. A characteristic of this type of antigens is that they are not presented to T cells during central tolerance. Therefore, as a consequence of cell death, these self-antigens may increase their visibility and abundance thus attracting and enhancing antigen-presenting cells or disturbing the cytokine balance (both locally and long distance) (1,3,52,61).

Fourth, the persistence of infection and a polyclonal response can trigger the appearance of ADs as a consequence of the **constant activation** of the immune response. In ADs, it is possible to find an accumulation of circulating immune complexes, which may affect specific tissues, for example, the case of mixed cryoglobulinemia caused by hepatitis C virus infection (1,61). Furthermore, the study of the relationship between infection and autoimmunity has been advanced by the description of **superantigens** They are bacterial, viral, or retroviral proteins that can activate a large quantity of T cells by binding the T cell antigen receptor to MHC molecules expressed on other cells. All this is done without antigen processing (61,62). The Vß region is sufficient for superantigen recognition in contrast to conventional epitopes that require a very specific interaction with the third hypervariable region of the TCR. Superantigens are not restricted to MHC class II, and as a result, they may cause T cytotoxic activity without antigen presentation. Therefore, superantigens are extremely potent immunostimulatory substances (61,62).

One of the consequences of the chronic inflammatory process is apoptosis or necrosis of the cells within the location where the infection took place. Some abnormalities such as deficiencies in the clearance of apoptotic bodies can cause a secondary necrotic step in which there may be an exposure of cryptic antigens. Consequently, these antigens may generate the autoimmune process. It is common in autoimmunity to find **clearance deficiency** of dying cell particles associated with disease development (Figure 3) (61,63). For example, there are deficiencies in the complement system in SLE patients. As a result, there are alterations of phagocytosis, clearance of apoptotic cells, and the recognition of potential pathogenic microorganisms. This causes the accumulation of substances which induces a pro-inflammatory microenvironment that includes the exposure of self antigens and the generation of autoantibodies against them (Figure 3) (61,63).

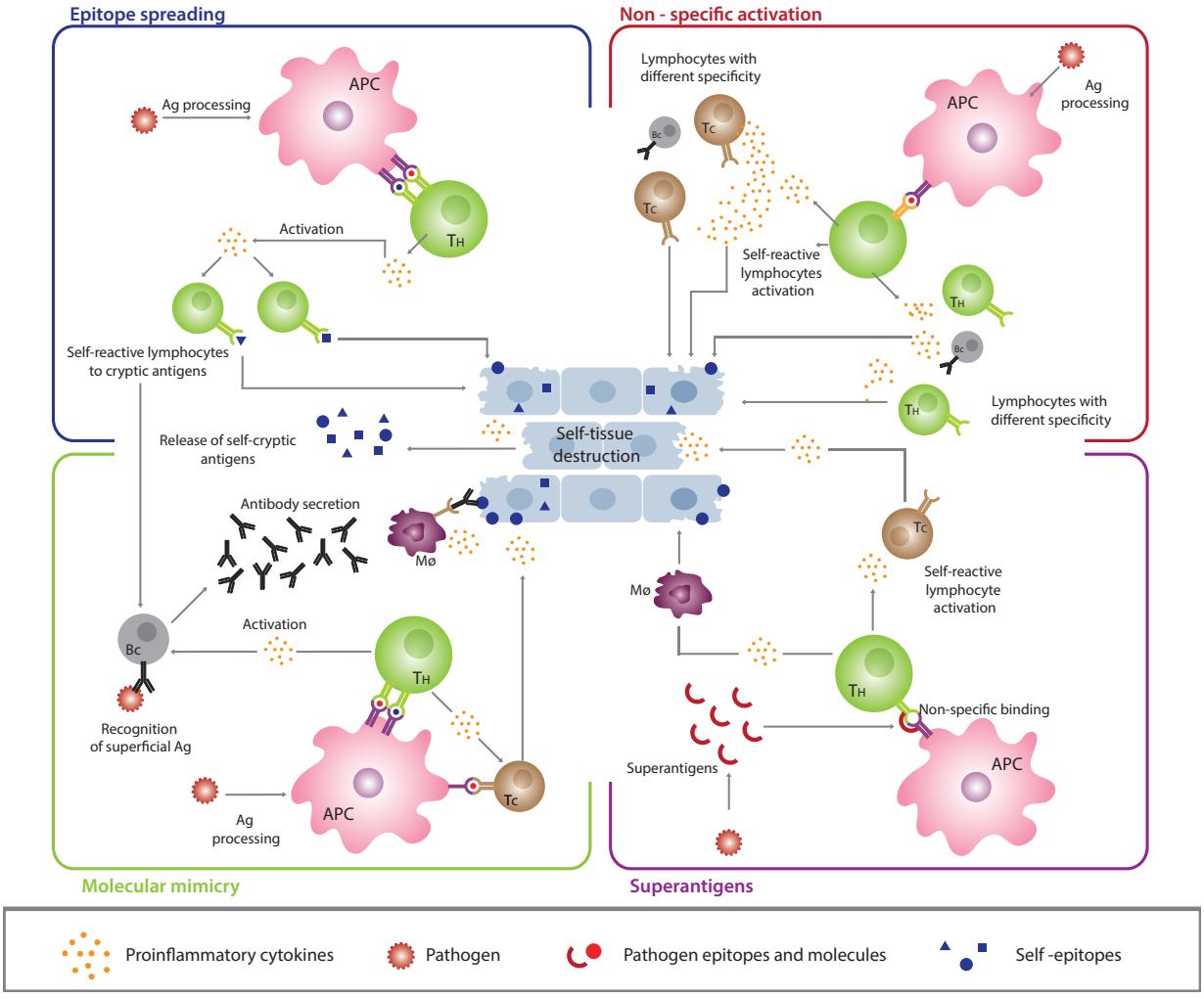

Figure 2. Infectious mechanisms of autoimmune disease. Molecular mimicry: Activation of self-reactive cells that may recognize pathogen and self-epitopes. Antigenic peptides are processed by antigen presenting cells (APC). As a result, peptides are presented to T helper cells (TH). Then, TH cells can activate T cytotoxic (Tc) and B cell (Bc). Finally, this concludes in the destruction of tissue and the release of self-antigens. Hence, auto-reactive cells may recognize self antigens and the activation of macrophages (Mφ). Epitope spreading: This is the result of the destruction of a tissue as a consequence of a persistent infection. The large quantity of inflammatory molecules can lead to the damage of the tissue and the release of self peptides that may be recognized by self-reactive cells that lead to the destruction of the tissue. Bystander activation: Refers to the activation of auto-reactive cells as a consequence of the constant stimulation of the immune system done by acute infection and the release of pro-inflammatory cytokines. Superantigens: These are proteins produced by pathogens that can induce the activation of T cells. Indeed, they induce the nonspecific binding of T cell receptor (TCR) and the HLA of the CPA. As a result, many T cells are activated, some of them potentially self-reactive. Adapted from Sfriso *et al.* (61).

β2-GPI AND TETANUS TOXOID: MOLECULAR MIMICRY

APS is an autoimmune multisystemic disease mainly characterized by recurrent fetal loss and thromboembolic phenomena together with the presence of antiphospholipid antibodies. These antibodies recognize negatively charged phospholipids, mainly through cryptic antigens of the β2-glycoprotein I (β2-GPI) that are exposed after the protein is unfolded. Molecular mimicry is one of the mechanisms that may induce experimental APS when it is associated with certain pathogens, e.g., *Haemophilus influenzae, Neisseria gonorrhoeae*, CMV, and the tetanus toxoid (22,23).

The β2-GPI molecule is involved in many important processes in the human body such as coagulation where it is related to induction of platelet aggregation, inhibition of prothrombinase activity, and production of platelet factor IX. β2-GPI has been identified as one of the most important antigens in APS and consists of 326 amino acids organized in five domains. The first four domains have conserved sequences, and the fifth contains a sequence that consists of 20 positively charged amino acids in the C-terminal loop ending. This loop constitutes the patch that determines the affinity for anionic phospholipids. It has been demonstrated

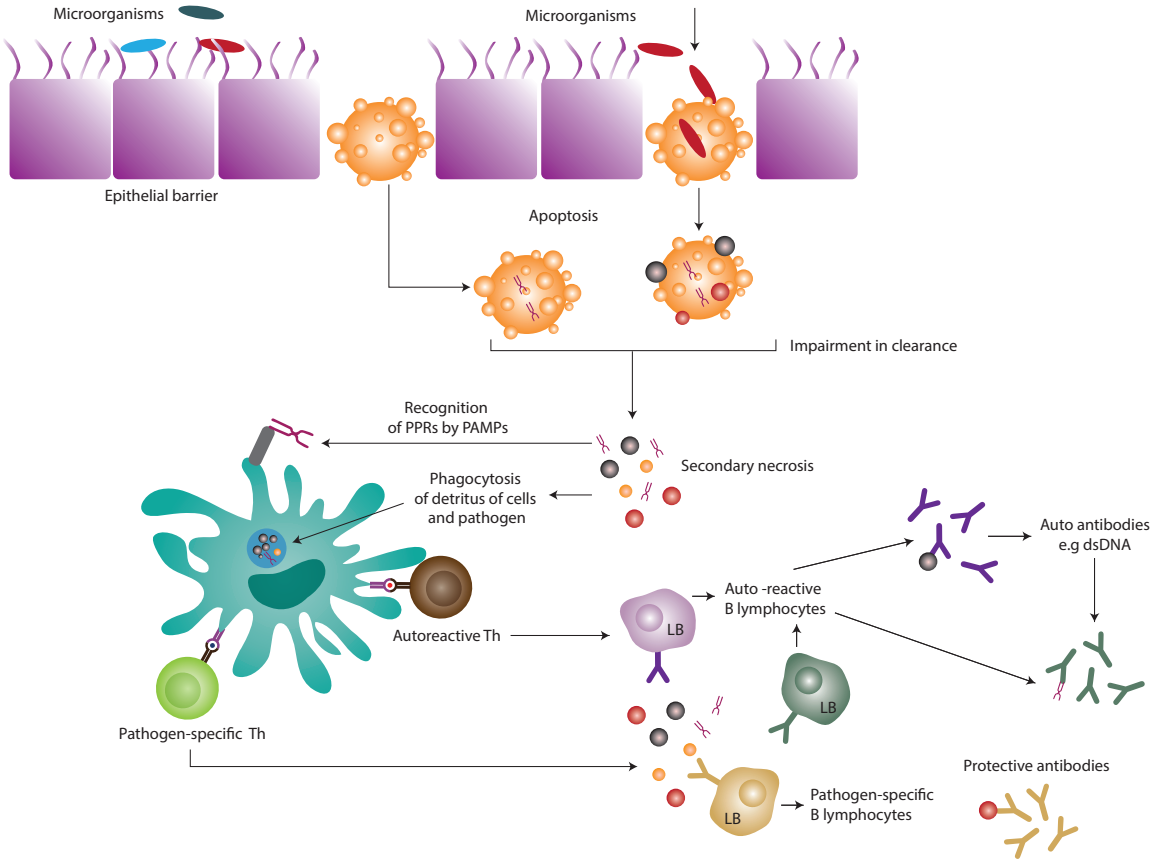

Figure 3. Clearance deficiency. Microorganisms may induce the apoptosis of cells as part of their pathogenic process. In systemic lupus erythematosus (SLE) there are defects in the mechanisms of clearance of apoptotic bodies. As a consequence, there is an accumulation of secondary necrosis material and release of auto-antigens and nuclear material. These auto antigens may be presented to auto-reactive T cells or recognized by auto-reactive B lymphocytes. Finally, this concludes in the autoimmune response, e.g., presence of anti-dsDNA. Adapted from Schulze *et al.* (63).

that the interaction between β2-GPI and phospholipids induces conformational changes resulting in the exposure of cryptic epitopes within other domains that will be recognized by antibodies. Indeed, this is essential to the exposure of a potential auto-antigen (23).

Tetanus toxoid, in turn, is a potent exotoxin produced by the bacteria *Clostridium tetani*. The active form of the toxoid is made up of two chains. It affects motor neurons by binding to cell membranes with its carboxyl terminal on the heavy chain. The infection may be prevented through a vaccine, which is composed of the tetanus toxoid and an adjuvant (usually aluminum hydroxide). However, the vaccine may induce the production of antibodies against the toxoid that can react to other auto-antigens, e.g., cardiolipins and antiphospholipids (23,64).

A human hybridome (H3) was produced from a boosted healthy individual with diphtheria and tetanus toxoid. The monoclonal antibody demonstrated a cross reaction with human β2-GPI. Consequently, the analysis of potential epitopes by a phage display library proved that the isolated monoclonal antibody recognizes a linear epitope (TLRVYK) that is found in the third domain of the β2-GPI (23,65–67). The TLRVYK was found to be located once on the β2-GPI and three times as conformational epitopes on the tetanus toxoid by bioinformatic analysis (Figure 4) (23,68). Therefore, molecular mimicry is a mechanism that favors APS development. In addition, studies with animal models have shown the induction of symptoms and the disease in the animals immunized with H3. Furthermore, murine model treated with this peptide did not develop the disease.

ADs PREVENTION: HELMINTHS AND INDUCTION OF TH2

Infections may prevent the development of autoimmunity or even withdrawal of an autoimmune process. This happens as a result of the interaction between microorganisms and the host (1,69). Part of the evolutionary goal of infectious agents is to stay in their host as long as possible. Thus, parasites such as helminths are a clear example of co-evolution. These microorganisms modulate the immune response towards an anti-inflammatory profile which favors their survival in the host. Thus, helminths are suppressors of the immunological pro-inflammatory process (69,70).

Over the last few decades, quality of life has improved in the developed countries. This has decreased the preva-

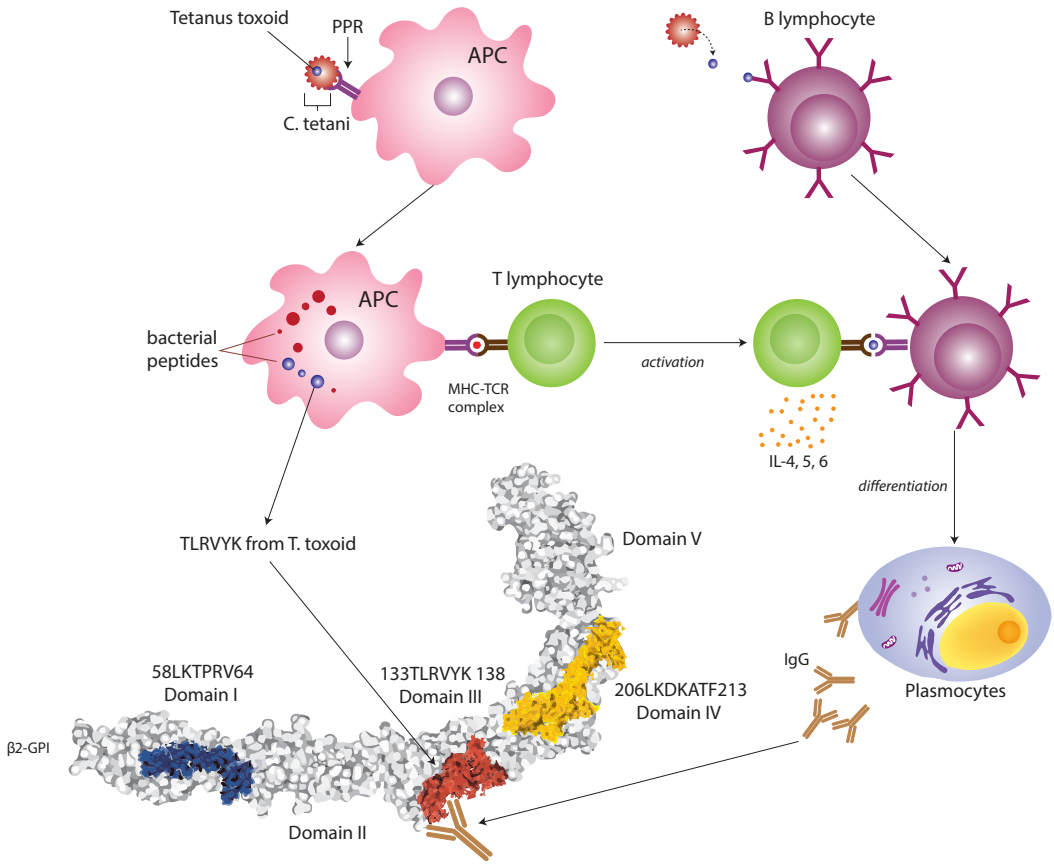

Figure 4. Molecular mimicry and anti-β2-GPI: Production of antibodies against β2-GPI by the presence of the tetanus toxoid. As a consequence of the recognition and destruction of the bacteria, there is presentation of bacterial peptides. The peptide that is shared by the toxoid and the β2-GPI may be among them. As a result, there is production of antibodies that recognize both molecules. Adapted from Cervera *et al.* (57).

lence of infectious diseases, especially those related to parasites. According to the hygiene hypothesis, the increase in atopic disorders, allergies, and even ADs are the result of the reduction in exposure to microorganisms and parasites during childhood (71,72). This was demonstrated by the geographic relationship observed worldwide. For instance, in places where there is a low prevalence of parasite infections, the prevalence of ADs is rising (71–74). Many inversed associations have been reported between parasitic infections and protection from ADs (Table 4). For example, the *Schistosoma mansoni* infection shows a protective effect against the development of T1D (70,75). Therefore, microorganisms should be recognized as not only a cause of infection and disease, but also potent immune system modulators.

Helminths may stay in their host for long periods of time making themselves successful pathogens. Essentially, parasites are able to change the cytokine profile from a pro-inflammatory to an anti-inflammatory profile. This change creates the perfect environmental conditions for them to survive and extend their lives within the host. Research using both human and animal models has shown that helminths can modulate the innate and the adaptive immune response. In the case of autoimmune and inflammatory diseases, the presence of parasites may induce an anti-inflammatory profile that prevents the pathological inflammatory process. First of all, they may promote the inhibition of IFNα, IL-1β, and IL-17 to suppress the Th1 and Th17 response (see Chapter 9). Secondly, they promote the production of IL-4, IL-10, TGF-β, and the activation of regulatory cells including Treg, Breg, regulatory dendritic cells, and macrophages (74,76–78).

Helminths infect their host mainly through the gut. Characteristically, there is an abundance of macrophages in the intestine which may be activated by the parasites many different ways. In a helminth infection, parasites induce the production of IL-10 and Th2 cytokine profile including IL-4, IL-5, IL-9, and IL-13 (79). Furthermore, this cytokine profile stimulates macrophages to become alternative activated macrophages (AAM). AAM express specific molecules such as arginase-1, RELMa, YM11, and TGF-β. At the same time, they have a decreased expression of IL-12 and IFNγ (Th1 cytokines) (76).

AD	HELMINTH
RA	*Schistosoma mansoni* *Ascaris suum* *Heligmosomoides polygyrus bakeri* *Nocardia brasiliensis* *Schistosoma japonicum* *Hymenolepis diminuta*
MS	*Schistosoma mansoni* *Schistosoma japonicum* *Trichinella spiralis* *Fasciola hepatica* *Heligmosomoides polygyrus bakeri*
T1D	*Schistosoma mansoni* *Heligmosomoides polygyrus bakeri* *Taenia crassiceps* *Trichinella spiralis*
IBD	*Schistosoma mansoni* *Schistosoma japonicum* *Trichinella spiralis* *Heligmosomoides polygyrus* *Hymenolepis diminuta* *Enterobius vermicularis* *Necator americanus*
CD	*Schistosoma mansoni* *Heligmosomoides polygyrus bakeri* *Trichinella spiralis* *Hymenolepis diminuta* *Trichuris muris*

Table 4. Helminth anti-inflammatory effect in ADs. Adapted from Kuijk *et al.* (76) and Elliott & Weinstock (80). *AD*: Autoimmune disease, *RA*: rheumatoid arthritis, *MS*: Multiple sclerosis, *T1D*: diabetes type 1, *IBD*: inflammatory bowel disease, *CD*: Crohn's disease.

There is production of different molecules during helminth infections. Some of them are essential for the parasite life cycle or structure. These molecules include proteins, lipids, glycoproteins, and glycolipids which probably have a regulatory effect on the host immune system (Table 5). Helminths may also produce molecules that mimic host cytokines due to structural similarities. There are two clear examples of this mechanism. The first one is the transforming growth factor homologue-2 (TGH-2) which is produced by *Brugia malayi* and binds to the TGF-β receptor (77,78). Secondly, some helminths produce macrophage migration inhibition factors (MIFs) which activate AAM. The result is that the anti-inflammatory profile (Th2) is induced which favors the survival of the parasite and the eosinophilic response (77).

The dendritic cell is the pivotal point to determine the adaptive immune response. This response will depend on the stimulation of a specific receptor (TLR, NLR, NOD-like, or CLRs). CLRs, in particular, recognize specific glycan parasite profiles, and after their activation, the result is Th2 cytokine polarization and the anti-inflammatory process (79,80). For example, the phosphorylcholine (PC) has been shown to be a potent immunomodulator. It contains the glycoprotein ES-62 from *Acanthocheilonema viteae*, which suppresses murine arthritis in vivo and improves the maturation of DC towards a Th2 cytokine profile (80).

Finally, regulatory cells are activated due to all these interactions and have a direct effect on the auto reactive B and T cells (Figure 5). T regulatory cells increase after a helminthic infection. This has been reflected in higher amounts of CD4+CD25+FOXP3+ cells that can release IL-10 and TGF-β.

MICROBIOTA AND ADs

There are different microorganisms that populate the gut known as intestinal microbiota. This process starts at the time of delivery and breastfeeding, and it plays an important role in the homeostasis of the human body. Indeed, there are microorganisms in the microbiota that produce enzymes and molecules which are not generated by human beings. Therefore, microbiota is important to normal metabolism because it is capable of degrading the different components in food (81,82). However, microbiota may also influence other systems that do not seem to be related to the gut such as in the case of the immune system. Moreover, it actively participates in the education of the immune system. For example, the development of Th17 and Treg lymphocytes is highly dependent on the interactions of commensal bacteria with host cells in the intestine. That is why it is possible to establish a connection between microbiota and autoimmunity or inflammatory disorders (81–84).

Studies of germ free (GF) animal models have demonstrated deficiencies in their immune system. It is noteworthy that microbiota is the first barrier against pathogenic microorganisms. They may produce molecules against the pathogens during infection because they occupy the same niche, thus competing for the same place. GF mice show deficiencies in T lymphocyte differentiation within the lamina propria in the presence of IgA in mucosal layers and alterations in the homeostasis of Th populations (Th1, Th17, and Treg). Furthermore, GF mice spleens show few germinal centers which indicates abnormal development and maturation of cells in the lymphoid follicles (81–83). As a result, the cytokine production and the normal maturation process of immune cells are greatly affected (82).

Microbiota varies from one individual to another and even in the same individual over the course of his life. However, there are many factors that influence the composition of microbiota. Initially, newborns are sterile, and their microbiota depends on maternal transfer at the time of delivery, breastfeeding, and skin contact with the mother. For example, there are differences between children that were born by natural delivery and caesarean section, and also between children that were breastfed and those fed with formula. The latter group in both cases is colonized by potential pathogens and, in contrast with first group, they present a lack of beneficial commensal microorganisms (82,85). Nevertheless, their own genetic background plays an important role in determining the microbiota before contact with the mother's microbiota. For example, animal

HELMINTH	MOLECULE	IMMUNOREGULATORY MECHANISM
Schistosoma mansoni	LNFPIII and SEA	Interact with TLR4 to produce Th2 profile by DCs
	Lysophosphatidylserine	Interact with TLR2 to induce Treg polarizing DCs
Acanthocheilonema viteae	ES-62	Exert immunomodulatory effects on macrophages and DCs by a TLR4-dependent mechanism with consequent Th2 polarization
Nippostrongylus brasiliensis	NES	Potently induce Th2 type of response via DC
Brugia malayi (adult)	Cystatins - CIP-2	Interfere with antigen processing in human cells and inhibit B cells
	Cytokine homologue MIF-1/2	Alternatively activate macrophages
Brugia malayimicrofilariae	Serpins - SPN-2	Block neutrophil protease and promote Th1 type of response
Brugia malayi L3 (larvae)	ALT-1/2 proteins	Inhibit macrophage resistance and present a good filarial vaccine candidate
Toxocara canis	TES32—C type lectin	Inhibit TLR responses on DC and compete with host lectins for ligands, thereby blocking host immunity
Heligmosomoides polygyrus	HES	Induce regulatory T cells through TGF-β Receptor
Teladorsagia circumcincta	Excretory-secretory antigen	Induce generation of Foxp3+ regulatory T cells through TGF-β mimicking effect
Trichinella spiralis	AdES, NBL and MLCr	All antigens from different life stages induce polarization towards mixed Th1/Th2 with predominance of Th2 response, via semimatured DC
	Excretory secretory muscle larvae antigen	Induce mixed Th1/Th2 response with the predominance of Th2 component and elicit regulatory arm of immune response
	Excretory secretory muscle larvae antigen	Interfere with LPS-induced DC maturation and induce expansion of Foxp3+ regulatory T cells
Fasciola hepatica	Thioredoxin peroxides	Alternatively activated macrophages

Table 5. Helminth derived – molecules with immunoregulatory properties. Adapted from Aranzamendi *et al.* (78) *LNFPIII*: Lacto-N-fucopentaoseIII, *SEA*: soluble egg antigen, *TLR*: Toll like receptor, *DC*: dendritic cells, *NES*: Excretory-secretory antigen, *Cystatins*: cysteine protease inhibitors, *MIF*: migration inhibitory factor, *Serpins*: serine protease inhibitors, *ALT*: Abundant larval transcript, *HES*: Excretory-secretory antigen, *AdES*: Adult excretory-secretory antigen, *NBL*: newborn larvae antigen, *MLCr*: crude muscle larvae antigen, *LPS*: Lipopolysaccharide.

models with gene alterations related to the innate immune system have problems in the signaling pathways associated with PRR recognition (e.g., TLRs and NODs). These models also have important differences in the pattern of microbiota microorganisms compared to wild type animals (81–83).

In addition, the evolution of medical treatment, stress, and quality of life influence microbiota development. For example, antibiotics are the treatment for infectious diseases, but their use is linked with the loss of beneficial microorganisms. Moreover, they alter the microbiota ecology because they affect the equilibrium between different bacterial species in the gut, which allows opportunistic pathogens to attack the body (82). Finally, diet plays a central role in the homeostasis of the microbiota because it defines which microorganisms can survive in the gut due to differences in the preferences of microorganisms for energy sources. Thus, diet composition is extremely important in microbiota maintenance. Components from plants are the energy source for beneficial microbes and promote their growth over other microbes. It has been suggested that differences in the modern western diet could be causing the rapid increase in diseases such as asthma (82,86). For example, one study shows how a switching from a low fat, vegetable rich diet to a high fat, high sugar diet could alter the microbiota within one day (82,87).

Altogether, there is a lot of variability in microbiota between individuals, and it also varies based on the anatomic area of the human body (Table 6) (88). Fluctuations in microbiota population have been described in patients with ADs (81). In addition, most of the studies have established a relationship between the microbiota inhabiting the gut and its influence on health. Usually, microorganisms that live in the gut are not pathogenic under healthy conditions, and they have a positive effect on the host (83). Nevertheless, some commensal bacteria may drive the preferential development of Treg while others promote Th17 response and inflammation. These bacteria favor the production of regulatory molecules and cytokines, e.g., Foxp3 and IL-10, which characterize the regulatory cells, Treg in particular. Specifically, species such as *Bacteroides fragilis* and the genus Lactobacillus and *Bififobacterium* greatly promote the presence of Treg in the gut. In contrast, a pathogenic phenotype characterized by Th17 response and pro-inflammatory cytokines is promoted by segmented filamentous bacteria such as *Firmicutes* (Figure 6). Moreover, it has been demonstrated that this kind of bacteria is able to induce the production of IgA in the small intestine. Th17 response certainly has its own positive role in the case of infection control, but it is also critical in the development of inflammatory and autoimmune diseases (81–83,88).

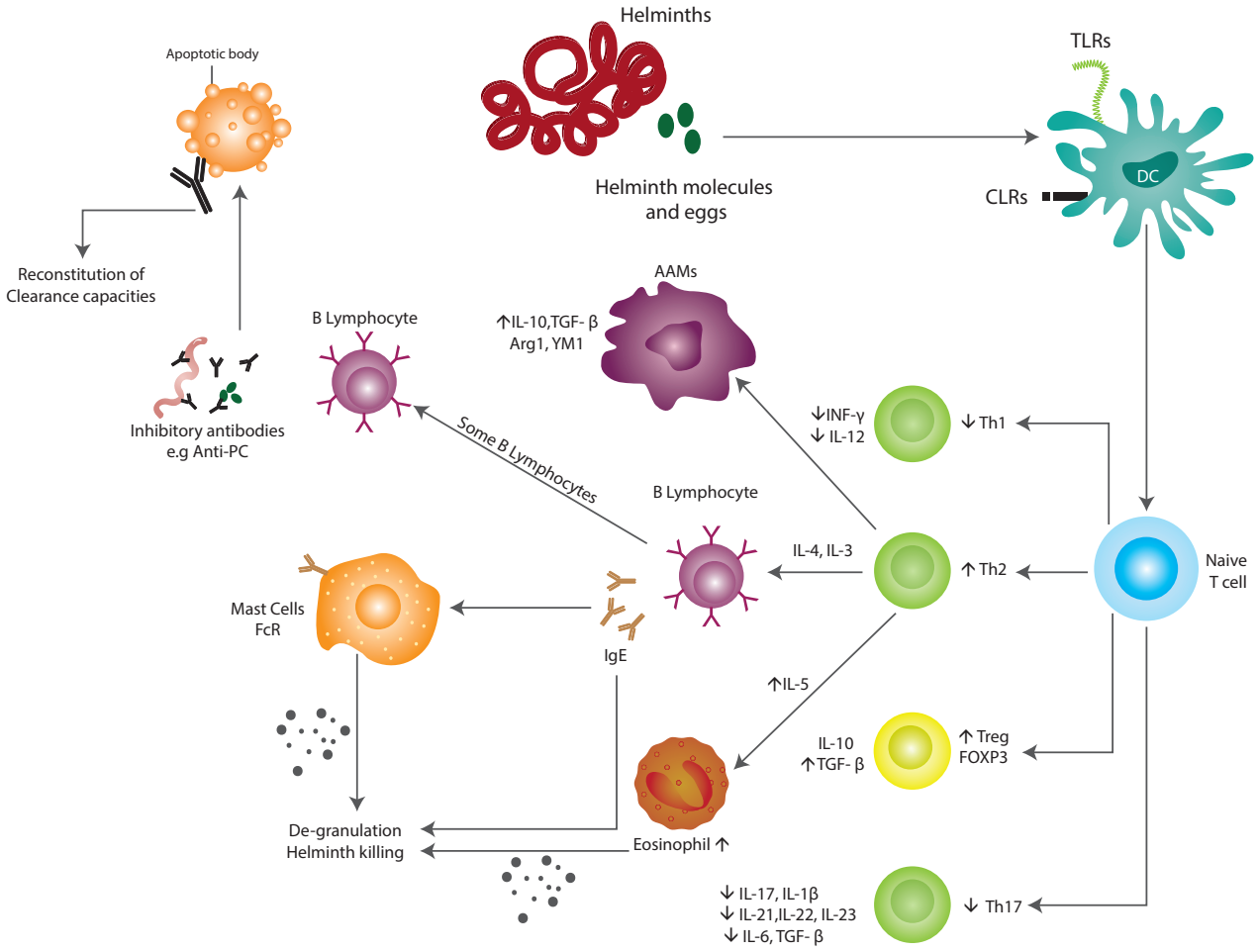

Figure 5. Immune response to helminths and protection from autoimmunity. The exposure to helminths induces changes in the innate immune system—mainly dendritic cells (DC) and macrophages that become alternative activated macrophages (AAM). These changes are mainly in the CLRs and TLRs expressed in membranes. As a consequence, recognized helminth molecules restrict the immune response to Th2 profile by blocking Th1 and Th17 response (pro-inflammatory cytokines). This usually leads to the activation of mast cells, production of IgE, and activation and de-granulation of eosinophils and AAMs—the typical response to parasites. Helminths, in turn, can stimulate a regulatory network characterized by Th2 profile and Treg activation that produce IL-10 and TGF-β. In addition, the production of antibodies to helminth molecules such as Phosphoril-choline (PC) may be helpful in the remediation of clearance deficiency (SLE). The apoptotic bodies and secondary material of dead cells have PC, and these molecules can be recognized by anti-PC. As a result, they can bind apoptotic bodies that can be degraded later. Adapted from Ben-Ami *et al.* (74).

	ACTINOBACTERIA	FIRMICUTES	PROTEOBACTERIA	BACTEROIDETES	CYANOBACTERIA	FUSOBACTERIA
Hair	++++++	++	++	+	+++	-
Nostril	+++++	++++	++	+	+	-
Mouth	++	++++	+++	++++	+	++
Oesophagus	+	++++++	+	+++	-	+
Skin	+++++	+++	+++	++	-	-
Stomach	++++	+++	++	++	-	+
Vagina	+	++++++	+	++	-	+
Colon	++	+++++	+	++++	-	-

Table 6. Anatomic site microbiota composition. Adapted from Cho & Blaser (88).

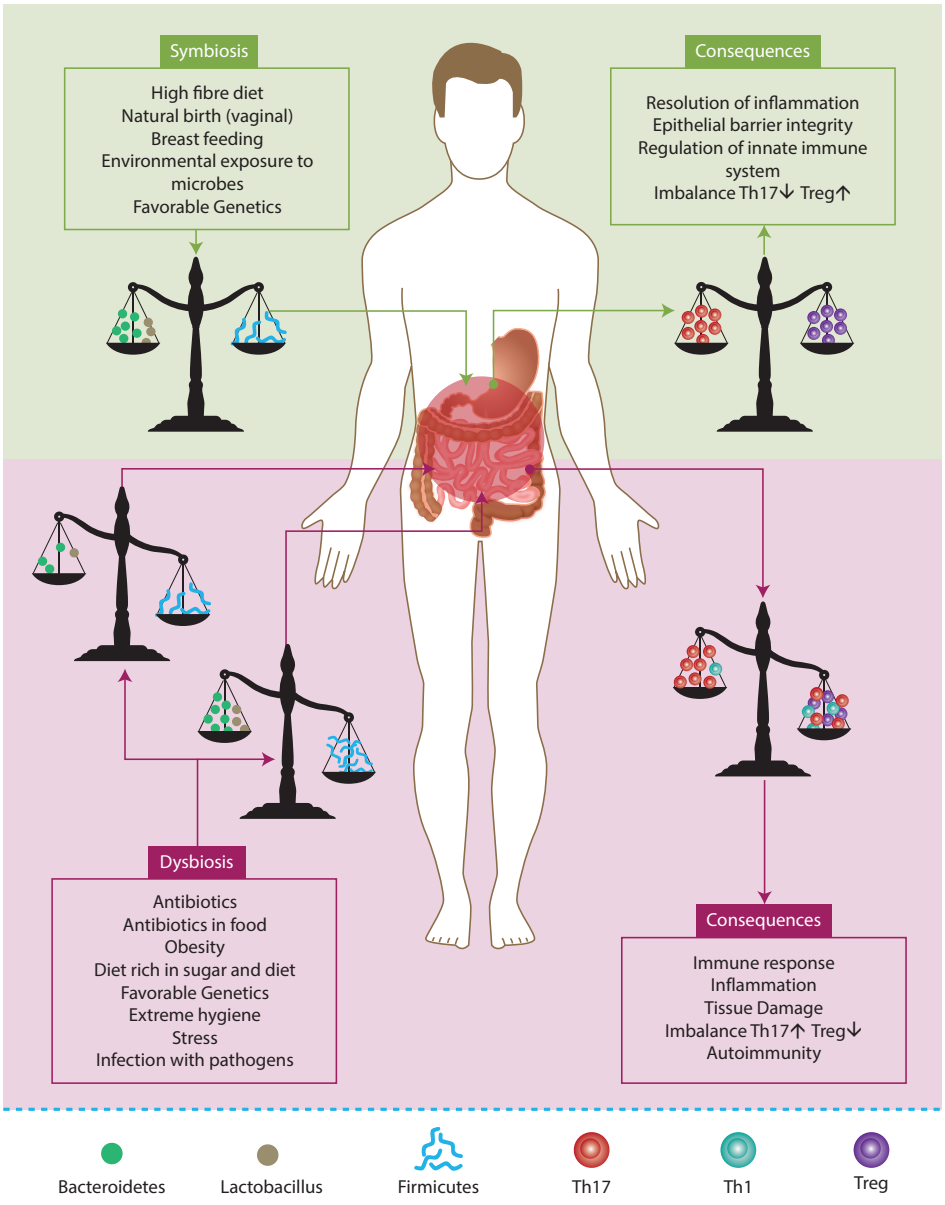

Figure 6. Factors influencing gut microbiota. A balancing microbiota in symbiosis influences the equilibrium between T regulatory cells (Treg) and effector T cells (Th1, Th17). As a consequence, the gut can respond to an infection but this also leads to the permanence of microbiota and favors the host. In contrast, if the environment induces the rupture of the equilibrium, it induces a state of dysbiosis. Hence, the percentage of anti-inflammtory microorganisms (e.g *Bacteroidetes* and *Lactobacillus*) may be lower than pro-inflammatory microorganisms (e.g, *Firmicutes*). Eventually, this may lead to an inflammatory profile that favors the development of autoimmunity. Adapted from Kranich *et al* (82) and Ivanov *et al.* (83).

Therefore, any change in their environment may induce a dysbiosis and a pathological event (Figure 6). At present, the use of antibiotics such as ampicillin, gentamicin, and vancomycin may affect the balance toward bacteria that stimulate the Th17 response. At the same time, the consumption of a diet rich in sugar and fat favors a rise in the population of *Firmicutes* and a reduction in the *Bacteroidetes* (81,82,88). Finally, the influence of microbiota on the immune balance does not depend exclusively on the presence of the microorganisms but also on molecules that are produced by microbes and can stimulate specific pathways associated with immune tolerance (82). An example is the anti-inflammatory effect of the short-chain fatty acids, e.g., acetate, propionate, and butyrate. These fatty acids can bind to the G protein/coupled receptor (GPR43 and GPR120) which is expressed in most of the innate immune system cells and thus produce an anti-inflammatory action (82). The anti-inflammatory events induce the maintenance of the epithelial barrier, regulate apoptosis, diminish oxidative DNA damages, and regulate cytokine production, phagocytosis and neutrophil recruitment. At the same time, the peptidoglycan and the polysaccharide A teach the immune system to recognize potential pathogenic bacteria, and they also help in the correct development of balanced T cell response in the gut. This balanced response promotes cell-cell interaction by modification of protein expression in immune cells (82).

METAGENOMICS

Metagenomics is the study of the genomes in a specific environment. Human beings can be considered either a su-

perorganism or an ecosystem. For instance, there are more microorganisms living in human bodies than cells themselves. Therefore, commensal and pathogenic microorganisms are considered biomarkers for specific conditions, and they may be a therapeutic option due to their potential immunoregulatory roles. As a consequence, different studies in the last few years described the human microbiome as a changing ecosystem that has many factors in the model (Figure 7a). Most of these studies try to determine what these microorganisms are and how they vary over time. However, the study of these microorganisms represents a challenge in terms of methodology because their identification was done by culture, and most of them cannot be grown in laboratory conditions. Therefore, most of them are now being studied with the help of DNA high-throughput analysis. Indeed, analysis of the entire DNA allows the study of all the organisms present in a sample (89,90).

There are different strategies for analyzing data. Currently, the sequencing of the 16S rRNA gene has made it possible to describe prokaryote taxonomic diversity. This gene has been used for this type of analysis for three main reasons. Primarily, it is present in every population member due to its essential role in protein translation in all prokaryotes. Secondly, 16S rRNA always and only differs between individuals with different genomes. Thirdly, it is considered an evolution marker between species because its function is extremely important, so any change in its sequence can be potentially lethal. Therefore, the 16S rRNA sequence analysis brings us to the construction of OTUs (operational taxonomic units) based on the percentage of similarity between sequences. Then, comparison between multiple databases leads to the identification of species within a sample. Finally, the population becomes a 16S rRNA sequence collection where the number of unique sequences represents the number of microorganisms. At the same time, the quantity of each one can show their distribution within the sample (89–92) (Figure 7b).

Furthermore, high-throughput DNA sequencing technologies have recently become very useful for metagenomic analysis (i.e., pyrosequenciation). Thus, different strategies for different types of genome assembly and annotation within a sample are used to ensure an accurate description (93). In addition, potential proteins encoded in each genome can be analyzed based on DNA sequence. As a result, it is possible to assign a role or function for a specific microorganism within the microbiome based only on their genome capabilities (89) (Figure 7b).

In the end, due to the current metagenomic results, the analysis of the microbiome is moving forward and new OMICS have been identified. However, the real involvement in host systems does not depend exclusively on the presence of a specific microbiome. For instance, metagenomics would be just a representation of what these microorganisms are. Moreover, molecules produced by microorganisms can interact with the host at different levels leading to diverse outcomes. In consequence, it is important to study gene expression (metatranscriptomics), proteins (metaproteomics), and metabolites (metametabolomics) from a microbiome. These analyses including possible interactions within the host system and under certain conditions can settle the real mechanisms by which specific microorganisms (present in a particular microbiome) may influence the host system and thus lead to complex disease modulation such as ADs (90) (Figure 7c).

CONCLUSION

The relationships between infection and ADs and their main mechanisms have been outlined (i.e., host-guest interaction). Nonetheless, most of the interactions and mechanisms that influence this relationship are still unknown. Microorganisms may alter and deregulate gene transcription, translation, and human metabolic processes. This means that the effect induced on the host by a microorganism is not caused by the presence of the microorganism itself but also by the metabolic and genetic polymorphism of the microorganism. In particular, intracellular pathogens may have a direct influence on gene regulation and protein expression inside host cells (85,94).

In the last few years, most of the large cohort studies have evaluated genetic factors that may predispose to ADs. In addition, expression and proteomic analysis have also been done, and most of them have tried to find a way to establish predictor genetic factors for the diseases. However, these studies do not take into consideration the DNA, RNA, and proteins from microorganisms that could be considered potential "contamination," and which should be considered a source of information that would help complete the overall picture of molecular interactions between infection and ADs and make it understandable (85,95,96).

Last but not least, familial autoimmunity and polyautoimmunity should be incorporated into the study of infection and ADs (97–99). ADs may be associated with a specific family group exposed to a particular environment. Within this environment, family members will be in contact with the same microorganism, and they will develop the same microbiota. As a new common mechanism for ADs, microbiota can be "inherited" by the child from parent, and it can also be shared among siblings (88).

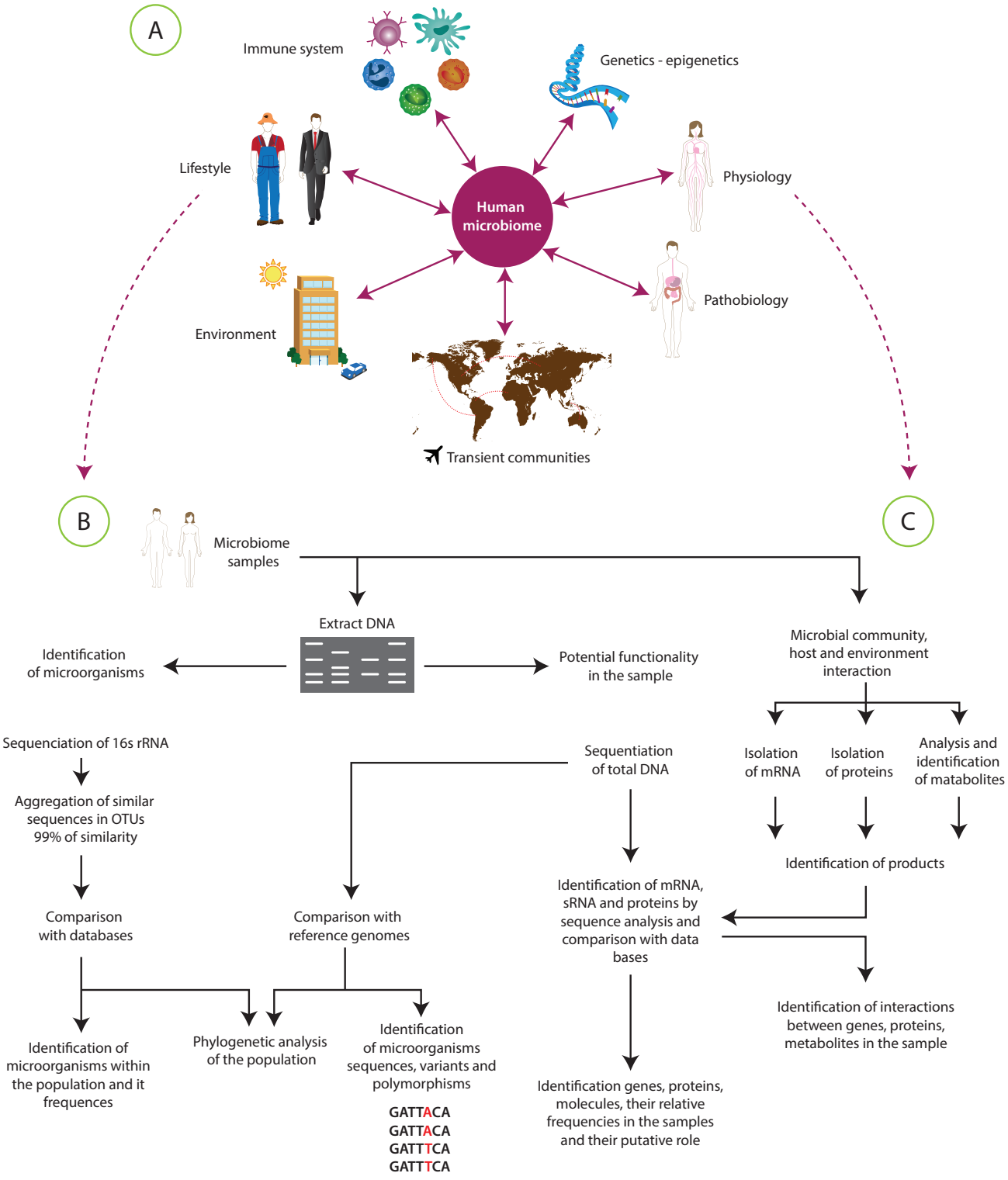

Figure 7. Metagenomic approximations. A. Factors that influence the human microbiome composition. B. Metagenomic analysis of a human sample allows species, function, and phylogeny identification. C. Metatranscriptomic, Metaproteomics and Metametabolomics: Other omic analyses that can help us understand the complex relationship between microbiome and host. Adapted from Morgan *et al.* (90).

ABBREVIATION LIST

- **AAM:** Alternative activated macrophages
- **AdES:** Adult excretory-secretory antigen
- **ADs:** Autoimmune diseases
- **ALT:** Abundant larval transcript
- **APC:** Antigen presenting cell
- **APS:** Antiphospholipid syndrome
- **β2-GPI:** β2 glycoprotein 1
- **Bc:** B cell
- **Breg:** B regulatory cell
- **CD:** Crohn's disease
- **CLR:** C-type lectin receptor
- **CMV:** Cytomegalovirus
- **Cystatins:** cysteine protease inhibitors
- **DC:** Dendritic cell
- **DNA:** Deoxyribonucleic acid
- **EBV:** Epstein-Barr virus
- **EA-EBV:** Early antigen of EBV
- **FOXP3:** Forkhead box P3 transcription factor
- **GF:** Germ free
- **GPR:** G protein/coupled receptor
- **H3:** Human hybridome 3
- **HES:** Excretory-secretory antigen
- **IBD:** Inflammatory bowel disease
- **IFNα:** Interferon alpha
- **IFNγ:** Interferon gamma
- **IL:** Interleukin
- **LNFPIII:** Lacto-N-fucopentaoseIII
- **LPS:** Lipopolysaccharide.
- **MBP:** Myelin basic protein
- **MIF:** Macrophage migration inhibition factor
- **MLCr:** Crude muscle larvae antigen.
- **MS:** Multiple sclerosis
- **Mφ:** Macrophages
- **NBL:** Newborn larvae antigen
- **NES:** Excretory-secretory antigen
- **NLR:** Nod-like receptor
- **NOD:** Nucleotide-binding oligomerization domain receptor
- **OTUS:** Operational taxonomic units
- **PC:** Phosphorylcholine
- **RA:** Rheumatoid arthritis
- **RNA:** Ribonucleic acid
- **rRNA:** Ribosomal ribonucleic acid
- **SEA:** Soluble egg antigen
- **Serpins:** Serine protease inhibitors
- **SLE:** Systemic lupus erythematosus
- **SS:** Sjögren's syndrome
- **T1D:** Type I diabetes
- **Tc:** T cytotoxic cells
- **TGF-β:** Transforming growth factor beta
- **TGH-2:** Transforming growth homologue-2
- **Th:** T helper cells
- **TLR:** Toll like receptor
- **Treg:** T regulatory cell

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20

AUTOIMMUNE ECOLOGY

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INTRODUCTION

Environment now extends far beyond the bounds of the local environment. It now includes the intimate enclosure of the individual or a local human population and the global domain of the human species. *Ecology* is a discipline focused on studying the interactions between an organism of some kind and its environment. Ecology was first described as a separate field of knowledge in 1866 by the German Zoologist Ernst Haeckel, who invented the word Ecology for "The body of knowledge concerning the economy of Nature –the investigation of the total relations of the animal to its inorganic and organic environment." The word ecology is derived from the Greek *oikos*, meaning "household," and *logos*, meaning "study." Thus, the study of the environmental house includes all the organisms in it and all the functional processes that make the house habitable. Literally, then, ecology is the study of "life at home" with emphasis on "the totality or pattern of relations between organisms and their environment" (1, 2).

Perhaps the best way to delimit modern ecology is to consider the concept of levels of organization seen as an ecological spectrum (Figure 1) and as an extended ecological hierarchy. Hierarchy means "an arrangemet into a graded series." Interaction with the physical environment (i.e., energy and matter) at each level produces characteristic functional systems. According to the standard definition, a system consists of "regularly interacting and interdependent components forming a unified whole." Systems containing living (i.e., biotic) and nonliving (i.e., abiotic) components constitute biosystems and range from genetic systems to ecological systems. This spectrum may be conceived of or studied at any level, as illustrated in Figure 1, or at any intermediate position convenient or practical for analysis. For example, host-parasite systems or a two-species system of mutually linked organisms are intermediate levels between population and community (1).

Human ecology is the study of human populations, their social organization, the characteristics of the environments they inhabit, and the technologies they employ. Human ecology is concerned with systems rather than their components in isolation. From that perspective, disease is an integral part of the ecosystem and its eradication may significantly disturb an ecosystem (3).

Understanding patterns of health and disease requires that the focus in public health be on not only personal behaviors, biological traits and specific risks but also characteristics of the social and physical environments that shape human experience. At the end of the 20th century a challenge to dominance of biomedicine and the need to give greater emphasis to the ecological context in the study of human health and disease was growing. The emerging new paradigm of public health is based on a holistic perspective on health and on creating social and environmental conditions conducive for health. The new ecological model for fostering health (i.e., an integrated, "ecohealth" approach) focuses attention on both individual and social environmental factors as targets for fostering health. It addresses the importance of interventions directed at changing interpersonal, organizational, community, and public policy (4-6).

This chapter provides a brief overview of what we call the "Autoimmune Ecology" which includes all of the above mentioned aspects of the global science of ecology applied to the development of autoimmunity. At present, current scientific methods are inadequate to tackle the causal puzzle for multiple related conditions [e.g., autoimmune diseases (ADs), obesity, metabolic syndrome, cardiovascular disease, type 2 diabetes, and Alzheimer's and Parkinson's diseases], that may be associated with lower socioeconomic circumstances (i.e., with poorer diet and housing and greater stress)

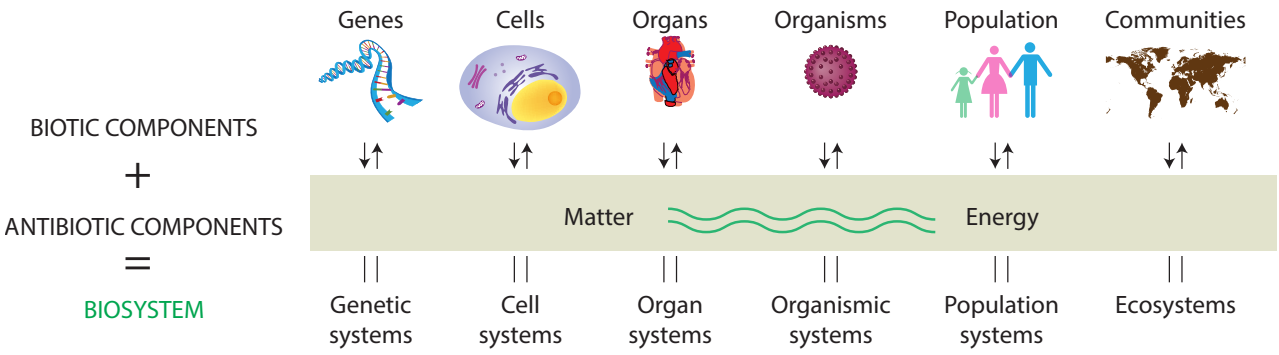

Figure 1. Ecological levels of organization emphasizing the interaction of living and nonliving components.

as well as with multiple potential contributory environmental factors (e.g., air pollution, heavy metals, and various endocrine disrupting chemicals). The focus will be on a group of specific toxicants and associated mechanisms that may be contributing to the development of the group of ADs.

CRITERIA AND EPIDEMIOLOGY FOR ENVIRONMENTALLY ASSOCIATED AUTOIMMUNE DISEASES

The autoimmune phenotype varies depending on the target cell and the affected organ, gender, ancestry, trigger factors and age at onset (7). Several environmental factors are common risk factors for ADs. Infectious agents are important in the pathogenesis of ADs since they are a major part of the ecology and environmental autoimmunity trigger (see chapter 19). Recently, a group of researchers from the National Institute of Environmental Health Sciences Expert Panel Workshop (8) has reviewed approaches for defining environmentally associated AD in three contexts: 1) identifying the necessary and sufficient data to define environmental risk factors for AD meeting current classification criteria; 2) establishing the existence of and criteria for new environmentally associated AD that do not meet current disease classification criteria; and 3) identifying specific environmental agents that induce AD in individuals, typically in a clinical setting. They highlight that additional efforts in all these areas are needed to achieve true consensus in this relatively undeveloped field and to define classification criteria that can distinguish environmentally associated AD cases from others with high sensitivity and specificity.

The panel of experts stresses that traditional epidemiologic approaches have relied on a number of features of association that were initially outlined by Hill in 1965. These include the strength of the association, consistency of findings across multiple studies, specificity, temporality, a biological gradient or dose response relationship, plausibility, coherence with current principles of biology and medicine, experiments showing that elimination of the suspect agent results in decreased disease incidence in the population, and analogy to similar conditions. These factors are shown in Table 1.

APPROACH	ELEMENTS TO CONSIDER	COMMENTS
Defining risk factors for autoimmune diseases meeting current classification criteria through epidemiology	Strength of association - Is the level of difference noted between risk of disease in exposed and unexposed populations strong?	Consensus on the number and types of the elements needed for confident, likely or unlikely associations is yet to be determined
	Consistency - How similar are results from multiple studies?	
	Specificity - How exclusive is the given association?	
	Temporality - Does the exposure precede the disease?	
	Biological gradient - Is there a dose response relationship?	
	Plausibility - Is there a biologic rationale?	
	Coherence - Are the findings consistent with current principles of biology and medicine?	
	Experiment - Does elimination of the suspect agent result in decreased disease in the population?	
	Analogy - Are there similar findings in related areas?	

Table 1. Epidemiologic approaches to identify environmental exposures and the development of autoimmune diseases as current classification criteria. Adapted from Miller *et al.* (8).

AGENT	COMMENTS
Chemical agents	Silica, asbestos, metals, pesticides, industrial chemicals and solvents, smoking, and personal care products (e.g., cosmetics and hair dyes)
Physical agents	Ionizing radiation, ultraviolet radiation (i.e., sunlight), and electric and magnetic fields
Biologic agents	Infectious agents, foods and dietary contaminants, molds, mycotoxins, and other toxins
Lifestyle and Social factors	Diet, exercise, stress, socioeconomic status, family, friends, health, employment
Psychological factors	Self-esteem, self-concept, relationships with family and friends, stress, cultural beliefs

Table 2. Environmental agents associated with autoimmune diseases. Adapted from Miller *et al.* (8).

Compared to the extent of genetic research, studies on environmental risk factors have received limited attention in many of these diseases. However, some areas of environmental research are relatively well developed, such as studies of the roles of silica and smoking, as detailed below. Miller *et al.* (8) have recently reviewed what is known with regard to the epidemiology of the relationship between environmental exposures and AD, and have prioritized topics for additional research. They divided environmental exposure into three broad classes (Table 2) and we included two additional aspects in order to establish the definition of autoimmune ecology (Figure 2).

Just as multiple genetic risk factors are important in the development of a given AD, it is likely that multiple environmental risk factors may need to be present at different periods of life, or in a specific temporal sequence, to induce the immune perturbations that result in AD (10). It is also likely that there are many ways or mechanisms by which similar clinical AD phenotypes develop, and that a given AD could have multiple environmental risk factors. Certain environmental agents could be risk factors for multiple AD. These concepts support the hypothesis that autoimmune ecology is a crucial component of the autoimmune tautology and reinforces that ADs share several common mechanisms (7-9).

AUTOIMMUNE ECOLOGY AND ITS PROPOSED MECHANISMS

Over the past 10 years, the National Institute of Environmental Health Science has participated in trans-NIH committees and sponsored a number of workshops examining the role of the environment in the development of AD. Despite the recommendations for research initiatives and the ongoing accumulation of research data, there are still numerous gaps in our knowledge of this field. In an expert panel workshop, Selmi *et al.* (10) analyzed the major categories of putative environmental agents involved in the process of ADs (Table 3).

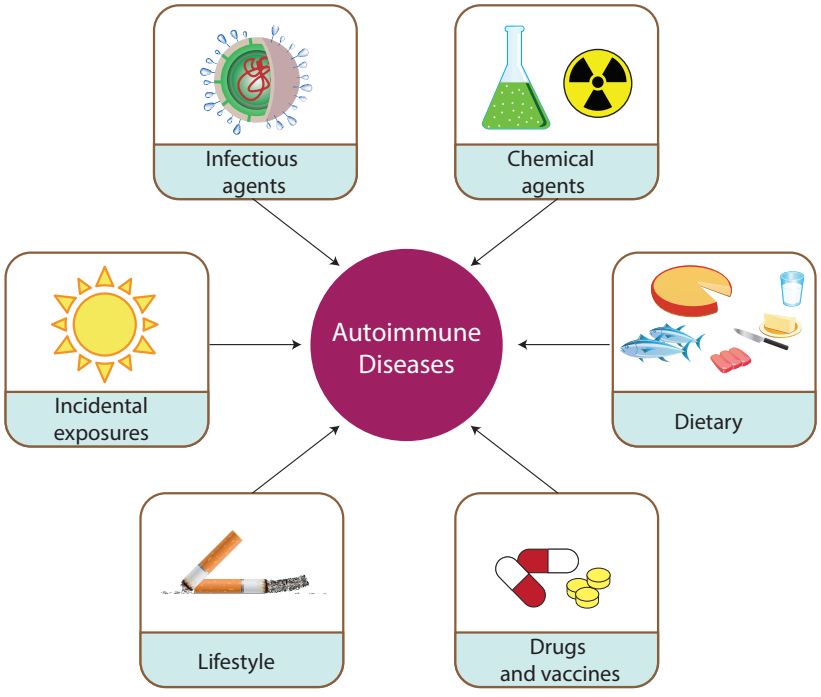

Figure 2. Environmental exposures associated with autoimmune diseases.

POSIBLE MECHANISMS
Polyclonal B cell activation
Direct effect impairing the immune response (e.g., Th17 cells)
Effects on innate immunity (e.g., TLR, adjuvants)
Direct interaction with regulatory cells (e.g., T regulatory cells)
Modification of self-antigens (i.e., post-translational modifications)
Alterations of DNA methylation (i.e., epigenetics)

Table 3. Proposed mechanisms involved in the breakdown of immune tolerance. *Th17*: type 17 lymphocytes; *TLR*: toll like receptors. Adapted from Selmi *et al*. (10).

Induction of systemic autoimmunity by drugs and chemicals requires a source of self-antigens. However an unanswered question, especially for intracellular antigens, is how and in what form the inciting self-antigens are made available to the immune system. For some toxicants this is very likely via mechanisms of cell death that may be reflective of the toxicant rather than an established biological process such as classical apoptosis (11).

Studies of idiopathic and environmentally induced systemic autoimmunity show that they are mediated by common adaptive immune response genes. In contrast, although the innate immune system is indispensable for autoimmunity, there are clear differences in the molecular and innate cell components that mediate specific systemic ADs, suggesting distinct autoimmune-promoting pathways. In idiopathic autoimmunity, some specific molecules (i.e,. type I IFN) are needed for disease with plasmacytoid dendritic cells (DC) being the primary cells involved. In contrast, in toxic-induced autoimmunity (i.e., pristane) the pathways involving IFN and proinflammatory cytokines do not require DC, but rather immature monocytes. These findings from several environmentally induced models suggest that environmental triggers can induce autoimmunity through diverse innate pathways. Figure 3 shows some of the main related interactions. A greater understanding of the specific innate processes that initiate or exacerbate disease will be

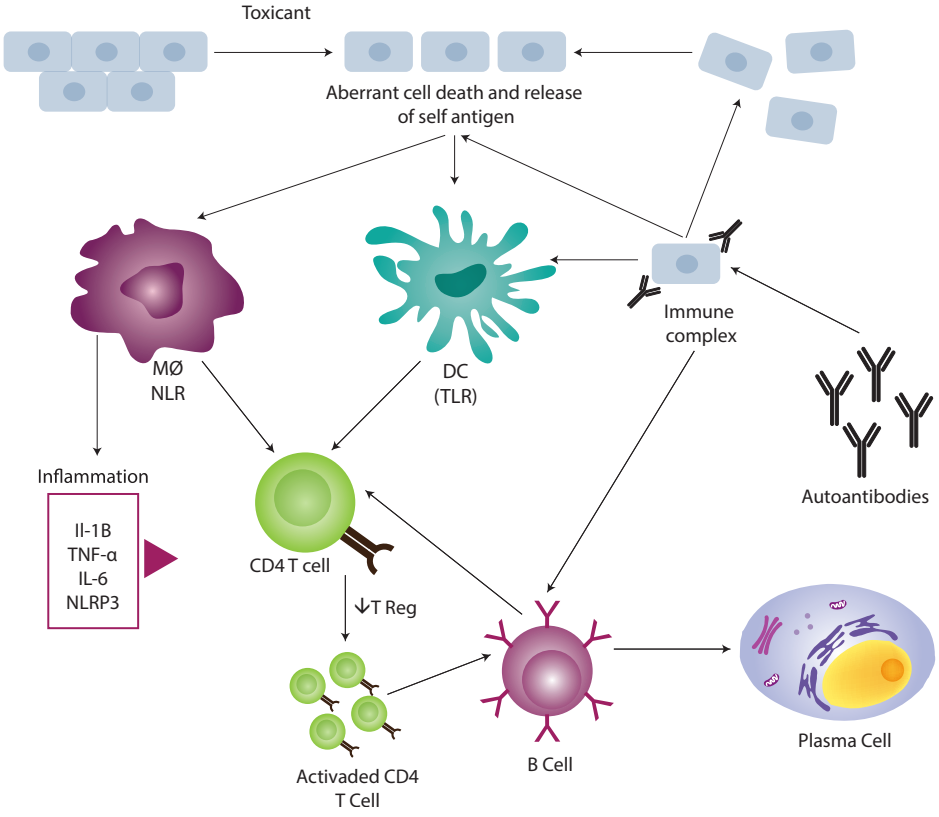

Figure 3. Putative mechanisms of drug/chemical induced autoimmunity. Exposure to a toxicant results in aberrant cell death making cell material persist which activates Nod-like receptors (NLR) and Toll-like receptors (TLR). In the case of NLR activation, this leAD to NLRP3-inflammasome activation and proinflammatory events including production of proinflammatory cytokines such as IL-1β, IL-6, and IFN-γ. For TLR-mediated response, activation of TLR by nuclear material is essential for autoantibody responses to nuclear antigens such as chromatin and RNA/protein complexes. Activation of self-reactive T cells proceeds via breaking of self-tolerance which may be mediated by a number of mechanisms. Reduction in the expression of regulators of T cell activation enhances T cell responses and promotes the activation of autoantibody producing B cells. The binding of autoantibodies to self-antigen leAD to immune-complex formation and tissue injury which in turn can release cellular material to amplify the response. Self-antigen containing immune complexes can also be taken up by B cells and other antigen presenting cells [e.g., dendritic cells (DC)] and amplify activation of autoreactive T cells. Adapted from Pollard *et al*. (12).

key to understanding the role of environmental factors in autoimmunity (12).

In a recent Expert Panel Workshop, Germolec *et al*. (13) reviewed the animal models used to examine the role of the environment in the development of AD. They did a review of the literature and identified individual chemical, physical, or biological factors that have been shown to either induce autoimmunity in non-autoimmune-prone strains or exacerbate disease in inducible or spontaneous autoimmune models. Their main conclusions are shown in Table 4.

CIGARETTE SMOKING

The relationship between cigarette smoking (CS) and poor health outcomes has been a main topic in epidemiologic studies, which have shown evidence of associations with cancer, cardiovascular disease, and overall mortality. Despite staggering evidence and a death rate of 6 million people each year, nicotine addiction and the tobacco industry have made CS the direct cause of the diminished health of millions of people around the world. Every effort to reduce tobacco consumption is needed to ameliorate this public health issue. ADs and CS share a complex relationship, mainly due to the diverse compounds found in cigarette smoke, and the fact that any single one of them could be responsible for altering the immune response.

In general, it is hypothesized that the effect of cigarette smoke is seen four different ways: direct tissue damage, perpetuation of inflammation, immunosuppression and anti-estrogenic effects (14). However, the mechanisms that originate this impairment are not very clear, due to the extensive number of organic compounds found in CS.

Chronic CS induces macrophage and neutrophil activation, and the release of proteolytic enzymes that causes degradation of the connective tissue. For instance, elastases are involved in the destruction of the extracellular matrix and accumulation of $CD4^+$ and $CD8^+$ lymphocytes in the alveoli and are responsible for the development of emphysema (15). Lymphocytes release chemokines, e.g., CXCL-9, CXCL-10 and CXCL-11 (16). These may be associated with an increased number of sputum neutrophils and protease concentration in past smokers compared to those who have never smoked (17). Radicals found in cigarette tar are capable of causing DNA damage (18), which

BASED ON EXISTING EVIDENCE, THE FOLLOWING WAS CONSIDERED CONFIDENT AND RELIABLE
Defining risk factors for AD meeting current classification criteria through epidemiology
Forms of inorganic mercury induce systemic AD in rats (transient) and mice, and exacerbate systemic AD in lupus-prone mice
Several mineral oil components and certain other hydrocarbons can induce an acute inflammatory arthritis in some rat strains
The mineral oil component 2,6,10,14-tetramethylpentadecane (TMPD or pristane) can induce chronic lupus-like disease and inflammatory arthritis in several strains of mice
For a limited number of pathogens, there is a clear association between infection and the development of specific AD
Excess iodine increases the incidence of autoimmune thyroiditis in genetically predisposed animal models
BASED ON EXISTING EVIDENCE, FOLLOWING WAS CONSIDERED LIKELY BUT REQUIRING CONFIRMATION
Gold causes (transient) nephropathy in rats. Gold and silver cause autoimmune responses, not autoimmune disease, in mice; but the ability of silver and gold to exacerbate spontaneous AD requires study
Silica exacerbates AD but more studies are needed using more species/strains and a wider range of doses and exposure routes
TCE exacerbates systemic autoimmunity although responses are often limited and transient. More studies are needed with additional species/strains to examine induction of autoimmune liver disease and in developmental studies
There is some indication that TCDD can promote autoimmunity when exposure occurs during fetal or early neonatal development
Organochlorine pesticides have been reported to enhance lupus-like disease in a predisposed mouse strain
UV radiation exacerbates lupus in genetically prone mice
THE FOLLOWING BROAD THEMES SHOULD BE PURSUED IN FUTURE INVESTIGATIONS
Responses of animals to environmental exposure should not be the only driving force for human studies. Studies should be "shaped by what is observed in humans, not by what is pOSible in mice"
A single mouse strain is clearly unable to encompass the heterogeneity of the human population. Thus studies should not be restricted to the identification and/or use of a "gold standard" animal model. Rather models should be investigated that best reflect human genetic heterogeneity, and/or ask specific mechanistic questions that a particular model is able to address
When using spontaneous disease models it is important to consider whether environmental exposures directly impact idiopathic autoimmunity, or reflect environmental factor-specific autoimmunity
More studies on the effects of environmental factor exposure on expression of autoimmunity during different stages of life (i.e., gestational to adulthood) are needed

Table 4: Animal models, environment interactions, and autoimmune diseases development: Conclusions of the Expert Panel Workshop literature review. *AD*: autoimmune disease; *TCE*: trichloroethylene; *TCDD*: aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-P-dioxi; *UV*: ultraviolet. Adapted from Germolec *et al*. (13).

in turn generates a systemic inflammatory process (19). This is made evident by the association of smoking and elevated levels of C-reactive protein and other inflammatory markers (e.g., fibrinogen, plasma viscosity and white blood cell count) in active smokers (20). Effects on the immune system are related to modifications in the response to external pathogens, which are achieved through the reduction of IgA, altered expression of tumor necrosis factor-α (TNF-α) and IL-6, and reduced response to lipopolysaccharides (21). Moreover, these mechanisms are responsible for the loss of immune tolerance (22) by inducing T cell senescence (23, 24) and auto-reactivity. Endothelial dysfunction is the first step towards atherosclerosis and has been associated with free radicals caused by CS. Likewise, high concentrations of angiotensin II, smooth-muscle hypertrophy, peripheral resistance, lower availability of nitric oxide and oxidation of low density lipoprotein cholesterol (LDL), are effects secondary to smoking (25).

In the clinical setting, there are three main outcomes involving CS and AD: disease onset, progression (Table 5) and treatment response.

RHEUMATOID ARTHRITIS

Since 1987, a clear causal relationship between tobacco and rheumatoid arthritis (RA) has been established (26). One of the main mechanisms underlying smoking-evoked autoimmune response in RA is the production of antibodies recognizing citrullinated proteins (27). Inert particles in smoke tar stimulate epithelial macrophage activation in the lung and translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkβ). RA patients who smoke show an increased expression of peptidyl arginine deiminase (PADI) and the presence of citrullinated proteins in the lung (28). In the synovial tissue of smoking patients with RA, an increase in the proportion of citrullinated proteins including filaggrin, vimentin, enolase, and fibrinogen has been shown (29). Likewise, an altered production of TNF-α has been observed (30). Moreover, the effect of smoking may also be related to tetrachlorodibenzo-P-dioxin (TCDD), which up-regulates the expression of IL-1β, IL-6, and IL-8 by binding to the arylhydrocarbone receptor, the effect of which is transmitted via the NFkβ and extracellular signal-regulated kinase signaling cascades. TCDD induces inflammatory cytokines and may, therefore, exacerbate the pathophysiological mechanisms involved in RA (31, 32). Figure 4 is a representation of the interaction between CS and RA.

A systematic review demonstrated the highest association for male current smokers with rheumatoid factor (RF) positive RA (33). Gender is not the only determinant of the increased risk observed, since, in an appropriate genetic background, CS facilitates the development of RA. Thus, a strong association between CS and the presence of shared epitope (SE) alleles (i.e., *HLA-DRB1*01, HLA-DRB1*04*) has been noted for RA patients who have positive RF and anti citrullinated peptide/protein antibodies (ACPA). The *HLA-DRB1* SE can accommodate citrulline in its antigen-anchoring pockets and thus, stimulate citrullinated protein-specific T cell responses,

DISEASE	DISEASE ONSET			EVOLUTION			REFERENCES
	EVER	CURRENT	PAST	EVER	CURRENT	PAST	
RA	POR 1.89 (1.56-2.28)* POR 1.27 (1.12-1.44)**	POR 1.87 (1.49-2.34)* POR 1.31 (1.12-1.54)**	POR 1.76 (1.33-2.31)* POR 1.22 (1.06-1.40)**	NA	EULAR response POR 0.72 (0.57-0.91)	DAS28 remission POR 0.78 (0.63-0.96) NA	(33)
SLE	NA	POR 1.50 (1.09-2.08)	POR 0.98 (0.75-1.27)	Mean SLEDAI 15.63 (12.96-18.3)	Anti DNA seropositivity OR 4.0 (1.6 -10.4)	Mean SLEDAI 9.64 (7.61-11.67)	(46-49)
PBC	AOR 1.57 (1.29-1.9)	AOR 0.49 (0.38-0.65)	NA	NA	Smoking intensity was significantly higher in patients with F3–F4 stage***	NA	(53, 54)
MS	AOR 1.32 (1.10–1.60)	NA	NA	NA	Progression to SPD HR 2.5 (1.42-4.41)	NA	(50, 51)
AITD	POR 1.90 (1.42-2.55)	POR 3.30 (2.09-5.22)	POR 1.41 (0.77-2.58)	Development of GOP POR 2.53 (1.70–3.77)	Development of GO POR 2.18 (1.51–3.14)	NA	(56)

Table 5. Association between smoking status and disease onset and evolution. *AITD*: autoimmune thyroid diseases; *AOR*: adjusted odds ratio; *DAS28*: disease activity score-28; *EULAR*: European League Against Rheumatism; *GO*: Graves' ophthalmopathy; *HR*: hazard ratio; *MS*: multiple sclerosis; *NA*: not available; *PBC*: primary biliary cirrhosis; *POR*: pooled odds ratio; *RA*: rheumatoid arthritis; *SLE*: systemic lupus erythematosus; *SLEDAI*: systemic lupus erythematosus disease activity index; *SPD*: secondary progressive disease. All results in parentheses correspond to 95%CI. *men ** women ***METAVIR stages of fibrosis.

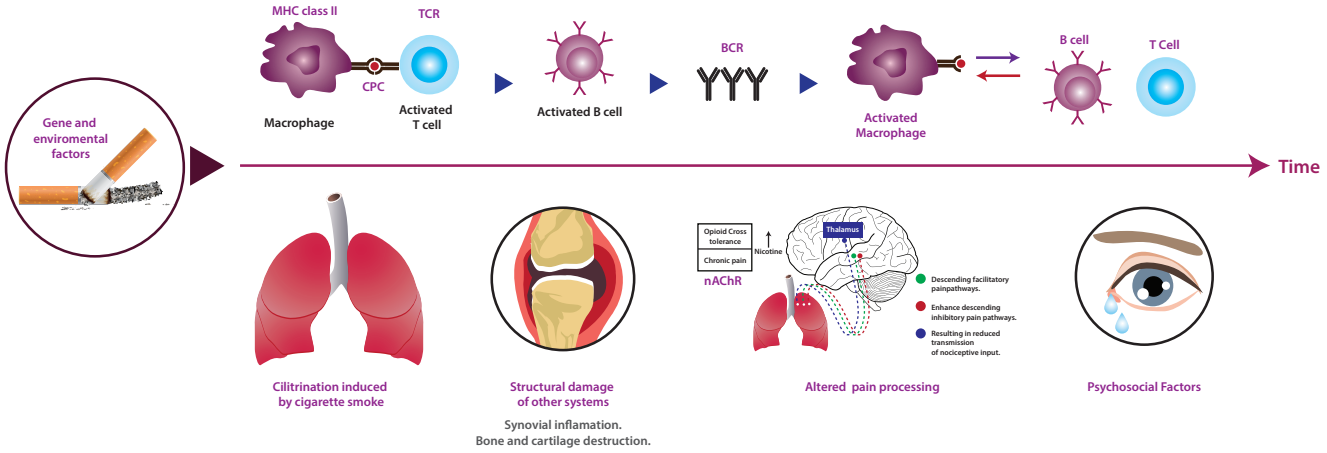

Figure 4. Complex relationship between cigarette smoking and rheumatoid arhtritis explains its role in development and poor treatment response. Acting at diverse levels including citrullination of proteins in the lungs of smokers, an inflaammatory state that perpetuates tissue damage, nociception alteration and psychosocial factors. *MHC*: Major Histocompatibility Complex. *TCR*: T cell receptor. *BCR*: B cell receptor

especially in smoking patients (34). The presence of SE can confer a RR as high as 15.7 when the subjects are carrying two SE copies (35). The number of copies of the SE that an individual carries can modify the risk of acquiring RA in smokers. The risk of RA increases with the intensity (i.e., pack per day) and duration of cigarette use. Heavy CS has been linked to a substantial increase in the susceptibility to RA. Karlson *et al*. (36) demonstrated that the highest risk was in heavy smokers with a double copy of SE. Moreover, the risk of RA increases after 20 pack-years of smoking (33) and remains elevated up to 20 years after smoking discontinuation.

Patients with tobacco exposure have increased titters of ACPA. A study done with 241 RA patients found a statically significant difference between ACPA concentrations in patients with a history of smoking and those who have never smoked (37). It also raises the possibility that smoking-induced citrullinated proteins may serve as a link in the process, possibly as neoantigens. Likewise, CS is associated with an increased prevalence of RF (38), which has been found up to 30 years prior to clinical diagnosis of RA. It is more likely to be associated with early age at onset (39), extraarticular manifestations, (e.g., rheumatoid nodules) (40), higher disease activity scores, and diminished response to treatment (e.g., particularly to anti-TNF therapy) (41). In 2006, Manfredsdottir *et al*. (42) followed 100 patients with early RA for 24 months, and determined that current smokers had elevated disease activity scores. The main differences found in swollen joint counts, tender joint count and pain visual analogue scale in all of which smokers had the worst features.

Regarding progression of erosion, data are inconclusive and current research shows evidence of an increased association with CS. The ESPOIR cohort evidenced a reduction in erosion progression after 1 year in patients with early RA (43). Other studies in turn have demonstrated a higher radiographic progression in current smokers versus nonsmokers (44).

In an ongoing systematic review, the effect of smoking on disease progression and treatment response was summarized. Smokers disclose a diminished response to treatment as defined by EULAR criteria, and CS was associated with a higher erosion index. A higher rate of radiographic progression was not demonstrated in smokers. Out of a total of 2,215 articles retrieved, 12 contained information about disease activity score 28 (DAS28) and 17 about radiographic progression. There was a negative association between smoking and EULAR response, and DAS28 remission. DAS28 as well as the erosive score was significantly higher in current smokers. Data were ambiguous for progression of erosion during follow-up. These results reflect the difficulty of evaluating the effect of tobacco on RA. Clinically observed effects may be the result of any interaction between smoking habit, genetic background, and other environmental factors. (See chapter 24)

SYSTEMIC LUPUS ERYTHEMATOSUS

Like RA, CS increases the risk of developing SLE. An observational study reported that women who start smoking before the age of 19 were at a higher risk for SLE with an incident risk rate of 1.9 (95%CI 1.0-3.6) (45). Moreover, a meta-analysis of seven existing case-control and 2 cohort studies revealed a statistically significant association between current smoking and development of SLE (46).

Current smokers are more likely to have anti-DNA antibodies than non-smokers. When compared to non-smokers, current smokers had an increased association with an OR 4.0 (47). This finding is related to the increased disease activity (i.e., SLEDAI) observed in LES patients that

are current smokers; among 111 patients, current smokers had a significantly higher SLEDAI score when compared to past smokers and non-smokers (48). Regarding clinical outcomes, CS reduces the onset from lupus nephritis diagnosis to end-stage renal disease (ESRD), as shown in an inception cohort in which median time to ESRD in months for smokers was 145 compared to 273 in non-smokers (49).

MULTIPLE SCLEROSIS

Although not widely studied, CS is associated with a higher incidence of multiple sclerosis (MS). An analysis of MS patients determined that the case subjects were more likely to have smoked than paired controls, with an OR 1.32 (95% CI 1.10–1.60, p = 0.003) (50). Furthermore, CS increases the risk of progressive disease from the moment of diagnosis as was demonstrated on patients that start smoking at age 15 or below (51). Also, current smokers progress from relapsing-remitting disease to secondary progressive disease faster than nonsmokers.

PRIMARY BILIARY CIRRHOSIS

There is an association between CS and the development of primary biliary cirrhosis (PBC). A population based, case-control study demonstrated that past smoking constituted a risk factor for developing PBC as ever smokers represented 76% of the cases, and controls represented 57% with an OR 2.4 (52). This observation was confirmed by a broader study where the adjusted OR for past smoking was 1.57 (95% CI 1.29-1.91) (53). CS is associated with an increased risk of liver fibrosis in PBC. A questionnaire completed by 223 patients assessed this association and established that smoking history and smoking intensity are independent risk factors for advanced fibrosis. (54).

GOODPASTURE SYNDROME

Goodpasture syndrome (GS), a disease associated with a specific set of auto-antibodies directed against the glomerular basement membrane (anti-GBM), is characterized by pulmonary hemorrhage and renal failure secondary to acute glomerulonephritis. It is an infrequent condition, and there is no clear association due to the lack of epidemiological studies, but there are observational studies that highlight the increased prevalence of smokers in GS. A series of 28 cases described a prevalence of current smoking status of 89%. Also CS seems to be associated with the development of salveolar hemorrhage in patients with positive anti-GBM (55).

AUTOIMMUNE THYROID DISEASE

CS has been linked to several thyroid diseases. A meta-analysis done of 8 observational studies showed an association between Graves' disease (GD) and, current and ever-smoking status, with an OR 3.02 (95% CI 2.09-5.22) and 1.9 (95%CI 1.42-2.55), respectively. There are two studies, in turn, that reported the association between Hashimoto thyroidits and CS with a reported OR of 1.92 (95% CI 1.25–2.93) (56). CS is a confirmed risk factor for Graves' ophthalmopathy (GO) (56). In a large study from the Netherlands, the OR for GO was 7.7 in smokers compared to non-smokers (57). Even though the exact mechanism is unknown, it has been suggested that smoking increases the connective tissue around the orbit through stimulation of the synthesis of glycosaminoglycans (58).

ORGANIC SOLVENTS

Definition. Solvents are capable of dissolving other substances (solutes) (59). In addition to this, organic solvents (OS) contain carbon in their molecular structure. There are different classes of organic solvents. They could be either aliphatic hydrocarbons, aromatic hydrocarbons, amines, esters, ethers, ketones, or nitrated/chlorinated hydrocarbons.

The uses for OS are multiple, ranging from industrial to domestic. They can be found in products such as: degreasing agents, varnish, paint, glue and many others.

OS are recognized as carcinogens, teratogen agents, and neurotoxins. Despite this, they have a wide use due to their effectiveness and inexpensiveness.

Background of the relationship with autoimmunity. Reports of the effect of OS on ADs appeared for the first time in 1957 when patients developed a scleroderma-like syndrome after exposure to vinyl chloride, epoxy resins, trichloroethylene (TCE), perchloroethylene, and other mixed solvents (60).

Initially research focused on the relationship between OS and scleroderma (SSc). Since the information was scarce and inconsistent, mainly due to sample sizes in each study, the data were sensitized in a single meta-analysis and the final OR for SSc was 2.4 (95% CI 1.7-3.4) (61). These findings opened a discussion on whether or not OS played a role in the development of ADs.

Current concept of autoimmunity and OS. In a meta-analysis done by our group (62), results indicated that OS are a risk factor for developing ADs as a whole. When ADs were considered separately, results showed a significant association with the development of MS, primary systemic vasculitis (PSV), and scleroderma (SSc). Furthermore, the direction and significance of this association did not change when all ADs considered as a single trait, showing a significant association (OR 1.54; 95% CI 1.25-1.92).

The clinical presentation of ADs determines the classification criteria given to them, and it is driven by the complex interactions of genetic and environmental factors. This comprises the term: mosaic of autoimmunity. Therefore, the effect of OS is subject to analysis across all ADs (62).

In industrial settings, OS alter cell proliferation, apoptosis, and tissue function. In addition, both amount and duration

of exposure to OS are crucial to the development of related pathologies. Chronic exposure to OS may cause tissue deposits and facilitate an immune response that may evolve into an AD during later stages. OS are capable of producing configurational changes in proteins which make them immunogenic, initiate an inflammatory reaction, and cause tissue destruction. Therefore, we have a heterogeneous group of compounds with similar biological effects, and a heterogeneous group of diseases that share common etiological mechanisms. Molecular mechanisms are represented in figure 5.

Besides the general aforementioned effect, OS may be involved in disease-specific mechanisms. Some examples of this are lymphocyte infiltration and immunoglobulin deposits in SLE and enzymatic alteration and scleroderma-specific antibody subsets in SSc (62).

Ketones are employed for domestic and industrial ends. Acetone, the simplest ketone, is used for cleaning purposes, and it is one of the active ingredients in nail polish remover and paint thinners. Acetone is related to the development of PBC, which is explained by a xenobiotic theory that suggests that its development is associated with certain halogenated compounds. The theory is that substances like ketones have the ability to boost the immunogenicity of mitochondrial proteins and induce anti-mitochondrial antibodies in animal models (63). In humans, a scientific study evaluated the relationship between PBC and nail polish exposure. Although not statistically significant, there was a tendency towards a positive association (53). The conclusion was reached that since it is a common compound related to a rare but serious AD, epidemiological studies evaluating this association are necessary .

Another example is autoimmune hepatitis (AIH), where prolonged exposure to OS seems to create a significant hepatic mononuclear infiltration ultimately causing autoimmune hepatitis. This behavior of cell infiltration of exposed tissues is present in different organs and models. As shown by the work of Cai *et al*. (64), lymphocyte infiltration was present in the pancreas, lungs, kidneys, along with the liver of exposed mice which suggests that this phenomena may not only be related to AIH but also to several other ADs.

In autoimmune thyroid disease (AITD), it is likely that solvents may alter iodine transportation, causing oxidative stress that results in an inflammatory response of the thyroid gland (65), and predisposing this tissue to the development of an autoimmune response.

Animal models have helped clarify the mechanisms involved in the autoimmune response initiated by OS. The exposure of mice that are MRL+/+, a breed predisposed to autoimmunity, to trichloroethylene produces an increased serum IgG concentration, antinuclear antibodies (ANAs) positivity, and anticardiolipin autoantibodies (66). Likewise, *in-vitro* experiments done with human tissue have supported these findings. An experimental model that employed trichloroethylene showed that keratinocytes exposed to this compound have an increased nitric oxide synthesis by nitric oxide synthase (67), an effect related to an AD with one of the strongest associations with OS: SSc.

The mechanism accountable for the development of environmentally induced autoimmune disorders is yet to be found. Although there are several hypotheses for the development of autoimmune phenomena after specific

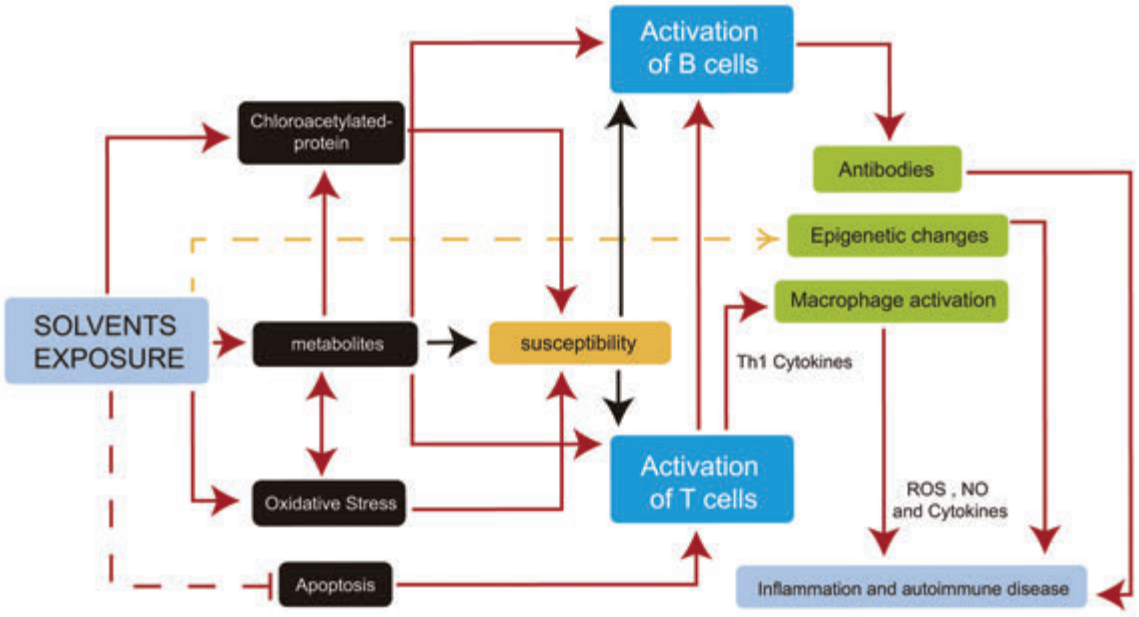

Figure 5. Potential molecular mechanisms implicated in solvent autoimmune disease development. Solid red arrows represent known paths. Yellow dashed arrow represents hypothetical mechanisms (warranting future research), and red dashed line represents an inhibited process. In susceptible individuals, activation paths are stronger (black arrows). See text for details. *NO*: Nitric Oxide. *ROS*: Reactive oxygen Species. Adapted from Barragan-Martinez *et al.* with permission (62).

environmental exposure, there is no strong epidemiological evidence congruent with causality criteria. Nevertheless, experimental models and observational studies have pointed towards this association and towards the mechanisms that may have effects on molecular signaling pathways, immune responses, or regulation mechanisms actively involved in disease etiology, specifically autoimmunity.

Finally, as described in the theory of the autoimmune/inflammatory syndrome induced by adjuvants, the toxic effect influences the development of ADs, in genetically vulnerable subjects.

SILICA

Silicon dioxide is a transparent, tasteless crystal, found in nature as sand, quartz, chalcedony, flint and tridymite–all common minerals. Environmental and industrial exposure to silica can be due to mining, tunneling, masonry, and pottery.

Inhalation of crystalline silica induces silicosis and silica related diseases such as tuberculosis and lung cancer (68). In addition to the aforementioned risks, silica has been associated with a series of autoimmune and auto-inflammatory disorders, e.g., SSc, SLE, RA, and sarcoidosis. These associations are supported by case reports and a series of epidemiological studies.

PATHOGENESIS

Silica is capable of initiating an inflammatory response once in contact with pulmonary tissue. This starts with the phagocytizing of silica particles by alveolar macrophages (AM) (69) and recruitment of inflammatory cells by chemokines. Chronic exposure perpetuates this inflammatory response that progresses even after exposure ceases because the destruction of silica particles can not be achieved in the lysosomes of macrophages. This is the general scenario that is thought to induce a loss of tolerance and a subsequent autoimmune response.

Despite the epidemiological and experimental association of silicosis and self recognition by the immune system (70), biological mechanisms are not entirely clear, and they may involve diverse pathways in the immunological response. The biological effects of silica include direct ones on several pathways such as cell-to-cell signaling, cell interaction, cell movement, and those secondary to persistent inflammatory activation of resident macrophages.

Observed effects of silica are not limited to lungs. Autoimmune/auto-inflammatory responses are documented along with others in joints, skin, and kidneys. An effect on an organ that is not in direct contact with silica dust may be due to a reproduction of the inflammatory response, but in some cases, it may be related to the mobilization of particles through the reticuloendothelial system.

By releasing reactive oxygen (ROS) and nitrogen species, silica induces tissue damage. The effect is independent of the amount of exposure, and it is followed by the activation of caspase-3 and caspase-9 with subsequent apoptosis of AM. A common agent in autoimmune response, ROS are capable of the prodcution of lipid peroxidation, disruption of lipid rafts, activation of tyrosine kinases, and the translocation of transcription factors, e.g., NFkβ or the nuclear factor of activated T cells (NAFT) to the nucleus (71) lead to the production of several pro-inflammatory cytokines such as IL-1β, IL-8, TNF-α and IL- 6.

These immune alterations lead to diverse phenotype expressions categorized as ADs. The first described example of these autoimmune phenotype is Caplan syndrome, which described in 1953 in a group of Welsh coal miners (72) that had rheumatoid radiological lesions in the lungs that were associated with an increased risk for RA in a later study (73). Other research done on this association has reinforced the role of silica in the pathogenesis of RA. Epidemiological studies in this topic (68, 74-84) are summarized in Table 6.

BERRILYUM

A hard metal found in rocks, coal, soil, and volcanic dust, beryllium is a rare element used in the aerospace industry, where it is employed in alloys that are resistant and have a relatively low density. Furthermore, it is relatively transparent to x-rays, which makes it useful in research and radiological appliances. Although an uncommon metal, exposure to high concentrations of beryllium is common in industries where it is used.

Chronic exposure to beryllium causes, in a proportion of cases, sustained inflammation and granuloma formation in the lung, a condition known as chronic beryllium disease. Characteristic histological findings are $CD4^+$ T lymphocytes and macrophages (85). In these cases, beryllium may act as a hapten, transforming peptides that will later bind to specific HLA. An increased risk for chronic beryllium disease is observed in carriers of *HLA-DPB1* Glu69-positive alleles, a finding that was later reproduced *in-vitro* models.

ADULTERATED RAPESED OIL

From 1981 to 1982, a toxic oil syndrome affected more than 20,000 people with 2% mortality, especially in Spain. This disorder was characterized by a resemblance to eosinophilia-myalgia syndrome and diffuse fasciitis with eosinophilia. Other clinical manifestations included vasculitis, sicca syndrome, neuropathy, SSc, Raynaud's phenomenon, and musculoesqueletal inflammation. Finally, the toxic oil syndrome was attributed to a branch of rapessed oil contaminated with aniline, oleylanilide, and other fatty acid anilides (86).

DRUGS

D-PENICILLAMINE

D-penicillamine (D-pen) is a compound derivate from penicillin hydrolysis that contains a sulfhydryl group (thiol). Due to its use as a copper-mobilizing agent and chelation with many metal ions, D-pen has been used at the clinical level in treatment of Wilson's disease and heavy metal poisoning. In

AUTHOR, YEAR	STUDY DESIGN	DIAGNOSIS	EVENTS	ASOCIATION	95% CI
Steenland and Brown 1995 (74)	n= 3328 gold miners through 1940-1965 in South Dakota	Arthritis. Non ACR criteria for RA	17	SMR 2.19	1.27 – 3.50
Steenland *et al*. 1992 (75)	n=991 granite cutters who died after 1960 compared with causes of death in U.S. population	Arthritis deaths. Non ACR criteria	17	PMR 2.01	1.17 – 3.21
Rosenman and Zhu 1995 (76)	Cohort 1990–1991	Women RA cases	0		
		Men RA cases	3	SIR 3.2	1.1 – 9.4
Sluis-Cremer 1985 (77)	Case-control n=79 systemic sclerosis miners n=79 miners without systemic sclerosis	Silicosis	79	OR 1.18	0.26 – 5.3
Sluis-Cremer 1986 (78)	Case-control study of silicosis in 157 white gold miners diagnosed with "definite" or "probable"	Miners "definite" RA	91	OR 3.79	1.72-8.36
		Miners "probable" RA	66	OR 1.94	0.81 – 4.63
Nuyts *et al*. 1995 (79)	Case-control n=16 patients diagnosed with Wegener's granulomatosis at six Belgian renal units between June 1991 and June 1993. Each patient was matched (by age, sex, and region of residence) with two controls randomly selected from lists of voters	Patients with Wegener's granulomatosis (renal involvement) and reported occupational exposure to silica	5	OR 5.0	1.4 – 11-6
Rafnsson *et al* 1998 (80)	Population-based Case control. Residents in a district with a diatomaceous earth processing plant. Population included 8 sarcoidosis patients who were linked to a file of all past and present workers employed at the plant after it opened in 1967. 70 controls were randomly selected from the district population	Sarcoidosis patients with occupational exposure to diatomaceous earth and cristobalite at the community plant	6	OR13.2	2.0 – 140.9
Klockars *et al*. 1987 (81)	Cohort morbidity study n=1,026 granite workers	Awarded disability pensions for RA	17	RR 5.08	3.31 – 7.79
		Receiving pensions for RA at end of study period	10	NA	NA
		Receiving free medication for RA at end of study period	19	NA	NA
Cowie. 1987 (82)	Cohort study of incidence of scleroderma in black gold miners seen by the medical service from July 1981 to June 1986	Miners with scleroderma that met diagnostic criteria	10	81.1:1000000	NA
burns *et al*. 1996 (83)	Population-based case-control study n= 274 women with confirmed m=1,184 female controls matched by race, age, and geographic region	Abrasive grinding or sandblasting	3	0.34	0.10 – 1.10
		Sculpting or pottery making	20	1.53	0.89 – 2.65
		Working in a dental laboratory	3	1.52	0.44 – 5.26
		Working with or near silica dust, sand, or other silica products	12	1.50	0.76 – 2.93
Bovenzi *et al*. 1995 (84)	Case-control study n=527 diagnosis of musculoskeletal disorder or connective tissue disease. Each scleroderma case was matched by age and gender to two controls	Women	16	0	NA
		Men	5	OR 5.20	0.48 – 74.1

Table 6. Epidemiological studies on silica and AD. *ACR*: American College of Rheumatology; *CI*: confidence interval; *NA*: not available *OR*: odds ratio; *PMR*: proportionate mortality ratio; *RA*: rheumatoid arthritis; *RR*: relative risk; *SIR*: standardized incidence ratio; *SMR*: standardized mortality ratio; *SRR*: standardized risk ratio. Adapted from NIOSH document 2002-129 (68).

addition, D-pen has been described over time in many other clinical settings such as cystinuria and ADs e.g., RA, and SSc.

D-pen possesses three prominent functional groups: an amine, a carboxyl, and a sulfhydryl. The biological effects of D-pen are regulated by the reactions of these functional groups with endogenous compounds resulting in formation of disulfide links, thiazolidine rings, or metal complexes (i.e., chelates) (87). Autoimmunity induced by D-Pen is an important complication of treatment with this drug and has been described in RA, SSc, and other non-ADs, e.g., Wilson's disease.

Underlying Mechanisms of autoimmunity induced by D-Pen. Drug induced autoimmunity is an immune-mediated process, but the mechanism by which autoimmunity is induced remains unclear. D-pen murine models have emerged to explain this phenomenon despite the idiosyncrasies of this process (88).

Murine models. Brown-Norway (BN) rats are the main animal model used to test D-Pen induced autoimmunity because it is a susceptible strain. Although the incidence is 50-80% at a daily dose of 20 mg/day, it can be influenced by other factors and reach 100% with the addition of poly_IC [synthetic polymer that stimulates macrophages via toll-like receptos (TLR)] or drop to 0% with one dose of misoprostol (88). Analysis of different doses of D-Pen showed that doses lower than 20 mg/day induces immune tolerance to the higher doses. Thus, tolerant animals treated with D-pen at a dose of 20 mg/day showed an increased production of regulatory cytokines (i.e., TGF-β and IL-10) by $CD4^+$ T cells. This does not occur in naïve rats suggesting the involvement of regulatory T cells (89).

Changes in mRNA expression and macrophage activation. Early changes in hepatic mRNA expression were described after 6hrs of treatment with D-pen. However, the autoimmune process was clinically evident after 3 weeks of treatment and showed the involvement of other unknown mechanisms (88). Murine models have shown that macrophage activation is an important characteristic of D-pen induced autoimmunity. D-pen binds to aldehydes on the surface of macrophages. Imine bonds formed by aldehyde groups on macrophages and amine groups on T cells are the interactions that have been associated with the induction of an immune response (90). Also, D-pen reacts with aldehyde groups to form a thiazolidine ring (91). It is likely that D-pen would react with these aldehyde groups on the cell membrane of macrophages and this reaction could modulate functions of macrophages even becoming fully activated (91). Studies on BN rats and macrophage cultures have demonstrated that D-pen can induce the production of different cytokines, including TNF-R, IL-6, and IL-23 (92). The fact that D-pen causes rapid macrophage activation while the onset of autoimmunity occurs after about 3 weeks of treatment strongly suggests that macrophages play a role in the initiation of the immune response leading to autoimmunity (92). Moreover, partial depletion of macrophages decreased the incidence of autoimmunity in BN rats (90).

Other Proposed Mechanisms. D-pen can bind to aldehyde groups to form a thiazolidine ring. Formation of an imine bond between aldehyde groups on antigen-presenting cells (APC) and amino groups on T cells is one of the activation signals. The formation of a thiazolidine ring by D-pen is irreversible and could lead to activation of APC, inducing autoimmunity, although it remains unclear (88).

AUTOIMMUNE DISORDERS RELATED TO D-PEN

Myasthenia Gravis (MG). It is a rare complication of treatment with D-pen. The clinical and electromyographical pattern between induced and non-induced MG is indistinguishable. However, association with *HLA-Bw35* and *HLA-DR1* in Caucasians has been reported (93). There are reports which describe that, after 8 months of therapy, most of the patients experienced ocular and bulbar symptoms, that can lead to generalized weakness and respiratory muscle involvement unrelated to the administered dose (94). Autoimmune pathogenesis is probable. D-pen binds to the acetyl choline receptors (AchR) and can cause the formation of AchR antibodies (95). The presence of these autoantibodies and symptoms are related to D-pen administration and withdrawal (94). This complication can evolve after years of treatment and may take over a year to resolve. Management of D-pen induced-MG includes drug withdrawal. After that, the treatment is the same as for non-induced MG. After the drug was discontinued, 70% of the individuals experienced complete remission in 6-10 months compared to 8% in the first year of idiopathic MG (94).

Dermatomyositis Polymyositis. This is an uncommon complication of treatment with D-Pen, and it has been described more frequently in RA patients than in others. Recovery is usually rapid after cessation of D-pen although glucocorticoid therapy may be required. Rechallenge with D-pen is discouraged because there are reports of exacerbation of polymyositis with a second challenge (96). Although the etiopathogenesis of D-pen induced polymyositis is unknown, D-pen is well-recognized for its ability to induce autoantibodies including ANA, anti-striational muscle, anti-DNA, RF, and anti-AChR but not antibodies to extractable nuclear antigens (ENA) (97). The development of D-pen induced polymyositis is associated with the presence of *HLA-B18*, *HLA-B35*, and *HLA-DR4* alleles while idiopathic polymyositis is associated with *HLA-B8* and *HLA-DR3* alleles thus showing a different genetic component (98). Therefore, this complication, although rare, can be fatal.

Systemic Lupus Erythematosus. D-pen is recognized by its ability to induce SLE. Main clinical findings are fever, rash, polyserositis, and anti-DNA presence, but other manifestations can appear (e.g., hemolytic anemia and hypocomplementemia). The association of D-pen with lupus syndromes

has been recognized in various clinical settings, e.g., Wilson's disease, cystinuria and RA (99). Also, neurologic alterations and glomerulonephiritis are described more frequently in drug-induced SLE. Presence of anti-histones antibodies is a typical laboratory finding associated with drug-induced SLE. Treatment includes D-pen cessation and glucocorticoids or immunosuppressive therapy.

PROCAINAMIDE AND HYDRALAZINE

The antiarrhythmic, procainamide (PRC), and the antihypertensive, hydralazine (HDZ) have been associated with drug-induced SLE (100), with reported incidences about 20% and 5-8% respectively (101).

Mechanism of Autoimmunity Induced by PRC and HDZ. The principal mechanism of action of PRC-induced SLE appears to be through the formation of highly reactive intermediates, including procainamide hydroxylamine (PAHA) (102). Thus, mice injected with PAHA showed a lOS of central T Cell tolerance and production of anti- (H2A-H2B)-DNA antibodies (103). PRC and HDZ inhibit DNA methylation, which in turn induces transcription, leading to lymphocyte activation (88). Theories about autoimmunity induced by drugs propose that, in mature T cells, hypomethylation of DNA correlates with an increase in gene expression, causing autoreactive T lymphocytes (100). In addition, T cells treated with DNA methylation inhibitors have the same pattern that T cells from patients with active SLE (104). Moreover, over expression of LFA-1 (leukocyte function associated antigen) as a result of hypomethylation of DNA in T cells has been found to be enough to cause autoimmunity (105).

Clinical and Immunological Findings. The main clinical findings in PRC-induced SLE are arthralgia, arthritis and myalgias in 80 – 85% of patients, followed by fever, weight lOS and fatigue. Also, pleuritis and pericarditis have been reported. HDZ-induced SLE typically includes arthralgias, myalgias, constitutional symptoms, fever, rash, pleuritis and leukopenia (101). Vasculitis induced by HDZ has been described also, with more severe course than hydralazine-induced SLE (106). Anti-histone antibodies are developed in HDZ-induced SLE, and the most common autoantibody detected in PRC-induced SLE patients was against a component of the nucleosome consisting of an H2A-H2B dimer.

OTHER DRUGS RELATED TO AUTOIMMUNITY

Minocycline. A previous review of literature reported four minocycline-induced syndromes: serum sickness, drug-induced SLE, autoimmune hepatitis, and vasculitis, of which, minocycline-induced SLE was the most frequent with positive ANAs in 100% of the patients. Arthralgias, arthritis, and fever were the main clinical manifestations in these patients. Anti-histone antibodies were only positive in 13% of the SLE patients. Except for serum sickness, in which the gender ratio was shared, all autoimmune manifestations were more frequent in women, and they affected younger patients (107).

Sulfonamides. Sulfasalazine and mesalazine are used in treatment of different pathologies such as RA, spondyloarthropathies (SpA) and inflammatory bowel disease (IBD). Antibiotics that contain sulfate also are part of this group of drugs and other sulfonamide nonantibiotic medications are widely available such as diuretics, sulfonylureas, anti-inflammatory drugs and other agents, e.g., sumatriptan, topiramate, Ibultide, sotalol and dapsone (108). Since 1945, when the first case of drug-induced SLE was reported associated with sulfasalazine, many cases have been identified and articles published defining the syndrome which was typically characterized by fever, rash, arthralgia, arthritis, and serositis. Remission on withdrawal of sulfasalazine, and recurrence with rechallenge is a characteristic finding of this entity (109). Other sulfonamide agents such as mentioned above have been related to autoimmune phenomenon as the mesalazine and some types of diuretics (i.e., thiazides) (110).

Anticonvulsants. Many types of anticonvulsants have been reported to be responsible for inducing autoimmune disorders. Some of these are phenytoin (111), carbamazepine (112), ethosuximide (113), valproic acid (114), and lamotrigine (115), and, in some cases, they have had an effect even many years after start of treatment.

Anti-TNF. Since the introduction of biological therapy in the treatment of AD and other conditions, there has been an increase in reports about biologics-induced autoimmune disorders, most of them involving anti-TNF (116). SLE is the most frequently reported AD related to anti-TNF therapy. It is overwhelmingly associated with RA in 72% of the cases and with SpA and IBD to a lesser extent (117). Other disorders related to use of anti-TNF therapies are vasculitis (118), demyelinating central nervous system diseases (119) and peripheral neuropathies (117), among others.

IFN. Different disorders have been related to IFN therapy including SLE, RA, polymyositis, psoriatic arthritis, and reactive arthritis (109).

Other. Drugs related to induced autoimmunity are antibiotics (e.g., quinolones, isoniazid), leukotriene antagonists (e.g., zafirlukast), statins, diuretics (e.g., thiazides), propylthiouracil, and herbal medicine (e.g., alfalfa, yohimbine, Kava-Kava) (109).

DRUG-INDUCED SLE

SLE is perhaps the most studied drug-induced AD, and the drugs responsible for it have been categorized based on their ability to induce SLE. Table 7 summarizes the main

Drugs definitely able to induce SLE	Hydralazine Procainamide Isoniazid Methyldopa Chlorpromazine Quinidine Minocycline Sulfasalazine
Drugs possibly causing SLE	Anticonvulsants carbamazepine, etosuximide, phenytoin, diphenylhydantoin, pirimidine, trimethadione, valproate, and zonisamide. Antithyroid drugs: propylthiouracil, methimazole, and thiamazole. Terbinafine Statins: lovastatin, simvastatin, and fluvastatin. Penicillamine Beta-blockers: practolol, acebutolol, labetalol, propranolol, pindolol, atenolol, metoprolol, and timolol Hydrochlorothiazide IFN-α Fluorouracil agents (fluorouracil, tegafur/UFT)
Drugs suggested to induce SLE	Gold salts, penicillin, streptomycin, tetracycline, phenylbutazone, estrogens and oral contraceptives, reserpine, lithium, para-aminosalicilic acid, captopril, griseofilvin, calcium channel blockers, ciprofloxacin, rifampin, clonidine, hydroxyurea, interferon, and Gemfibrozil
Drugs recently reported to induce SLE	Interleukin-2, clobazam, clozapine, tocainide, Lisinopril, zafirlukast, etanercept, and infliximab

Table 7. Drugs able to induce systemic lupus erythematosus. Adapted from Katz *et al*. (120).

medications implicated in drug-induced SLE based on the probability of inducing SLE. More than 80 different medications are related to drug-induced SLE. The incidence varies depending on the probability that drugs will induce SLE. This probability is 20% per year for procainamide, 5-8% per year for hydralazine, less than 1% per year for quinidine, and less than 1 % per year for other drugs (120).

VACCINES AND AUTOIMMUNITY

Vaccine-associated autoimmunity is a rare phenomenon that occurs after a long time after vaccination which puts limits on determining causality (121). Areas of debate are strength of association, prone population, vaccine combinations and adjuvants that can be related to the development of autoimmune manifestations (see Chapter 21) (121).

Proposed mechanisms for vaccine-induced autoimmunity. Molecular mimicry, epitope spreading, bystander activation and polyclonal activation are mechanisms by which autoimmune phenomena after vaccination with agents containing antigens from infectious agents can be explained (121).

Another important mechanism is related to vaccines that include adjuvants, which can stimulate the immune system without any specific antigenic effect of their own (122). Adjuvants exert their immunological effect through different means of action including 1) translocation of antigens to the lymph nodes where they can be recognized by T Cells; 2) antigen concealing which grants a prolonged exposure; 3) grater the ability to cause local reactions at the injection site; 4) inducing the release of inflammatory cytokines, and; 5) interacting with pattern recognition receptors (PRRs), specifically TLRs, on accessory cells (122).

Autoimmunity is a rare complication of vaccination (121), but autoimmune disorders have been related to vaccines. For example influenza vaccine is related to Guillain-Barré syndrome (123), oral polio vaccine to transverse myelitis (124) *Haemophilus influenza* B (HIB) vaccine to type 1 diabetes mellitus, and a combination of vaccines such as diphtheria-tetanus-pertussis (DTP) and measles-mumps-rubella (MMR) which are related to arthritis (121).

In a literature review, Batista-Duharte (125) reported selected cases describing association between autoimmune manifestations and vaccines. These associations are shown in Table 8.

Risk factors in patients with ADs. Evidence of vaccine-induced autoimmunity is scarce. However, studies reported that there is no association between vaccination and exacerbation of an existing AD (126). Even vaccines such as influenza and pneumococcal vaccines are safe for patients with SLE or RA (127).

HORMONES AND AUTOIMMUNITY

There is a widely held view that sexual steroid hormones–particularly estrogens–may increase SLE activity. This traditionally accepted view is based on different clinical and empirical observations: SLE is a predominantly a female disease (128-131). The female to male ratio of incidence rates, has been reported to be as high as 15:1, especially during reproductive years. Altered estrone metabolism has been demonstrated in males and females affected with the disease, and there is also a considerable amount of literature regarding disease flares in SLE women receiving oral contraceptives (OC) and hormone replacement therapy (HRT). However evidence has been conflicting (131-136).

Experiments with animal models have shown that male prepuberal orchidectomy makes the expression of disease comparable to that of females. In animals that already have the disease, estrogen administration increases autoimmunity and mortality while androgen administration reduces

VACCINE	RELATED AUTOIMMUNE MANIFESTATION
Hepatitis B	Rheumatic: RA, ReA, lupus flares, SS, vasculitis, crioglobulinemia, polyartrhalgia, polyarteritis nodosa, fatigue. Cutaneous: erythema nodosum, multiform erythema, cutaneous lupus, localized scleroderma, lichen planus, Neurologic: encephalitis, acute aseptic meningitis, TV, ON, brachial and lumbar plexus neuropathy, GBS, BP, acute cerebellar ataxia, myasthenia gravis, MS Hematologic: thrombocytopenia, AHA, aplastic anemia, Others: nephrotic syndrome, uveitis, alopecia, acute pericarditis, GD
Hepatitis A	AIH
Influenza	Rheumatic: HSP, microscopic polyangiitis, ReA, giant cell vasculitis, rheumatic polimyalgia, crioglobulinemia, polyarthralgia, fatigue. Neurologic: meningoencephalitis/encephalitis, ON, TV, brachial neuritis, GBS, BP Hematologic: lymphopenia, thrombocytopenia, AHA Cardiac: acute myocarditis, acute pericarditis. Others: T1DM, nephrotic syndrome, uveitis, myositis, multiform erythema.
MMR	Rheumatic: acute arthralgia and arthritis, chronic arthritis, myositis Neurologic: encephalitis, aseptic meningitis, myelitis, ON, GBS Hematologic: thrombocytopenia/acute thrombocytopenic purpura, HUS, AHA
Varicella vaccine	Rheumatic: arthralgia and arthritis, vasculitis (usually HSP) Neurologic: encephalitis, aseptic meningitis, myelitis, ON, GBS, cerebellar transitory ataxia, TV, BP Hematologic: thrombocytopenia, aplastic anemia Cutaneous: multiform erythema
OPV	Rheumatic: acute arthiritis Neurologic: encephalitis, GBS Hematologic: AHA
Rabies vaccine	Neurologic: GBS, meningitis, myelitis, white matter autoantibodies, myelin basic protein autoantibodies, anticardiolipin antibodies Others: serum sickness like disease
Smallpox vaccine	Neurologic: postvaccinal encephalopathy, encephalomyelitis Others: multiform erythema, myopericarditis, dilated cardiomyopathy
Diphteria/Tetanus Toxoid	Neurologic: CNS demyelinating diseases, GBS
Lyme disease vaccine	Chronic arthiritis
BCG	Arthritis, Reiter's disease
Yellow fever	Neurotropic and viscerotropic disease (unspecified mechanism)

Table 8. Suspected or confirmed associations between vaccines and autoimmune manifestations. *AIH*: autoimmune hepatitis; *AIH*: autoimmune hepatitis; ; *BP*: Bell palsy; *CNS*: central nervous system; *GBS*: Guillain-Barré syndrome; *GD*: Graves´ disease; *HSP*: Henoch-Shoenlein purpura; *HUS*: hemolytic uremic syndrome; *MMR*: measles-mumps-rubella; *MS*: multiple sclerosis; *ON*: optic neuritis; *OPV*: oral polio vaccine; *RA*: rheumatoid arthritis; *ReA*: reactive arthritis, *SS*: Sjögren's syndrome; *T1DM*: type 1 diabetes; *TV*: transverse myelitis. Modified from Batista-Duharte *et al*. (125).

Anti-DNA antibody production and ameliorates disease activity (137-139). Estrogen effects on murine models of SLE have shown that they may be either harmful or beneficial depending on the way they affect immune responses. It has been demonstrated that some estrogens can stimulate B cell activity and thereby worsen complex-mediated glomerulonephritis, but they can also suppress some T cell-mediated responses and improve sialoadenitis, renal vasculitis, and periarticular inflammation (140-142). In fact, an imbalance between hormone relationships can result in lower immune-suppressive androgens and higher immuno-enhancing estrogens. Women with SLE tend to have lower androgen levels than healthy women (131).

Recently, the evidence of an association between exposure to exogenous sex hormones (i.e., estrogens) and SLE was evaluated through a systematic review and meta-analysis (131). Computerized databases of the Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE, EMBASE, SciElo, and BIREME (1982 to July 2012) were assessed. The search was done for studies showing the association between estrogen therapy (i.e., HRT and OC), exposure, and SLE development using Medical Subject Headings (MeSH), Descriptors Health Sciences (DECS) terms and keywords, and following the PRISMA guidelines. Two reviewers abstracted the data independently and selected studies without language restrictions. A random-effects model was

used to combine data on SLE development [American College of Rheumatology (ACR) criteria]. A common effect size was calculated and stratified analyses were done (Comprehensive Metaanalysis Version 2.0 software).

In a PubMed search, 1,781 articles were identified, and 5,098 additional records were identified through other sources. Finally, a total of 10 full-text articles related to OC exposure and 12 to HRT met the criteria and were included in the meta-analysis. For all exposures, 32 meta-analyses were developed. Thirteen evaluated HRT and 19 OC exposures respectively. A significant association between HRT exposure and increased risk of developing SLE was found. The final common effect size, based on a random model, was statistically significant (Rate Ratio: 1.96; p <0.001. Q-value: 3.37; degree of freedom (Q):5; p = 0.643; I-squared: 0%; Tau-Squared 0. Significant publication bias was not identified using the Egger test (p-value 2-tailed: 0.48; intercept: 1.61). When the analysis was run in search of an association between HRT exposure and SLE development including the case control studies, the results were not significant (OR: 0.84; 95% CI: 0.51-1.39; p = 0.51).

There was no association between HRT exposure and specific outcomes of SLE (OR or RR calculations). Six meta-analyses were run evaluating different outcomes: death, all flares, multiple flares, major flares, thrombosis (arterial or venous compiled), and coronary disease. None of them were significant. Analyses carried out searching for an association between OC exposure and different outcomes of SLE were not significant with the exception of a marginal result in a meta-analysis including the SLE outcome limited to patients with ¨ever use¨ status and stratified by age. The final common effect size, based on a random model, was statistically significant (OR: 1.44; 95% CI: 1.00-2.08; p = 0.047). However, if all the groups were included, the results were not significant.

In conclusion, after doing several analyses including different studies on patients with HRT exposure, it was demonstrated that this exposure increases the risk of SLE development in healthy women. Although marginal, there was a significant result when analyzing the risk for SLE with OC exposure (stratified by age). Studies with different designs have found conflicting results. While some have found a significantly higher risk of developing SLE among OC users (143, 144) others have not (145, 146), However, the present meta-analysis did not find a significant association.

The relevance of these results rely on the fact that a relation between SLE and hormonal exposure, especially involving HRT, is significant. It is well known that SLE is a complex and clinically heterogeneous AD characterized by autoantibody production, complement activation, and organ-specific tissue destruction. Genetic predisposition has been implicated in the pathogenesis of SLE. In fact, SLE has a relatively strong genetic component (sibling risk ratio, λs, ~30) compared to many other autoimmune diseases (147). There is substantial information supporting the possible pathways involved in the causality of SLE which include environmental and hormonal factors such as estrogen exposure (131, 148-150). The role of estrogens has been evaluated in different ADs and displays a dual interaction an anti-inflammatory activity by inhibiting many proinflammatory pathways of innate immunity, adaptive immunity, and inflammatory tissue responses. Arguments in favor of a proinflammatory response are the antiapoptotic effects on immune cells, promotion of neoangiogenesis, and stimulation of B cells, which has been delineated to be an unfavorable factor in B cell-driven diseases such as SLE (151).

Nevertheless, identifying individual risk factors predisposing healthy individuals to develop an AD such as SLE has to be given special attention in those who are planning to begin HRT (131).

CONCLUSIONS

The effects of autoimmune ecology on the development, phenotype, and progression of ADs constitute one of the pillars of the autoimmune tautology. The clinical significance of the concepts reviewed in this chapter highlights the plausibility of environmental exposures as risk factors for the emergence of severe subphenotypes, treatment failure, and a worse prognosis.Therefore, an approach based on the identification as well as the intensity and frequency of these exposures should be encouraged in our daily practice in order to prevent the occurrence of ADs, determine clinical outcomes, and personalize the treatment. The generation of public policies, which are focused on the establishment of autoimmune ecology as an essential health component, should be applied as an instrument in the clinical management of patients with ADs resulting in a positive impact on the quality of life and survival rates.

ABBREVIATION LIST

- **ACPA:** anti-citrullinated peptide/protein antibody
- **AchR:** acetylcholine receptor
- **AD:** autoimmune diseases
- **AIH:** autoimmune hepatitis
- **AM:** alveolar macrophage
- **ANA:** antinuclear antibodies
- **Anti-GBM:** anti-glomerular basement membrane antibodies
- **BN:** Brown-Norwat rats
- **CS:** cigarette smoking
- **DAS28:** disease activity score 28
- **DC:** dendritic cells
- **D-Pen:** D-penicillamine
- **ESRD:** end-stage renal disease
- **EULAR:** European League Against Rheumatism
- **GD:** Graves' disease
- **GO:** Graves' ophthalmopathy
- **GP:** Goodpasture syndrome
- **HDZ:** hydralazine
- **HLA:** human leukocyte antigen
- **HRT:** hormone replacement therapy
- **IBD:** inflammatory bowel disease
- **IFN:** interferon
- **MG:** myasthenia gravis
- **MS:** multiple sclerosis
- **NFkB:** nuclear factor kappa-light-chain-enhancer of activated B cell
- **OC:** oral contraceptives
- **OR:** odds ratio
- **OS:** organic solvents
- **PAHA:** procainamide hydroxylamine
- **PBC:** primary biliary cirrhosis
- **RA:** rheumatoid arthritis
- **RF:** rheumatoid factor
- **SE:** share epitope
- **SLE:** systemic lupus erythematosus
- **SLEDAI:** SLE disease activity score
- **SpA:** spondyloarthropathies
- **SSc:** scleroderma
- **TCDD:** tetra chlorodibenzo-P-dioxin
- **TCE:** trichloethylene
- **TLR:** toll-like receptor
- **TNF:** tumor necrosis factor

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21 MOLECULAR MIMICRY IN AUTOIMMUNITY AND VACCINATIONS

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INTRODUCTION

The healthy immune system is tolerant to the molecules which are the building bricks of our body. Braking the self tolerance due to clonal escape, DNA rearrangement epigenetic and environmental factors, all in concert with genetic predisposition, may lead to an autoimmunity termed "The mosaic of autoimmunity" (2). This kaleidoscope of autoimmunity may lead to a variety of autoimmune diseases or to autoimmune (autoinflammatory) syndrome induced by adjuvant (ASIA) (3). One of the mechanisms involved in induction of an autoimmune response is molecular mimicry.

During the last few decades, molecular mimicry was demonstrated between self and non-self molecules that lead to an autoimmune response (4-26). As a prelude, a shared sequence/structure between a non-self microbial/viral infection or a drug and host antigen entails a particular inflammatory state in order to induce an autoimmune state. The severity of the inflammation is influenced by the strength of the infection or perturbation of the immune system. Studies in animal models support the view that a specified infection determines the inflammatory state. To establish the autoimmune nature of the inflammation, it is important to show that it persists in the absence of the inciting microbe. Although the microbe may have been cleared long before disease manifestations appeared, a common infecting agent, may provoke a disease only in combination with genetic or environmental elements, or it may just prime/stimulate the immune system, being a second virus or a nonspecific adjuvant as a second "hit." Viruses, microbes, and parasites may brake peripheral self-tolerance and induce and maintain autoimmunity via several overlapping mechanisms such as epitope spreading, bystander activation, viral persistence, or post-translational modifications of self and altered proteins (3-10). The shared epitopes between the pathogens and autoantigen may induce or trigger chronic inflammation, which is mandatory for establishing all the above listed mechanisms that contribute to an autoimmune response by unveiling "hidden" self-epitopes, cross-reactive peptide presentation, determinant spreading, upregulation of Major Histocompatability Complex (MHC), adhesion, co-stimulatory molecules on antigen presenting cells, upregulation of cellular and extracellular processing, apoptosis, infection of professional Antigen Presenting Cells (APCs), autoantibody production subversion of T cell responses and the immunological homunculus networks (1-11). Excluding shared epitopes between a pathogen and self molecules, structural mimicry can be exemplified also between drugs and self molecules. Molecular mimicry can be an inducer of an autoimmune response or a protector of an autoimmune scenario.

INDUCTION/TRIGGERING OF AUTOIMMUNITY BY MOLECULAR MIMICRY

Various examples of molecular mimicry in different autoimmune systems were described. The main ones are summarized in Table 1. Some examples which put the basis for this mechanism as inducer of an autoimmune scenario are discussed here.

MIMICRY BETWEEN A PATHOGEN AND SELF MOLECULES

Rheumatic fever is a classic example for molecular mimicry, post infection with *Streptococcus pyogenes* (group A streptococcus). The mimicry is between the infecting agent

AUTOIMMUNE DISEASE	SELF ANTIGEN	PATHOGEN MIMETIC
Rheumatic fever	Cardiac myosin, tropomyosin laminin, vimentin, actin, keratin, *N*-acetyl-glucosamine	*Streptoccocus pyogenes* M protein and N-acetyl-glucosamine
Guillain-Barre'	Gangliosides	lipo-oligosaccharide of *Campylobacter jejuni*
Multiple sclerosis Experimental autoimmune encephalomyelitis	Myelin basic protein (MBP) Myelin oligodendrocyte glycoprotein (MOG) 18-32 Myelin proteolipid protein (PLP)peptide 139-151; MBP89-101	Corona, measles, mumps, EBV, human herpes Semliki Forest Virus (SFV) E2 peptide 115-129 Acanthamoeba castellanii (ACA)
Myasthenia gravis	Acetylcholine receptor, neurofilaments	Herpes virus, *Hemophilus influenzae*
Chagas' cardiomyopathy	Human beta 1-adrenergic receptor; Cardiac myosin, Cha antigen Common glycolipid antigenson nervous tissue	*Trypanosoma cruzi* -Ribosomal P0; B13 protein; - shed acute-phase antigen (SAPA) ; 160-kDa flagellum; -trypomastigote stage-specific glycoprotein
Systemic lupus erythematosus	Ro 60 kD, Sm, NMDA, dsDNA	EBV,HERV, pneumococcal polysaccharide
Antiphospholipid syndrome	β2-glycoprotein-I	*Hemophilus influenza*, *Neisseria gonorea*, Tetanus toxin, CMV
Ankylosing spondylitis	HLA-B27, type I, II, IV collagen	*Klebsiella pneumoniae*, chlamydia
Lyme arthritis	*DRB1*0401* or *HLA-DRB1*0101* alleles. Human leukocyte function–associated antigen1α (hLFA-1)	*Borrelia burgdorferi* (outer surface protein A - OspA)

Table 1. Molecular mimicry between pathogens and self antigens in autoimmune diseases. Adapted from (3, 27).

streptococcal M protein and/or a carbohydrate, N-acetyl-beta-d-glucosamine (GlcNAc) and self antigens such as myosin peptides located in the S2 hinge region of the human cardiac myosin leading to rheumatic fever and/or Sydenham's chorea, respectively (12, 13). The disease is characterized by damage to the heart valves (myocarditis/valvulitis), joints and the brain structures, affecting the central nervous system (Sydenham's chorea). The molecular mimicry was proven by three main points: a) Activated T cells isolated from a damaged valve recognize various sequences of the bacterial wall M protein in the bacteria cell wall and support B cells secreting anti-heart myosin Abs. b) Purified antibodies from patients with rheumatic fever cross react with streptococcal M protein and heart myosin, laminin, and vimentin. c) Rats immunized with heart myosin developed myocarditis. In Sydenham's chorea and it's possible variant pediatric autoimmune neuropsychiatric disorder associated with streptococci (PANDAS), autoantibodies present in Sydenham's chorea bind to brain gangliosides, signal neuronal cells activating calcium calmodulin-dependent protein kinase II (CaMKII) in neuronal cells and recognize the intracellular protein biomarker tubulin. These Abs cross react with cardiac myosin in the heart's extracellular membrane antigens such as laminin on the valve surface endothelium.

An additional classical molecular mimicry induces autoimmunity is the case of **Guillain-Barré Syndrome (GBS)**. It is exemplified by damage to the peripheral nervous system mediated by an immune reaction and is characterized by paralysis of the extremities and additional motor damage. Clinical manifestations often appear 1 to 3 weeks after infection by *Campylobacter jejuni* or a viral infection such as EBV, cytomegalovirus (CMV), or *Mycoplasma pneumoniae*. Four conditions were determined that prove the molecular mimicry between an infecting agent and a self component (14,15):

a. High titers of Abs cross-reactive with C. jejuni and gangliosides (Galactose-N-Acetyl-GD1a-1, GD1a, GMIb, GMI). An infection with CMV induces synthesis of Abs that cross-react with GM2 whereas M. pneumoniae induces synthesis of Abs that cross-react with galatocerebroside. Pathogenic monoclonal and polyclonal Abs, specific against gangliosides were obtained from patients with GBS. These Abs have biological pathogenic activities such as damage at the ends of motor nerves mediated by complement proteins, ion-gate blocking and damaged the blood-nerve barrier.

b. Presence of GBS CD4+ T cells specific to the shared epitopes. CD8+ cells expressing receptors for αβ or γ, which react against protein components in *C. jejuni*, were isolated from nerves and peripheral blood samples of GBS patients. The common structure between *C. jejuni* and nerve cells was identified. Lipo-oligosaccharide (LOS) of *C. jejuni* imitates the structure on the ganglioside. Abs obtained from patients with GBS bind to LOS from *C. jejuni*. Immunization of mice with LOS or infection with

C. jejuni induces GBS which resembles the disease in humans. The mice developed T cell response and high titers of Abs against gangliosides that had activities similar to Abs obtained from GBS patients.

The last example of molecular mimicry that induces autoimmune disease relates to **antiphospholipid syndrome (APS)**. The disease is characterized by recurrent fetal loss, repeated thromboembolic phenomena, thrombocytopenia, and prolonged coagulation time. These diverse clinical pictures are associated with elevated levels of circulating antiphospholipid β-2-glycoprotein-I (β2GPI)-dependent Abs mainly. Employing a peptide phage display library, our group have identified three hexapeptides that react specifically with the anti-β2GPI monoclonal Abs located on the β2GPI molecule as mimotopes. All three peptides specifically inhibit the biological functions of the corresponding anti-β2GPI monoclonal Abs (for example *in-vitro* endothelial cell activation and *in-vivo* induction of experimental APS) (16). Using the Swiss Protein database revealed high homology between the hexapeptides with different bacteria and viruses. The direct proof of induction of experimental APS by peptidomimetic between a pathogen and self came from passive transfer experiments in which mice were immunized with pathogens sharing amino acid sequences with human β2GPI molecule. Experimental APS was induced by passive transfer of affinity purified Abs from the immunized mice on a column composed of the shared peptidomimetic between the infection agent and the β2GPI molecule to naïve mice (16). The mice developed enhanced fetal loss associated with prolonged coagulation time and thrombocytopenia (17). Pierangeli et al (18) demonstrated that immunization of naive mice with CMV and β2GPI shared peptide induced generation of mouse thrombogenic anti-β2GPI Abs in *ex vivo* models of thrombosis as well as fetal loss. Based on the above studies, molecular mimicry between a pathogen and β2GPI molecule provided proof for the etiology of APS.

A mimicry scenario was proposed by Pender (19) for **Epstein-Barr virus (EBV)** mediated latent infection of B cells. Human chronic autoimmune diseases, including lupus, multiple sclerosis, Sjogren's syndrome, rheumatoid arthritis, autoimmune thyroiditis, autoimmune hepatitis, and cryptogenic fibrosing alveolitis, are based on infection of auto reactive B-lymphocytes by EBV. Latent EBV infected auto reactive memory B cells may lodge in organs where their target antigen is expressed and act as antigen presenting cells. When CD4+ T cells that recognize antigens within the target organ are activated in lymphoid organs by mimicry with infectious agents, they migrate to the target organ but fail to undergo activation induced apoptosis because they receive a co-stimulatory survival signal from the infected B cells. The auto reactive T cells proliferate and produce cytokines, which recruit other inflammatory cells with resultant target organ damage and chronic autoimmune disease.

EBV mimicry was proven in lupus patients and experimental models by the shared epitope amino-acid sequences between Epstein–Barr virus nuclear antigen (EBNA-1), a DNA binding protein that maintains replication of the EBV genome within infected cells and is required for maintaining viral latency, and the major lupus specific Smith rib nucleoprotein complex (Sum B/B', D1, D2, and D3) (20). The PPPGRRP motif of EBNA-1 share homology with Sum proline-rich motif, PPPG-MRPP and are recognized by sera from lupus patients. Mice immunized with the entire EBNA-1 protein vector produced anti-Sum suggesting that the anti-Sum response occurs as a consequence of antigenic mimicry through EBNA-1 antibodies. Immunizing mice and rabbits with EBNA-1-Sm homologous peptides resulted in epitope spreading and lupus-like autoimmunity. SmD195-119 Abs and EBNA-135–58 region show similarity whereas EBNA-2 shares similarity to the C-terminal domain of SmD1 and SmD3. All the EBNA and Sum/Sad similarities are recognized by lupus patient sera. Ro autoantibodies are among the first to appear in SLE. The 60 kea Ro169-180 cross-react with EBNA-158-72 epitopes. Animals immunized with either the first epitope of 60 kea Ro or the cross-reactive EBNA-1 epitope progressively develop autoantibodies to Ro and acquire clinical symptoms of lupus such as leukopenia, thrombocytopenia, and renal dysfunction. However, this EBNA-1 sequence does not share significant homology with the Ro 169–180 sequence. Rather there must be an immunological structural relationship between EBNA-1 and Ro that is sufficiently powerful to persist across species barriers among mammalian immune systems.

The ability of these peptidomimetics shared between EBNA-1, 2, and Sm/SmD or Ro antigens to cause lupus like response in animal models supports the notion of molecular mimicry between EBV and self nuclear antigens as a possible inducer of lupus.

Additional EBV molecular mimicry induces autoimmune response, mediated by shared epitopes on MHC-dependent pathways. An example of that was shown in rheumatoid arthritis (RA). Several autoantigens which undergo citrullination were described in RA such as filaggrin, fibrinogen, vimentin, collagen type II, and α-enolase. Upon citullination, anti-citrullinated peptide/protein Abs (ACPA) are triggered and show high specificity to RA. ACPA are found in RA patients with HLA-DRB1 alleles. The products of these alleles encode a 5 amino acid sequence in a peptide-binding pocket, the so-called shared epitope (SE). Citrullination increases self-antigen immunogenicity through increased binding affinity to SE-containing HLA-DR molecules. HLA molecules carrying the amino acid sequence QKRAA, QRRAA or RRRAA at positions 70–74 of the DRβ1 chain are associated with ACPA positive RA. The QKRAA determinant is also expressed on the EBV protein gp110 and has been shown to be a target of humoral and cellular immune responses in humans (21). One group found decreased T cell response to EBV gp110 in peripheral blood which correlates with disease activity and severity in patients with rheumatoid arthritis (20). In addition, several sequences are found in EBNA-6 and HLA-DQ*030228: a six-amino acid sequence is shared by EBV reading frame BPLF1 and 65 kD heat shock protein, a protein that induced arthritis in an animal model of adjuvant arthritis (22).

T cell receptor (TCR) from patients with multiple sclerosis (MS) recognized both a DRB1*1501-restricted myelin basic protein (MBP) and DRB5*0101-restricted EBV peptide. Crystal structure of the DRB5*0101-EBV peptide complex revealed a marked degree of structural equivalence to the DRB1*1501-MBP peptide complex at the surface presented for TCR recognition [22]. CD4+ T cells found in cerebrospinal fluid of MS patients cross react with EBV DNA polymerase peptide EBV627-641 and immunodominant MBP85-99 peptide [22]. The viral LMP1 has homology to MBP suggesting natural cross-reactivity. Thus, antibodies induced against LMP1 during EBV infection might act as an inflammatory trigger to reacting to MBP through a mimicry mechanism (23).

T CELL EPITOPE PEPTIDOMIMETICS

Mimicry can also take place at the level of the T cell. Disease inducing peptidomimetics are those peptides of autoantigens that can be presented by major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APCs) to autoreactive CD4+ T cells (or alternately by HLH subtypes.)

Autoimmune hepatitis (AIH) is a chronic, progressive liver disease, characterized by hepatocellular inflammation and necrosis. A subgroup of AIH patients presents specific autoantibodies to soluble liver antigen/liver-pancreas (SLA/LP) protein. Autoantigenic SLA/LP peptides are targeted by CD4+ T cells and restricted by the allele HLA-DRB1*03:01, which confers disease susceptibility. A positively charged residue at position 71 has been indicated as critical for AIH susceptibility in all of the HLA alleles identified. Molecular mimicry between SLA/LP and viral/bacterial antigens was illustrated using an *in-silico* strategy (24). The immunodominant region of SLA/LP was used as the query in databank searches to identify statistically significant similarities with viral/bacterial peptides. Homology modeling and docking was used to investigate the potential interaction of HLA-DRB1*03:01 with the identified peptides. A statistically significant structural similarity between the immunodominant regions of SLA/LP and a region of the surface antigen PS 120 from Rickettsia spp. has been detected. The interaction of the SLA/LP epitope and the corresponding Rickettsia sequence with the HLA-DRB1*03:01 allele has been simulated.

Primary biliary cirrhosis (PBC) is an autoimmune cholestatic liver disease characterized by the presence of antimitochondrial Abs and inflammation of interlobular bile ducts. Shimoda et al. (25), showed that human PDC-E2163-176 peptide (GDLLAEIETDKATI) is an immunodominant autoreactive T cell epitope in PBC patients that is restricted by HLA DRB4*0101. Different T cell clones derived from PBC patients reactive to the human PDC-E2163-176 peptide, were reactive to mimicry peptides derived from microbial proteins. These results proved the involvement of molecular mimicry in PDC-E2 (EQSLITVEGDKASM) peptide from diverse pathogens such as *Escherichia coli, Acholeplasma laidlawii, Pseudomonas putida, Neisseria meningitis outer membrane and Clostridium difficile Toxin B* protein P64K. Therefore, molecular mimicry was postulated to be a possible mechanism in the development of PBC.

An additional example for T cell epitope peptidomimetics is a systemic disease such as Lyme arthritis which results from infection by the tick-borne spirochete *Borrelia burgdorferi*, which results in inflammatory joint disorder that resembles rheumatoid arthritis (26). The majority of individuals with the antibiotic treatment resistant disease have the HLA-DRB1*0401 or HLA-DRB1*0101 alleles and high titer of IgG specific for outer surface protein A (OspA) of *Borrelia burgdorferi*. Th1 cells reactive to OspA are often found as well. Immunization with recombinant OspA has been effective in preventing Lyme disease in two clinical trials and is now available as a vaccine. These findings suggest that HLA-DRB1*0401 or HLA-DRB1*0101 restricted immune response, that is OspA-specific, somehow precipitates joint-specific autoimmunity. The severity of joint swelling and the duration of Lyme arthritis after antibiotic treatment are associated with T cell responses to specific epitopes of OspA. The nine-residue peptide OspA165–173 was predicted to be the peptide most effectively bound by HLA-DRB1*0401. In addition, when injected with OspA protein, transgenic mice for HLA-DRB1*0401 responded primarily to the OspA165–173 peptide as did T cells from an HLA-DRB1*0401 antibiotic-resistant patient with Lyme arthritis, which were challenged *In-vitro*. A search of the Genebank protein Database identified one human protein, leukocyte function–associated antigen 1α (hLFA-1α), which contains the peptide hLFA-1α (L332–340). hLFA-1α (L332–340) has homology to the dominant epitope of OspA and was predicted to bind strongly to HLADRB1* 0401 (this was eventually confirmed experimentally).

Human endogenous retroviruses (HERV) and molecular mimicry. Retroviruse-derived elements in the human genome constitute 90% of non-coding mobile sequences and contribute substantially to the architecture of the human genome. Five to ten percent of the eukaryotic genome consists of elements of retroviral origin. The HERVs were integrated into the human genome 30 to 40 million years ago and are present in primates with the exception of gorillas. HERVs were coined as the key molecular link between the host genome and infectious viral particles. Epigenetic status of the genome (hypomethylation, histone acetylation), UV, chemicals/drugs, injury/stress, hormones, infections, all as a single cause or in concert may modulate HERVs involvement in pathogenic processes. Infection can promote HERV expression by molecular mimicry or by functional mimicry. Several reports have indicated that HERV activation followed by the expression of HERV proteins may play an important role in the induction of autoimmune diseases such as SLE. Clone 4-1 is a member of the HERV-E family and shows sequence homology with Molony murine leukaemia virus; it has ~8.8 kb of sequence including open reading frames in the gag and env regions . Anti-clone 4-1 gag and env product autoantibodies were detected in 48.3 and 10.7% of Japanese SLE patients respectively. These antibodies were not detected in the serum of normal individuals. This finding indicates that transcription of HERV genes may be facilitated and virus particles or components may

be produced in some SLE patients. Furthermore, a computer search of current entries in sequence libraries revealed that there are extremely high homologous sequences between clone 4-1 gag region and the E antigen of HLA class I molecules. The homology between clone 4-1 gag protein and E antigen may contribute to the escape of endogenous retrovirus production from the killing effects of CTL and/or NK cells.

Another example is the EBV mediated latent infection of B cells. EBV transactivates HERV-K18 superantigen via docking to the human complement receptor 2 (CD21) on primary B cells and induces HERV-K18 env gene in resting B lymphocytes (27). The env protein encoded by HERV-K18 has a superantigen activity that strongly stimulates a large number of T cells.

MOLECULAR MIMICRY BETWEEN A DRUG AND A SELF-COMPONENT

Many drugs are processed endogenously by the liver. Consequently, new foreign antigens termed neo-antigens may be created in the form of protein adducts. These new antigens may resemble self-antigens. The metabolism of the anesthetic halothane elevates levels of trifluoroacetylated (TFA) proteins in humans or in mouse models. Only 1 of 3,000 people develop hepatic injury (halothane hepatitis). These people cannot tolerate the presence of TFA proteins and create cross reacting Abs that identify the TFA proteins and pyruvate dehydrogenase (PDH) (28). Molecular mimicry has been defined as TFA-lysine in TFA proteins that imitate clusters of prostatic lipoic acid of the subunit E2 of PDH (27). Interestingly. Patients with halothane hepatitis did not properly express the cross reacting component E2.

Therefore, in this instance, molecular mimicry played a protective role by canceling the cross reacting specificity. These findings raise the possibility that proper introduction of self components (such as PDH) may induce immune tolerance to a self-component and to other foreign antigens that present similar structures, for example, TFA proteins created spontaneously during halothane metabolism.

MOLECULAR MIMICRY VACCINATION AND INFECTION

TETANUS TOXIN AND APS

In 2002, our group was the first to decipher the enigma of the infection-APS relationship, demonstrating the molecular mimicry between some β2GPI peptides and the tetanus vaccine (17, 29). Antibodies directed to the shared epitope induce an experimental APS in naïve mice. Human anti-β2GPI monoclonal Abs (mAbs) from an APS patient with recurrent fetal loss were introduced into a hexapeptide phage display library, resulting in the identification of three synthetic peptides which had homology to diverse bacteria viruses, parasites, and tetanus toxoid (TT). One of the synthetic peptides, TLRVYK, was found by our group to be the common structure for β2GPI and TT molecules (appears three times in the TT, not as a linear peptide but as conformational mimotopes) as illustrated by ribbon three-dimentional structures of β2GPI and TT. Naïve BALB/c mice, immunized with TT, developed antibodies directed to β2GPI and to diverse structures of TT and became sick. Therefore, in order to study the effect of TT-β2GPI-related antibodies on the induction of experimental APS, we isolated the TT/β2GPI antibodies. The Abs from the TT-immunized mice were affinity purified on a column composed of TLRVYK synthetic peptide. Anti- TT/β2GPI Abs bound β2GPI and TT with high affinity dose-dependently. Passive transfer of the affinity purified anti-TLRVYK Abs to naïve mice induced the experimental model of APS manifested by a high, significant percentage of fetal loss, prolonged coagulation time, and thrombocytopenia. Nine years later, Zivkovic et al (30), confirmed the association between TT and experimental APS. TT hyperimmunized mice and passive mice transferred with anti-TT monoclonal antibody cross-reactive with β2GPI had increased fetal loss and low fecundity in BALB/c mice. Furthermore, hyperimmunization of BALB/c mice with TT in aluminium hydroxide, glycerol, or CFA resulted in elevated circulating antibodies to TT, β2GPI, gangliosides, laminin, and induced fetal loss. Last year, 2012, Dimitrijevic' et al. (31) reconfirmed the molecular mimicry between the 3D conformation of the linear sequence TLRVYK of TTd and β2GPI. The authors succeeded in inducing antiphospholipid syndrome (APS) in two different non-autoimmune prone mouse strains, BALB/c and C57BL/6, by tetanus toxoid (TTd) hyperimmunization using different adjuvants. Both molecular mimicry and polyclonal B cell activation occur in APS induction with molecular mimicry effects being dominant in BALB/c mice, and polyclonal cell activation being dominant in C57BL/6 mice. Confirmation of molecular mimicry effects, which in the condition of T cell stimulation generated fetal resorptions in the BALB/c strain, was achieved by passive infusion of monoclonal antibody (mAb) T-26 specific for TTd and anti-β2-glycoprotein I obtained after TTd hyperimunization.

MYCOBACTERIUM AND MS, RA AND UVEITIS

Molecular mimicry between microbial antigens and host-proteins is one of the etiological enigmas for the occurrence of autoimmune diseases. T cells that recognize cross-reactive epitopes may trigger autoimmune reactions. Association of *Mycobacterium tuberculosis (M. tuberculosis)* has been implicated in different autoimmune diseases including rheumatoid arthritis and multiple sclerosis. Employing bioinformatic tools, Babu Chodisetti et al. (32) have identified potentially cross-reactive T cell epitopes restricted to predominant class I and II alleles of human leukocyte antigens (HLA). These are similar to peptides of mycobacterial proteins and considerable numbers of them are promiscuous. Some of the identified antigens corroborated with established autoimmune diseases linked with mycobacterial infection. The authors' analysis showed several peptides from mycobacterial HSP60 (also known as HSP65) homologous to human HSP60 and it relatives that bind to

many different alleles. One particular peptide, KPLVIIAEDVD-GEALSTLVLN, promiscuously bound to many alleles including HLA-DRB1*15:01 with high affinity. This is suggestive of the fact that such cross-reactive epitopes may initiate the pathogenesis of MS. Another autoimmune disease that has been frequently associated with the occurrence of tuberculosis is RA. Garip et al. (33) investigated the cellular immune response in uveitis developing after intravesical Bacille-Calmette-Guérin (BCG) applications suggesting possible antigenic mimicry of mycobacterial and retinal antigens. A 72-year-old HLA-B27-negative patient with bilateral granulomatous anterior uveitis that developed during the third cycle of intravesical BCG applications. The patient's peripheral T cell reactivity to ocular autoantigens was compared with the response to purified protein derivative (PPD) from Mycobacterium tuberculosis. T cell proliferation and cytokine and chemokine secretion were measured *in vitro*. The authors demonstrated proliferation to PPD, interphotoreceptor retinoid-binding protein (IRBP), and IRBP-peptide R16 as well as secretion of proinflammatory cytokines in response to PPD, retinal soluble antigen (S-Ag), IRBP, cell retinal-binding protein (CRALBP), and some S-Ag and IRBP peptides. Amino acid sequence alignments revealed homologies, similar and even identical regions of 5 to 11 amino acids, between proteins from M. tuberculosis, BCG, and retinal antigens suggesting antigenic mimicry as a potential cause of uveitis in this patient.

VACCINATION WITH NY-ESO-1 ANTIGEN AND AUTOIMMUNE THYROIDITIS

Immunotherapies and targeted therapies are frequently associated with thyroid dysfunction, which is in contrast with the rare thyroid abnormalities induced by cytotoxic agents. Vita *et al.* (34) describe a case of Graves' disease triggered by NY-ESO-1 in a HLA-A2 negative woman. A 32-year old woman with a synovial sarcoma, received radiotherapy, chemotherapy, and finally NY-ESO-1 vaccine. The patient typed HLA A11/A33 (19), B13/B56 (22), Cw3/-. One month after the beginning of immunotherapy, thyroid dysfunction was clinically suspected and Graves' disease was biochemically confirmed. The authors hypothesized that NY-ESO-1 shared partial homology with thyroid autoantigens and that at least one pair of homologous sequences contained amino acid sequence binding motif(s) to a restricted number of HLA molecules. They used the software BLAST to search for amino acid sequence homologies between NY-ESO-1 and thyroid autoantigens [TSH-receptor (TSH-R), thyroperoxidase (TPO) and thyroglobulin (Tg)] and the HLA ligand/motif database to look for HLA/T cell receptor binding motifs in the regions of NY-ESO-1 and thyroid autoantigens that were homologous. They found 15 epitopic regions of NY-ESO-1 homologous to 15 regions of thyroid autoantigens, some of which were epitopic: 5 of TSH-R, 8 of Tg, 2 of TPO. These homologous sequences contain binding motifs belonging to several HLA class I antigens including HLA A2 and the patient's A11 and A33. This case demonstrates again that genetically predisposed patients are at risk to develop thyroid dysfunction after vaccine immunotherapy for malignancies.

HBV VACCINE AND MYELIN MOLECULAR MIMICRY

Hepatitis B vaccination (HBV) is a non-infectious viral subunit consisting of the small hepatitis B virus surface antigen (SHBsAg). Over the years, a link between HBV administration and development of demyelinating disorders leading to multiple sclerosis (MS) was demonstrated. Cellular and humoral immunity against myelin basic protein (MBP) and oligodendrocyte glycoprotein (MOG) results in myelin damage in multiple sclerosis (MS). Surfing the protein database, Bogdanos found that known epitopic regions on SHBsAg share extensive homologies with MBP and MOG. Moreover, normal subjects undergoing HBV vaccination developed responses to the shared homologies between the SHBsAg and MOG antigens. The selective appearance of viral and myelin cross-reactive responses in some but not all of the mimicking pairs supports the biological significance that vaccine plays a possible role as an immunomodulator of viral and self cross-reactivity (35).

DNA VACCINATION AND AUTOIMMUNE HEPATITIS

Autoimmune diseases have been generated after infection by LCMV in transgenic mice expressing LCMV-nucleoprotein (NP) or glycoprotein in β cells of the islets of Langerhans or their oligodendrocytes (36). These transgenic mice did not develop any immunopathology in the absence of the LCMV challenge. These experiments showed that molecular mimicry between self peptides and viral proteins can be responsible for initiating and maintaining the autoimmune process. Diabetes and CNS autoimmune disease in transgenic mice were mediated by CD8+ cells, and a critical number of activated CTLs were necessary to induce the disease. Djilali-Saiah et al. (37) took this further by demonstrating an autoimmune hepatitis in a transgenic mouse model vaccinated with a neo-self antigen in the liver. The transgenic mouse model proposed in this study was based on the hypothesis that infectious agents have the potential to initiate autoreactivity through molecular mimicry. A transgenic mouse expressing lymphocytic choriomeningitis virus nucleoprotein (NP) in a H-2b background developed liver injury when vaccinated with plasmids expressing NP as an intracellular or a secretory protein. Coinjection of plasmids coding for NP and IL-12 facilitated the induction of a Th1 phenotype as detected by a specific B lymphocyte response characterized by a predominance of IgG2 subclass anti-NP Abs. CTLs activated in peripheral lymphoid organs by DNA vaccination migrated to the periportal and lobular areas of the liver. Their presence was associated with a significant degree of cytolysis as evidenced by elevated transaminases several weeks after immunization. As activated specific T lymphocytes proliferated in the periphery and caused cytolysis of target cells, this study suggests that autoimmune hepatitis can be triggered by molecular mimicry, and that local injury may not be essential to initiate autoreactivity in the liver.

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22

EPIGENETICS AND AUTOIMMUNE DISEASES

Paula Quintero-Ronderos
Gladis Montoya-Ortíz

INTRODUCTION

Epigenetics was defined by Conrad Waddington in the early 1940s as *The branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being* (1). Currently, epigenetics is defined as the *study of changes in gene function that are heritable and that do not entail a change in DNA sequence* (2). As has been mentioned before, all these mechanisms are heritable thus the epigenetic marks have the ability to persist during development and potentially be transmitted from offspring to offspring. These mechanisms play an essential role in: regulation of gene and microRNA (miRNA) expression, DNA-protein interactions, cellular differentiation, embryogenesis, X-chromosome inactivation, and genomic imprinting (3).

One of the main functions of epigenetics is gene regulation. Gene regulation plays an important role in determining individual gene function and activity, sets of genes which are functional in each specific cell type, cell type development and differentiation, and metabolic plasticity of the cell that allows it to adapt itself to environmental changes. But it is important to note that epigenetics is not the only determinant in gene function. There are intrinsic components that are stable over time and are the same in each cell type. These intrinsic components such as polymorphism and mutations are one of the mechanisms that affect gene expression. Also, the environment (virus, hormones, nutrition, and chemicals) influence epigenetics and the intrinsic components thus altering gene function (4).

The interaction between environment and epigenetics is only one of the mechanisms by which a large range of different phenotypes arise from the same genotype such as in the case of monozygotic twins. Monozigotic twins have an identical DNA sequence, but studies had found some phenotypical differences that may be the consequence of different exposure to environmental stressors. This exposure produces alterations in the DNA methylation pattern and histone modification (5,6). This approach may be one of the causes of the differences found in the concordance rate of autoimmune diseases between homozigotic twins (Table 1) (7–22).

Another example of how epigenetics interact with the environment is in the study of *Agouti* pregnant rodents. In this study, researchers fed *Agouti* pregnant rodents food rich in methyl donors and they found that the offspring of these rodents had a different coat color because of an altered DNA methylation process in comparison to the offspring of pregnant rodents fed a normal diet (Figure 1) (23). Other researchers showed that Dutch who were exposed prenatally to

AUTOIMMUNE DISEASE	CONCORDANCE RATE	REFERENCES
Systemic lupus erythematosus	11 - 25 %	Jarvinen P *et al* (1992) (7) Deapen D *et al* (1992) (8)
Type I diabetes mellitus	13 – 48 %	Kyvik KO *et al* (1995) (9) Kaprio J *et al* (1992) (10) Kumar D *et al* (1993) (11) Matsuda A *et al* (1994) (12) Olmos P *et al* (1988) (13)
Rheumatoid arthritis	12 – 22 %	Aho K *et al* (1986) (14) Silman AJ *et al* (1993) (15) Bellamy N *et al* (1992) (16)
Grave's disease	22.2 %	Brix TH *et al* (1998) (17)
Multiple sclerosis	9 – 31 %	Kinnunen E *et al* (1987) (18) Mumford CJ *et al* (1994) (19) Sadovnick AD *et al* (1993) (20) Ebers GC *et al* (1986) (21)
Celiac disease	75 – 83 %	Greco L *et al* (2002) (22)

Table 1. Concordance rate of autoimmune diseases between monozygotic twins.

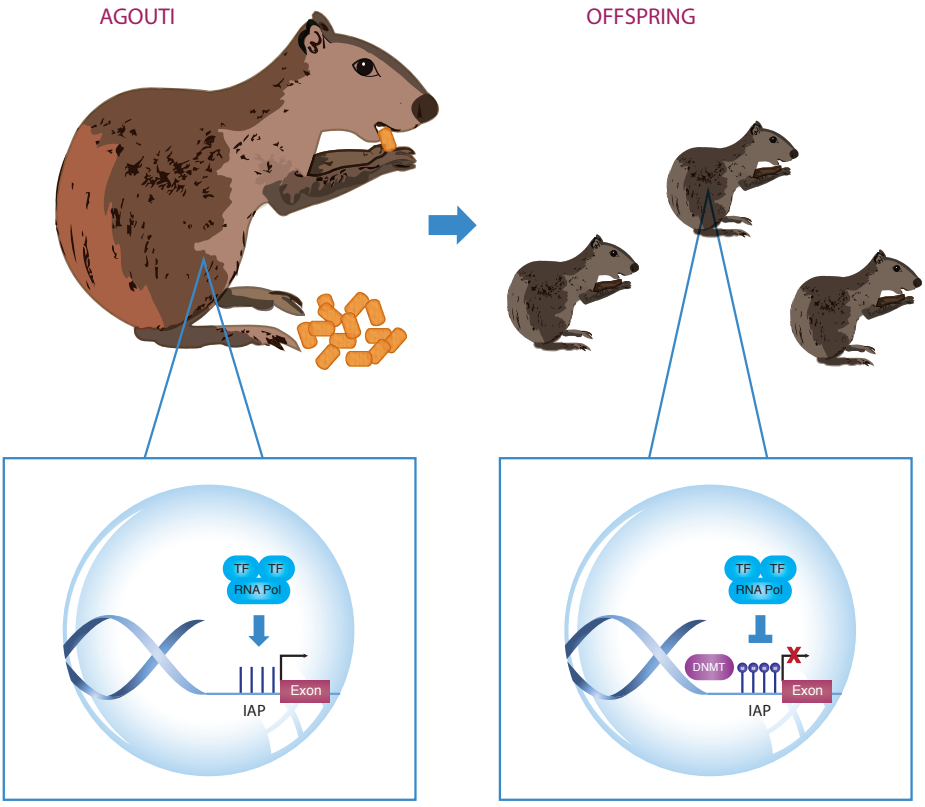

Figure 1. Epigenetics – environment Interaction. Offsprings from *Agouti* pregnant rodent fed with food rich in methyl donors such as folic acid, had a different coat color because of an altered DNA methylation process.

famine during Dutch Hunger Winter in World War II. Because of the lack of nutrients during the prenatal life of these individuals, there was a deficiency in methyl donors such as the amino acid methionine that causes the hypomethylation of the maternally imprinted insulin-like growth factor II (IGF-2) differentially methylated region (DMR) in comparison to unexposed and same sex siblings. The IGF-2 gene plays a key role in human growth and development, thus this finding supports the fact that early mammalian development is important for establishing and maintaining epigenetic marks (24,25).

Many studies had been done with the Dutch Hunger Winter cohort from World War II. One of them looked for differences in birth weight between offspring of mothers who were exposed to famine in early and late gestation. The authors found that epigenetic differences were found in individuals who were exposed to famine in early gestation but individuals also were born with a normal birth weight. In contrast, individuals exposed to famine in late gestation were born with low birth weight, but they didnt have any epigenetic changes (23). At the same time, other studies had demonstrated that those individuals exposed to famine during the gestational period have a higher risk of developing schizophrenia and dyslipidemia. One of these studies demonstrated that there are sex-specific differences in the pattern of atherogenic lipids at the age of 58. Women showed elevated serum concentration of total cholesterol, LDL, and tiglycerides in comparison with unexposed women (26,27). Also, it was found that exposed women had a wide range of indexes of body mass and thus had a higher risk of obesity and developing chronic diseases (28–30). Other studies have shown that individuals exposed to famine in early gestation have an increased risk of schizophrenia in both males and females, but individuals who were exposed in later gestation have a higher risk of developing affective disorder in the schizophrenic spectrum (31–33).

Nowadays, literature about how environmental factors may affect epigenetic mechanisms is increasing. Indeed in the last few years, some studies have investigated the relationship between socio-economic status (SES) and epigenetic differences, and their impact on risk and disease development. McGuinness D. *et al*., studied the global methylation content in individuals from the Glasgow-Based pSoBid cohort to elucidate differences in prevalence between more and less privileged groups. This work showed global DNA hypomethylation in the most deprived participants in comparison to more privileged ones, thus showing a relationship between hypomethylation status and biomarkers of cardiovascular disease and systemic inflammation such as an increase in IL-6 levels (34). Another study was done with forty adult males from the 1985 British Birth Cohort Study to look for the methylation state of promoters in individuals with extreme SES. Some limitations on the study are: individuals are only males and the blood samples were taken only in adulthood. However, authors found differential methylation in promoters for basic cell functions and signaling between SES groups (35). Tehranifar

P.*et al.*, used a cohort of US birth women to examine if early life and adult SES were associated with methylation of repetitive elements Sat2, Alu, and LINE-1. The results showed higher Sat2 and Alu methylation in individuals with low family income at birth which may predispose to disease development (36). As the study mentioned before, a limitation on the study was the use of a cohort of only women. Although all these studies give us a point of view of how SES affects the methylation status, it is important to mention that there is a lack of studies on gene-specific methylation status and SES. Also, it is important to remember that a variety of social conditions may affect methylation such as: diet, physical activity, alcohol intake, organic solvents, and pollutants in water and air (37,38). These studies are of great importance because it is known that cells may be influenced during development by environmental stressors that alter the epigenome and persist throughout life because it is maintained after mitosis. This could be an explanation for altered phenotypes and disease development (39).

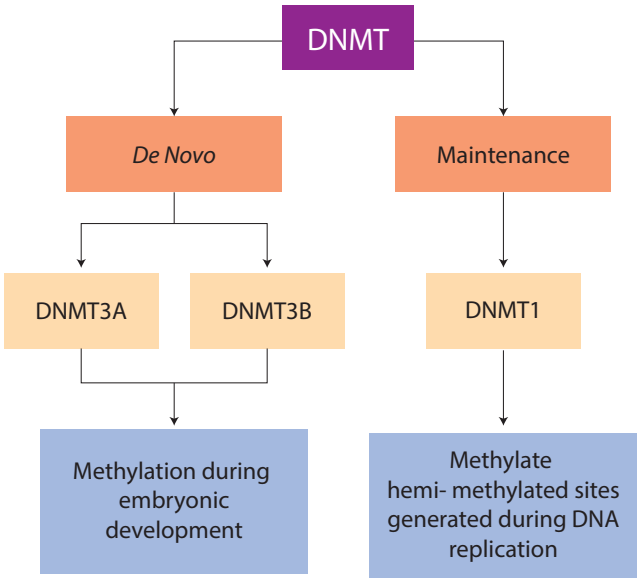

Figure 2. Classification of DNMTs. DNMTs can be classified in de *novo* and maintenance. *de novo* DNMTs are involved in methylation during embryonic development and maintenance DNMTs are involved in methylation during DNA replication.

EPIGENETIC MECHANISMS

There are different epigenetic mechanisms that regulate gene expression by activating or repressing gene expression: DNA methylation, histone modification, nucleosome positioning and RNAi (miRNAs and siRNAs) (2). It is important to mention that all these epigenetic mechanisms act together at the same time to regulate gene expression and not separately.

DNA METHYLATION

DNA methylation occurs in different regions of the genome and it is important in the embryogenesis, cellular differentiation, and tissue-specific development. It is noteworthy that DNA methylation varies among tissues and cellular types because of a dynamic process involving methylation and demethylation events (40–42). Once there is an alteration in DNA methylation and demethylation patterns, it will give rise to dysfunctional cells and consequently disease. Methylation is mediated by the DNA methyltransferase (DNMT) family which is in charge of donating a methyl group to DNA 5-cystosine producing a 5-methylcytosine (5-mC). This family of enzymes has 5 members: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. At the same time, DNMTs can be classified into *de Novo* and maintenance DNMTs (Figure 2) (2). *De Novo* DNMTs are DNMT3a and DNMT3b, and they are in charge of methylation during embryonic development. DNMT1 is the maintenance DNMT, which is in charge of methylate hemi-methylated sites that are generated during DNA replication. DNMT2 acts on transfer RNA and DNMT3L acts on embryogenesis (43).

The other mechanism that counteracts DNA methylation is demethylation. Demethylation can be passive or active (2). The first one is induced by inhibition of DNMT activities such as in the case of several drugs that are used as therapeutic compounds to erase aberrant hypermethylation. This inhibition produces the passive removal of methyl groups by the absence or dilution of the enzyme (44). Active demethylation, in turn, occurs in cell differentiation and has been found in the activation of immune cells (45). This process depends on cytosine deaminase in which its activation induces cytidine deaminase (AICDA) that deaminates 5-methylcytosine (46). Even though this was the first mechanism suggested, there is now evidence about three enzymatic families that activate demethylation: Ten-Eleven translocation family (TET), AID/APOBEC family, and the base excision repair glycosylases (BER). TET proteins (TET 1, 2, and 3) catalyze the conversion of 5-mC to 5-hidroxymethylcytosine (5-hmC). Furthermore this product is oxidized to 5-formylcytosine (5-fC) and 5-carboxylmethylcitosine (5-caC) (47). This conversion is followed by a deamination caused by the AID/APOBEC family of 5-hmC to 5-hidroxymethyluracyl (5-hmU). Here the active process of demethylation starts. The product 5-hmU is then replaced through the BER enzyme family to an unmethylated cytosine. The BER family is composed of a glycosylase member family such as thymine DNA glycosylase (TDG) and single-strand selective monofunctional uracil DNA glycosylase 1 (SMUG1). The function of these enzymes is DNA repair by the replacement of unstable products with unmethylated cytosines. They also interact with transcription factors, histone acetyltransferases, and *de novo* DNMTs (48–50). Moreover, there is evidence that RNA polymerase II interacts with 5-fC and 5-caC producing a signal for the recruitment of TDG and BER (51). All this information opens the door to new evidence about the demethylation mechanisms in gene imprinting. Hackett J. *et al.*, demonstrated that

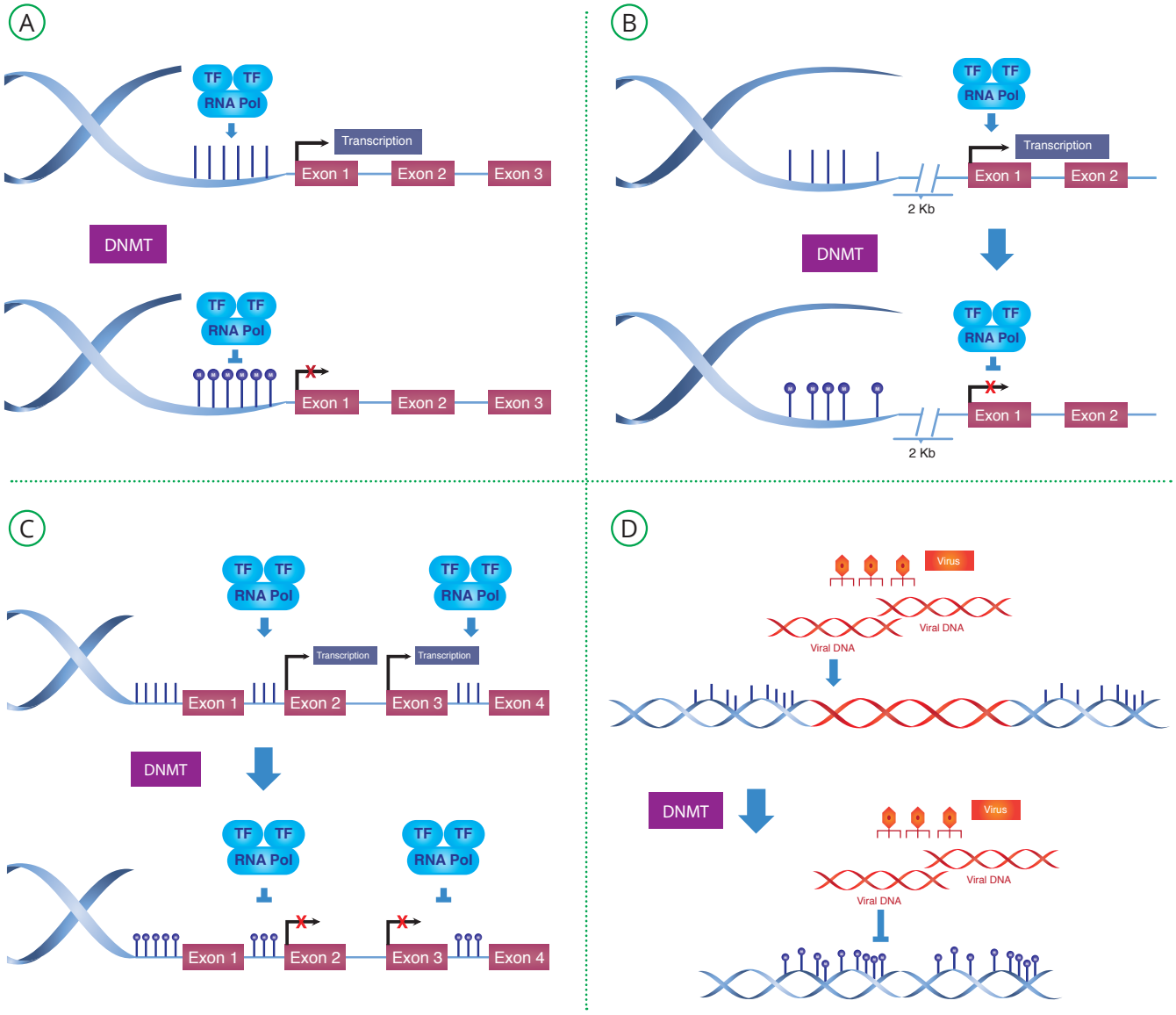

Figure 3. DNA methylation patterns. (a) Basal state of CpG islands is been unmethylated to allow the transcription, but when they are methylated at promoter regions of genes the transcription will be inhibited. (b) At the same time, CpG island shores located up to ~2 kb of CpG islands have a similar methylation pattern to the CpG islands in which methylation is closely associated with transcriptional inactivation. (c) Gene bodies are methylated to prevent spurious transcription initiations. (d) Repetitive sequences which are hypermethylated to protect chromosomal integrity by preventing reactivation of endoparasitic sequences that causes chromosomal instability.

demethylation in primordial germ cells occurs by TET1 and TET2 enzymes and this conversion to 5-hmC occurs in a temporal order depending on the imprinted Differentially Methylated Regions (DMR) (52).

It is important to understand that when there is a methylation state, transcription will be repressed; in contrast, when there is an unmethylated state, transcription will be permitted. Transcription inhibition is achieved because methyl groups interfere with the binding of transcription factors that activate transcription from a specific gene. Many of these transcription factors recognize mainly CpG sequences, but when these sequences are methylated they are unable to bind DNA. An additional mechanism of transcriptional repression involves proteins that are attracted to methylated CpG sequences. These proteins are part of a family methyl-CpG-binding domain (MBD), and they recognize methylated sequences to provide a further signal to alter chromatin structure by formation of a co-repressor complex (53).

There are four possible DNA methylation patterns. The first methylation pattern and the most widely studied is the methylation of CpG islands in promoter regions of genes. These CpG islands are regions of more than 200 bases

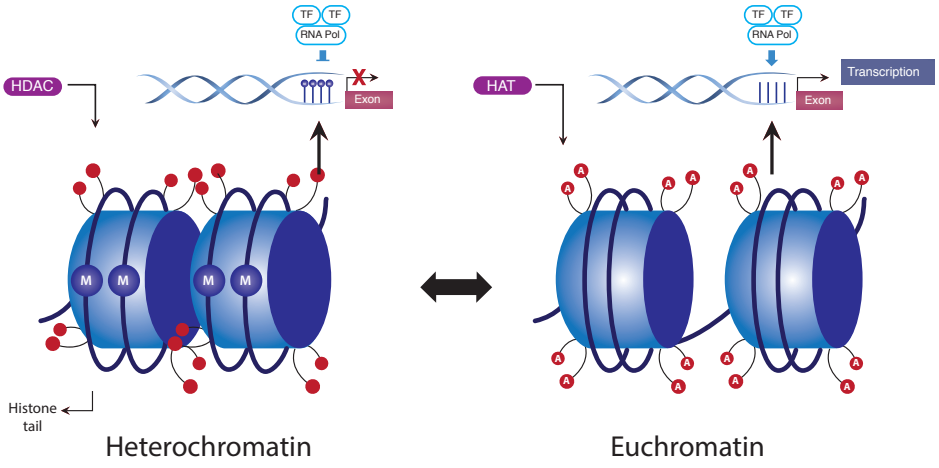

Figure 4. Histone Modification. To form heterochromatin, histone deacetylation of histone tails caused by HDACs enzymes in association with DNA methylation (M) confers a dense configuration of DNA that prevents its transcription. In the euchromatin state, there is an acetylation of histone tails (A) by HATs enzymes in association with DNA demethylation to promote gene expression.

with a G + C content of at least 50%. Many human gene promoters (60%) are associated with CpG islands and their basal state is unmethylated to allow transcription (Figure 3a) (53,54). The second pattern is DNA methylation of CpG island shores which are regions of lower CpG density in close proximity (~2 kb) to CpG islands. This pattern is similar to the CpG island methylation pattern in which methylation is closely associated with transcriptional inactivation. It is important to note that most of the tissue-specific DNA methylation occurs in these regions (Figure 3b) (53,55).

In contrast to both above mechanisms mentioned, the third pattern occurs in gene bodies where their basal state is to be methylated to avoid transcription, thus preventing spurious transcription initiations (Figure 3c) (56). In disease, gene bodies are demethylated to allow transcription initiation at incorrect sites. DNA methylation also take place at CHG and CHH (H = A, C or T) sites in the human genome. This methylation has been predominantly found in stem cells and seems to be enriched in gene bodies directly co-rrelated with gene expression. The last pattern is hypermethylation of repetitive sequences that protect chromosomal integrity by preventing reactivation of endoparasitic sequences, thus causing chromosomal instability, translocations, and gene disruption (Figure 3d) (57).

HISTONE MODIFICATIONS

Histones are conserved proteins that package and order DNA. These proteins can be grouped in core histones (H2A, H2B, H3 and H4) and linker histones (H1and H5). The linker histones bind to the DNA to seal off the nucleosome at the location where DNA enters and leaves (58).

Histones suffer some post-translational modifications such as lysine acetylation and methylation, phosphorylation, ubiquitination, SUMOylation, and ADPribosylation. Histone modifications play an important role in transcriptional regulation, DNA repair, DNA replication, and chromosome condensation (58,59). Of all these modifications,

NAME	CLASS	LOCALIZATION	TISSUE EXPRESSION
HDAC-1	I	Nucleus	All tissues
HDAC-2			
HDAC-3		Cytoplasma and shuttle to nucleus	
HDAC-8			
HDAC-4	II	Cytoplasma and shuttle to nucleus	Brain, Heart, Muscle
HDAC-5			Brain, Heart, Muscle
HDAC-6			Testis
HDAC-7			Thymocytes
HDAC-9			Brain, Heart, Muscle
HDAC-10			Liver, Spleen, Kidney
SIRT-1	III	Nucleus	All tissues
SIRT-2		Cytoplasm	
SIRT-3		Mitochondria	
HDAC-11	IV	Nucleus	All tissues

Table 2. **Histone Deacetylation Enzymes (HDAC).** Adapted from (62).

the one most widely studied is lysine acetylation. In this process histones are acetylated and deacetylated on lysine residues in the N-terminal tail. These reactions are catalyzed by histone acetyltransferases (HATs) or histone deacetylases (HDACs), respectively (60,61). HATs promote gene expression by transferring an acetyl group to lysine and HDACs promote gene repression by removing an acetyl group from the lysine tail (Figure 4). At present, it is known the presence of 4 classes of HDAC. Class I HDACs are localized in the cellular nucleus whereas class II shuttles between cytoplasm and nucleus. Class III HDACs are members of sirtuin family, and they are structurally different to the other ones because their activity depends on the cofactor NAD+. The last HDACs is the class IV which its only member, is HDAC-11 and it is found in the nucleus and has structural similarities with class I and II that are also metallohydrolyases dependent on Zn++ (Table 2) (62).

Another group of enzymes playing a role in histone methylation are histone methyltransferases (HMTs) and histone demethylases (HDMTs). HMTs can add methyl groups to lysine residues at three sites to form a mono-, di-, or trimethylated lysine. Also the methyl group can be donated to an arginine residue. It is important to mention that the site where the methyl group is added and the number of methylation may affect in a different way the chromatin structure and the gene expression. One of the most studied histone methylation is the methylation at lysine 9 on histone 3 (H3K9)(63). Activation of gene transcription is associated with H3K4, H3K36, and H3K79 whereas gene silencing and chromatin condensation is related to H3K9 and H3K27 (62,64,65). There are also two groups of demethylating enzymes which remove the mono- and dimethylations or all methyl groups from lysines. This family of enzymes is composed by the lysine-specific demethylase 1 (LSD1). LSD1 is a monoamine oxidase which uses a flavin adenine dinucleotide (FAD) as a cofactor to oxidize the amine group of methylated lysine (66–68).

It is important to note that histone modification may act together with DNA methylation states. An example of how these modifications act on transcriptional regulation is the histone deacetylation with the association of 5′ methylcytosine in the DNA which confers a heterochromatin configuration that makes DNA inaccessible to transcription factors. On the other hand, acetylation of histone tails (H3K9) and DNA demethylation causes euchromatin configuration which is accessible to transcription machinery (69). It is important to mention that many post-translational modifications can occur at the same histone tail and at same time to produce the repression or the activation of gene expression (70). For example, during the cell cycle, there is a regulatory relationship between methylation of histone H3 lysine 9 (H3K9) and phosphorylation of H3 serine 10 (H3S10). Phosphorylation of H3S10 is required for chromosomal condensation. During early prophase and anaphase, there are high quantities of H3S10 phosphorylation; in contrast, during late anaphase dephosphorylation occurs and H3K9 methylation re-emerges. Therefore, H3S10 phosphorylation blocked methylation of H3K9 but not demethylation in the same residue permitting the access of transcription factors to DNA during mitosis. Also, phosporylation preserves methylation patterns during cell division (71).

NUCLEOSOME POSITIONING

Nucleosome is the complex produced by the histones and the packaged DNA. There are nucleosome positioning patterns that have an important role in transcriptional regulation. Depending on the position that nucleosomes have around transcription start sites (TSSs), they block the access of activators and transcription factors to the DNA strand thus inhibiting elongation of the transcripts. Active gene promoters have a nucleosome free region at the 5′ and 3′ UTR to facilitate the assembly and disassembly of the transcription machinery (72). For example, nucleosome displacements of as few as 30 bp at TSS have been implicated in changes in the activity of RNA polymerase II. When there is a loss of a nucleosome upstream of the TSS, transcription factors bind to the TSSs and gene expression is achieved. In contrast, when there is an occlusion of the TSS by a nucleosome, transcription machinery do not bind to the TSSs and gene repression occurs. Also, DNA methylation influences nucleosome positioning because they are associated with formation of heterochromatin where DNA is condensed into positioned nucleosomes to prevent transcription (73).

miRNAs

miRNAs are 18 – 23 nucleotide RNAs in length that function as post-transcriptional regulators. They regulate mRNA translation by binding to complementary sequences that are cleavaged or repressed. Many miRNAs are transcribed from intergenic regions or from introns of protein-coding genes and, sometimes, they are expressed at the same time that is transcribed the protein gene. Just a few miRNAs have been located in exons of protein-coding genes. Of all these miRNAs, the intergenic miRNAs are the ones which have their own gene promoter and regulatory region (74).

The translational repression and target degradation of mRNAs is achieved by the level of complementarity between miRNA strands and the site in the 3′ UTR targets. If there is a complete complementation, there is a cleavage of the mRNAs and it will produce degradation. On the other hand, if there is an incomplete complementation, translation will be prevented by taking the transcripts into P bodies to keep them in a silenced state using proteins that prevent translation or removal of the cap structure. Another mechanism by which miRNAs affect gene expression is by histone modification and DNA methylation of promoter sites. This mechanism occurs thanks to RNA-induced transcriptional silencing (RITS) complex (Figure 5). This protein complex binds to miRNAs to do post-translational modification of histone tails such as methylation of lysine 9 of histone 3 to form heterochromatin and to cause transcriptional repression (74,75).

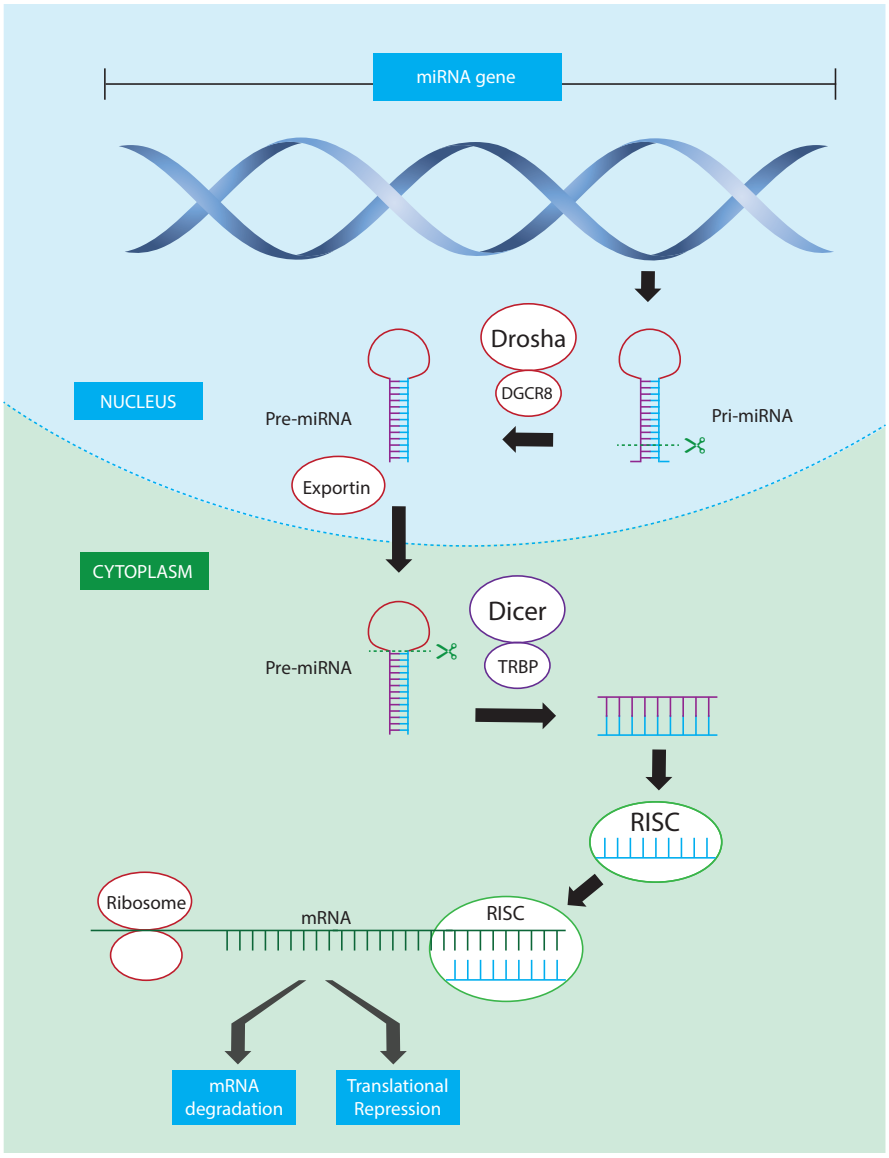

Figure 5. microRNA biogenesis. miRNA genes are transcribed by RNA Polymerase II in the nucleus forming a primary miRNA (pri-miRNA) with 100 to 1000 nucleotides in length. This pri-miRNA is recognized by nuclear enzymes Drosha, Pasha or DGCR8 (in humans) which cleave it about 11 nucleotides to produce hairpin structures known as pre-miRNA with ~ 70 nucleotides in length. Once pre-miRNA hairpins are made, they are exported from the nucleus to the cytoplasm by the Exportin-5 enzyme. In the cytoplasm, Dicer enzyme cleaves pre-miRNAs to form a duplex miRNA of 18 - 23 nucleotides in length. Of these 2 strands, the one with lower stability in the 5' end is the guide strand and it will be associated with the RNA-induced silencing complex (RISC), where miRNAs interact with the mRNA targets. The RISC complex needs to interact with other proteins to function appropriately such as Argonaute (Ago) proteins and TRBP. The translational repression and target degradation of mRNAs can be achieved by the level of complementarity between microRNAs strand and the site in the 3' UTR targets. If there is a complete complementation, there is a cleavage of the mRNAs and it will produce the degradation. On the other hand, if there is an incomplete complementation, translation will be repressed by taking the transcripts into P bodies to maintain them in a silenced state.

EPIGENETICS AND AUTOIMMUNITY

Autoimmune diseases are a complex group of diseases with different epidemiology, pathology, and symptoms but with a common origin (76). All autoimmune diseases share immunogenetic mechanisms that are part of a pleiotropism of several repertoires of genes. Many studies over the years have shown that these diseases are caused by alterations at many loci and in genes of the human genome (77). But until recent years, epigenetic studies have been focusing on autoimmune diseases. Therefore, is important to underline that autoimmune diseases may be generated by many alterations in the same epigenetic mechanism. Also, it is essential to understand that epigenetics is not the only mechanism that may cause autoimmunity; instead there are intrinsic and extrinsic components (mutations, polymorphisms and environmental factors) that predispose to autoimmunity.

DNA METHYLATION AND AUTOIMMUNE DISEASES

As we mentioned at the beginning of this review, DNA methylation is the most widely studied mechanism in autoimmune diseases. Several studies done so far have found that some diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) have a global hypomethylation in promoter regions of DNA in their target cells (Table 3). The other autoimmune diseases are just beginning to be studied for methylation pattern.

SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is a systemic multiorgan autoimmune disease characterized by autoantibody response to nuclear and/or cytoplasmic antigens. Several studies have shown that there is a global hypomethylation of promoter regions that contain

the genes that are overexpressed in the disease such as: *ITGAL*, *CD40LG*, *PRF1*, *CD70*, *IFGNR2*, *MMP14*, *LCN2*, and in ribosomal RNA gene promoter (18S and 28S) (78–82). The DNA hypomethylation may also affect the chromatin structure of T-cells thus enhancing the overexpression of these genes. This gene overexpression will cause cellular hyperactivity, perpetuation of the immune response and, consequently, perpetuation of inflammatory response (83–85).

An example of how hypomethylation alters gene expression in SLE is the hypomethylation of e1B promoter of CD5 in resting B cells. CD5 is a protein found in B cells that serves to mitigate activating signals from the BCR so that B cells are only activated by strong stimuli and not by normal tissue proteins. CD5 has two isoforms: e1A which is expressed on the membrane and e1B which is retained in the cytoplasm. The hypomethylation of e1B promoters may be the consequence of a reduced expression of DNMT1. Therefore, there is an increase in the expression of this CD5 isoform that will cause impairment in cell receptor signaling and thus promote autoimmunity (86). There are other studies where hypomethylation of the IL-10 and IL-1R2 genes is found in SLE patients and their relationship with disease severity. It is important to mention the importance of IL-10 for inhibition of T-cell function and encouragement of the B cell mediated function, while IL-1R2 interferes with IL-1 binding to its receptor IL-1R1 (87). Also, there is evidence of the abnormal expression of DNMT1 and MBD2 in PBMCs from SLE patients, in whom a decrease in the expression of DNMT1 and higher level of MBD2 mRNA was found in those patients. This finding is consistent with the state of global hypomethylation found in SLE patients (88). Moreover, in murine prone lupus models it is shown alteration within methylation pathway and S-adenosylmethionine (SAM) metabolisms, which is the enzyme in charge to donate the methyl group to cytosine. The product of SAM metabolism 5-deosy-5methylthioadenosine (MTA) may inhibit T cell activation, polarization to Th1 and Th2 and TCR-related signaling pathways and it was found decreased in these murine models. Thus, treating those mice with MTA enzyme ameliorated signs of lupus such as splenomegaly, lymphadenopathy, autoantibodies levels and IgG deposition (89).

Another example is in Lupus like disease caused by procainamide and hydralazine. These two drugs are DNA methylation inhibitors, thus they produce hypomethylation of DNA (90). In the case of procainamide, it is a competitive inhibitor of DNMT1 (91). Instead, hydralazine inhibits T and B cell signal-regulated kinase pathways (92). The kinase signaling pathway has in important role in the regulation of methylation (93). At the end, these two mechanisms produce a reduction in DNMTs that will enhance the gene expression of adhesion molecules on lupus drug-induced lymphocytes (94–96).

RHEUMATOID ARTHRITIS

RA is a disease characterized by the progressive destruction of joints by invasive synovial fibroblasts. The RA synovial fibroblasts (RASFs) have a major role in the initiation and perpetuation of the disease (97). They are the reason of why several epigenetic studies of RA are focused in these synovial cells. Researchers have found a global hypomethylation of these cells which could be the responsible of overexpression of inflammatory cytokines in the synovial fluid (98–100).

Some examples of hypomethylation in RA are in CpG islands upstream of an L1 open-reading frame and IL-6 promoter gene in monocytes. L1 is one of the major classes of repetitive element that are interspersed in the genome. They are used as a marker because in normal synovial tissue they are methylated. In synovial tissue from patients with RA, L1 is hypomethylated as a consequence of reduce expression of DNMTs. This reduction of methylation in inflammatory response promoter genes causes an overexpression of growth factors and receptors, adhesion molecules and cytokines. At the end they will cause irreversible phenotypical changes that occur in synovial fibroblast (100,101).

The other example is the hypomethylation in CpG island within IL-6 promoter gene in monocytes. IL-6 is a pro-inflammatory cytokine that participates in B cell response. When this promoter is hypomethylated, there is an overexpression of IL-6 that will cause, at the same time, an overexpression of pro-inflammatory cytokines that will be associated with a local hyperactivation of the inflammation circuit (102). But there is evidence that in monocytes we can find also a mechanism of hypermethylation such as in the case of the CpG island within the promoter of death receptor 3 (DR-3). DR-3 is a protein that causes apoptosis and activation of transcription factor NF-kB, but when there is a downregulation of this protein because of the hypermethylation of its promoter, RA synovial cell will be resistant to apoptosis (103–105). Even though many of the studies have been done on PBMCs from RA patients, there are other studies that evaluate DNA methylation patterns from fibroblast like-synoviocytes (FLS). These studies have shown the association between differentially methylated loci with altered architecture and inflammation such as in the case of CHI3L1 (cartilage specific antigen), STAT3 (associated with IL-6 activation), TRAF2, TIMP2, ADAM12, CAPN8, TNFAIP8, CCR6, IL-6R, DPP4, and IL-1. All the genes found in this methyloma were involved in relevant pathways such as cell movement, adhesion, and trafficking (106,107). Another study shows that cytokine milieu may play an important role in epigenetic modification of FLS in RA. Authors showed that proinflammatory cytokines such as Interleukine 1 beta (IL-1β) and Tumor Necrosis Factor alpha (TNF-α) could suppress DNMT gene expression in FLS giving more evidence to global hypomethylation status of some key genes in RA pathogenesis (108).

Moreover, the enzyme MeCP2 in charge of gene silencing by DNA methylation and histone modification is found to be up-regulated in FLS isolated from RA murine models. Thus, this study found that MeCP2 may influence in the activation and maintenance of the Wnt pathway responsible of synovial hyperplasia, inflammation, pannus formation, and cartilage erosion during RA pathogenesis (109,110). Also,

there is evidence of the influence of methylation states in other promoter regions that may be produced by exposure to some environmental hazards. It is known the influence of tobacco in the development of RA. An explanation of how this environmental toxin may influence in RA is because a DNA methylation change in the promoter region of GSTA2 gene which has a key role in detoxification of electrophilic compounds. In this same study was found some differentially methylated positions within MHC region that could influence in the genetic risk to develop RA (111).

TYPE 1 DIABETES (T1D)

T1D is a T cell-mediated autoimmune disease that develops in genetically susceptible individuals affecting their endocrine pancreas. There are some mechanisms by which epigenetics may play an important role in T1D: by modulating lymphocyte maturation and cytokine gene expression and by differentiation of subtype T helper cells ruled by epigenetic controls. In this autoimmune disease in contrast to SLE and RA, there is a global hypermethylation activity caused by altered metabolism of homocysteine (112).

Glucose and insulin levels are determinants of methylation (113). They alter homocysteine metabolism by increasing cellular homocysteine production by its inhibition of trans-sulfuration (114,115). When there is an increase levels of homocysteine, methionine in cells will be catalyzed in DNA methyltransferases (DNMTs) by S-adenosylmethionine. This will enhance DNMTs activity that subsequently led to increased global DNA methylation. Also, an increase maternal homocysteine during pregnancy by a low protein diet can produce an alter methionine metabolism that will cause decrease islet mass and vascularity in the fetus with a subsequent glucose intolerance in adult life (116,117).

Moreover, studies in T1D have shown hypermethylation of FOXP3 promoter region in CD4+ T cells and higher levels of DNMT3b mRNA (118). In contrast, another study found hypomethylation of CpG-19, -135, and -234 proximal to TSS in the insulin promoter region that may be associated with the development of the disease (119). Epigenomic wide association studies (EWAS) have also shown methylated variable position in twins with T1D, suggesting that this difference may be one of the causes of different concordance rates (120).

MULTIPLE SCLEROSIS (MS)

MS is an inflammatory chronic disease characterized by myelin destruction followed by a progressive grade of neurodegeneration. Recent studies have shown that the promoter region of peptidyl arginine deiminase type II (PAD2) is hypomethylated (121). PAD2 has a key role in the citrullination process of myelin basic protein (MBP). This citrullination process has important biologic effects: promotes protein autocleavage increasing the probability to create new epitopes and, also, modulates the immune response. In MS we will find an increase demethylase enzyme activity which will cause hypomethylation of PAD2 promoter region (122). Because of this hypomethylation, it will be an overexpression of PAD2 that will increase MBP citullination process with subsequent increase production of immunodominant peptides. These peptides will increase the autocleavage of MBP causing irreversible changes in its biological properties producing proteolytic digestion, myelin instability, and a chronic inflammation response (123–125). Another protein with a role in MS pathogenesis is SHP-1, which functions as a negative regulator of cytokine signaling through STATs and NF-kB. In MS patients was found a hypermethylation of promoter 2 of this protein (126). Methylation also may influence in the state of the disease. A study found differences in DNA methylation pattern in Relapsing-Remiting Multiple Sclerosis (RRMS) between remission and relapsing patients (127).

SJÖGREN'S SYNDROME (SS)

There is little information about the role of methylation in SS disease. Even though, there is a study that shows BP230 hypermethylation and decrease in mRNA levels in labial salivary gland biopsies. This result shows that alteration in expression of genes coding for hemidesmosomes type I proteins (α6β4 integrins, BP180 and BP230) may cause basal lamina disorganization and modifications in localization and distribution of α6β4 integrins. As a consequence of these alterations, there will be an abnormal extracellular matrix – acinar cell communication, thus contributing to alterations in survival responses and cell death (128). Another study demonstrated the hypomethylation and upregulation in the expression of CD70 (TNFSF7) gene in CD4+ T cells from SS patients. It is important to mention the function of CD70 as a B cell co-stimulatory molecule that interacts with CD27 to initiate immunoglobulin production and cell differentiation (129).

HISTONE MODIFICATIONS AND AUTOIMMUNE DISEASES

SYSTEMIC LUPUS ERYTHEMATOSUS

Histone modifications in SLE have been studied in murine models and in humans. These studies have found that during apoptosis, histones are modified making them immunogenic. It is noteworthy that in the pathogenesis of SLE antibodies are directed against components of the cell nucleus which are exposed at the cell surface during apoptosis (130,131).

The nucleosomes, the primary inciting antigen in SLE, are released in patients with SLE as a result of a disturbed apoptosis or an insufficient clearance of apoptotic debris. During apoptosis, the nucleosome is modified, thereby creating more immunogenic epitopes. Subsequently, epitope spreading will lead to the formation of autoantibodies against unmodified chromatin components (132,133). Histone modifications such as H3K4 trimethylation, H4K8 triacetylation, H3K27 trimethylation, and H2BK12 acetylation will cause increase apoptotic nucleosomes (Table 3). These

apoptotic nucleosomes will generate autoimmunogenicity that causes activation of antigen-presenting cells and autoantibody production with a subsequent inflammatory response (134–137).

There are other studies that have shown a global acetylation pattern of histone H3 and H4 in active SLE CD4+ T cells (137). Also, it is shown that monocytes, which are important in SLE renal disease, have altered acetylation pattern of histone H4 thus increasing the expression of interferon genes that have a key role in SLE pathogenesis (138–140). Nowadays, there is evidence about a possible mechanism that could link histone modification to NETosis with a subsequent induction of autoimmunity. This study showed how NETs were enriched with histone methylation, citrullination, and acetylation marks such as mono-, di-, and tri-methyl H3K4, 9, 27, 36, H4K20, and H4K5-16 acetylation (141).

RHEUMATOID ARTHRITIS

RA synovial tissue is characterized by a misbalance between HAT and HDAC activity. Cartilage destruction is thought to be mediated by matrix metalloproteinases (MMPs) and enzymes from the ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) family. Many of these genes are regulated by modifications in theβ chromatin including acetylation of histones (142–144). It is noteworthy that levels of HDAC activity are increased in RA patients before starting anti-TNF treatment (145)

Many studies have shown that HDAC inhibitors inhibit cartilage degradation blocking the induction of key MMPs by proinflammatory cytokines at both the mRNA and protein levels. Also, ADAMTs enzymes are inhibited at the mRNA level (146). In fact, hyperacetylation of synovial cell histones induces p16 and p21 (cyclin-dependent kinase inhibitors that regulates cell cycle) expression with a subsequent decrease in TNF-α synthesis. All this mechanisms will inhibit join swelling, synovial inflammation and joint destruction in murine RA models (144,147). Also, the hyperacetylation of histones will downregulate HIF-1a (hypoxia inducible factor) and VEGF to block angiogenesis in synovial cells (Table 3) (148).

It is noteworthy that HDAC inhibitors may therefore be novel chondroprotective therapeutic agents in arthritis by its ability to inhibit the expression of destructive metalloproteinases, ADAMTs, and cytokine production in synovial tissue (149–151).

TYPE 1 DIABETES

There are just few epigenetic studies associated with histone modifications and the pathogenesis of the T1D. Patients with T1D show a subset of genes with increase in H3K9me2 in lymphocytes. This subset of genes includes the *CLTA4*, which is a type 1 diabetes susceptibility gene, and has increase methylation of H3K9 in its promoter region. Other genes that have altered H3K9me2 are transforming growth factor-beta (TGF-B), nuclear factor-kB, p38, a mitogen-activated protein kinase, toll-like receptor, and interleukin-6 (Table 3). The transcription factor NF-kB is also upregulated by H3K4 methyltransferase, thus causing increase in inflammatory gene expression in diabetic mice. All these genes are associated with autoimmune and inflammation-related pathways (152–154). Moreover, a study shows variation in the levels of H3K9Ac in the upstream regions of HLA-DRB1 and HLA-DQB1 genes in the insulin-dependent locus, suggesting an important role in the regulation of these genes (155).

On the other hand, histone modifications are also part of the mechanisms that causes cardiovascular complications in T1D patients. Chemical modification of the H3 histone tail of lysine 4 and 9 has recently been identified with gene expression conferred by hyperglycemia. Transient hyperglycemia promotes gene-activating epigenetic changes and signaling events critical in the development and progression of vascular complications. These epigenetic changes are H3K4 and H3K9 methylation in genes associated with vascular inflammation (156–158).

MULTIPLE SCLEROSIS

The oligodendrocyte identity is modulated by post-translational modifications of histones. In rodents, histone deacetylation produces oligodendrocyte differentiation whereas acetylation is associated with transcriptional inhibitors of differentiation. In patients with MS, there is a shift toward histone acetylation in the white matter. Thus, hyperacetylation of H3 at the promoter region of inhibitory genes will produce high levels of transcriptional inhibitors of oligodendrocyte differentiation such as *TCF7L2*, *ID2*, and *SOX2* (Table 3) (159). Moreover, polymorphisms in genes expressing HDACs such as SIRT4, HDAC-9, and HDAC-11 may be correlated with brain volume changes in MS patients (160).

NUCLEOSOME POSITIONING AND AUTOIMMUNE DISEASES

There are not many studies about how nucleosome positioning causes autoimmune diseases. But in RA, histone variant macroH2A interferes with the binding of transcription factor NF-kB impeding the action of some proteins that restructure nucleosomes (161). Also, it has been reported that a SNP in the region 17q12-q21 associated with high risk of T1D, Chron's disease and Primary Biliary Cirrhosis leads to allele-specific differences in nucleosome distribution (162).

miRNAs AND AUTOIMMUNE DISEASES

SYSTEMIC LUPUS ERYTHEMATOSUS

Studies have shown that most lupus-related genes contain at least one miRNA target site for more than a hundred miRNAs. In SLE, there is evidence of the key role of some miRNAs in its pathogenesis (Table 3). For example, miR-146a is a negative regulator of TLR signaling and its expres-

sion was decreased in patients with SLE. Also, this miRNA is a negatively regulator of type I interferon (IFN) pathway by targeting IFN regulatory factor (IRF)-5 and STAT-1. Therefore, decreased expression of miR-146a in PBMC may contribute to the enhanced type I IFN production in SLE (163). Other studies identified that miR-21 and miR-148a were upregulated in CD4+ T cells. A possible mechanism of how these miRNAs act in SLE is because they produce hypomethylation of some promoters by repressing DNMTs thus increasing the expression of autoimmune-associated methylation-sensitive genes, CD70, and lymphocyte function-associated antigen (164).

Additional studies have found that miR-125a was reduced in patients with lupus. This miRNA is expressed in T cells and is a critical transcription factor in the regulation of the chemokine RANTES. The decreased expression of miR-125a results in the upregulation and the elevation of the inflammatory chemokine RANTES in lupus T cells (165). Also, it was found a downregulation of miR-125b in T-cells and this may contribute to lupus nephritis pathogenesis and an increase gene expression of ETS1 and STAT3 (166). There are other miRNA which function is to regulate B and T cell immunity such as miR-155. Therefore, the upregulation of miR-155 in lupus B and T lymphocytes may lead to abnormal B-cell activation and abnormal inflammatory T-cell development and cytokine production in patients with lupus (167,168). Another miRNA that interfere with immune response is the miR-126 which reduces the levels of DNMT1 and this fact produces the overexpression of CD11a and CD70 genes in SLE T cells (169). There is other study that shows the expression of some miRNAs and active nephritis. These authors found an upregulation of some miRNAs such as miR-142-3p and miR-181a, and a downregulation of miR-106a, miR-17, miR20a, miR-92a, and miR-203. All these miRNAs were involved in TGF-b signaling pathways, apoptosis, cytokine receptors, T-cell development and cytoskeletal organization (170).

RHEUMATOID ARTHRITIS

miRNAs are also critical in RA pathogenesis (Table 3). For example, miR-155 and miR-146 are overexpressed in RASFs. miR-155 expression is enhanced by TNF-α and IL-1β, and they produce an inhibitory effect on metalloproteinases expression in synovial fribroblasts (171). Additional, miR-146 is a miRNA that is upregulated by pro-inflammatory cytokines and its function is to downregulate NF-kB pathway in monocytes. In contrast, another study showed that TNF-α induces the expression of miR-17-92 in RASFs which play a role in cartilage destruction and chronic inflammation through NF-kB signaling, thus producing upregulation of metalloproteinases and pro-inflammatory mediators. This miRNA have a strong correlation with the levels of TNF-α and IL-17 (172–175). Another miRNA in RA is miR-203 which also causes repression of several metalloproteinases and inhibition of IL-6 (176).

Another miRNA implicated in RA is miR-124, which targets cyclin-dependent kinase 2 (CDK-2) and it is decreased in RA synovial tissue. At basal state, it represses cell proliferation and arrested the cell cycle at G1 phase. Also it targets monocyte chemoattractant protein 1 (MCP-1) which is responsible of the recruitment of mononuclear phagocytes into the joint. Thus in RA, this miRNA produces increase in cell proliferation and MCP-1 production (177,178).

There is evidence about novel miRNA that may be playing a role in RA pathogenesis such as miR-503, miR-625, miR-550, miR-500, miR-202-3p, miR-30b, and miR551b which may be associated with altered gene expression. It is important to mention that it may be a combined action of miRNA and DNA methylation in which they may have antagonistic effects in the gene expression control (107,179).

MULTIPLE SCLEROSIS

Currently, many studies have been focusing in miRNAs involved in MS pathogenesis (Table 3). A recent study found that miR-326 plays a critical role in the pathogenesis of MS upregulating the differentiation Th-17 cell by targeting Ets-1 which is a negative regulator of Th-17 differentiation. This miRNA was significantly upregulated in patients with RRMS producing an increase in Th-17 cell number and more severe symptoms (180). Other miRNAs involved in MS and miR-34a are miR-155 which are upregulated in active MS lesions and contributed to MS pathogenesis by targeting CD47 to release macrophages from the inhibitory control signal thus causing increased phagocytosis of myelin. Also, miR-155 promotes development of inflammatory Th1 and Th17 cells (171,181). Moreover, miR-29b is increases in CD4+ T-cells promoting a chronic inflammation caused by Th1 (182).

In addition, differentially expressed miRNAs such as miR-17-5p, miR-497, miR-193, and miR-126 have been identified in different lymphocyte subsets including CD4+ T cells, CD8+ T cells, B cells, and CD4+ CD25+ Treg cells from patients with MS. Nevertheless, direct involvement and contribution of dysregulated miRNAs in MS have largely remained unknown and needs additional investigation (183). It is noteworthy that all miRNAs are involved just in the pathogenesis of the disease. There are miRNAs that can serve as prognostic markers. For example, the expression of miR-18b and miR-599 is related to relapse and miR-96 is involved in remission (184). Also, BCL2 in the apoptosis process has been shown to be modulated by the downregulation of miR-15a and -16-1 in CD4+ T-cells from RRMS patients (185). Other miRNAs are brain-specific such as miR-124 which is expressed in microglia but not in peripheral monocytes or macrophages. Its function may be related to reduce activation of myelin-specific T cells with a marked suppression of disease being a key regulator of microglia quiescence and a good prognostic factor for MS (186).

Nowadays, evidence about new miRNAs playing a role in MS pathogenesis is growing. miR-922, miR-181c, and miR-633 are differentially regulated in cerebro-spinal fluid (CSF) from MS patients and the last two miRNAs are differentially regulated in the RRMS phenotype (187).

TYPE 1 DIABETES

There are just a few studies related with miRNAs and T1D pathogenesis. But there are some hypotheses that T cell regulator (Tregs) functions are influenced by changes in the expression of specific miRNAs (Table 3). In Tregs of T1D patients there is an increased expression of miRNA-510 and miR-326 and decreased expression of both miRNA-342 and miRNA-191. On the other hand, there are studies in which demonstrate that miRNAs may be the cause of cytokine-mediated beta-cell cytotoxicity. This cytotoxicity is achieved when IL-1β and TNF-α induce the expression of miR-21, miR-34a, and miR-146a in pancreatic islets thus producing beta-cell failure by increasing proinflammatory cytokines (188–190). Moreover, miR-21a and miR-93 are found to be downregulated in PBMCs from patients with T1D (191). In a study made with sera from children with new onset of T1D was demonstrated upregulated miRNAs such as miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25 and miR-200a. All these miRNAs were related to beta-cell function and glycemic control (192).

SJÖGREN'S SYNDROME

This syndrome is characterized by an inflammation and dysfunction of salivary and lacrimal glands which causes dry mouth and eyes. miRNA is the most study mechanism in this disease and it is known that the majority of this are concentrated in exosomes in serum and saliva, instead of whole serum (193). In SS seems to play an important role some miRNAs: miR-547-3p, miR-168-3p, miR-146a/b, miR-150, and miR-149 (Table 3). The three first ones are overexpressed in salivary gland; instead the last ones are upregulated in salivary glands and lymphocytes. Their overexpression may be the cause of the downregulation of some mRNAs that are important for correct immune function and for the downregulation of pro-inflammatory cytokines (112,194–196). Also, there is a role of SS antigensSSB/La as a pre-miRNA binding protein and as a global regulator of miRNA processing *in vitro* by the requirement of the RNA-binding motifs (LAM, RRM1 and RRM2) of this auto-antigen for the correct functioning of miRNA processing (197).

EPIGENETICS MECHANISMS AND AUTOIMMUNE DISEASES		
DNA Methylation		
Systemic Lupus Erithematosus	**Global Hypomethylation of promoter region of genes**	
	ITGAL	Lu Q. *et al*. (2007) (79)
	CD40LG	Kaplan M. *et al*. (2004) (80)
	PRF1	Oelke K. *et al*. (2004) (81)
	CD70	Javierre BM. *et al*. (2010) (82)
	IFGNR2	Javierre BM. *et al*. (2010) (82)
	MMP14	Javierre BM. *et al*. (2010) (82)
	LCN2	
	Ribosomal RNA gene promoter (18S and 28S)	Javierre BM. *et al*. (2010) (82)
	e1B promoter of CD5 in resting B cells	Garaud S. *et al*. (2009) (86)
	IL-10	Lin SY. *et al*. (2012) (87)
	IL-1R2	Lin SY. *et al*. (2012) (87)
Rheumatoid Arthritis	**Hypomethylation**	
	CpG islands upstream of an L1 open-reading frame	Neidhart M. *et al*. (2000) (100)
	IL-6 promoter gene in monocytes	Nile CJ. *et al*. (2008) (102)
	CHI3L1	Nakano K. *et al*. (2013) (106)
	STAT3	Nakano K. *et al*. (2013) (106)
	ADAM32	Nakano K. et al. (2013) (106)
	CASP1	Nakano K. *et al*. (2013) (106)
	IL-6R	Rica L. *et al*. (2013) (107)
	TNFAIP8	Rica L. *et al*. (2013) (107)
	CAPN8	Rica L. *et al*. (2013) (107)
	IL-10	Lin SY. *et al* (2012) (87)
	IL-1R2	Lin SY. *et al* (2012) (87)

DNA Methylation		
Rheumatoid Arthritis	**Hypermethylation**	
	Promoter of death receptor 3 (DR-3)	Takami N. *et al*. (2006) (105)
	ADAMTS2	Nakano K. *et al*. (2013) (106)
	CD55	Nakano K. *et al*. (2013) (106)
	PTPN14	Nakano K. *et al*. (2013) (106)
	TIMP2	Nakano K. *et al*. (2013) (106)
	MAP3K1	Rica L. *et al*. (2013) (107)
	DPP4	Rica L. *et al*. (2013) (107)
	CCR6	
Type 1 Diabetes	Global hypermethylation FOXP3	Li Y. *et al*. (2011) (118)
	Hypomethylation of CpG-19, -135 and -234 in the insulin promoter region	Fradin D.*et al*. (2012) (119)
Multiple Sclerosis	Hypomethylation of promoter region of peptidyl arginine deaminase type II (PAD2)	Mastronardi FG. *et al*. (2007) (121)
	Hypermethylation of SHP-1 promoter 2	Kumagai C. *et al*. (2012) (126)
Sjögren's Syndrome	Hypermethylation of BP230	González S. *et al*. (2011) (128)
	Hypomethylation of CD70 (TNFSF7)	Yin H. *et al*. (2010) (129)
Histone Modification		
Systemic Lupus Erithematosus	**Predisposition to apoptotic nucleosomes:**	
	H3K4 trimethylation H4K8 triacetylation	van Bavel CC. *et al*. (2010) (133,136,137)
	H3K27 trimethylation H2BK12 acetylation	van Bavel CC. *et al*. (2010) (133,136,137)
	Global acetylation of histone H3 and H4 in active CD4+ T cells	Hu N. *et al*. (2008) (134)
	NETosis	
	mono-, di-, and tri-methyl: H3K4, -K9,-K27,-K36 and H4K20 Acetylation: H4K5 and -K16 Citrullination: H3Cit (2, 18, 17) and H4Cit3	Liu CL. *et al*. (2012) (141)
Rheumatoid Arthritis	**HDAC inhibitors**	
	Block induction of Metalloproteinases Repress of ADAMTs enzymes	Young DA. *et al* (2005) (146)
	Hyperacetylation of histones induces p16 and p21	Nishida K. *et al*. (2004) (147)
Type 1 Diabetes	**Increase H3K9me2 in lymphocytes genes** CLTA4 TGF-B NF-kB p38 IL-6	Miao F. *et al*. (2008) (152)
	Hyperglycemia causes H3K4 and H3K9 methylation	Cooper ME. *et al*. (2010) (156)
Multiple Sclerosis	Hyperacetylation of H3 promoter region in white matter	Pedre X. *et al* (2011) (159)
	Polymorphisms in SIRT4, HDAC-9 and -11	Inkster B. *et al* (2013) (160)

microRNAs		
Systemic Lupus Erithematosus	**Decreased expresion**	
	miR-146a	Tang Y. *et al*. (2009) (163)t
	miR-125a	Zhao X. *et al.* (2010) (165)
	miR-125b	Zhao X. *et al* (2013) (166)
	miR-106a	Carlsen A.*et al* (2013) (170)
	miR-17	Carlsen A.*et al* (2013) (170)
	miR-20a	Carlsen A.*et al* (2013) (170)
	miR-92a	Carlsen A.*et al* (2013) (170)
	miR-203	Carlsen A.*et al* (2013) (170)
	Upregulation	
	miR-21 and miR-148a	Pan W. *et al*. (2010) (164)
	miR-155	Calame K. (2007) (167)
	miR-126	Zhao S. *et al* (2011) (169)
	miR-142-3p	Carlsen A.*et al* (2013) (170)
	miR-181a	Carlsen A.*et al* (2013) (170)
Rheumatoid Arthritis	**Overexpression**	
	miR-155	O' Connell RM. *et al*. (2008) (168)
	miR-146	Li J. *et al*. (2010) (172)
	miR-203	Stanczyk J. *et al.* (2011) (176)
	miR-551b	Rica L. *et al.* (2013) (107)
	Downregulated	
	miR-124	Nakamachi Y. *et al.* (2009) (177)
	miR-625	Rica L. *et al.* (2013) (107)
Multiple Sclerosis	**Upregulation**	
	miR-326	Du C. *et al.* (2009) (180)
	miR-34a	Junker A. *et al*. (2009) (181)
	miR-155	Junker A. *et al.* (2009) (181)
	miR-29b	Smith K. *et al* (2012) (175)
	Downregulation	
	miR-15a	Lorenzi JC. *et al* (2012) (185)
	miR-16-1	Lorenzi JC. *et al* (2012) (185)
	Expression in Treg cells: miR-17-5p, miR-497, miR-193 and miR-126	De Santis G. *et al.* (2010) (183)
	Disease Relapse: miR-18b and miR-599	Otaegui D. *et al.* (2009) (184)
	Disease Remission: miR-96	
	Brain-specific: miR-124	Ponomarev ED. *et al.* (2011) (186)
Type 1 Diabetes	**Upregulated:**	
	miRNA-510	Hezova R. *et al.* (2010) (188)
	miR-326	Sebastiani G. *et al.* (2011) (198)
	miR-152	Nielsen L. *et al*. (2012) (192)
	miR-30a-5p	Nielsen L. *et al*. (2012) (192)
	miR-181a	Nielsen L. *et al.* (2012) (192)
	miR-24	Nielsen L. *et al.* (2012) (192)

microRNAs		
Type 1 Diabetes	miR-148a	Nielsen L. *et al.* (2012) (192)
	miR-210	Nielsen L. *et al.* (2012) (192)
	miR-27a/b	Nielsen L. *et al.* (2012) (192)
	miR-29a	Nielsen L. *et al.* (2012) (192)
	miR-26a	Nielsen L. *et al.* (2012) (192)
	miR-25	Nielsen L. *et al.* (2012) (192)
	miR-200a	Nielsen L. *et al.* (2012) (192)
	Decreased expression of miRNA-342, miRNA-191 and miR-93	Hezova R. *et al.* (2010) (188) Salas-Pérez F. *et al.* (2012) (191)
	Beta cell failure: miR-21, miR-34a, and miR-146a	Roggli E. *et al.* (2010) (190)
Sjögren's Syndrome	Overexpression: miR-547-3p , miR-168-3p and miR-146a/b	Alevizos I. *et al.* (2010) (194) Zilahi E. *et al.* (2012) (195)
	Upregulated: miR-150 and miR-149	Alevizos I. *et al.* (2010) (194)

Table 3. Summary of epigenetic mechanisms involved in autoimmune diseases.

CONCLUSIONS

Epigenetic research has grown to provide new insights into autoimmune diseases and this is possible thanks to advances in technological development which are enabling epigenomic analysis on a large scale. All this improvement in the genetic field has permit us to find new causes that may explain the etiology of autoimmune disease, showing us one more time that this group of diseases is not caused by a single altered component.

The candidate gene approaches have identified a small set of genes that undergo aberrant DNA demethylation and overexpression in systemic lupus erythematosus and rheumatoid arthritis which are the autoimmune diseases most widely studied in the last years. This identification of cell-specific targets of epigenetic deregulation in autoimmune rheumatic disorders will provide clinical markers for diagnosis, disease progression and response to therapies. But to achieve this, high-throughput approaches are necessary for screening epigenetic alterations in autoimmune disease related to specific tissue and cell types that are relevant to disease pathogenesis.

Once we have mapped all the altered epigenetic mechanisms that produce each one of the autoimmune diseases, we can research even more for therapeutic potential of compounds directed against those epigenetic mechanisms. But to do this, detailed human DNA methylomes, histone modification and nucleosome positioning maps in healthy and diseased tissues are needed.

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23 IMMUNOLOGICAL COMPUTATION

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INTRODUCTION

The natural immune system has remarkable information processing properties that have served as an inspiration to generate models and applications for the so called artificial immune systems (AIS). This opened up a new branch of Computational Intelligence which emerged in the 1990s.

This paper summarizes the main developments in the AIS field. First, some general concepts in immunology which have served as a basis in AIS modeling are summarized. Then, a brief historical revision of the main models of AIS is presented. Main AIS models have focused on: negative selection, artificial immune networks, clonal selection algorithms, Danger Theory, and dendritic cells. Other properties and processes in the biological immune system have also motivated the development of other recent models and problem solving methods.

BASIC CONCEPTS ON IMMUNOLGY

The natural immune system consists of molecules, cells, and organs distributed throughout the body. There is no main organ that controls the functions of the immune system. One important task carried out by the immune system is to monitor the body, for instance, and search for foreign elements that may cause diseases. Accordingly, an important role of the immune system is to discriminate self from non-self (1).

The immune system works on three different levels: physical barriers (e.g., the skin), the innate immune system, and the adaptive immune system. Most of the artificial immune system models developed so far are inspired by the last, which one presents the desirable properties for a computational intelligence system such as learning and memory (2).

Antigens are usually proteins or molecules external to the body, which are derived from pathogens or malignant cells. Such antigens are characterized by regions called epitopes. In defining antigens, two main properties should be distinguished: antigenicity, the ability of a given antigen to be recognized by the antigen-specific receptors expressed by T or B cells; and immunogenicity, the ability of the antigen to induce an immune response (3).

The adaptive immune response consists of the cell and humoral responses (4):

A. Cell Immune Response: A cell infected by a virus can degrade this virus and transport sections of its proteins to the membrane in order to present them. This type of cell is called an antigen presenting cell (see Chapter 10). Helper T cells can detect proteins that are being presented and are thus activated. Such activated T cells circulate throughout the body destroying infected cells.

B. Humoral Immune Response: This response is initiated by macrophages, which engulf antigens, e.g., bacteria and viruses. Then, they process such antigens to take them to their cell membrane and present them to T cells. Again, T cells detect such antigens that are being presented and activate themselves. Activated T cells are cloned to produce identical copies. Subsequently, T cells help B cells to differentiate into antibody producing cells (plasma cells) (See Chapters 5 and 6). If all of these antigens remain at the same location, phagocytes must approach the antigen identified by the antibodies and destroy it (5).

The biological immune system has developed the ability to generate a set of detectors, so that those that recognize an antigen are selected. This system presents an almost unlimited capability to detect any chemical agent, either natural or artificial (6).

Complement system, macrophage, and dendritic cell interactions:

- The interaction between complement molecules, macrophages, and dendritic cells (DC) is deeply explained in Section I of this book. In summary, recognition of a pathogen-associated molecular pattern (PAMP) by pattern recognition receptors (PRR) (e.g., toll-like receptors-TLRs) generates signals that activate either the macrophage or the DC for phagocytosis of the pathogen. The expression of PRR is not clonal, i.e., all such receptors displayed by cells of a given type (e.g., macrophages) have identical specificities. Macrophages and DCs have receptors for antibodies and complement molecules (e.g., CR3 and CR4 complement receptors on the DC surface) (7), so that the coating of microorganisms with antibodies or complement molecules (or both) enhances phagocytosis (8). The engulfed microorganisms are subjected to a wide range of toxic intracellular molecules such as superoxide anion, hydroxyl radicals, etc.

Innate responses frequently involve complement, acute-phase proteins, and cytokines. The early events of complement activation, which are based on an enzymatic amplifying cascade comparable to that seen in blood clotting, can be triggered by one of three pathways (9). The classic pathway is activated by antigen-antibody complexes, the alternative pathway by microbial-cell walls, and the lectin pathway by the interaction of microbial carbohydrates with mannose binding protein in the plasma (10). Irrespective of the source of activation, the outcome is the generation of a number of immunologically active substances. For example, a proteolytic cleavage fragment of complement component C3, the C3b molecule, is deposited on the surface of microorganisms. This event enhances phagocytosis of the microbe because phagocytic cells such as macrophages have cell-surface receptors for C3b (11).

HISTORY OF AISs

AISs have proved to be a good alternative in many applications. It is noteworthy that the artificial immune system area of research bridges immunology, computer science, statistics, and engineering. The contributions in this field have also involved AISs immune modeling, theoretical AISs, and their applications. Immune modeling includes developing models and simulations of natural and artificial immune systems. The theoretical aspects of AISs, in turn, include proposing new algorithms and analyzing their convergence, performance, and complexity. There is a recent and comprehensive survey on theoretical AISs in Timmis et al. (12). A survey on the recent developments in the AIS field can be found at Yu et al. (13).

The immune system has drawn significant attention as a potential source of inspiration for novel approaches to solving complex computational problems. The main properties that are used as inspiration include: learning capabilities, memory, feature extraction, pattern recognition features, and being highly distributed, adaptive, and self-organized.

In the seminal paper "self–non-self discrimination in a computer" Forrest et al. (14) proposed a negative selection algorithm for distinguishing self from other which is based on the generation of T cells in the immune system. In this paper, the method was applied to the problem of computer virus detection. Since then, several variants of the negative selection algorithm have been developed though the essential characteristics of the original negative selection algorithm still remain.

Artificial immune networks (AINs) were inspired by Farmer et al.'s immune network model (15). The first immune network algorithm was proposed by Ishida (16). Timmis et al. (17) re-defined and re-implemented the artificial immune network model and introduced a model called AINE (Artificial Immune NEtwork) (18). In an artificial immune network (AIN/AINE) a set of B cells, which interact among themselves, undergo some cloning and mutation operations.

In 2000, Castro et al. (19) proposed the clonal selection algorithm (CSA) that was based on clonal selection and affinity maturation principles (20). CSA was later known as CLONALG. In CLONALG, cells are generated and evolve through selection, clone, mutation, reselection, and population replacement. The variation of candidate cells in CLONALG is to some degree similar to a genetic algorithm (GA). Applications of CLONALG include binary character recognition and multimodal optimization. Also, CLONALG has the capability of learning and develops a high quality memory.

More recently, other areas of immunology have inspired other algorithms and computational tools, namely, humoral immune response (21), Danger Theory (22), dendritic cell functions (23), and the pattern recognition receptor model (24).

In the last two decades, the AIS community has produced a diverse set of immune inspired algorithms, which have been applied to computational and other real world problems. An extensive body of literature has been produced in this field (25). The first volume in AIS titled "Artificial Immune Systems and Their Applications" was edited by Dasgupta in 1999 (26). This volume provided an overview of the immune system from the computational viewpoint and summarized the main work done in this field as of 1998. Later, in 2002, Castro and Timmis (27) wrote the book titled "Artificial Immune Systems: A New Computational Intelligence Approach." This book provided a very accessible introduction to the biological principles of the natural immune system as well as a comprehensive presentation of the main AIS algorithms. It also included a comparative analysis of the immune system as well as other biological systems and processes. In 2003, Tarakanov et al. (28) published their book "Immunocomputing: Principles and Applications." This book presented an introduction of immunocomputing, the mathematical basis of the immunocomputing, and various applications of immunocomputing.

Most recently, Dasgupta and Nino's book (29) "Immunological Computation: Theory and Applications" provided an overview of fundamental immunological concepts and some theoretical models of immune processes. It also presented up-to-date immunology-based computational techniques that were developed by their own research group and by other groups around the world.

MAIN ARTIFICIAL IMMUNE SYSTEM ALGORITHMS

Four major AIS algorithms have been under constant development: 1) Negative Selection Algorithms (NSA), 2) Artificial Immune Networks (AINE), 3) Clonal Selection Algorithms (CLONALG), 4) The Danger Theory and Dendritic Cell Algorithms (DCA). The four AIS algorithms are summarized in this section.

NEGATIVE SELECTION ALGORITHM (NSA)

In the biological immune system, T cell precursors move to the thymus from the bone marrow, and T cell development occurs in the thymus. T cell precursors do not express any of the T cell markers such as T cell receptor. The stages of T cell development are identified by the expression of specific TCR. Direct cell to cell interaction between Pre-T cells and thymic cells induces Pre-T cell proliferation and also differentiation. At this point, the alpha-chain of TCR undergoes generic rearrangement. These T cells undergo the processes of negative selection to eliminate those T cells that are strongly activated by self MHC plus self peptides in the thymus. (30)

The main characteristics of this method were well described by Forrest et al. (14) and still remain the same including negative representation of information, distributed generation of the detector set, and one class classification. Data representation is one of the main aspects of negative selection algorithms. Typically, they use either string or real-valued vector representation. Furthermore, a negative selection algorithm is distinguished by a particular matching rule which is based on a distance or similarity measure. Note that a matching threshold is also thought to model partial matching for the purpose of approximation and generalization.

Gao et al. (31) introduced a "genetic algorithm based negative selection algorithm detector optimization scheme." They focused mainly on optimizing the non-overlapping detectors to obtain the maximal nonself space coverage.

ARTIFICIAL IMMUNE NETWORKS (AINE)

The immune network theory suggests that the immune system is capable of achieving immunological memory by the existence of a mutually reinforcing network of B cells. The B cells not only stimulate each other but also suppress connected cells to regulate the overstimulation of B cells in order to maintain a stable memory. The paratopes of a B cell can be matched against idiotopes on other B cells. The binding between idiotopes and partopes has the effect of stimulating the B cells which the immune network called, idiotypic networks is made up of (32).

Based on Farmer et al.'s immune network model (15), the artificial immune network algorithm proposed by Ishida (16) can be considered the earliest work in the field of AIS. These earlier works were further improved by Hunt et al. (33), who proposed a system that was composed of a bone marrow object, a network of B cell objects, and an antigen population. The B cell object population is randomly initialized by the bone marrow object. When the antigen population is loaded into the system, it is randomly picked up and inserted at a randomly chosen point in the B cell network. If the B cell can bind to the antigen population, many new B cell objects will be cloned and the clones with the highest affinity for the cells already in the network will be added to the existing B cell network (30).

CLONAL SELECTION ALGORITHM (CLONALG)

Clonal selection theory states that a clonal expansion of the original lymphocyte occurs when the original lymphocyte is activated by binding to the antigen. However, any clone of the activated lymphocyte with antigen receptors specific to molecules of the organism's own body (self-reactive receptors) is eliminated during the development of the lymphocyte. During the clonal expansion of B cells, the average affinity increases for the antigen that triggered the clonal expansion through a process of affinity maturation. Therefore, the memory B cells are developed to produce a more effective immune response to antigens that had been encountered. Affinity maturation is caused by a somatic hypermutation and a selection mechanism. Somatic hypermutation results in a diversity of antibodies by introducing random changes to the genes that encode for them. The selection mechanism guarantees that only those clones (antibodies) with the highest affinity for the encountered antigen will survive (30).

DANGER THEORY AND DENDRITIC CELL ALGORITHMS (DCA).

The Danger theory has become popular among immunologists over the last decade. Its chief advocate, Polly Matzinger, proposed this theory in 2002 (34). She pointed out that the "foreignness" of a pathogen is not the important feature that triggers a response, and "selfness" is no guarantee of tolerance. The central idea in the Danger Theory is that antigen presenting cells (APCs) are activated by danger/alarm signals from injured cells such as those exposed to pathogens, toxins, mechanical damage, and so forth. However, danger/alarm signals should not be sent by healthy cells or by cells undergoing normal physiological deaths. Alarm signals can be constitutive or inducible, intracellular or secreted, or even a part of the extracellular

matrix. Cells that die necrotically release their contents. Any intracellular product could potentially be a danger signal when released. Inducible alarm signals could include any substance made, or modified, by distressed or injured cells.

According to the Danger Theory, a cell that dies unnaturally sends out the danger/alarm signal. The danger signal establishes a danger zone around itself. As a result, the antigens near the cell that emits the danger signal are captured by APCs such as macrophages and are taken to the local lymph node. Then, the antigens are presented to lymphocytes. The antibodies secreted by B cells match the antigens, but only those that match the antigens in the danger zone will be activated or stimulated and undergo the clonal expansion process. Those that do not match or are not in the danger zone will not be stimulated (30).

APPLICATIONS

Although there have undeniably been a lot of successful applications of AIS, there are still very few examples that really stand out as instances of AISs being used in industry seriously. Successful applications of AISs include computer security, optimization, data mining, anomaly detection, fault detection and diagnosis, robotics and control software testing, web mining, and scheduling. In this section, some applications developed by the authors are briefly described.

AIS was used in the extraction and representation of knowledge in Romero et al. (31). They present an approach to knowledge extraction and representation based on an artificial immune system. The main purpose is to extract the important concepts from a set of text documents and find the relationships between such concepts. The final step is to develop a graph representation, which is intended to present a picture of the contents of the document.

The generation of textual information has grown considerably. Many people and organizations need to work with huge amounts of data. These data can be in the form of corporate documents and e-mail, etc. This situation raises the need to use computational tools that facilitate the management of such large amounts of information reliably, securely, and efficiently so that this information becomes helpful to people's daily work. The problem is selecting the documents that are helpful and provide appropriate information. Therefore, it is very important to have tools that can help in the process of getting the essential meaning of the documents and determining whether they are relevant to the user or not. They have presented a proposal for knowledge extraction and visualization which can be used to get the most important concepts from text documents and the relationships between these concepts. This process is based on concepts which simulate the biological immune system, specifically its pattern recognition and memory capabilities (35).

Figure 1 presents the graph for the medical category. In this graph, some clusters can be seen, which means that the documents in this category contain several important concepts.

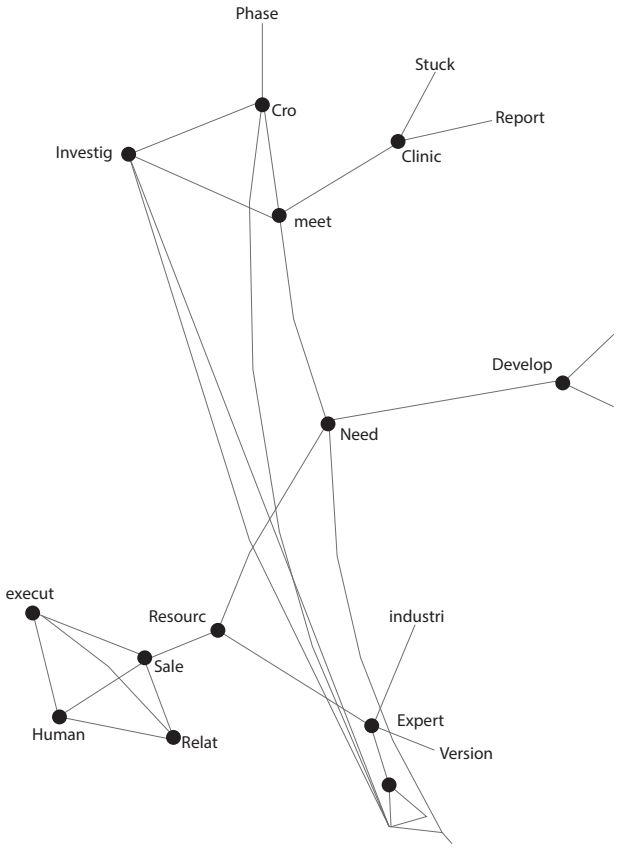

Figure 1. Graph for the Medical Theory (35).

In this approach, a model inspired by the biological immune system has been used to solve problems in the field of knowledge management. Its ability to recognize patterns and memory, in particular, have proven to be useful for detecting the important concepts in text documents efficiently.

Additionally, Balachandran et al. (36) presented a framework to generate multi-shaped detectors with valued negative selection algorithms (NSA). In particular, detectors can take the form of hyper-rectangles, hyperspheres, and hyper-ellipses in the non-self space. These novel pattern detectors (in the complement space) are developed using a genetic search (the structured genetic algorithm) which uses hierarchical genomic structures and a gene activation mechanism to encode multiple detector shapes. This genetic search (the structured GA) makes it possible to maintain diverse shapes while contributing to the proliferation of the best suited detector shapes in expressed phenotype. The results showed that a significant coverage of the non self space could be achieved with fewer detectors compared to other NSA approaches (using only singleshaped detectors).

A new cognitive model for multiagent object transportation was proposed by Muñoz, et al. (37). This model is inspired by the innate immune response. Some relevant characteristics of macrophages, dendritic cells, and the complement system are

modeled and incorporated into this artificial immune system. The function of pattern-recognition receptors and complement receptors on macrophages as well as DCs to detect an antigen and the function of complement molecules to flag antigens are used. The goal of the multi-agent system is to transport scattered objects from an unknown dynamic environment to a specific storage room. Therefore, the exploration and mapping problems are handled by agents that emulate the behavior of complement molecules and the object transportation is done by agents that emulate the behavior of a macrophage and a dendritic cell.

The dendritic cell algorithm is an immune-inspired technique for processing time-dependent data. Julie et al. (38) proposed a possible solution for a robotic classification problem. The dendritic cell algorithm is implemented on a real robot, and an investigation is done of the effects of varying the migration threshold median for the cell population. The algorithm works well on a classification task with very little tuning. Ways of extending the implementation to allow it to be used as a classifier within the field of robotic security are suggested.

PERSPECTIVES

Recent work on AIS systems shows the tendency of developments in this field. Work has focused, in particular, on modeling some new processes of the natural immune system. In addition, more complex models that attempt to implement a closer representation of natural immune processes have been proposed. As in other fields of computational intelligence, some hybrid models that combine immune based models with other computational intelligence models such as genetic algorithms and neural networks have been proposed.

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Section III

Autoimmune Diseases

24

RHEUMATOID ARTHRITIS

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INTRODUCCION

Rheumatoid arthritis (RA) is the most common inflammatory arthropathy worldwide. It is a chronic, complex, and heterogeneous autoimmune disease (AD). It is characterized by the presence of long-standing inflammation of the diarthrodial joints resulting in symmetric polyarthritis and synovial membrane hypertrophy with progressive joint damage, bone and cartilage destruction as well as deformity. The autoimmune compromise is systemic, leading to extra-articular manifestations (EAM) (1–3). Comorbidity is frequent (4,5). Therefore, disability (6,7), impaired quality of life (8,9), and premature mortality, which is two times the that of the general population (10,11), characterize the disease. The prediction of RA risk is one of the major challenges of personalized medicine and uses gene-environment interactions, cytokine measurements, and detection of autoantibodies (12–14).

EPIDEMIOLOGY

The disease has a worldwide distribution affecting all races. As with of the ADs, it predominanly affects women with a sex ratio between 2:1 and 4:1 (15–17). The age at onset is commonly situated around 30s with a peak in the fifth decade of life. RA with disease onset at ages over 65 years is called Late-Onset RA (LORA) while RA starting at earlier ages (i.e., 17-65) is called Young-Onset RA (YORA) (18). The prevalence increases with age, and gender differences diminish in the older age group (16,17,19). In North European and North American populations, the prevalence is 0.5-1.1% (16,19). Studies from developing countries report a lower prevalence (between 0.01-0.5%) even in Latin American (LA) (16,20). In African-Colombian patients a period-prevalence of 0.01% was observed (21), in agreement with low prevalence in Black Africans (16). In contrast, a higher prevalence has been reported for certain Native Americans (16), highlighting the influence of ancestry on the risk of acquiring the disease. The worldwide incidence ranges from 0.01 in South Europe to 0.3 in Asia (16). The incidence increases with age and seems to reach a plateu starting from the age of 60 years (17). The incidence of RA in the USA is estimated to be 25 per 100,000 persons for men and 54 per 100,000 persons for women (22) (Table 1).

Mortality rates are higher among RA patients than in the general population. Life expectancy for RA patients is three to ten years less than that for the general population depending on the severity of the disease, age at onset and comorbidities (23). Nevertheless, the causes of death do not differ significantly between RA patients and the general population, but RA patients die at a younger age. Cardiovascular disease (CVD) is one of the major EAM (24) and a major predictor of poor prognosis. CVD, accounts for 30-50% of all deaths in patients with RA (5,25–27).

POPULATION	PREVALENCE RATES	INCIDENCE RATES
Asia	0.1-0.5	0.04-0.09
Africa	0-0.3	ND
North America	0.9-6.0	0.02-0.89
North Europe	0.4-1.1	0.02-0.04
South Europe	0.2-0.7	0.01-0.02
South America	0.01-0.5	ND

Table 1. Prevalence and incidence rates of RA worldwide (case per 100 inhabitants). *ND*: No data.

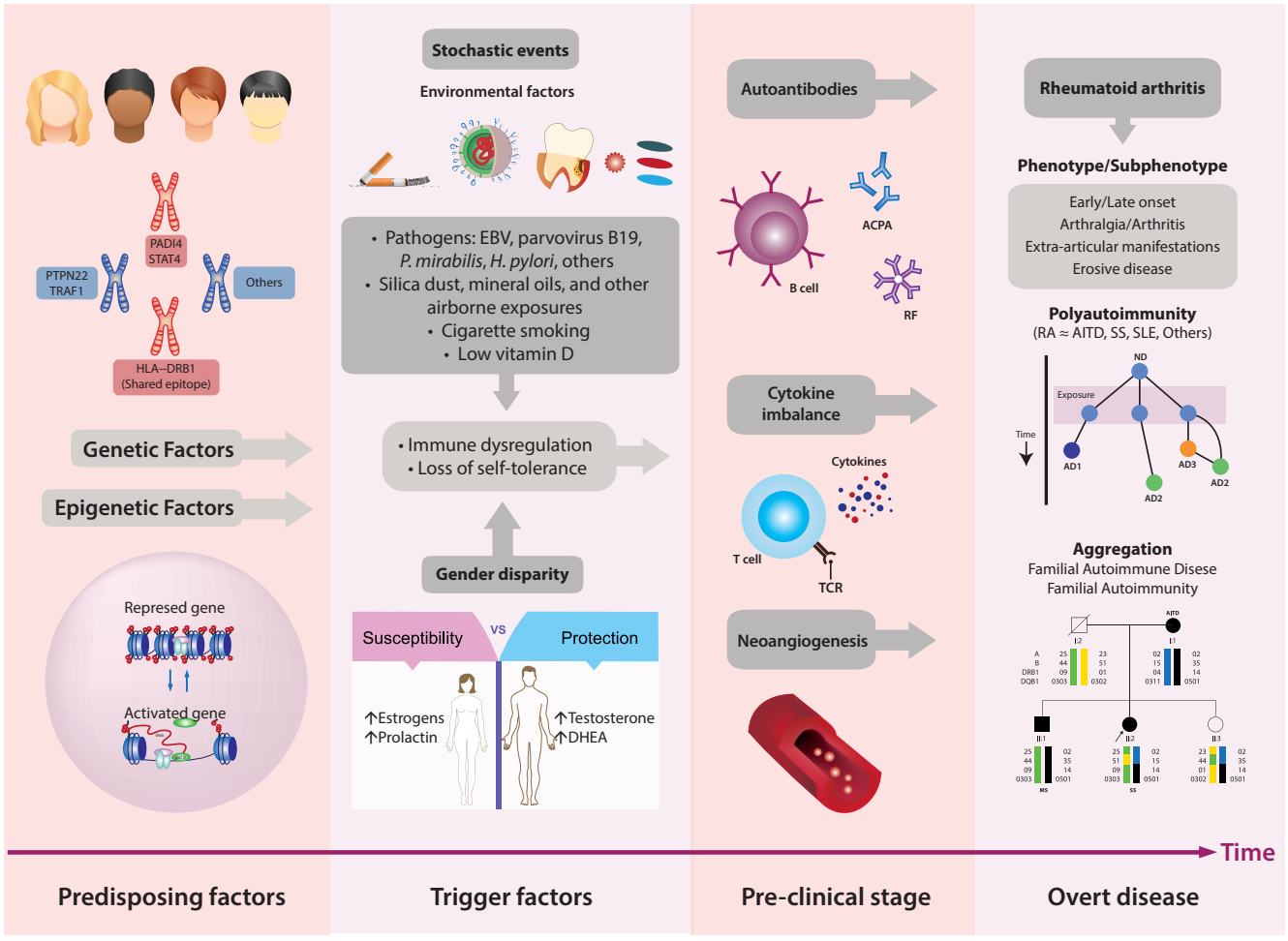

Figure 1. Risk factors for RA. The etiology of RA is multifactorial, in which a mosaic of predisposing and stochastic factors play in concert for the induction of loss tolerance and subsecuent organ damage. The pre-clinical stage is characterized by the presence of autoantibodies in serum, and pro-inflammatory markers. The clinical stage has a broad spectrum of subphenotypes. *ACPA*: anti-citrullinated protein antibodies; *AD*: autoimmune disease *AITD*: autoimmune thyroid disease; *DHEA*: dehydroepiandrosterone; *EBV*: Epstein-Barr virus; *MS*: multiple sclerosis; *PADI*: peptidyl arginine deiminase; *PTPN22*: protein tyrosine phosphatase, non-receptor type 22; *RA*: rheumatoid arthritis; *RF*: rheumatoid factor; *SLE*: systemic lupus erythematosus; *SS*: Sjögren's syndrome; *STAT4*: signal transducer and activator of transcription-4; *TCR*: T cell receptor; *TRAF1*: tumor necrosis factor receptor associated factor-1.

ETIOLOGY AND RISK FACTORS

A risk factor is any factor (i.e., genetic, epigenetic, environmental, or personal) that increases the risk of developing a disease. RA is a multifactorial disease in which the interaction between susceptibility genes and environmental factors (e.g., infections, lifestyle, and toxics, etc.) lead to disease (Figure 1).

GENETIC FACTORS

RA is a complex and thus polygenic disease. The heritability of RA has been estimated to be about 60-65% (28). Genetic factors including class II human leukocyte antigens (HLA-II) (29) as well as non-HLA genes have been implicated in the pathogenesis of RA and its outcome (30). Certain gene profiles or signatures have also been associated with response or nonresponse to therapy (31,32). Recent genome-wide association studies (GWAS) have enabled the simultaneous assessment of thousands of genes leading to more consequent results of genetic associations (33) (Table 2).

HLA-genes. In 1969, researchers noticed that peripheral blood lymphocytes from patients with RA were nonreactive in so-called mixed lymphocyte cultures to cells of the same type from other patients with RA (34). Peter Stastny (29,35) first found the association between the so-called B cell alloantigen DRw4, now known as *HLA-DR4*, and RA. According to the current nomenclature, *HLA-DRB1*04* denominates the allele group corresponding roughly to the archaic serotypical classification DR4, while the next appending digit set defines a specific allele (e.g.,*HLA-DRB1*0404*) (36). A decade after Stastny's discoveries, further characterization of the HLA locus

identified multiple RA risk alleles within *HLA-DRB1*. Gregersen *et al*. (37) showed that molecules encoded by RA associated *HLA-DRB1* alleles share a common amino acid (a.a) sequence, comprising residues 70–74 in the third hypervariable of the DRβ1 chain. This finding led to the 'shared epitope' (SE) hypothesis. HLA molecules that contain this 5 a.a sequence (i.e., QKRAA, QQRAA, and KKRAA), which is encoded by SE alleles and is arranged around the antigen-binding groove, are associated with the development of anti-citrullinated protein antibodies (ACPA), and, mostly, with ACPA-positive RA (38). SE alleles (*HLA-DRB1*01, DRB1*04, DRB1*10*) exert the strongest association with disease susceptibility, accounting for about 30% of the total genetic component (39). Thus, SE alleles *HLA-DRB1*1001* can accommodate citrulline in their antigen-anchoring pockets and thus stimulate citrullinated protein-specific T cell responses, especially in smoking patients (40). Further investigations have established more RA-associated alleles, mainly *HLA-DRB1*0401, *0404,* and **0408* in Caucasians; *HLA-DRB1*0405* in Spaniards, Japanese, and Jews; *HLA-DRB1*0101/2* in Israelis; *HLA-DRB1*1402* in some Native Americans; and *HLA-DRB1*1001* in Greeks (41). In Latin Americans, RA is associated with SE and DR4 positive *HLA-DRB1* alleles, mainly *HLA-DRB1*0404* (42). HLA associations with RA were initially related to ACPA-positive patients; however, new perspectives about the SE have recently been raised by Viatte *et al*. (36) and Mackie *et al*.(41) including new associations between HLA and ACPA-negative RA. Vignal *et al*. (43) showed two non-SE alleles strongly associated with RA: *HLA-DRB1*0301* with ACPA-negative RA and *HLA-DRB1*0701*, the latter regardless of autoantibody status.

A new classification system for the SE has been proposed and validated by French researches (44). Briefly, the susceptibility risk represented by the RAA motif is modulated by a.a at positions 70-71. Thus, at position 71, lysine (K) confers the highest risk, arginine (R) an intermediate risk while alanine (A) and glutamic acid (E) confer a lower risk. At position 70, glutamine (Q) and R represent a higher risk than aspartic acid (D). Based on the type of a.a at positions 70-71, the new classification system divides SE alleles into S1, S2, S3P, and S3D groups and denotes all non-RAA motifs as X. A positive association with RA was found for S2 and S3P allele carriers, while S1, S3D, and X are low risk alleles, which are pooled together as L alleles (44). The presence of S2 or S3P alleles has been correlated with ACPA production, whereas the presence of S3D and S1 alleles appeared to be protective. Stahl *et al*. (45) demonstrated that the risk of RA associated with the *HLA-DRB1* gene correlates most strongly with the a.a residue in position 11, located at the bottom of the DRβ1 antigen-binding groove. Viatte *et al*. (36) found independent RA risk alleles in *HLA B* and *HLA-DPB1*. In both cases, signals from these regions were best explained by a variation in a single a.a site at the bottom of their respective antigen-binding grooves. That is, these genetic variants in *HLA B, HLA-DRB1*, and *HLA-DPB1*, affecting a total of 5 a.a positions, almost completely explained the variance in RA risk caused by the HLA region (46,47). *DRB1*1301, *1302, *1304, *0103*, and **0402* alleles carry the DERAA motif, which is rather responsible for protective effects (20,47,48). A large European meta-analysis in 2010 confirmed *HLA-DRB1*1301* as a protective allele for RA (44). For further details about HLA, see Chapters 10 and 17.

Non-HLA susceptibility genes. In addition to *HLA-DR* alleles, several association studies have confirmed the role of non-HLA genes in susceptibility to RA (Table 2). Begovich *et al*. (49) identified a non-synonymous SNP in the protein tyrosine phosphatase, non receptor type 22 gene (*PTPN22*), which codes for lymphoid tyrosine phosphatase (Lyp), a downregulator of T cell receptor (TCR) signaling. The *PTPN22* C1885T polymorphism leads to an a.a change from arginine (arg) to tryptophan (Trp) at a.a position 620. This variant remains one of the most strongly RA associated SNP identified to date, right after *HLA-DRB1*, with an odds ratio (OR) of 1.8 for ACPA-positive RA. This SNP has been associated by many groups with ACPA and rheumatoid factor (RF) positive RA and probably worse prognosis. The presence of *PTPN22* C1885T polymorphism, in addition to SE and ACPA status, strongly supports the early diagnosis of RA. In contrast to SE, *PTPN22* may not be closely associated with smoking (50,51).

Suzuki *et al*. (52) described a SNP in the third intron of the peptidyl arginine deiminase type 4 enzime (*PADI4*) gene, responsible for increasing the stability of *PADI4* mRNA transcripts and associated with ACPA-positivity in patients with RA. This enzyme mediates the citrullination of proteins (i.e., conversion of arginine residues to citrulline). Citrullinated peptides bind with higher affinity to *HLA-DRB1 SE* molecules, are naturally processed and, importantly, are immunogenic. Thus, it seems that increased translation of *PADI4* mRNA variant boosts production of citrullinated peptides, which act as autoantigens and elicit profound adaptive immune responses (53). Whereas many other risk loci seem to be connected to several ADs, the *PADI4* locus seems to be specific to RA (36). However, the association between *PADI4* and RA is mostly observed in Asian cohorts (52,54).

On the basis of recent GWAS data, the tumor necrosis factor (TNF) receptor-associated factor 1 (*TRAF1*) in the *TRAF1-C5* region may be the third most strongly RA associated locus (55). This region has also been associated primarily with ACPA positive RA. TRAF1 is an adaptor protein that links TNF family members, such as TNF-α, to downstream signaling networks. TRAF1 has been implicated in cell growth, proliferation, apoptosis, and in the overall pathogenesis of RA. TRAF1 has been related to increased radiological progression; however, TRAF1-C5 may not be associated with RA mortality (55,56).

The association of RA with the signal transducer and activator of transcription 4 (*STAT4*) gene is relatively modest in comparison to the previous genetic factors discussed above. STAT4 exerts a distinct role in the signaling of cytokines, primarily interleukin (IL)-12, through janus kinase-2 (JAK-2) enzymes. Interestingly, different SNP in *STAT4* gene may increase susceptibility to both ACPA positive and negative RA (57,58).

Other important and confirmed loci include, Fc gamma receptor IIIA (*FCGR*) (58,59), *CD40* (58,60,61), chemokine receptor 6 (*CCR6*), *CTLA4, IRF5, IL6ST, IL2RA, IL2RB, CCL21 MBL2, IL6R, IL-10, IL-18, TNFRS*, and *TNFAIP3* (48,62,63). For more details, see table 2. In summary, several HLA and non-HLA genes have been implicated in susceptibility to or protection against RA. To date, more than 40 genes have been associated with the disease and these genetic factors account for about 50% of the genetic variants linked to RA susceptibility (36,38,58).

EPIGENETICS

The disparity between the presence of susceptibility genes and the development of a disease such as RA in twins clearly states that carrying susceptibility genes is not enough to acquire the disease. Epigenetics may explain the low rate of concordance of RA between identical twins (see chapters 1 and 22). Using variance modeling, RA heritability is about 60% based on the higher monozygotic twin concordance rates (12–15%) compared to dizygotic twins (2–4%) (28,64). In a cohort of 91 monozygotic twins' pairs, increased concordance for RA was observed in SE positive pairs (RR: 3.7). In addition, a 5-fold risk for RA concordance was seen in twins who were "homozygous" for the SE as compared with those negative for the SE (65). Familial aggregation (i.e., greater disease occurrence in relatives of probands than in healthy controls) has been consistently observed in RA. It is estimated that familial aggregation of RA ranges between 2% and 17%, depending upon the disease prevalence in the population used as reference (20,28,39). However, aggregation of diverse ADs, also known as familial autoimmunity (FA), has been overlooked in RA patients (66). Analysis of DNA methylation in T cells has revealed global hypomethylation in cells derived from patients with RA compared with those from healthy controls (67). DNA hypomethylation has also been observed in RA fibroblast-like synoviocytes (FLS), as compared with normal FLS. Nakano *et al*. (68) performed a genome-wide evaluation of FLS derived from patients with RA and osteoarthritis (OA). As many as 1,859 loci, relevant to cell movement, adhesion, and trafficking were differentially methylated in RA (732 hypomethylated and 1,127 hypermethylated) (68). In a gene-targeted approach, Nile *et al*. (69) investigated DNA methylation patterns in the promoter region of *IL-6* in peripheral blood mononuclear cells derived from patients with RA and healthy controls. This study iden-

CANDIDATE GENE	ENCODED PROTEIN	FUNCTION	POPULATION	REFERENCE
PTPN22	Protein tyrosine phosphatase, non-receptor type 22	Lymphocyte-specific non-receptor tyrosine phosphatase involved in regulation of activation threshold of lymphocytes	American, Caucasian	(49–51)
PADI4	Peptidylarginine-deiminase 4	Enzyme that converts arginine to citrulline, creating autoantigens in RA	Asian	(36,52–54)
TRAF1	TNF receptor-associated factor 1	Regulator of TNF-α–receptor superfamily signaling (i.e., to NF-κB and JAK)	American, Caucasian	(55,56)
STAT4	Signal transducer and activator of transcription 4	Transducer of cytokine signals that regulate proliferation, survival, and differentiation of lymphocytes	Asian, Caucasian	(57,58)
FCGR	Fc gamma receptor	Low-affinity IgG Fc receptor that regulates macrophage and neutrophil activation, and immune-complex clearance	Caucasian	(58,59)
CD40	Cluster of differentiation 40	Co-stimulatory molecule that enhances interactions between T and B cells, and increases autoantibody production	Caucasian	(58,60,61)
CCR6	Chemokine receptor 6	B-lineage maturation and antigen-driven B cell differentiation, and it may regulate the migration and recruitment of dendritic and T cells during inflammatory and immunological responses	American, Caucasian	(36,62,63)
IRF5	Interferon-related factor 5	Modulation of cell growth, differentiation and apoptosis	Caucasian	(38,45)
IL2RA, IL2RB	IL-2 receptor A and B	High-affinity receptor for IL-2 on lymphocyte subsets	American, Caucasian	(38,45)
CCL21	CC chemokine ligand 21	Chemokine implicated in germinal-center formation	Caucasian	(48,62,63)

Table 2. The most relevant susceptibility alleles in RA according to GWAS studies and related population. *Fc*: fragment crystallizable region; *GWAS*: genome-wide association studies; *IL*: interleukin; *JAK*: janus kinase; *RA*: rheumatoid arthritis; *TNF-α*: tumor necrosis factor alpha; *NF-kβ*: nuclear factor kappa-light-chain-enhancer of activated B cells.

tified a single CpG motif 1,099 base pairs upstream from the *IL-6* transcription start site that was less methylated in patients with RA than in controls. Increased expression of microRNA 115133 and microRNA 203134 has been observed in RA FLS compared with OA FLS, and this increase correlates with elevated levels of matrix metalloproteinase 1 (MMP 1) and IL-6. MicroRNA (miRNA) is a small non-coding RNA molecule, which functions in transcriptional and post-transcriptional regulation of gene expression (see chapter 1). It is important to note that the expression of miRNA 115132 and of miRNA 203134 is inversely correlated with levels of DNA methylation (Figure 1).

AGE

It has been suggested that YORA might have a poorer prognosis as manifested by more persistent disease activity, more radiographic deterioration, systemic involvement, and a rapid functional decline. Aggressive disease is largely restricted to those patients with high titers of RF (70). Pease *et al*. (71) found that LORA patients had longer stiffness in the morning. It has also been reported that older patients have more acute onset in both large and small joints and usually present polymyalgia rheumatica-like symptoms (72). These patients can present more constitutional features like weight loss, myalgia, rheumatic nodules, and neuropathy (73). Turkcapar *et al*. (73) reported that proximal interphalangeal (PIP), metacarpophalangeal (MCP), elbow, metatarsophalangeal (MTP), and ankle joints are more associated with YORA. Classical hand deformities, interstitial lung disease, lung disease, and Sjögren syndrome (SS) are observed more frequently at this group. Rojas-Villarraga *et al*. (74) found ACPA can be detected at early disease stages and may be used as indicators of RA progression and prognosis, as well as, family history of RA and *HLA-DRB1* SE are consistently associated with joint damage. Recently, we showed that YORA is associated with female gender, higher educational level, higher joint involvement, and EAM while LORA is related to environmental exposure, CVD, and higher body mass index (BMI) (75).

GENDER

Probably, the striking gender differences in RA patients are due to the effects of sex hormones, fetal microchimerism, and sex chromosomes (76,77). Women have an enhanced antibody production and increased cell mediated responses following immunization while men produce a more intense inflammatory response to infectious organisms. Further, women have higher $CD4^+$ T cell counts than men contributing to an increased CD4/CD8 ratio, higher levels of plasma IgM, and greater T helper 1 (Th1) cytokine production (77,78). The predominance of RA in females suggests a role for hormonal imbalances. In fact, the peak age at RA onset is the fifth decade, which coincides with hormonal changes in women (77). Estrogens, androgens, and prolactin, have been the first proposed candidates to have important roles in the sex bias observed in RA, due to their capacity of modulating the immune response via androgen and estrogen receptor. Morning activity of RA is correlated with prolactin plasma levels observed at that time (79) (Figure 1). A history of child-bearing may protect against RA while nulliparity has been suggested as a risk factor for the disease (80). Pregnancy results in changes in disease activity. Signs and symptoms of RA decrease during pregnancy while postpartum may favor disease exacerbation (81). Postpartum worsening may be associated with the return to the Th1 environment (82). After pregnancy, a flare may be induced in RA by breastfeeding through the actions of prolactin (83). However, recent data show that, overall, women who breastfeed their infants have a decreased risk for RA (84). In contrast, women who report a postpartum onset of RA, breastfeeding, especially after the first pregnancy, increased the risk of RA fivefold (85).

ENVIRONMENTAL FACTORS

Smoking. Tobacco smoke is a widely known risk factor for RA (86–89) (Figure 1 and 2). Tobacco smoking affects both the innate and adaptive immunity. One of the main mechanisms underlying smoking-evoked autoimmune response in RA is via the production of antibodies recognizing citrullinated proteins (77). Citrullination promotes the transformation from self-antigens to autoantigens (90). The number of copies of the SE that an individual carries can modify the risk of acquiring RA in smokers which suggests a gene environment interaction. Smokers who have two copies of the SE have a 21-fold higher risk of developing RA than non-smokers who do not carry the SE (86). The risk of RA increases with the intensity (i.e., pack per day) and duration of cigarette use. Heavy cigarette smoking has been linked to a substantial increase in the susceptibility to RA. A recent metaanalysis disclosed that both smoking and the *PTPN22* risk allele are associated with the risk of ACPA positivity (91).

Alcohol and coffee consumption. Some studies have found alcohol consumption to be associated with significant reduction in the risk of RA, particularly ACPA-positive (92). The observed inverse association between alcohol intake and the risk of RA, and the demonstration of a preventive effect of alcohol in experimental arthritis, indicate that alcohol could protect against RA (93). On the contrary, coffee consumption has been implicated as a risk factor for seropositive RA in longitudinal data from Finland as has decaffeinated coffee in USA (94), but neither of these associations has been replicated.

Vitamin D. A high vitamin D intake has been associated with a lower risk of RA since vitamin D is an important modulator of the inflammatory response through the vitamin D receptor. Despite its confirmed importance in other ADs such as type 1 diabetes mellitus (T1DM) and multiple sclerosis (MS),

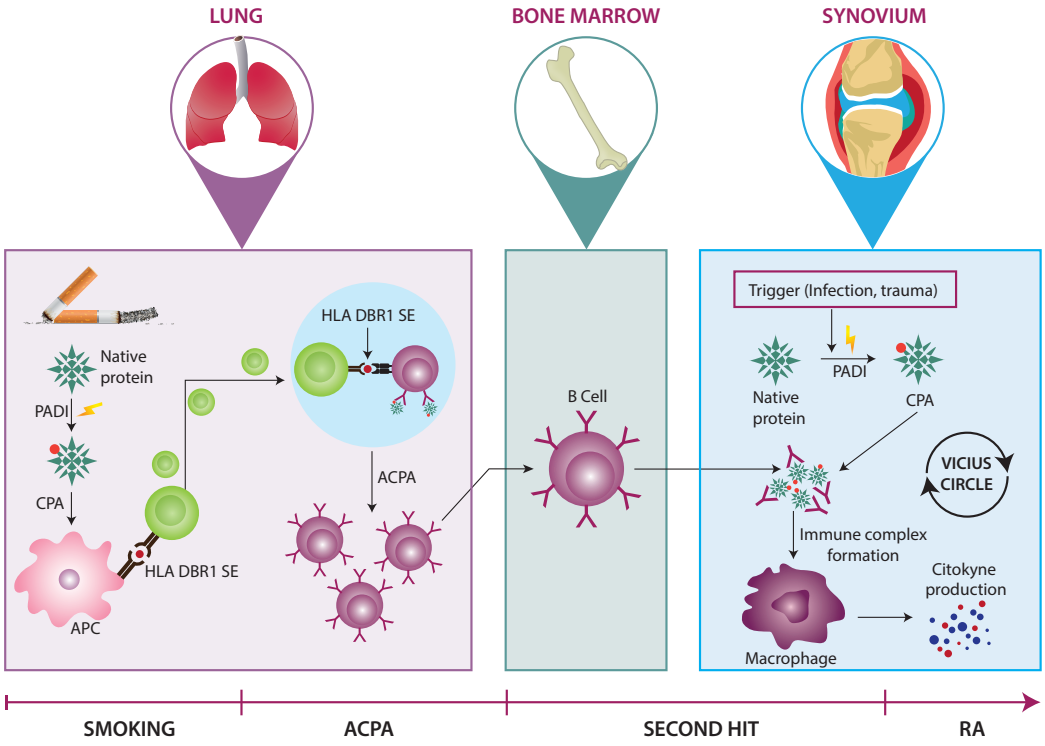

Figure 2. Gene-environment interaction: Smoking. Long-term smoking induces a local activation of PADI enzymes, with subsequent citrullination of peptides present in the lungs; later the activation of APC, in response to signals delivered by toxic components in the smoke, leads to increased uptake, processing and presentation of citrullinated peptides in the context of SE to CPA-specific pathogenicCD4+ T cells, expressing the PTPN22 risk allele, which initiates proliferation, differentiation, cytokine production, somatic hypermutation, epitope spreading and the formation of a CPA-specific T cell memory. A second inflammatory event occurs in the synovium,with local activation of PADI enzymes and citrullination of proteins and peptides in the synovium, following the recruitment of ACPAs of multiple specificities to the joint, the formation of CPA-ACPA immune complexes, with binding to Fc receptors expressed on ACP, leads to the production of pro-inflammatory cytokines and an increased antigen presentation of citrullinated peptides, which in turn leads to further activation of pathogenic T cells, B cell help and increased ACPA production. Subsequently, a vicious circle has been established, entertaining a chronic synovial inflammation and the development of RA. *ACPA*: anti-citrullinated protein/peptide antibody; *APC*: antigen presenting cells; *CPA*: citrullinated peptide antigen; *PADI*: peptidyl arginine deiminase; *RA*: rheumatoid arthritis; *SE*: shared epitope.

its role on the risk of RA is not unanimous (95). The Iowa Women's Health Study (96) analyzed data from a prospective cohort study of 29,368 women aged 55–69 years. The study found that greater intake of vitamin D might be associated with a lower risk of RA.

Diet and other vitamins. The effect of diet on the risk of RA is controversial. A diet rich in fish, olive oil, and cooked vegetables has been shown to protect against RA due to the high content of omega 3 fatty acids (i.e., Mediterranean diet) (77,97). Vitamin C was associated with a reduced risk of inflammatory polyarthritis, suggesting that antioxidants may protect against RA development (98). There is also some evidence suggesting that copper and selenium deficiency are linked to RA.

Others. Silica dust, mineral oils, and other airborne exposures have been included as potential risk factors for RA (77). Air pollution has been associated with increased risk of RA. Populations living near roads of high traffic density show an increased risk of the disease. As with cigarette smoke, inhaled particulate matter may induce both local lung inflammation and systemic inflammation. Indirect support for this hypothesis comes from the established link between air pollution and inflammation. Organic solvents have been shown to increase the risk of ADs (99).

Infectious agents. Several microbial agents have been associated with risk of developing RA (Figure 1), including parvovirus B19, Epstein-Barr virus (EBV), retroviruses, *M. tuberculosis*, *E. coli, P. mirabilis*, and mycoplasmas. The evidence for the participation of microorganisms in the induction of RA include the presence of increased serum levels of antibodies against microbial epitopes, a greater number of cells carrying the genome of some of these viruses and the demonstration, by polymerase chain reaction technique, of bacterial or viral genes in the rheumatoid synovium. It is possible that in many cases these agents act triggering the disease, or helping to perpetuate the disease. The most accepted mechanism is the molecular mimicry (100). Mimicry is also possible

respect to shared conformational epitopes (see chapter 19). Whether the SE is itself presented as an antigenic peptide to allow molecular mimicry is far from clear. It is much more likely that it governs the antigenic peptides that are presented to the immune system (100). Several studies have shown both *P. mirabilis* infection (mostly urinary) and antibody titers against these bacteria are higher in RA patients than in healthy controls. In addition, the association with RA and the immune response against the bacteria has been demonstrated at the molecular level (101), mainly as a consequence of the similarity between its α-hemolysin, urease, and self-epitopes. Bioinfomatic analysis has shown that SE ("EQ/KRRAA") has high structural similarity with a linear peptide from the bacterial hemolysin ("ESRRAL"). Besides, there was also found a molecular similarity between the peptide "IRRET" found in the type XI collagen and a peptide from the bacterial urease ("LRREI") (102).

Patients with periodontal disease (PD) seem to have an increased likelihood ratio (LR) of suffering RA (103–105). Conversely, RA patients show increased likelihood of PD, a relationship that could not be attributed simply to inadequate tooth cleaning in RA patients (106,107). ACPA titers have been correlated with PD severity (108). Elevated anti-*P. gingivalis* antibody titers were associated with higher serum concentrations of C-Reactive protein (CRP) and ACPA (109). There are experimental data suggesting that periodontitis and RA influence each other's pathogenesis (110–113).

Socioeconomic status (SES): The World health organization (WHO) suggests a number of individual and system factors that can influence and determine health outcomes. Among those, access and use of health-care services and SES are recognized as independent determinants of health, and are particularly relevant for patients living with chronic conditions. Disparities in health resulting from lack of access to care are avoidable and unfair, and can therefore be referred to as inequities (114). SES has been associated with the risk of RA although not uniformly.

PATHOGENESIS

The seat tissue of the inflammatory process is the synovium. There are three independent, but interacting pathological processes in the RA joints: chronic inflammation, hyperplasia of synovium (i.e., pannus), and increased osteoclastogenic activity. Chronic inflammation is characterized by an infiltrate of mainly mononuclear cells including lymphocytes, monocytes/MΦ, and dendritic cells (DC). The synovial membrane is transformed into a secondary lymphoid organ with the presence, in many cases, of germinal centers where RF and antibodies against self-proteins are produced. As a result, there is an increased production of proinflammatory cytokines and chemokines, which contribute to the recruitment of new cells and progressive joint damage. The cartilage destructive process is carried out directly by the action of MMPs, produced by fibroblasts and other cellular subpopulations in pannus-cartilage interface. Cells in the inflammatory infiltrate, predominantly T cells, activate the process of osteoclastogenesis leading to an increased bone resorptive process causing juxta-articular osteopenia and the appearance of erosions (1,22,60,115).

IMMUNE RESPONSE

The innate and the adaptive immune response contribute in a highly interactive form to the pathogenesis of RA. The most likely mechanism for the environmental component is repeated activation of innate immunity. This process can take many years with evidence of autoimmunity increasing gradually until some unknown process tips the balance toward clinically apparent disease. It is possible that the initial inciting event involves the product interaction of a microbial agent, bacterial or viral, with receptors on cells of the innate immunity. When they are activated, these cells invoke the adaptive immune response by providing the second signal of stimulation to the lymphocytes T and B. The discovery of Toll-like receptors (TLRs) and other intracellular receptors from nucleotide-binding oligomerization domain (NOD) group, classified within the pattern-recognition receptors (PRR) [i.e., receptors that recgonize pathogen-associated molecular patterns (PAMPs)], allowed to better understand possibe triggers of early events of the disease.The PRR are present on cells of the innate immune response as DCs, monocytes/macrophage. They are activated upon recognition of PAMPs from viruses or bacteria, and generate a promoter cascade of local inflammation. This inflammatory reaction involves engaging adaptative response cells, mainly T and B lymphocytes, generating antibodies against self-molecules and cellular responses mediated by antigen-specific clones in the joint (116) (Figure 3).

Adaptive immune response. After onset of clinical disease, the normally hypocellular synovial membrane becomes hyperplastic, comprising a superficial lining layer with a high density of FLS and MΦ, overlying an interstitial zone that contains a marked cellular infiltrate, arranged in the subintimal, as diffuse infiltrates, perivascular localization or in germinal centers (106). The inflamed synovium invades adjacent cartilage and promotes articular destruction, which is mediated by the activities of osteoclasts, chondrocytes, and FLS. The underlying bone marrow also exhibits an inflammatory infiltrate, containing T cell–B cell aggregates, and so the bone probably receives a bidirectional insult (117). Articular damage, in turn, probably generates a rich source of neo-antigens to promote further autoimmune reactivity. In addition, the articular environment is profoundly hypoxic and angiogenesis is a characteristic feature of rheumatoid joints (118). All these cells express cytokines, HLA-II, and co-stimulatory molecules. Cytokines are implicated in each phase of the pathogenesis by promoting autoimmunity (including during the pre-articular phase), by maintaining chronic inflammatory synovitis and by driving the destruc-

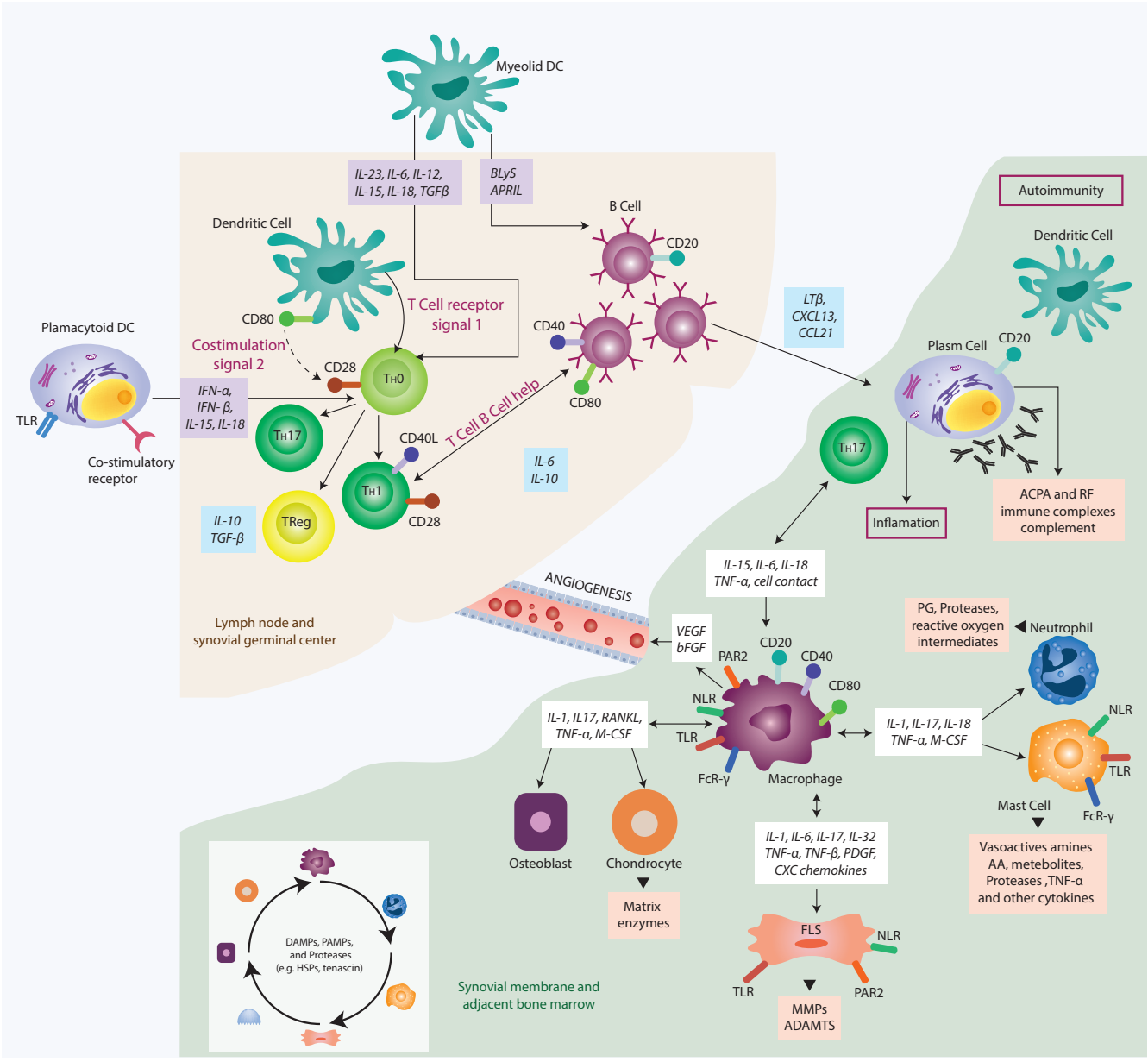

Figure3. Adaptive and innate immune response: synovial interactions in rheumatoid arthritis. The costimulation-dependent interactions among DCs, T cells, and B cells are shown as occurring primarily in the lymph node. Pivotal cytokine pathways are depicted in which activation of these cells and macrophages underpins the dysregulated expression of cytokines that in turn, drive activation of effector cells (i.e., neutrophils, mast cells, endothelial cells and synovial fibroblasts). These events generate an autoimmune response to citrulline-containing self-proteins. In the synovial membrane and adjacent bone marrow, adaptive and innate immune pathways integrate, through positive feedback mediated by the interactions shown among cells and cytokines, together with the molecular products to promote tissue remodeling and damage, drive the chronic phase in the pathogenesis of RA. *AA*: arachidonic acid; *ADAMTS*: a disintegrin and metalloprotease with thrombospondin-1–like domains; *ACPA*: anti-citrullinated protein/peptide antibody; *APRIL*: a proliferation-inducing ligand; *bFGF*: basic fibroblast growth factor; *BLyS*: B-cell activating factor; *CCL21*: CC-chemokine ligand 21; *DC*: dendritic cell; *CXCL13*: CXC-chemokine ligand 13; *DAMP*: damage-associated molecular pattern; *FcR*: Fc receptor; *FcγR*: Fc receptor for IgG; *FGF*: fibroblast growth factor; *HSP*: heat-shock protein; *IFN-α/β*: interferon-α/β; IL: Interleukin; *LTβ*: lymphotoxin-β; *M-CSF*: macrophage colony-stimulating factor; *MMP*: matrix metalloproteinase; *NLR*: nucleotide-binding oligomerization domain–like receptor; *PAMP*: pathogen-associated molecular pattern; *PAR2*: protease-activated receptor-2; *PDGF*: platelet-derived growth factor; *PG*: prostaglandins; *RANKL*: receptor activator of nuclear factor κ-b ligand: *RF*: rheumatoid factor; *TGF-β*: transforming growth factor-β; *Th*: T helper; *Th0*: type 0 helper T cell; *Th1*: type 1 helper T-cell; *Th17*: type 17 helper T cell; *TLR*: toll-like receptor; *TNF-α*: Tumor necrosis factor-alpha; *TReg*: regulatory T cell; *VEGF*: vascular endothelial growth factor.

MOLECULE OR SIGNAL MEDIATOR	CELL EXPRESSION	DISEASE RELEVANT FUNCTIONS
Cytokines		
IL-1α and IL-1β	Monocytes, B cells, synovial fibroblasts, chondrocytes	Increase synovial fibroblast cytokine, chemokine, MMP, iNOS and PG release, monocyte cytokine, aggrecanase, ROI and endothelial-cell adhesion molecule expression Osteoclast, leukocytes and endothelial-cell activation. Decrease GAG synthesis. Mediate fever. Enhance glucose metabolism. Reduce cognitive function
IL-6	Monocytes, synovial fibroblasts, B cells, T cells	B cell proliferation and antibody production; haematopoiesis and thrombopoiesis; T cell proliferation, differentiation and cytotoxicity. Activation of osteoclasts. Increase hepatic acute-phase response and neuroendocrine effects (implicated in hypothalamic–pituitary–adrenal axis dysfunction and fatigue)
IL-7	Synovial fibroblasts, monocytes?	Promote and maintain T cell expansion and survival. Macrophage activation. Haematopoietic regulation. Thymic regulation and NK-cell maturation. Block apoptosis, and maintain T cell–macrophage cognate interactions
IL-10	Monocytes, T cells, B cells, DCs, epithelial cells	Increase macrophage cytokine release, iNOS and soluble receptor expression, and B cell isotype switching. Decrease ROI, T cell cytokine release, MHC expression, anergy induction, TReg-cell maturation and effector function (?), DC activation, cytokine release, and synovial fibroblast MMP and collagen release
IL-12	Macrophages, DCs	TH1-cell proliferation and maturation, T cell and NK-cell cytotoxicity, B cell activation
IL-15	Monocytes, synovial fibroblasts, mast cells, B cells, PMNs, DCs	Promote T cell chemokinesis, activation and memory maintenance, B cell differentiation and isotype switching, NK cell activation and cytotoxicity, synovial fibroblast activation, macrophage activation/suppression (dose dependent), PMN activation, adhesion molecule expression and oxidative burst. Block apoptosis, and maintain T cell–macrophage cognate interactions
IL-17A and 17F	Th17 cells, synovial fibroblasts	Act synergistically to enhance activation of synovial fibroblasts, chondrocytes, and osteoclasts. Increase MMP release, haematopoiesis and leukocyte cytokine production. Reduce chondrocyte GAG synthesis
IL-18	Monocytes, PMNs, DCs, platelets, endothelial cells	Promote T cell differentiation (TH1 cells with IL-12; TH2 cells with IL-4), NK-cell activation, cytokine release and cytotoxicity, PMN activation, cytokine release and migration; pro-angiogenic for endothelial cells. Decrease chondrocyte GAG synthesis, iNOS expression; monocyte cytokine release and adhesion molecule expression
IL-21	T cells, NK-cells	Activates Th17 and B cell subsets
IL-23	Macrophages, DCs	Expands Th17
IL-32	Epithelial cells, monocytes(?), synovial fibroblasts(?)	Activates cytokine production by several leukocytes, promotes osteoclast differentiation, PG and MMP release
IL-33	T cells, mast cells	Activates mast cells and neutrophils
Type I IFNs	Widespread	Antiviral response, broad immunomodulatory effects. Increase MHC expression, macrophage activation, lymphocyte activation, differentiation, survival (antiproliferative) and cytoskeletal alterations
LTα and/or LTβ	T cells, monocytes, synovial fibroblasts	Peripheral lymphoid organ development; otherwise similar bioactivities to TNF
Oncostatin M	Monocytes, activated T cells	Promote megakaryocyte differentiation. Increase synovial fibroblast TIMP and cytokine release, acute-phase reactants, protease inhibitors, neuroendocrine effects and corticosteroid release; osteoblast modulation(?). Decrease monocyte TNF release and IL-1 effector function

Table 3. Key molecules and signal mediators implicated in the pathogenesis of RA. *APRIL*: a proliferation-inducing ligand; *BLyS*: B-lymphocyte stimulator; *BMP*: bone morphogenetic protein; *BTK*: Bruton's tyrosine kinase; *COX*: cyclooxygenase; *DTH*: delayed-type hypersensitivity; *DC*: dendritic cell; *FFA*: free fatty acid; *FGF*: fibroblast growth factor; *GAG*: glycosaminoglycans; *GM-CSF*: granulocyte–macrophage colony-stimulating factor; *IFN*: interferon; *IL*: interleukin; *iNOS*: inducible nitric-oxide synthase; *JAK*: janus kinase; *LT*: lymphotoxin; *M-CSF*: macrophage colony-stimulating factor; *MIF*: macrophage migration-inhibitory factor; *MMP*: matrix metalloproteinase; *NK*: natural killer; *PDGF*: platelet-derived growth factor; *PG*: prostaglandin; *PI3K*: phosphatidylinositol 3-kinase; *PLA2*: phospholipase A2; *PMN*: polymorphonuclear leukocyte; *RANKL*: receptor activator of nuclear factor-κB (RANK) ligand; *ROI*: reactive oxygen intermediate; *SyK*: spleen tyrosine kinase; *TCR*: T cell receptor; *TGF-β*: transforming growth factor-β; *Th17*: type 17 helper T cell; TIMP, tissue inhibitor of MMPs; *TNF*: tumor-necrosis factor; *TReg*: regulatory T cell; *VEGF*: vascular endothelial growth factor. Adapted from McInnes and Schett (115, 119).

MOLECULE OR SIGNAL MEDIATOR	CELL EXPRESSION	DISEASE RELEVANT FUNCTIONS
TNF-α	Monocytes, T cells, B cells, NK cells, PMNs, mast cells, synovial fibroblasts, osteoblasts	Increase monocyte activation, cytokine and PG release, PMN priming, apoptosis and oxidative burst, T cell apoptosis, clonal regulation and TCR dysfunction, endothelial-cell adhesion molecule expression, MMP, cytokine and chemokine release, adipocyte FFA release, endocrine effects. Decrease synovial fibroblast proliferation and collagen synthesis. Activation of osteoclasts and resorption of cartilage and bone. Mediates metabolic and cognitive dysfunction
Growth and differentiation factors		
APRIL	Monocytes, T cells	Activate B cells and have a role in the maturation of B cells, enhancement of autoantibody production, isotype switching and survival. T cell co-stimulation
BMP family (BMP2–BMP15)	Epithelial cells, synovial fibroblasts, mesenchymal embryonic tissues	Regulate crucial chemotaxis, mitosis and differentiation processes during chondrogenesis and osteogenesis. Tissue morphogenesis
BLyS	Monocytes, T cells, DC	Activate B cells and have a role in the maturation of B cells and enhancement of autoantibody production
FGF Family	Synovial fibroblasts, monocytes	Growth and differentiation of mesenchymal, epithelial and neuroectodermal cells
GM-CSF	Macrophages, T cells, mast cells, NK cells, endothelial cells	Enhance differentiation of granulocyte and myeloid-lineage cells in the bone marrow and synovium
M-CSF	Monocytes, granulocytes, endothelial cells, and fibroblasts	Enhance differentiation of granulocyte and myeloid-lineage cells in the bone marrow and synovium
MIF	Macrophages, activated T cells, synovial fibroblasts	Enhance macrophage phagocytosis, cytokine and NO release. Increase T cell activation, DTH, fibroblast proliferation, COX expression, PLA2 expression and intrinsic oxidoreductase activity ('cytozyme')
PDGF	Platelets, macrophages, endothelial cells, synovial fibroblasts	Paracrine and/or autocrine growth factor for various lineages. Wound healing
RANKL	Stromal cells, osteoblasts, T cells	Stimulates bone resorption via osteoclast maturation and activation, modulates T cell–DC interactions
TGFβ	Synovial fibroblasts, monocytes, T cells, platelets	Wound repair, matrix maintenance and fibrosis. Increase TH17- and TReg-cell proliferation, early phase leukocyte chemoattractant, gelatinase and integrin expression, early macrophage activation then suppression. Decrease iNOS expression, NK cell proliferation and effector function; initial activation then suppression of inflammatory responses
VEGF	Monocytes, endothelial cells, synovial fibroblasts	Angiogenesis
Intracellular signaling molecules and transcription factors		
BTK		Plays important role in the activation of B cells, macrophages, mast cells, and neutrophils, through regulation of B cell receptor and Fc receptor signaling as appropriate
JAK		Tyrosine kinase that regulates cytokine-mediated leukocyte maturation and activation, cytokine production, and immunoglobulin production
NF-kβ		Helps integrate inflammatory signaling and is important for cell survival
PI3K		Mediates signals that drive proliferation and cell survival
SyK		Tyrosine kinase that regulates immune-complex–mediated and antigen-mediated activation of B and T cells and other Fc receptor–bearing leukocytes

Table 3 continuation. Key molecules and signal mediators implicated in the pathogenesis of RA. *APRIL*: a proliferation-inducing ligand; *BLyS*: B-lymphocyte stimulator; *BMP*: bone morphogenetic protein; *BTK*: Bruton's tyrosine kinase; *COX*: cyclooxygenase; *DTH*: delayed-type hypersensitivity; *DC*: dendritic cell; *FFA*: free fatty acid; *FGF*: fibroblast growth factor; *GAG*: glycosaminoglycans; *GM-CSF*: granulocyte–macrophage colony-stimulating factor; *IFN*: interferon; *IL*: interleukin; *iNOS*: inducible nitric-oxide synthase; *JAK*: janus kinase; *LT*: lymphotoxin; *M-CSF*: macrophage colony-stimulating factor; *MIF*: macrophage migration-inhibitory factor; *MMP*: matrix metalloproteinase; *NK*: natural killer; *PDGF*: platelet-derived growth factor; *PG*: prostaglandin; *PI3K*: phosphatidylinositol 3-kinase; *PLA2*: phospholipase A2; *PMN*: polymorphonuclear leukocyte; *RANKL*: receptor activator of nuclear factor-κB (RANK) ligand; *ROI*: reactive oxygen intermediate; *SyK*: spleen tyrosine kinase; *TCR*: T cell receptor; *TGF-β*: transforming growth factor-β; *Th17*: type 17 helper T cell; TIMP, tissue inhibitor of MMPs; *TNF*: tumor-necrosis factor; *TReg*: regulatory T cell; *VEGF*: vascular endothelial growth factor. Adapted from McInnes and Schett (115, 119).

tion of adjacent joint tissue. Therefore, cytokines integrate the immune-regulatory and tissue-destructive events that underlie the clinical presentation and progression of RA (119) (Figure3).

Innate Immune System. A variety of innate effector cells, including MΦ, mast cells, and NKs are found in the synovial membrane, whereas neutrophils reside mainly in synovial fluid (Figure 3). Macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), and granulocyte–macrophage colony stimulating factor (GM-CSF) enhance maturation of these cells, their efflux from the bone marrow, and transfer to the synovium (120). In particular, MΦ are central effectors of synovitis (121) and act through release of cytokines (e.g., TNF-α,IL-1,6,12,15,18,23), reactive oxygen intermediates, nitrogen intermediates, production of prostanoids and matrix-degrading enzymes, phagocytosis, and antigen presentation. This pattern of expression of proinflammatory cytokines and inducible nitric oxide synthase suggests a predominant M1 MΦ phenotype. Macrophages are activated by TLRs (i.e., TLR 2/6, 3,4,8), NOD-like receptors (NLRs), cytokines, immunecomplexes, lipoprotein particles, liver X–receptor agonists [i.e., oxysterols, oxidized low density lipoprotein (LDL), serum amyloid A-rich, and high-density lipoprotein (HDL)] (119,122). Moreover, microRNA species (i.e., microRNA-155) have been implicated in the regulation of synovial cytokine expression (123). Neutrophils, mast cells, and NK cells are present in high concentrations during the process. Mesenchimal tissue responses, soluble products, cytokines, complement activation, immune complexes, and additional factors, including nitric oxide, neuropeptides (e.g. substance P), arachidonic acid metabolites, clotting factors and fibrinolysis play a key role in the pathogenesis of RA (106,115,124-126) (Figure 3).

AUTOANTIBODIES

Rheumatoid factors are antibodies directed against the Fc portion of IgG. Initially, the RF was described by Waaler and Rose in 1944, and is commonly measured in clinical practice as an IgM-RF; however, other immunoglobulin types, including IgG and IgA, have been identified (127). An abnormal immune response appears to select, via antigenic stimulation, high affinity RF from the host's natural antibody repertoire. Testing for RF is primarily used for the diagnosis of RA. However, RF may also be present in a number of inflammatory diseases characterized by chronic antigen exposure (e.g., infections and others AD) (128–131) (Table 4). Normal human lymphoid tissue commonly possesses B lymphocytes with RF expression on the cell surface. Nevertheless, RF is not routinely detectable in the circulation in the absence of an antigenic stimulus. Modified IgG could be a stimulus to RF production and could be an important component of RA pathogenesis; this concept is supported by studies that observed an association of RF and more severe RA with autoantibodies to advanced glycated end product damaged IgG or agalactosyl IgG (132,133). In fact, compared to those with seronegative RA, patients with polyarticular symmetrical arthritis who have a persistently positive test for RF are likely to have more erosions of bones and joints, more EAM, and worse function (134). The production of RF results in part from the help provided from a specific subset of T cells to RF precursor B cells. Since T cells reactive with autologous IgG have not been identified in patients with RA, it is likely that these T cells react with antigen(s), and then bind to specific B lymphocytes, which proliferate. Co-stimulation of B cells, perhaps mediated by TLRs, may allow B cells with low affinity receptors for IgG to become activated (134). Another factor that amplifies the inflammatory potential of RF is the propensity for IgG RF to self-associate into large lattice-like complexes. These complexes can be found in all tissues of the rheumatoid joint, and may help concentrate additional material within this structure (e.g., superficial layers of articular cartilage) (135).

A high correlation for RF has been noted among identical twins with RA, suggesting that genetic factors influence both RF function and disease development. However, some

OTHER PATHOLOGY	FREQUENCY (%)
ADs	
PM/DM	5-10
SLE	15-35
Types II and III mixed cryoglobulinemia	40-100
Primary biliary cirrhosis	45-70
Non-differentiate autoimmune disease	50-60
SS	75-95
Infection	
Leprosy	5-58
Syphilis	5-13
Tuberculosis	8-10
Other viral infection	15-65
Hepatitis B or Hepatitis C	20-75
Parasitic diseases	20-90
Bacterial endocarditis	25-50
Pulmonary disease	
Sarcoidosis	3-33
Interstitial pulmonary fibrosis	10-50
Asbestosis	25-30
Silicosis	30-50
Miscellaneous diseases	
Malignancy	5-25
After multiple immunizations	10-15

Table 4. Presence of rheumatoid factor. *ADs*: autoimmune diseases; *PM/DM*: polymyositis/dermatomyositis; *SLE*: systemic lupus erythematosus; *SS*: Sjögren´s syndrome.

studies have shown that patients with RF-negative RA have HLA susceptibility alleles similar to those in RF-positive patients. Furthermore, there may be a similar immunogenetic predisposition to RA in these patients that is independent of RF (131). The reported incidence may be higher in older subjects without rheumatic disease, ranging from 3-25%. Part of this wide range may be explained by a higher incidence of RF among chronically ill older adults as compared with healthy older patients (5-25%) (136,137). Population-based studies have shown that some healthy people with a positive RF develop RA over time, especially if more than one isotype is persistently elevated and if the level of RF is high (138). In fact, Nielsen *et al*. (139), demonstrated that individuals in the general population with elevated RF have up to 26-fold greater long term risk of RA, and and up to 32% 10 year absolute risk of RA.

Estimates of the sensitivity and specificity of RF vary depending upon the populations being examined, and this will also affect the calculated predictive value (130,131) (Table 5). This difference may reflect classification criteria that led published series of patients with RA to be biased toward more severe (and more seropositive) disease, thereby overestimating the sensitivity of RF in RA. The specificity depends substantially upon the choice of the control group (130,137,140). The specificity with respect to disease control populations is substantially lower, especially if the disease control populations include patients with rheumatic and other diseases associated with RF (131) (Table5).

As with any diagnostic test, the predictive value is also affected by the estimated LR of disease prior to ordering the test and by the proportion of patients with a non-rheumatic disorder associated with RF production. The positive predictive value of RF was 24% for RA and 34% for any rheumatic disease. The calculated negative predictive value of the RF tends to be high in a population with a low pre-test probability of RA. However, a negative RF in this setting may not be particularly useful clinically. The negative predictive values for RA and for any rheumatic disease were 85% and 89% respectively (141). The titer of RF should be considered when analyzing its utility. The higher the titer, the greater is the LR that the patient has a rheumatic disease. There are, however, frequent exceptions to this rule, as noted above. Some have suggested that erosive disease may be accurately predicted by analyzing the combination of *HLA-DRB1* and RF status among patients with RA (47). However, these tests are of limited value in an individual patient as almost one-half of "high risk" patients had no erosions at one year.

The presence of RF can be detected by a variety of techniques. These include agglutination of IgG-sensitized sheep red cells or of bentonite or latex particles coated with human IgG, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and nephelometry (142,143). Measurement of RF is not standardized in many laboratories (leading to problems with inconsistent results). Although no one technique has clear advantage over others, automated methods such as nephelometry and ELISA, tend to be more reproducible than manual methods.

Anti-citrullinated peptide/protein antibodies (ACPA). These include anti-cyclic citrullinated peptides (ant-CCP) which are present in both RF positive and negative RA (144). ACPAs are present in the earliest stages of disease in almost 70% of patients (145). The optimal clinical use of ACPA testing and its relationship to RF testing remain uncertain (146,147). Although ACPA and RF have similar sensitivity for the diagnosis of RA, ACPA is a more specific marker (148) (Table 5). Of note, the 2010 revised classification criteria for RA include both RF and ACPA (149). The immunoreactivity of citrullinated fibrin with IgA and IgM in the RA synovium, and the colocalization of PADI and citrullinated peptides, support the notion that arginine residues on fibrin and fibrinogen may be citrullinated and become potential autoantigens of RA (147,150–152). Intracellular citrullinated proteins colocalized with the deimidase in most of RA synovial samples (153).

The RA-associated *HLA-DRB1*0404* allele is also associated with production of antibodies to citrullinated fibrinogen, and T cell proliferation in response to fibrinogen peptides is frequent in RA patients but rare in controls (154). In contrast, in another study the SE was associated with antibodies to a citrullinated peptide derived from vimentin but not to a fibrinogen-derived citrullinated peptide (155,156). Comparisons of the SE frequencies on *HLA-DRB1* alleles in healthy populations with RA patients who do or do not harbor ACPA have shown that the SE is associated only with ACPA-positive disease and not with ACPA-negative disease. This indicates that the *HLA-DRB1* alleles encoding the SE do not associate with RA as such, but rather with a particular phenotype, disease with ACPA (157).

A strong association between cigarette smoking, a known risk factor for RA, and the presence *HLA-DBR1*0404*, as mentioned above exists (158,159). In case-control study, Klareskog *et al*. (158) demonstrated that the RR of developing RA was increased 20-fold in those who had two alleles for the SE, had ever smoked cigarettes, and were anti-CCP.

Gene-environment interaction (e.g., smoking), and its role in the citrullination process (158,159) (Figure 2). The lack of an association between smoking and risk of RA in those who are ACPA-negative, suggests that these disease subsets (i.e., ACPA-positive vs. ACPA-negative) differ in their pathogenesis. However, a large collaborative study that included 2,476 Caucasian RA patients from North America confirmed a strong association between the presence of ACPA and the SE, but found only a weak association between ACPA formation and smoking (160). On the other hand, Verpoort *et al*. (161) in a study of 216 patients demonstrated a strong association between ACPA and tobacco exposure, irrespective of the presence of the SE. A second system of antibodies against proteins modified by carbamylation rather than citrullination has been described in ACPA-negative patients with RA (162).

Anti-mutated citrullinated vimentin (MCV) antibodies. Anti MCV recognize a naturally occurring isoform of citrullinated vimentin, which can be found in patients with RA and in which arginine residues are replaced by glycine (163,164). Vimentin is a widely expressed intermediate filament. It becomes citrullinated through deamination, which occurs in MΦ undergoing apoptosis. The diagnostic and prognostic value of anti-MCV antibodies in RA was analyzed in a 2010 systematic review of 14 studies, most of which used a commercially available assay. Findings disclosed a similar performance than anti-CCP antibody testing (165). A subsequent report evaluated a longitudinal cohort of 238 patients with RA over 10 years and found that anti-MCV predicted joint damage damage similar to anti-CCP (166). The OR for radiographic progression was increased in the presence of either anti-MCV or anti-CCP. Similar observations were previously made in this cohort with anti-CCP antibodies. In patients with undiagnosed early inflammatory arthritis or established RA, the diagnostic and prognostic value of adding anti-MCV antibody testing to anti-CCP and RF testing, or substituting anti-MCV for other tests, remains uncertain (167).

Other autoantibodies such as anti-galactose IgGs (168,169), and antibodies to glucose-6-phosphateisomerase may be associated with more active disease and correlate with EAMs (170–172)

NEOANGIOGENESIS

One of the earliest histopathologic responses in RA is the generation of new synovial blood vessels, which covers the increased metabolic demands of the proliferating cells that form the pannus (173). This event is accompanied by the transudation of fluid and the transmigration of both lymphocytes into the synovium and of neutrophils into the synovial fluid. In the mature RA synovium, the mass of tissue is too much for even the multiple new capillaries to nourish, and local tissue ischemia is the result (106) (Figure 3). Relative synovial hypoxia is associated with an increased production of hypoxia-inducible factor-1 (HIF-1) that activates transcription of genes that are of fundamental importance for angiogenesis (e.g., VEGF) (174). Without new blood vessels, there would be no scaffold upon which synovitis could grow. RA can therefore be considered an "angiogenesis-dependent disease" (175). RA patients show an imbalance between pro-angiogenic factors and antiangiogenic factors in favor of the first.

AUTOANTIBODY	SENSITIVITY	SPECIFICITY
RF	26-90%	85-90%
ACPA	55-80%	90-98%
MCV	64-84%	79-96%

Table 5. Sensitivity and Specificity of autoantibodies. *ACPA*: anti-citrullinated protein antibodies; *MCV*: muted citrullinated vimentin. *RF*: rheumatoid factor.

Cell migration. As the new vessels develop, cytokines produced in the synovium in response to the driving force of TNF (including IL-1, IL-6, IFN-γ, and substance P) activate endothelial cells to produce adhesion molecules such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1 (VCAM-1), P-selectin, and E-selectin (176). A positive correlation can be made between the expression of adhesion molecules on endothelial cells and the appearance of these cells on histologic sections. Both vascular proliferation and high endothelial venules are found in rheumatoid joints with proliferating, invasive synovitis, but not in clinically uninvolved joints. Although much attention has been focused on the presence of lymphocytes in the synovium, similar lymphoid accumulation also occurs in the juxta-articular bone marrow (106). Targeting chemokines has been attempted in RA, including anti-chemokine antibodies or chemokine receptor antagonists, but few have been successful. This is most likely due to the redundant nature of the chemokine system, which makes it difficult to block cell recruitment. In one phase II trial, an anti-CXCL10 antibody demonstrated efficacy when used in combination with methotrexate (MTX) (177).

THE PANNUS

The normal synovial membrane consists of two main layers: the inner layer or coating, formed by two cell types of distinct lineages, MΦ or type A synoviocytes, fibroblasts or type B synoviocytes, and subintima layer (1). A feature of RA is the enormous growth of the synovial membrane, which becomes hyperplastic by accumulation of FLS in the inner layer (178). These cells confer to the synovial membrane of RA patients an aggressive behavior at the transition pannus/cartilage by their excessive production of pro-inflammatory cytokines, MMPs and angiogenesis promoting substances. Synovial hyperplasia could also reflect increased influx of mesenchymal cells (179), thus when FLSs are cultured on articular cartilage, MMP-13 (i.e., collagenase-3) is produced by the cells; this may be the mechanism by which the rheumatoid synovial pannus is attracted to and begins invading cartilage at the periphery of inflamed joints (180). The subintimal layer contains the inflammatory aggregates arranged in diffuse form, creating granulomas in some parts or organized in germinal centers in others and gives the rheumatoid synovial the appearance of a secondary lymphoid organ. This layer is the seat of chronic inflammatory component, responsible for intimal hyperplasia due to the effect of cytokine-enriched environment on synoviocytes (106,119). No one knows what causes this phenotypic transformation of synovial fibroblasts; some studies suggest mutations of the antiproliferative protein p53 (178). In this front it is concentrated an increased number of substances with lytic ability of the components of the cartilage extracellular matrix, predominantly MMPs (119).

STRUCTURAL DAMAGE: EROSIVE DISEASE

Cartilage degradation: Articular cartilage is composed of a non-mineralized surface layer and a deep mineralized layer adjacent to bone. Only the resorption of the mineralized layer is osteoclast mediated. Both layers contain chondrocytes, which determine cartilage metabolism. A hyperplastic synovium is the major contributor to cartilage damage in RA. Loss of the normally protective effects of synovium alter, the protein-binding characteristics of the cartilage surface, promoting FLS adhesion and invasion (115,181). The chondrocytes themselves synthesize cytokines or respond to local cytokine release, particularly IL1-B, 17, 18 and TNF-a; this accelerates the switch from an anabolic matriz-synthetizing state to a catabolic state that is characterized by the formation of MMPs and ADAMTS, which cleave collagen fibers and the cartilage component proteoglycan, respectively, and thus, further diminish cartilage integrity.

Moreover, matrix-degrading enzymes are also released by FLSs, PMNs, and mast cells, which are closely located to articular cartilage (106).

Bone erosions: Bone erosion occurs rapidly, affecting 80% of patients within 1-2 year after diagnosis and is associated with prolonged, increased inflammation (182). Normal physiological processes ensure a balance between bone formation and bone resorption to maintain skeletal homeostasis. This balance is perturbed in RA for bone resorption. Bone resorption depends on osteoclasts (183). In RA, osteoclasts at the interface between synovial tissue and articular bone induce bone resorption, which in turn, permits invasion by cells of the synovial membrane and results in pannus formation, as mentioned above (184). This process depends on the influx of osteoclast precursors into inflamed synovial tissue and the differentiation of these cells into mature os-

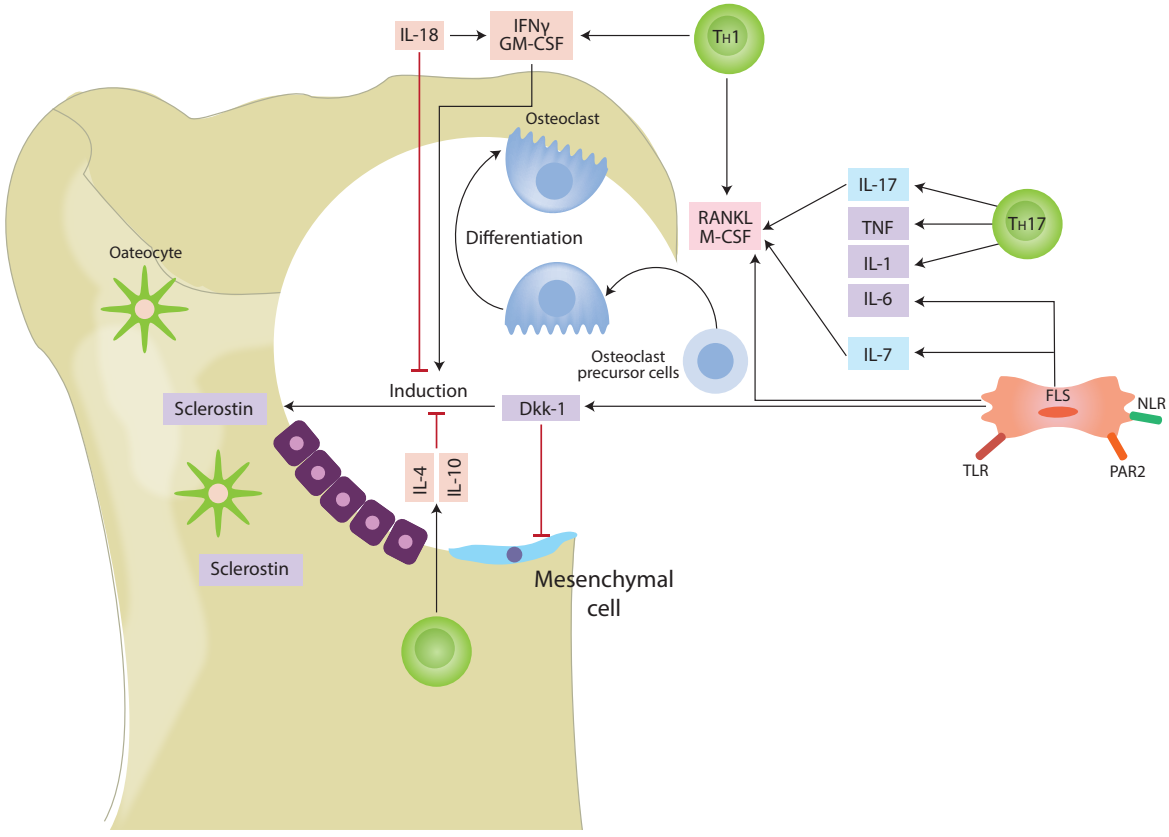

Figure 4. Mechanism of erosive disease. Inflammation within synovial tissue induces osteoclastogenesis through increased expression of RANKL, and M-CSF, the essential cytokine mediators of erosive RA, which are expressed by synovial fibroblasts, Th1 and Th17 cells. Osteoclast differentiation is achieved by the actions of proinflammatory cytokines such as, TNF, IL-1, as well as of IL-17, and IL-7. By contrast, IL-4, IL-10, GM-CSF and IFN-γ, inhibit osteoclast differentiation. In addition, expression of Dkk-1by synovial fibroblasts leads to inhibition of osteoblast differentiation and consequently of bone formation. *Dkk-1*: dickkopf-related protein 1; IL: interleukin; *FLS*: fibroblast-like synoviocytes; *IFN-g*: interferon-g; *M-CSF*: macrophage colony-stimulating factor; *NLR*: nucleotide-binding oligomerization domain–like receptor; *PAR2*: protease-activated receptor 2; *RANKL*: receptor activator of nuclear factor-k β; *Th*: T helper; *Th17*: Type 17 helper T cell; *TLR*: toll-like receptor; *TNF*: tumor-necrosis factor.

teoclasts. Their metabolic activation to resorb bone requires complex cellular interactions between cells of the osteoclast lineage with mesenchymal cells and lymphocytes. These interactions are controlled by cytokines (Figure 4): M-CSF and RANKL [receptor activator of nuclear factor-κβ (RANK) ligand] wich are essential for the differentiation of osteoclasts from their precursor cells, promote invasion of the periosteal surface adjacent to articular cartilage, and a lack of either molecule is sufficient to block osteoclast formation completely (119,185). RANKL, a member of the TNF superfamily, is expressed by mesenchymal cells, and activated synovial T cells (184). RANKL expression is regulated by inflammatory cytokines such as TNF, IL-1β, 6, and 17, but is also influenced by non-cytokine inflammatory mediators (e.g., PGE2) (186). The interaction of RANKL with its receptor RANK is modulated by osteoprotegerin (OPG), a soluble decoy receptor, which is expressed by mesenchymal cells in the RA synovium (187). In RA, an imbalance between OPG and RANKL expression promotes RANKL-induced bone loss (188). Osteoclasts have the acidic enzymatic machinery necessary to destroy mineralized tissues, including mineralized cartilage and subchondral bone; destruction of these tissues leads to deep resorption pits, which are filled by inflammatory tissue. Mechanical factors predispose particular sites to erosion. Thus, mechanically vulnerable sites such as the second and third metacarpals are prone to erosive changes (189). Breach of cortical bone permits synovial access to the bone marrow, which causes inflammation of the bone marrow [osteitis as observed on magnetic resonance imaging (MRI)], in which T cell and B cell aggregates gradually replace marrow fat (190). Eroded periarticular bone shows little evidence of repair in RA, unlike bone in other inflammatory arthropathies.

The total process, molecules, and mechanism of the pathogenesis and erosive RA are shown in the Figres 3 and 4, and Table 3 (191-195).

CLINICAL FEATURES

RA not only compromises the joints, but it also involves other organs and has an adverse impact on the biopsychosocial sphere. Classically, the clinical manifestations are divided into articular and systemic manifestations (i.e., EAMs). Patients commonly present with pain and stiffness in multiple joints, although one third of patients initially experience symptoms at just one location or a few scattered sites. In most patients, symptoms emerge over weeks to months, starting with one joint and often accompanied by prodromal symptoms of anorexia, weakness, or fatigue. Weakness is commonly out of proportion to pain on examination. Low-grade fever, fatigue, malaise, and other systemic complaints may arise, especially in an acute presentation. In approximately 15% of patients, onset occurs more rapidly over days to weeks. In 8-15% of patients, symptoms begin within a few days of a specific inciting event such as an infectious illness (22).

CLASSIFICATION CRITERIA

Since 1958 different criteria for classification of RA patients have been established, and have been revised over time, the criteria established by the American College of Rheuma-

CRITERIA	DEFINITION	LR+	LR-	PERCENTAGE WITH RA IF CRITERIA IS	
				PRESENT	ABSENT
Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement.	1.9	0.5	39	14
Arthritis of 3 or more joint areas	Three or more of the following joints noted to befluid-filled or have soft tissue swelling (not bony overgrowth): wrist, PIP, MCP, elbow, knee, ankle, MTP, observed by a physician presenting simultaneously forat least 6 weeks.	1.4	0.5	32	13
Hand joint involvement	Wrist, MCP, or PIP joints among the symptomatic joints observed for at least 6 weeks.	1.5	0.4	33	12
Symmetric arthritis	Right and left joints involved for one or more of following: wrist, PIP, MCP, elbow, knee, ankle, MTP.	1.2	0.6	29	17
Rheumatoid nodules	Subcutaneous nodules over bony prominences, extensor surfaces, or in justa-articular regions, observed by a physician.	3.0	0.98	50	25
Rheumatoid factor	Detected by a method that is positive in fewer than 5% of normal controls	8.4	0.4	74	13
Radiographic changes	Typical of RA on posteroanterior hand and wrist radiographs; they must include erosions or unequivocalbony decalcification localized in or most markedadjacent to the involved joints (OA changes alone donot qualify)	11	0.8	79	21

Table 6. The 1987 ACR Criteria. At least four criteria must be fulfilled for classification of RA; patients with two clinical diagnoses are not excluded. *ACR*: American College of Rheumatology; *LR+*: positive likelihood ratio; *LR–*: negative likelihood ratio; *PIP*: proximal interphalangeal; *MCP*: metacarpophalangeal; *MTP*: metatarsophalangeal; *OA*: osteoarthritis. Modified of Rindfleisch and Muller (22).

Target population (Who should be tested?): Patients who 1) Have at least 1 joint with definite clinical synovitis (swelling) ± 2) With the synovitis not better explained by another disease† Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of 6/10 is needed for classification of a patient as having definite RA)‡	Score
A. Joint involvement§	
1 large joint*	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)**	2
4-10 small joints (with or without involvement of large joints)	3
> 10 joints (at least 1 small joints)#	5
B. Serology (at least 1 test result is needed for classification)††	
Negative RF and negative ACPA	0
Low-positive RF or low-positivity ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants (at least test result is needed for classification) ‡‡	
Normal CRP and normal ESR	0
Abnormal CRP or normal ESR	1
D. Duration of symptoms §§	
< 6 weeks	0
≥ 6 Weeks	1

Table 7. The 2010 ACR/EULAR classification criteria for RA.

±The criteria are aimed at classification of newly presenting patients. In addition, patients with erosivedisease typical of rheumatoid arthritis (RA) with a history compatible with prior fulfillment of the 2010criteria should be classified as having RA. Patients with longstanding disease, including those whose-disease is inactive (with or without treatment) who, based on retrospectively available data, havepreviously fulfilled the 2010 criteria should be classified as having RA.

† Differential diagnoses vary among patients with different presentations, but may include conditions such as systemic lupus erythematosus, psoriatic arthritis, and gout. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted.

‡ Although patients with a score of <6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time.

§ Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are excluded from assessment. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based on the pattern of joint involvement.

* Large joints refers to shoulders, elbows, hips, knees, and ankles.

** Small joints refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.

In this category, at least 1 of the involved joints must be a small joint; the other joints can include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (i.e.,temporomandibular, acromioclavicular, sternoclavicular, etc.).

†† Negative refers to IU values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay. Where RF information is only available as positive or negative, a positive result should be scored as low-positive for RF.

‡‡ Normal/abnormal is determined by local laboratory standards.

§§ Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis(i.e., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardlessof treatment status.

ACPA: anti-citrullinated protein antibodies; *ACR*: American College of Rheumatology; *CRP*: C-reactive protein; *ESR*: erythrocyte sedimentation rate; *EULAR*: European League Against Rheumatism; *RA*: rheumatoid arthritis; *RF*: rheumatoid factor. Adapted from Aletaha *et al*. (149).

tology (ACR) in 1987 are the most used (196). These criteria were developed from a cohort of subjects with long-standing RA. Validation studies conducted in the outpatient clinics confirmed that the criteria were an accurate method of classifying RA with sensitivity in the range of 77-95% and specificity in the range of 85-98%. Because of the nature of the cohort for which the criteria were developed, the criteria performs best at distinguishing subjects with long duration and active RA from those with other arthritis (197). Classification criteria generally allow categorization of patients in those groups with and without AR, providing a basis for a common approach to the definition of the disease and thus allow comparison across different studies (2) (Table 6).

Recent studies have demonstrated that early aggressive treatment for RA can halt or slow the progression of synovitis and bone erosions, decreasing disease-related dis-

ability and increasing the rate of disease remission (198). However, the 1987-ACR criteria do not perform as well for early RA (i.e., arthritis symptoms ranging from 4 weeks to 2 years) (199) compared with long-duration RA. Criteria such as joint erosions and rheumatoid nodules are often absent early in the disease, thus decreasing the sensitivity of the classification criteria. For these reasons, in the 2010, the collaborative work between the ACR and the European League Against Rheumatism (EULAR), has established a new classification criteria for RA (149). These new criteria have not been developed to be considered as a reference by the general practitioner, but as a tool to facilitate the study of patients in early stages of the disease. The goals set for the development of these new criteria were: to identify subjects at high risk of chronic disease and erosive joint damage, and use them as a basis for initiating disease modifying therapy, and not exclude the inclusion of patients who are in late stages of the disease (200). Validation of these criteria in three of the most important global cohorts showed that they were adjusted between the 87-97% of patients in which MTX was chosen as initial treatment (201). In accordance with the above, Radner *et al*. (200) in a systematic literature review found pooled sensitivity and specificity of 82% and 61%, respectively for these new criteria. The authors of these criteria emphasize that physicians should report whether there is a significant proportion of patients who do not meet the new criteria, but in whom there is a reason to be treated with disease modifying therapy or who in the following, without a change of their classification, developed erosive disease (201). In Table 7, the new criteria, their scores and notes for taking into account to do diagnosis are listed.

The diagnosis of RA may also be made in patients without all the criteria described above. Examples include the following: seronegative RA (38,202), recent onset RA (71,203), and inactive RA (204).

DIFFERENTIAL DIAGNOSIS

A number of disorders can closely resemble RA and must be ruled-out when the diagnosis of RA is being made. Infection-related reactive arthropathies, seronegative spondyloarthropathies, and other ADs such as SLE and SS, may have symptoms in common with RA, as may an array of endocrine and other disorders (Table 8).

QUANTITATIVE DIAGNOSIS AND PREDICTION

The art of RA diagnosis is based upon hypotheses generated with the information obtained from the clinical history and physical examination. These assumptions can be strengthened by an objective and percentage calculation of the probabilities of accurate diagnosis before and after performing a maneuver or diagnostic test –pre and post probabilities test (205). The probability that an individual will develop RA is equivalent to the prevalence of disease in the general population (206). This probability varies according to epidemiological data and characteristics of each individual as well as the symptoms, clinical signs, and diagnostic test results that have been requested for the patient.

DIAGNOSIS	COMMENTS
ADs	SSc, SS, SLE
Fibromyalgia	Evaluate for trigger points and social context
Hemochromatosis	Iron studies and skin coloration changes may guide diagnosis
Infectious endocarditis	Rule out murmurs, high fever, and history of intravenous drug use
Polyarticular gout	Joints often erythematous; podagra commonly found; gout and RA rarely coexist, but calcium pyrophosphate deposition disease can accompany RA
Polymyalgia rheumatica	RA, unlike polymyalgia rheumatica, rarely presents with pain in the proximal joints of the extremities only
RS3PE	Resembles seronegative RA in the elderly
Sarcoidosis	Granulomas likely, as are hypercalcemia and chest film findings
Seronegative spondyloarthropathies, reactive arthritis	Tend to be more asymmetric than RA. More commonly involve the joints of the spine. Evaluate for history of psoriasis, Reiter's comorbidities, inflammatory bowel disease.Reactive arthritis can be postinfective, sexually acquired, or related to gastrointestinal disorders
Thyroid disease	Consider thyroid-stimulating hormone level depending on symptoms
Viral Arthritis	Consider parvovirus, hepatitis B and C, rubella infection or immunization
Multicentric reticulohistiocytosis	Systemic proliferation of multinucleated lipid-laden histiocytes causes swelling in the small joints of the hands and a characteristic erosive pattern on radiographs. A typical "string of pearls" may be seen around the cuticle in some patients

Table 8. Differential diagnosis of RA. *ADs*: autoimmune diseases; *RA*: rheumatoid arthritis; *SLE*: systemic lupus erythematosus; *SS*: Sjögren´s syndrome; *SSc*: sclerodermia; *RS3PE*: remitting seronegative symmetric synovitis with pitting edema.

These characteristics have sensitivity and specificity, but by the ratio of these two can be calculated the LR, which increases or reduces the possibility of more accurately diagnosing disease (201).

Prediction models have been developed from prospective observational studies of treated patients with early RA (ERA). These models are designed to forecast outcomes in individuals with early arthritis who do not currently meet the 1987 criteria. Several factors can establish whether patients are likely to develop RA. However, in patients with ERA, the joint manifestations are often difficult to distinguish from other forms of inflammatory polyarthritis. The more distinctive signs of RA such as joint erosions, rheumatoid nodules, and other EAMs are seen primarily in patients with longstanding, poorly-controlled disease but are frequently absent on initial presentation (199). Nevertheless, ERA has not been full defined. Some authors have named it as one of less than a year of development and in some studies defining very early arthritis, as one of less than 3 months (134,207). In Table 9 a score applied to 3 independent cohorts of patients with undifferentiated arthritis is described and has an excellent discriminative ability for assessing the LR of progression to RA. The cut off point for RA is 8 points or more (208).

VARIABLE	SCORE
1. Age (multiply by 0·02)	
2. Sex (If is female)	1 point
3. Distribution of involved joints	
• Small joints hands and feet	0.5 point
• Symmetrical	0.5 point
• Upper limbs	1 point
• Upper and lower limbs	1.5 points
4. Morning stiffness (visual analogue scale)	
• 26–90 mm	1 point
• >90 mm	2 points
5. Number of tender joints	
• Four to ten	0.5 point
• 11 or more	1 point
6. Number of swollen joints	
• Four to ten	0.5 point
• 11 or more	1 point
7. C–reactive protein (mg/L)	
• Five to 50	0.5 points
• 51 or more	1.5 points
8. Rheumatoid factor positive	1 point
9. ACPA positive	2 points

Table 9. Format used to calculate the patient's prediction score. *ACPA*: anti-citrullinated protein antibodies; *L*: liters; *mg*: milligrams; *mm*: millimeters. Adapted from van der Helm-van Mil *et al.* (208).

ARTICULAR MANIFESTATIONS

The clinical evaluation of RA patients should consider the full joint study, including the systemic exhaustive evaluation given the condition of the disease with the possibility of compromise other than the joints. For this, it is necessary to perform a detailed medical history that extends from the presentation of the disease to the evaluation of the patient's functional class.

RA is a symmetric and inflammatory disease (arthralgia with morning stiffness, sagging throughout the day). The illness may begin with a monoarticular engagement in 35% of cases, oligoarticularin 25% of cases and polyarticular in 40% of cases (205). The main joint involvement is given at the hands and feet. However, there may be a joint compromise of medium and large size as elbows, ankles, shoulders, knees, and hips. Persistent inflammation leads to destruction of cartilage and bone tissue with the consequent of misalignment and joint deformity (201).

The involvement of RA in hands occurs at different levels. It frequently causes disruption of the MCP joints, leading to the destruction of soft tissue tendon favoring the imbalance and weakness of the active and passive forces with subsequent instability, pain, and deformity. The two most frequently associated deformities with MCP level are volar subluxation and ulnar deviation. The condylar structure of the MCF, which allows movement on two planes, makes them more volatile than the PIP, being then more noticeable the deformities at that level (Figure 5)

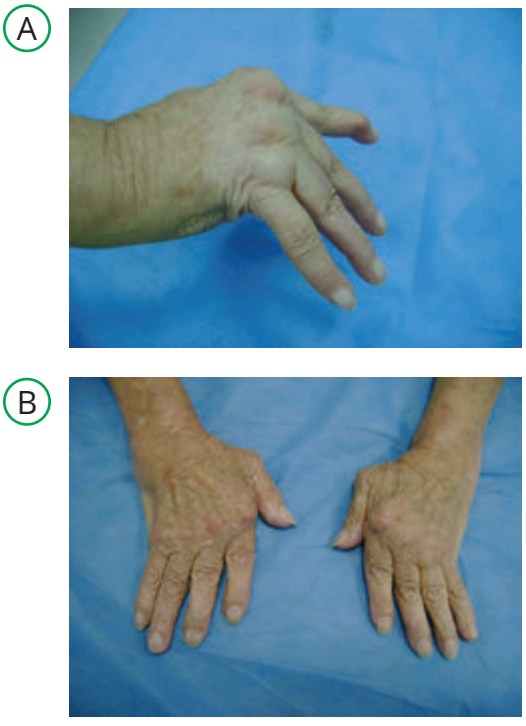

Figure 5. Metacarpophalangeal deformities. Ulnar deviation. *A*: lateral view; *B*: frontal view. Adapted from Rojas-Villarraga *et al.* (201).

In initial stages, the MCP capsule and ligamentous structures are expanded by synovial proliferation, leading to collateral ligament loss and decreased motility ligament with decreased stability of the joint. Normally, in the flexed position of the MCP there is a minimal lateral movement but with the increase of laxity of collateral ligament deviations of up to 45° occur. The volar subluxation is also caused by the weakness of the collateral ligaments and the dorsal extensor mechanism, especially when the extenders are displaced between any two heads of the metacarpals. As a result, there are no forces that counteract the flexor and then a bending in the contraction of MCF occurs with an evident prominence of the metacarpal heads (201).

In the early stages of RA, fusiform fingers occur by the presence of synovitis and joint effusion at the PIP. Among the most frequently reported deformities, in advanced stages of RA at finger level, is the **boutonnière** deformity and the swan-neck finger. These deformities are reversible in the early stages, but not in the advanced stages of the disease (Figure 6). The compromise of DIPs in RA is rare, and when it is present, it is the result of deformities and biomechanical forces given by the alterations of the PIP. At the level of the first finger, the feature deformity of an advanced stage is the finger at "Z" (Figure 7).

The wrist may present a severe involvement and destruction as a result of chronic synovitis preceded by pain, decreased functionality, and impact level of the fingers, by compromising the MCF and PIP. Alteration and destruction of tendons, which stabilize carpal at that level, manifest the ulnar commitment. The distal radioulnar joint can erode and parallel compromise to the dorsal capsule. A distal ulnar dorsal subluxation, subluxation of extensor carpi ulnaris tendon, and supination of proximal carpal bones can occur. In the radial portion scaphoid subluxation, ligamentous instability, ulnar translocation of the carpal bones and collapse with a height decreased of the carpal bones may occur (Figure 8). Clinically, the disease presents radial deviation of the hand with supinated position. The imbalance generates ulnar deviation of the metacarpals and phalanges (197,201,205).

The compromise on elbow is manifested by pain and limited range of motion, initially extension and then flexion. The ulna-trochlear joint often gets affected at the initiation and then undertakes the rotary motion of the forearm. The loss of bone consistency with or without stabilizer tendon destruction may result in instability symptoms. These alterations may lead to limitation of mechanical forces and mobility. The presence of effusion may be evident between the lateral olecranon and radial head, which can migrate to the capitelum-humeral in advanced stages of cartilage loss, thus limiting flexion and extension.

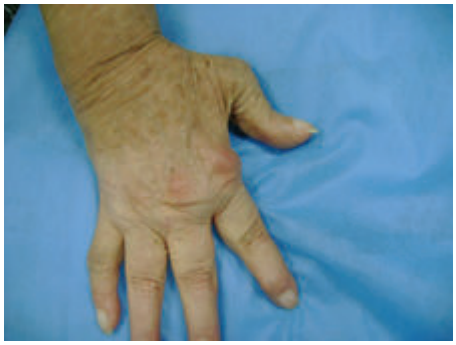

Figure7. Finger at "Z" from the first finger. Metacarpophalangeal subluxated and hyperextension of interphalangeal. Adapted from Rojas-Villarraga *et al*. (201).

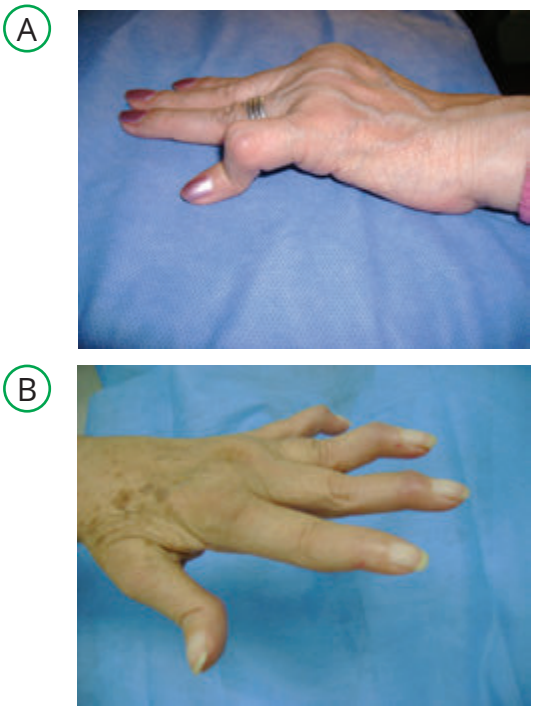

Figure 6. Boutonnière deformity and swan-neck finger. A: Boutonnière deformity: Flexion in the proximal interphalangeal and hyperextension in distal interphalangeal; B: Swan-neck finger: Hyperextensión of proximal interphalangeal and hyperflexion of distal interphalangeal. Adapted from Rojas-Villarraga *et al*. (201).

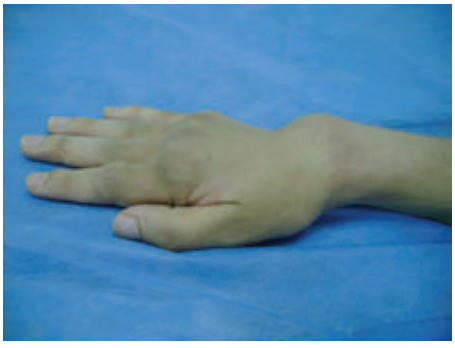

Figure 8. Wrist involvement. Lateral view of distal ulnar dorsal subluxation. Subluxation of extensor carpi ulnaris tendon and supination of proximal carpal bones with imbalance, generating ulnar deviation of metacarpals and phalangeas. Adapted from Rojas-Villarraga *et al*. (201).

The ulnar synovitis can generate ulnar compressive neuropathy. The presence of rheumatoid nodules is common in the ulnar extensor area of the elbow and in the olecranon area (Figure 9), region in which bursitis can also occur (201,209).

At shoulder level, the compromise is variable and often underestimated since in its early stages, it can be subtly evident from the clinical standpoint. This leads in many cases to late diagnosis. Up to 65% of RA patients mention symptomatology at shoulder level at some point in the disease. A hallmark of rheumatoid shoulder is that inflammation is difficult to be clinically recognized; it is evident in isolated cases of bursitis, rotator cuff injury, or severe arthritis (210). As a result of inflammatory involvement, an altered functional component can be found, resulting from the combination of pain, muscle weakness, and decreased range of motion. Chronic synovitis can lead to joint space narrowing (JSN), which predicts erosive involvement to the level that, over time ends in joint destruction. The degree of tendon compromise of the rotator cuff can be variable, even generating complete destruction with superior migration of the humeral head as a consequence. In Figure 10, a severe arthritis of the shoulder, a rare manifestation of RA, is presented (197,201,205).

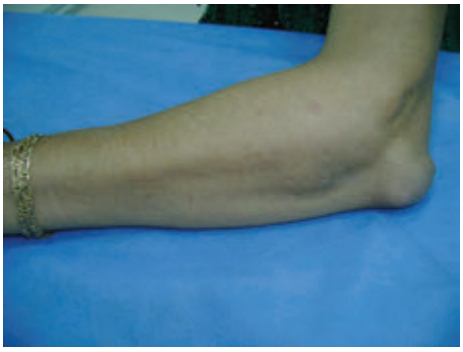

Figure 9. Rheumatoid nodules. Localized at level of elbow in the ulnar area. Adapted from Rojas-Villarraga *et al*. (201).

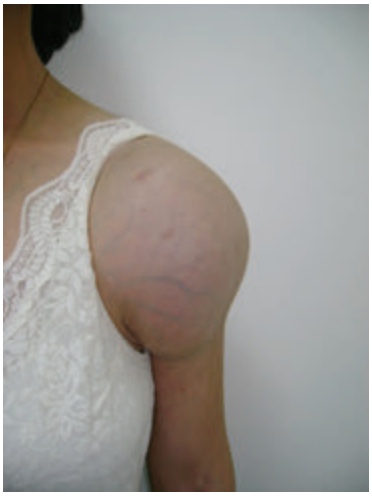

Figure 10. Shoulder compromise. Severe involvement of the shoulder as rare manifestation of the RA.

Between 5% and 15% of RA patients have hip compromise; this is in some cases a gradual and slow destructive process. In a few cases there is a rapid destruction of the femoral head with a severe inflammatory involvement. Generally the inflammatory process begins with synovitis and effusion, which causes a rearrangement of the femoral head by making it assume a position in flexion, external rotation, and abduction. Between the 15-20% of patients with hip compromise by RA develop acetabular protrusion, complicated with fracture of the acetabulum in some cases.

As in other joints, involvement of the knee has a broad spectrum. In the initial stages, the manifestations may range from secondary articular edema to synovial proliferation and effusion without evidence of cartilage damage. Usually the disease affects both kness symmetrically, but initial monoarticular presentation has been described. Compromise of flexo-extensor movements is concomitantly produced. The valgus deformity is common. Sometimes it is accompanied by flexion contracture; although less frequent, varus deformity may occur. This deformity can also accompany external rotation of the knee with an impact at the foot, leading to severe pronation. In some patients, the inflammatory exuberant compromise with synovial proliferation may persist for long periods, leading to chronic painful conditions. In advanced stages of the disease, there may be instability of the cruciate ligaments and collateral ligaments (201,205).

The foot is involved in 85-90% of patients and is the first manifestation in 15% of them (Figure 11). The rheumatoid foot involves primarily the forefoot. Symptoms begin with MTF joint involvement in 90% of the cases. Synovial chronic inflammation leads to distension of the joint capsule, collateral ligament compromise, and laxity of the plantar fascia leading to subluxation and dislocation of the metatarsal heads, which is a characteristic deformity in advanced stages of the disease. In the forefoot, there are also deformities such as hallux valgus, claw toe, hammertoe, mallet

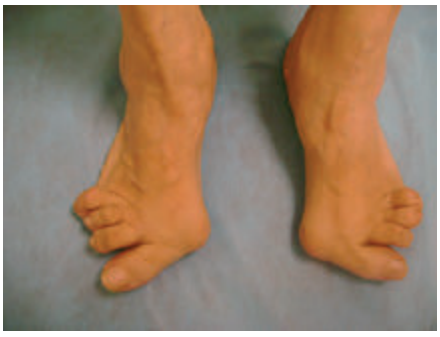

Figure 11. Severe rheumatoid foot. This involves the forefoot with deformity as hallux valgus, hammertoe and overriding the fourth on the fifth finger bilaterally. Adapted from Rojas-Villarraga *et al*. (201).

toe, and plantar hyperkeratosis areas that sometimes have perforations and are at risk of osteomyelitis. (197,205).

In studies of patients with ERA who have been evaluated for predicting clinical models forecasting the type of arthritis that the patient will follow, the positive lateral compression test of the MTP, i.e., squeeze test, with presence of pain has been found to be predictive of the development of persistent erosive arthritis. Therefore the performance of this simple clinic maneuver during physical examination is very informative (182,201) (Figure 12).

The RA also involved the hindfoot and ankle in about 30% to 60% of patients. The hindfoot valgus and flat feet are common. The inflammatory compromise of the subtalar-calcaneal-talar and tibiotalarjoints can also generate hindfoot valgus deformity. Talonavicular joints and calcaneo-cuboid can be seen to be involved in up to 25% of patients. The midfoot is not commonly affected. However, the first metatarso-cuneiform joint is frequently one of the most affected ones, causing instability (197,205).

Abnormalities at the foot are present in 82% of the patients, and the most common findings include hallux valgus (65%), flattening of the longitudinal arch (65%), claw toes, and hammer (39% and 25% respectively). A significant association between pain and physical examination of the forefoot is found, specifically in the subtalar and tibiotalar joints. Another positive association is the presence of the disease activity and disability, is measured through the HAQ and DAS28, respectively. In addition, the squeeze test was significantly associated with the risk of disability (9).

Other joints involved in RA are the temporomandibular, cricoarytenoid (i.e., causing hoarseness), and cervical. The latter may present with occipital pain and headache, neck pain, and, in advanced stages, movement limitation. The involvement of the atlanto-axial joint, given by laxity, presence of pannus and erosions, can lead to the development of subluxations in many directions; those changes could compromise the spinal canal, causing progressive quadriparesis and even endanger life. At the risk of compromise at that level, it is important to evaluate the atlo-axoideo space through radiography (i.e., no more than 3 mm between the atlas arch and odontoid process) and MRI (197).

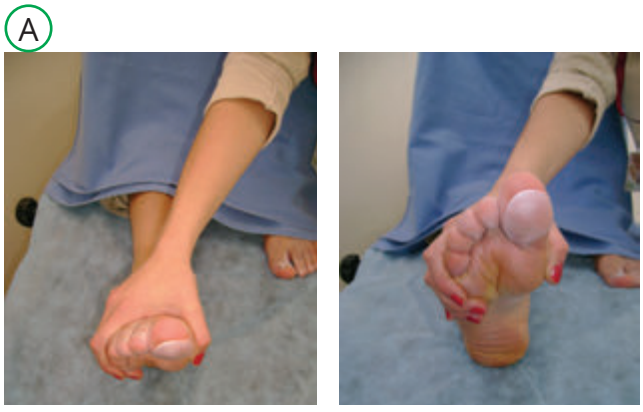

Figure 12. Squeeze test. A: dorsal view. B: plantar view. Adapted from Rojas-Villarraga *et al.* (201).

EXTRA-ARTICULAR MANIFESTATIONS (EAM)

A subgroup of patients with AR has been defined as extra-articular AR (EARA), which has been associated with poor prognosis and mortality increase. The presentation of the EAMs have been linked to genetic, clinical, immunological, and environmental factors (211,212). Regarding environmental factors, smoking has been reported as one of the most strongly associated (211,213,214). The EAM most frequently reported are the nodulosis, pleuritis, cutaneous vasculitis, even CVD (1,22,211,213,214) (Figure 13). About 15% of patients with RA during long-term follow-up develop EAM, corresponding to an estimated incidence of 1/100 person per year (215). In a recent study, carried out with 538 Colombian patients with RA, 32% had EAAR, specifically nodules and pulmonary involvement were present in 21% and 4% of patients, respectively. Patients with EAMs were older than those without it, presented longer disease duration, and higher titers of ACPA when compared with patients without EAM. Hypertension and thrombosis were significantly associated with EAM. The absence of smoking was a protective factor for developing these manifestations (214).

Cardiovascular. The main cause of mortality in patients with RA is CV events, which are the first cause of death in over 50% of patients and two times higher than the general population (214) (see Chapter 38).

Lung. The pulmonary compromise in RA is clinically infrequent; nevertheless, it has been detected in 5-63% of the patients (216). The most common types of described compromise are: interstitial lung disease (Figure 14), pleural effusion, rheumatoid nodules, and compromise of the airway. The pleural involvement is given by pleuritis (20%), pleural effusion, pleural thickening, and pneumothorax. Nodular pleural compromise has also been described (211,216). Other lung manifestations, less frequently presented, are: follicular bronchiolitis, diffuse alveolar damage, lymphoid interstitial pneumonitis, empyema, bronchopleural fistula, cricoarytenoid arthritis, bronchiolitis obliterans, follicular bronchiolitis, Caplan syndrome, vasculitis, pulmonary hemorrhage, pulmonary hypertension, respiratory muscle weakness, and amyloidosis (216).

Eye. Dry eye, as a manifestation of SS associated with RA, is one of the most common eye compromises (see Chapter 28). This polyautoinmunity occurs in up to 30% of patients with RA. Usually the SS that accompanies RA occurs after RA onset. However, in some adults, the SS may be the initial manifestation. The prevalence of SS in patients with RA varies according to the different populations studied and the criteria used for inclusion (217). We have such a condition (i.e., RA-SS) in 15% (210,218). Other causes of dry eye other than

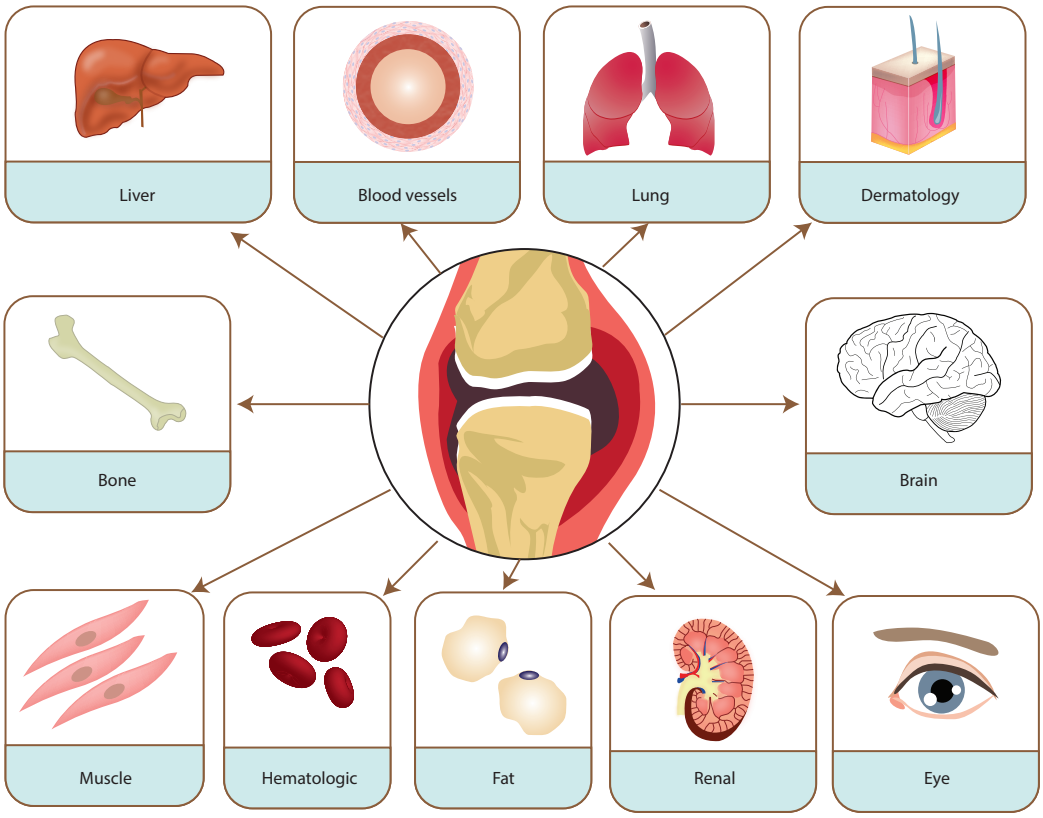

Figure 13. Rheumatoid arthritis as systemic disease. Systemic compromise may be observed in blood vessels (e.g., atherogenesis, myocardial infarctions), liver (e.g., elevated acute phase response and iron redistribution), lung (e.g., interstitial lung disease, pleuritis), eye (e.g., scleritis-episcleritis), skin (e.g., nodules), hematologic (e.g., anemia), kidney (e.g., amyloidosis-rare), bone (e.g., osteoporosis), fat tissue (e.g., release adipocytokine), brain (e.g., low stress tolerance, depression), and muscle (e.g., insulin resistance, weakness).

SS in RA patients, include age, use of anticholinergic medication, presence of diabetes, and hypoandrogenism. Clinical manifestations can range from ocular foreign body sensation, to filamentary keratitis, and corneal ulceration in severe cases (217,219). Another serious ocular manifestation in patients with RA is scleritis-episcleritis (211,217,219) (Figure 15). Other ocular complications in patients with RA are uveitis, retinal vasculitis, cataracts, retinal hemorrhage, and iridocyclitis (211,217,219).

Dermatology. Rheumatoid nodules (Figure 9) have been considered as classical components of the clinical manifestations of RA (149,196). Nodules have been reported worldwide in 24-34% of the cases (211,212) and recently were found in 29% of our cohortwith RA (5). Clinically, these are subcutaneous, more frequently on the extensor surfaces. Another dermatological manifestation present in RA is vasculitis, which can also occur in different organs. They have been described in 1-5% of patients and, in cases of systemic compromise, a high mortality up to 30% at five years, secondary to the disease or treatment complication has been reported (211,212). The dermal involvement can be skin ulceration, secondary to immobility, steroid or underlying vasculitis or neuropathy, digital arteritis

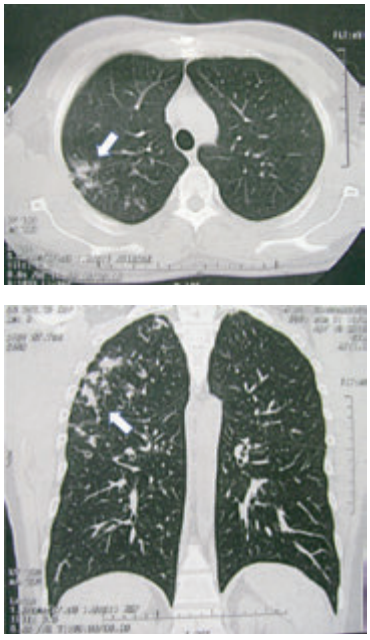

Figure 14. Interstitial lung disease seen on high resolution computed tomography. A: axial; B: coronal; Adapted from Rojas-Villarraga *et al.* (201).

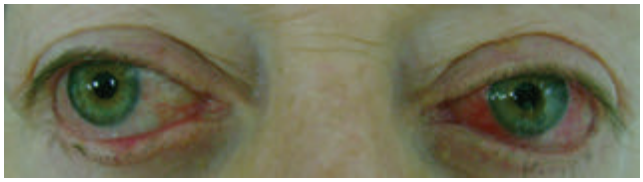

Figure 15. Scleritis-episcleritis. Scleral compromise, predominantly left eye. Adapted from Rojas-Villarraga *et al*. (201).

(e.g.,from splinter hemorrhages to gangrene), and palpable purpura, etc. (211) (Figure 16A and 16B). Other skin manifestations present in RA are white atrophy, livedo reticularis, palmar erythema, Raynaud's phenomenon, opportunistic skin infections, granulomatous neutrophilic dermatitis, psoriasiform lesions, and toxic manifestations associated with immunomodulatory treatment (220).

Hematologic. Anemia may be associated with RA, manifested as normocytic-normochromic. It must be differentiated from hypochromic microcytic anemia resulting from iron deficiency caused by multiple causes, including the use of anti-inflammatory drugs (NSAIDs), secondary gastrointestinal lossess and metrorrhagia (219). Another type of anemia present in RA is macrocytic, associated with folate deficiency by the MTX intake. Additionally, the mean corpuscular volume may be high as the result of deficiency of vitamin B12 secondary to concomitant polyautoimmunity with pernicious anemia or AITD. Autoimmune hemolytic anemia has also been as resulting from the use of some some disease-modifying antirheumatic drug (DMARD) or associated polyautoimmunity (201).

Felty's syndrome. It has been described as the triad of arthritis, splenomegaly, and neutropenia. The presence of the three components is extremely variable. This syndrome occurs most often in patients with RF, nodular arthritis, and *HLA-DRB1* SE (215). The main complication is the presence of infections associated with neutropenia in a third of the patients. Neutropenia can be occasionally found in patientes with DMARD or NSAIDs therapy. (212). Benign lymphadenopathy can be found in patients with active RA. In several retrospective studies it has been shown an increased presence of lymphoid malignancy -non Hodgkin's and Hodgkin-associated with RA, especially in up to one third of patients with Felty's syndrome. Other hematologic malignancies associated with RA are multiple myeloma and myeloid leukemia. The leuco-lymphopenia should lead to the study of polyautoimmunity with SLE or medicine reactions. Immune thrombocytopenia may be a manifestation associated with RA with a good response to steroid treatment. Thrombocytosis is usually a common manifestation of RA activity, although there have been reported thrombotic thrombocytopenic purpura cases (201,212,221).

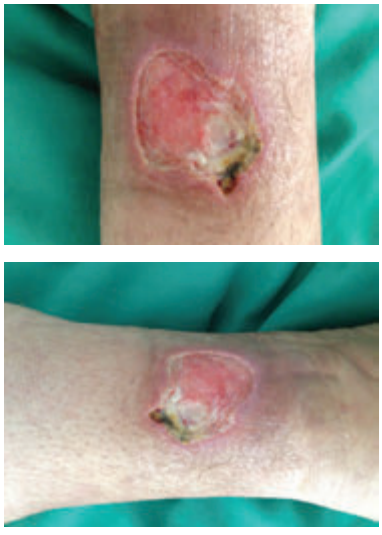

Figure 16. Skin compromise: Chronic ulcer on internal supramalleolar region.

Renal. The renal involvement in RA patients is rare. However, it may be the result of associated comorbidities. For instance, amyloidosis has been reported in up to 20% of patients with long-standing and severe RA. It is the most important cause of end-stage renal disease in some patient series (211). The use of several medicines may have a nephrotoxicity impact such as NSAIDs and DMARDs (e.g, gold salts, penicillamine, anti-TNF). The glomerular involvement in RA is rare and may be mesangial type, followed by minimal-change glomerulopathy and glomerulonephritis associated with ANCA (201,212).

Hepatic. Active RA may be associated with an increase in liver function abnormalities. With control of rheumatoid inflammation, the liver function abnormalities return to normal. Hepatomegaly might be observed (221). Serum alkaline phosphatase is increased in 18–46% of patients and gamma-glutamyl transaminase is raised in 23–77% of patients. Polyautoimmunity with autoimmune hepatitis should be considered in the presence of elevated liver enzymes. In this case hepatic compromise by medicines such as MTX should also be ruled out (218).

Nervous system. For various pathogenic mechanisms, the central and peripheral nervous system can be affected by RA and manifests in different ways, including entrapment neuropathies, (e.g., carpal tunnel, tarsal tunnel syndrome), which tend to occur when the nerve is compressed by the inflamed synovium against a fixed structure. Peripheral neuropathy, presenting as diffuse sensor/motor neuropathy or mononeuritis multiplex, occurs in a small subset of patients with RA. The underlying mechanism is small vessel vasculitis, which involves the vasa nervorum with ischemic neuropathy. Atlanto-axial subluxation caused by erosion of the odontoid process or the transverse ligament of C1 may allow the odontoid process to slip posteriorly and cause a cervical

myelopathy. Basilar invagination, with upward impingement of the odontoid process into the foramen magnum, can also result in cord compression. The presence of cord compression is indicated by a positive Babinski sign, hyperreflexia, and weakness. This complication require surgical stabilization (197,211,212).

Other extra articular events and comorbidities. The most common gastrointestinal disorders are related to the use of NSAIDs or steroids which produce gastric ulcer. The intestinal immune compromise associated with RA such as Crohn's disease and ulcerative colitis, has rarely been described (212,222). Depression, osteoporosis, muscle weakness, and infections are common complications or comorbidities (197,211,212,219,223,224).

POLYAUTOIMMUNITY AND FAMILIAL AUTOIMMUNITY

ADs share similar mechanisms. In clinical practice some conditions support these commonalities. One of these corresponds to polyautoimmunity, which is defined as the presence of more than one AD in a single patient (218,225). The importance of these terms is due to the fact that patients with polyautoimmunity may have a modified disease course and a modified clinical presentation (218,226). Several studies have consistently mentioned association and clustering between ADs (227). Recently, we have observed polyautoimmunity in up to 21% of RA patients (5, 218). Later, in a literature review, worldwide prevalence was reported between 0.5% in African population to 27% in Caucasian population (226). AITD was followed by SS, which was associated with RA in 11.8%. The factors associated with these conditions were female gender, CVD, and presence of ANAs. RA is more frequently associated with SLE, antiphospholipid syndrome, T1DM, scleroderma (SSc), biliary inflammatory disease, celiac disease, vitiligo, autoimmune hepatitis, myasthenia gravis, dermatomyositis, and pernicious anemia. (218,228).

FA is defined as the presence of any AD in first-degree relatives (FDRs) of the proband (225,228,229). Amaya-Amaya *et al*. (5) found 6.7% of FA in a cross-sectional analytical study in which 800 consecutive Colombian patients with RA were assessed. Recently, a systematic review and meta-analysis performed by Cardenas-Roldán *et al*. (66) found AITD, T1DM, SLE, SS, psoriasis, ankylosing spondylitis, pernicious anemia, SSc, and Wegener's granulomatosis significantly observd in relatives of RA patients. Moreover, FA confers additional susceptibility to CVD in RA patients (5). Conditions related to FA and CVD include radiographic progression, which denotes high disease activity and persistent increased inflammation. El-Gabalawy *et al*. (230) indicated that levels of multiple cytokines and high sensitivity CRP are higher in Amerindian patients with RA and their FDRs as compared to individuals from a non-AD. In the same way, familial autoimmune disease or family history of RA is defined as the presence of the disease in at least one FDR (74,228). This condition was found in 7% of the Colombian population and was associated, along with ACPA positivity, with an early erosive compromise, and therefore, a rapid progression of disease (74). Walker *et al*. (231) found an excess risk for AITD in RA multicase families compared with the general population.

MONITORING PATIENTS WITH RHEUMATOID ARTHRITIS

Patients should be seen on a regular basis for clinical evaluation, monitoring of clinical and laboratory assesment of diease activity and screening for drug toxicities. The initial evaluation and subsequent monitoring should also include periodic assessment of disease activity using a quantitative composite measure (232). Additionally, the ongoing evaluation and monitoring of patients with RA following the initiation of therapy also involves: patient and clinician assessment of symptoms and functional status, evaluation of joint involvement, EAMs, laboratory markers, and imaging techniques (233).

LABORATORY FINDINGS

Given the physical, social, functional, and psychological aspects of RA, it is necessary to closely monitor patients after the diagnosis, using an excellent and careful history and physical examination [i.e., presence and distribution of swollen joint count (SJC), tender joint count (TJC), visual analogue scale (VAS) of pain, and global assessment of the disease], and objective measures to evaluate the response to treatment. This should be done, usually every three months. For these reasons, several tests can provide objective data that allow disease progression to be followed. In laboratory measurements, it has been demonstrated that some are directly related to poor prognosis or early development of the disease. The ACR Subcommittee recommends that baseline laboratory evaluations include a complete blood cell count with differential, RF, and ESR or CRP. Baseline evaluation of renal and hepatic function is also recommended. Table 10 summarizes the test findings associated with the follow-up of RA (1,22,197).

ASSESSMENT OF DISEASE ACTIVITY

The TJC and SJC are a direct reflection of the amount of inflamed synovial tissue, establishing in a numerical form the joint compromise has certainly proven to be the most specific and simple clinical method to measure the activity of RA (234). Disease activity should be evaluated initially and at all subsequent visits. It is recommended that a structured assessment of disease activity using a composite measure such as those described here, should be performed initially, every three months (234). There are several scoring methods, including TJC, SJC, and it is mathematically combined with laboratory measurements and global evaluations to determine the degree of disease activity (235). Adjustments to treatment regimes should be made to quickly achieve and maintain

LABORATORY TEST	COMMENTS
CRP*	Typically increased; it is useful measure periodically in the assessment of the disease activity, thus may be used to monitor disease course
ESR *	Often increased to >30 mm per hour; it is useful measure periodically in the assessment of the disease activity, thus may be used to monitor disease course
Hemoglobin/hematocrit*	Slightly decreased; normochromic anemia, also may be normocytic or microcytic
Liver function*	Normal or slightly elevated, it is useful measure periodically in the assessment of the adverse effects from the treatment, thus may be used to monitor it
Platelets*	Usually increased
White blood count*	May be increased. Decreased could be seen in polyautoimmunity with SLE
Urinalysis	Microscopic hematuria, leukocyturia or proteinuria may be present in SLE
Complement levels	Normal or elevated. Decreased could be seen in polyautoimmunity with SLE
Immunoglobulins	Elevated alpha-1 and alpha-2 globulins possible
ANA**	Limited value as a screening study for rheumatoid arthritis, but these could be seen in polyautoimmunity with SLE
HLA-DRB1/SE	Screening HLA to evaluate diagnosis, severity, prognosis, and outcome
Others	Depending on the conditions associated or treatment: Screening infections (e.g;, hepatitis C, tuberculosis, hepatitis B, among others), potasium levels, uric acid, etc

Table 10. Laboratory tests in rheumatoid arthritis. * Recommended for initial evaluation for RA, together with RF and ACPA. ** ANA and other autoantibodies depending on clinical manifestations on search of polyautoimmunity. *ANA*: anti-nuclear antibodies; *CRP*: C-Reactive protein; *ESR*: erythrocyte sedimentation rate; *RA*: rheumatoid arthritis; *SLE*: systemic lupus erythematosus; *SE*: Shared epitope. Modified of Rindfleisch and Muller (22).

control of disease activity of targeted treatment goals (i.e., remission or low disease activity), rather than an undefined degree of improvement. Among more than 60 activity measures available for evaluation of patients with RA, the six measures noted below have been identified by the ACR as having the greatest utility in clinical practice because they accurately reflect disease activity; are sensitive to change; discriminate well between low, moderate, and high disease activity; have remission criteria; and are feasible to carry out in clinical settings (236). The choice of measure is based upon clinician preference; some measures require both patient and clinician input while others are based only upon patient-reported data. Measures that require both patient and clinician input as well as calculators for these measures, include the following:

- The Disease Activity Score derivative for 28 joints (DAS28) (237). It is made up of the TJC and SJC on 28 joints determined by physician and CRP (mg/L). The equation is as follows: DAS28 = 0.56* √(TJC28) + 0.28* √(SJC28) + 0.36* ln(CRP + 1)* 1.10 + 1.15.
- The Simplified Disease Activity Index (SDAI) (235) is the algebraic sum of the following parameters: TJC and SJC on 28 joints determined by the physician, CRP level (mg/dL), patient VAS-Global, and MD Global.
- The Clinical Disease Activity Index (CDAI) (238)is the algebraic sum of the SDAI items minus CRP level.

The patient-reported outcome (PRO) measures include:

- The Routine Assessment of Patient Index Data 3, 4, and 5 (RAPID3, 4, 5) (239,240): The RAPID 3 is a PROs-based index that uses the three core set criteria evaluated by the patient, that is, physical function (from MDHAQ), VAS-Pain, and VAS-Global (scale 0–10). The RAPID 4 includes the same variables as RAPID3 plus self-administered, RA disease activity Index (RADAI) (Scale 0–10), and RAPID5 includes the same variables as RAPID4 plus MD-Global (Scale 0–10).
- Similarly, the self-administered index is a tool counting SJC and TJC (Figure 17).

Recently, in a meta-analysis, PROs demonstrated moderate to high correlations and concordance with the objective measures done by the health care practitioners. These can be administrated in the clinical practice and clinical trials without pretending to replace the clinical concept but aimed at facilitating and optimizing the clinical consult and the patient outcomes (3,241,242). Table 11, summarizes different scales, questionnaires, and indexes used to evaluate RA patients.

IMAGING TECHNIQUES

X-RAY

Plain radiography (X-ray) is a valuable tool in dealing with RA; it has different uses, including: diagnostic, structural damage assessment, measurement of the severity and progression, establishment of treatment effects and objective measure of joint involvement. For proper radiographic interpretation it is

necessary to have adequate technical aspects in their decision and a correct interpretation (205). For the study of RA application, comparative x-ray of both hands and feet is essential for diagnosis and objective staging since most existing indices of damage quantification are performed at both levels. It is suggested that radiographic study of hands and feet be requested at the beginning of the disease with annual monitoring and then, every six months to asses radiographic progression (i.e., structural damage) (205). Proper radiographic evaluation of the hand, including the wrist and fingers, requires posteroanterior (PA) conventional projections, lateral and oblique. The PA projection is the most useful for the assessment of injuries as bad alignments, space narrowing, erosions, and soft tissue lesions (205). The oblique projection can be used to display other alterations on this plane that cannot be seen on the other two, but its use is not widely validated in follow-up studies of RA as if it is the PA projection (243). When a patient has asymmetric symptoms, it is important to apply a comparative hands study precisely because it allows physicians to compare and evaluate other possible alterations that are not clinically evident in the contralateral joint and so staging the damage. The Norgaard projection evaluates hands in their normal resting state making more visible the presence of subluxations and the appearance of early erosions in the PIP and MCP, but it presents technical difficulties in reproducibility by the position at the time of making the radiography to assess the progression and therefore it is not validated through most radiographic follow-up studies (244). Standard projections for evaluation of the foot are the anteroposterior, lateral and medial oblique, and the lateral load (support) which provide a better assessment of the hind foot. Practically all body areas likely to be compromised by the RA can be studied by plain radiography. The order of frequency of injury in RA is: hands, feet, knees, hips, cervical spine, and shoulders. In the hands mainly undertake MCP joints, wrists PIP, and feet the MTP, PIP, and retro-half foot (245). The assessment of damage (injury or radiographic structural alteration) in RA in small joints is a good proxy for assessing the total radiological damage (243).

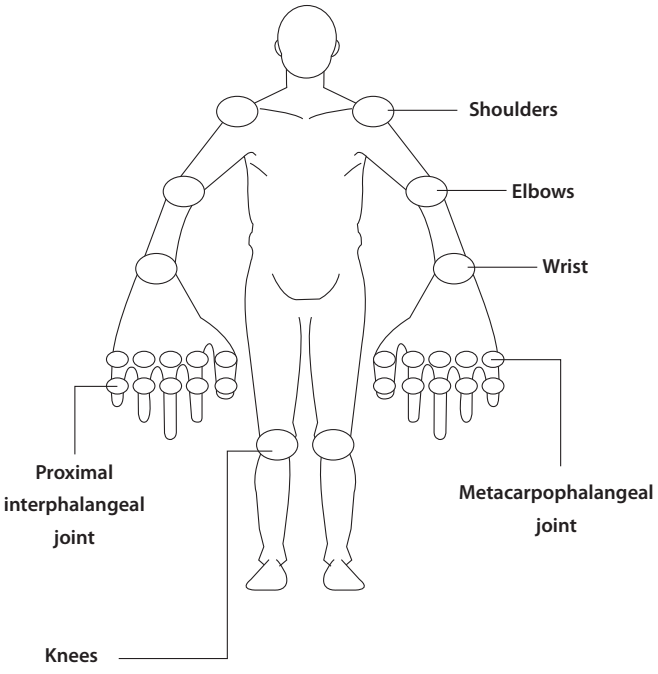

Figure 17. Disease activity index-28: Mannequin.

PROs:	VAS-Pain	
	VAS-Global	
	Patient self-reported-joints	SJC[&]
		TJC[&]
		RADAI[*]
	Functionality questionnaires	HAQ[#]
		AIMS
		MACTAR
	Quality of life and fatigue	RaQOL
		SF-36
		ALI
		FACIT-F
Clinimetry	VAS-Pain	
	VAS-Global	
	Articular index	SJC
		TJC
		RADAR
		Thomson
	Activity index	DAS28[‡]
		SDAI
		CDAI
		RAPID[¥]
	Response criteria	ACR Core set[€]
		EULAR

Table 11. Patients reported outcomes instruments and clinimetry. *ACR*: American College of Rheumatology; *AIMS*: arthritis impact measurement scale; *ALI*: arthritis and lifestyle index; *CDAI*: clinical disease activity index; *DAS28*: disease activity score-28 joints; *EULAR*: European League Against Rheumatism; *FACIT-F*: functional assessment of chronic illness therapy fatigue scale; *HAQ*: health assessment questionnaire; *MACTAR*: MacMaster Toronto arthritis patient preference disability questionnaire; *RADAI*: rheumatoid arthritis disease activity index; *RADAR*: rapid assessment of disease activity in rheumatology; *RAPID*: Routine assessment of patient index data; *RaQOL*: rheumatoid arthritis quality of life; *SDAI*: simplified disease activity index; *SF-36*: medical outcomes study short form; *SJC*: swollen joint count; *TJC*: tender joint count; *VAS*: visual scale analogue; [&]Using self-administered index on mannequins (Figure17); [*]Using self-administered index on text; [#]HAQ may be: HAQ-DI, MDHAQ, MHAQ, or CLINIHAQ; [¥]DAS28 may use ESR (i.e., DAS28-ESR) or CRP (i.e., DAS28-CRP); [¥]RAPID may be RAPID3, RAPID4, RAPID5, depending on number variable taking account; [€]ACR criteria response may be ACR20, ACR50, ACR70.

Joint injuries visualized by X-ray: the major structural evaluated changes are (246):

1. Soft tissue edema: Usually fusiform and evenly distributed around the joint.

2. Osteopenia: The term refers to a shortage quantity of the bone. The amount of bone to be lost before the osteopenia can be detected in an X-ray is about 33%. When osteopenia is found, it is important to define if it is juxta-articular or generalized (246).

3. Joint space narrowing: It is one of the most important measures of radiographic injuries in AR. JSN indicates loss of articular cartilage. In RA this loss is usually symmetrical. When there is total loss of space, it is called ankylosis (246) (Figure 18).

4. Erosion: It is defined as the loss of definition of the bone surface or loss of the continuity of the cortical in the bones that compromise the joint. Juxta-articular erosions are observed in the "bare spots" of bone, (i.e., in areas within the joint in which the bone is not protected by coating cartilage) (246,247). Erosions constitute one of the key measures to assess radiographic damage in RA.

5. Joint congruency: The loss of the congruency between the bones in a joint may be total (luxation) or partial (subluxation). These bad alignments are components of some of the deformities in RA (i.e., boutonnière deformity and swan-neck finger) (248) (Figure 6 and 7).

6. Subchondral cysts: Radiolucent areas with well-defined edges underlying the articular surface.

Of all the above structural changes, erosions and JSN are those that best reflect the pathophysiological process of joint damage in RA, and therefore, they have been used in most studies and quantified through indexes and validated scoring systems (247–249).

Radiographic indices in RA. Several methods have been developed to quantify radiographic progression in RA. Some of these methods provide a global assessment of joint disorders giving a score group and others provide detailed score based on independent findings (250).
A summary of the characteristics evaluated by different methods is found in Table 12. Sharp methods (251,252) and its modification by Van der Heijde (253) are the most widely used. The Larsen method (254) and its modifications (255,256) (i.e., Scott and Rau) have also been implemented in several studies (245,247,248).

Sharp-Van der Heijde (S-VdH) Index. The version of Sharp method modified by Van der Heijde Désirée (253,257) (S-VdH) removes an area of erosions in hand, leaving 16 areas

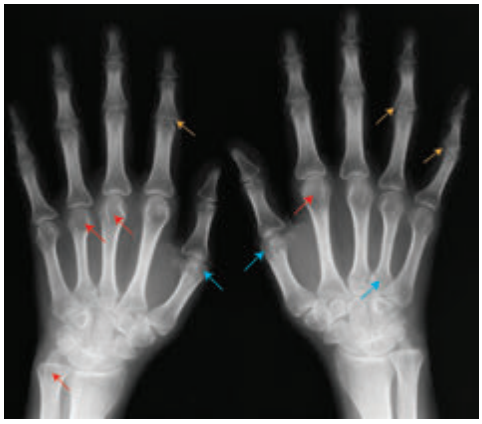

Figure 18. Comparative hands x-ray in RA. Posteroanterior view. Erosions are shown as red arrow. Yellow arrows indicate JSN, and loss of the joint congruency is exposed as blue arrow.

to evaluate, and eliminates three wrist areas to qualify the JSN, leaving 15 areas to be evaluated (Figure 19). The rating for erosions in the hands and JSN according to this method is found in Table 13. The maximum score is 160 in hands erosion and for JSN it is 120. This scoring system adds the qualification level of the feet including all MTP joints and the first bilateral PIP to assess erosions with a maximum score of 120 (maximum 10 for each joint) and a maximum score of 48 for evaluating JSN (maximum 4 per joint).

Simple Erosion Narrowing Score (SENS Index). This scoring system developed by D Van Der Heijde (258) is a simplified score version for erosions and JSN and has been validated and compared with traditional methods founding similar reliability. A total of 32 eligible joint areas of the hands and 12 in the feet are analyzed for erosions (total of 44), and 30 parts of the hands and 12 of the feet are analyzed for JSN (total of 42). Score by area is 1 and a score of 1 for the presence of JSN, for each joint the maximum score is 2 (1 for erosion and 1 for JSN), the total score then can be maximum 86.

Implementation in studies. There is no definitive method for scoring radiographic damage in RA. An important aspect is the reliability of scores that are evaluated by correlation coefficients intra-class and inter-observer when the same reader analyzed the radiographs at different times and several readers analyze and give their scores at the same time. These coefficients have proved to be adequate for detailed scores like Sharp or modification S-VdH. Suitable correlation coefficients for scores of Rau-Larsen and SENS have also been found (259). Sensitivity to change is another measure that can determine the sensitivity of a radiographic scoring method to detect real changes in RA. Over time, this has been validated and implemented by the OMERACT (Outcome Measures in Rheumatoid Arthritis Clinical Trials) by the concept of the smallest detectable difference and the concept of the minimum clinical important difference in radiography, or minimal detectable change (260).

METHOD	EROSION	JSN	OSTEOPENIA	SOFT TISSUE EDEMA	SUBLUXATION	ANKYLOSIS
Steinbrocker 1949						
Kellgren 1956						
Sharp 1971	√	√				√
Sharp 1985	√	√				√
Genant 1983	√	√		√		
Genant 1998	√	√			√	√
Kaye/Sharp 1986	√	√			√	√
Van Der Heidje-Sharp 1989	√	√			√	√
SENS 1999	√	√			√	√
Larsen 1977	√	√	√	√		
Larsen 1995	√	√				
Scott/Larsen 1995	√	√	√	√	√	
Rau-Larsen 1995	√	√	√	√		
Ratingen 1998	√	√				
SES 2000	√	√				

Table 12. Major indexes radiographic scoring in RA. Evaluation of several alterations in the bone and joint by different radiographic methods. *JSN*: joint space narrowing; *RA*: rheumatoid arthritis. Adapted from Anaya *et al.* (205).

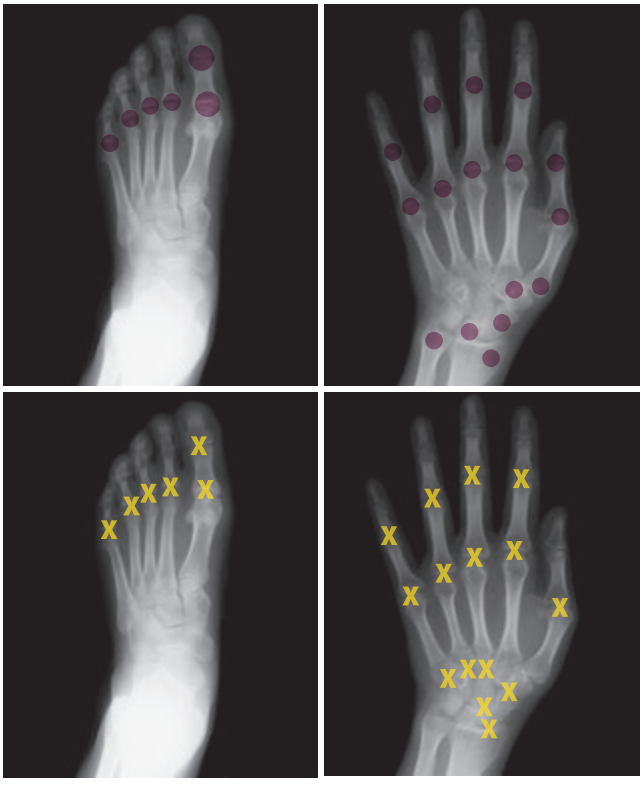

Figure 19. Hands, wrist and feet joints assessed by the method of Sharp - Van der Heijde (S-VdH) Index. Above: O = Erosions (16 areas in hands and 6 areas in feet); Low: X= JSN (15 areas in hands and 6 areas in feet).

Reading time using these scoring methods has been found between 3.9 minutes (Larsen), 19 minutes (Sharp), 25 minutes (S-VdH), and 7 minutes (SENS) (259). Adequate radiological monitoring for a RA patient must take into account the appropriate position at the time of shooting, the exposure time in the film, and the type of film used, and the reproducibility of the shooting, so that these measures ensure the accuracy of the appreciated alterations (259). Some studies have implemented the use of the digitized x-ray using scanning techniques to be read on the screen and can provide measures of bone density and porosity level at the site of the joint, developing indices that are being validated (261). Recently the OMERACT group (262) describes the use of computer programs (software) to accurately measure the JSN in millimeters with good reliability compared to conventional visual methods, hoping to have greater sensitivity and accuracy with the improvement of these programs and their implementation in clinical studies.

Radiographic progression and clinical correlation. Radiographic progression and structural damage are related to the disease activity and may be used to assess treatment effectiveness (250). At the same time, the radiographic damage progression relates to clinical outcome, defined as physical disability. There is also a relationship between clinical measures of disease activity and radiographic progression measures in different studies and a strong correlation between local inflammation and progression of joint damage has been established individually (243,250,263). The erosion and structural damage indicators are presented in the first or second years of disease onset, hence the importance of

SCORE	JSN*	EROSIONS
0	Normal	None
1	Focal or doubtful	Discreet
2	Generalized, respects> 50% original space	Bigger size as compromised articular surface
3	Generalized, respects<50% or subluxation	Spread half imaginary bone
4	Ankylosis or complete luxation	Greater than 3 <5 (In the 4 quadrants)
5	-	Full Face - Full Collapse (≥5 erosions)

Table 13. Score of erosions and JSN by the method of S-VdH. *If a joint had a surgery, the score will be the previous peak. *JSN*: joint space narrowing; *S-VdH*: Sharp - Van der Heijde.

treating the disease early (264). Radiographic progression was assessed (265) in patients with early RA, and it is possible to classify the outcome as progressive (onset of erosion) or non progressive (stability without radiographic appearance of erosions). This has served as a criterion for establishing the extent of therapeutic effect (both early and established RA) with different medicines, including inducing remission DMARD (i.e., MTX and others), leflunomide, and biological therapy (204,249,264,266,267). The moment the first erosion appears is important because it has prognostic implications. Both MTX (204) as well as new, more specific therapies for the treatment of the disease, have been shown to arrest the progression of radiological damage and repair radiographic erosions, thus improving the prognosis of patients (268).

Early arthritis The use of the x-ray in patients with early arthritis allows to classify the type of the disease and to perform differential diagnosis, especially in early RA predicting when used concomitantly with radiography of hands and feet, the latter increases the sensitivity of prediction (182). Using methods of scoring within the first three months of the disease can detect changes if chronological studies are performed (265,269). In some early arthritis studies, structural damage has not been demonstrated (e.g., erosions or JSN), but simply osteopenia, a finding that has low reliability due to its low reproduction in readings for the same or different observer (268). Therefore, software used to develop mathematical models for predicting early arthritis over time is currently being implemented (270). On contrast, there are studies that have shown that patients with early onset arthritis whom had ACPA have a higher scoring system (Larsen) at two years of the disease. Through logistic regression, predictability to develop more severe disease was demonstrated when these antibodies were positive at the beginning of the disease and caused greater radiological damage (271). The use of radiographic scoring methods, within the first three months of the disease, can detect early changes and, also, can let to begin DMARDs in a timely manner (199).

Weaknesses of the x-ray. The use of x-ray in dealing with RA has several weaknesses, including (250): variability in reading and interpretation by untrained observers, alterations in interpretation when the shooting technique is inadequate (i.e., projection, exposure time, proper film, reproducibility, among others), lack of sensitivity to detect early changes that are not erosive such as synovial thickening, synovial fluid, cartilage abnormalities and bone edema, development of erosions or decrease in space, prolonged reading time for account using a suitable damage index (S-VdH 25 minutes) and lack of standardization of computer methods through software to perform a more accurate measurement of the JSN and erosion; lack of standardization of the daily use of digitized radiographs.

ULTRASOUND

The indications for use of the ultrasound (US) are addressing the inflammatory joint condition, studying abnormal tendons, bursae, ligaments, muscles and synovial fluid. Furthermore, it enhances studies through ultrasonographically guided procedures, application of US-guided medicine, evaluation of the inflammatory activity of the disease, and monitoring disease progression (272, 273). US has proved to be a promising tool because of its superiority to conventional imaging study for evaluating the joints in RA; this assertion is based on the visualization of inflammatory and destructive changes (e.g., erosions) that are not detected by the clinic or radiography (Figure 20).

Alterations in RA evaluated by US

Synovitis. The US can distinguish between arthritis and tenosynovitis from a joint with effusion and also distinguish turbulence caused by mechanical disturbance, at the same time it can visualize structural changes in the synovial membrane (274). Synovial tissue is not seen through US unless there is a thickening of it, in which case it is seen as an intra-articular hypoechoic tissue. However, the exact appearance varies based on the amount of extracellular fluid that is in the synovial tissue. In some areas such as the radial or cubital aspect of MCP joint or prepatellar area, synovitis can be better appreciated. Most authors consider that evaluation of synovitis as a tool to define the therapeutic response (275).

Joint fluid. The US is extremely sensitive in detecting joint fluid, even in small joints (273). The distension of the joint

capsule is the main sonographic finding of the articular phlogosis. In recent onset synovitis, synovial fluid is characterized by homogeneous lack of echogenicity. In cases of prolonged flogosis, the presence of irregular echogenicity of the synovial fluid due to the presence of proteinaceous material and/or inflammatory cells is often revealed. These margins are different from those observed in cases in which synovial hypertrophy has a thickening and/or variable irregularity (proliferation) of the articular capsule wall (205,246).

Erosion. Several authors have described the ability of ultrasound to detect erosions (247,272). One of the main benefits of ultrasound is the ability to visualize the joint in different planes and thus obtain greater sensitivity in the detection of erosions (273). The bone surface is a barrier to US and it is seen as a hyperechoic structure with posterior acoustic shadowing. Through US, terosions have been detected 7.5 times more in patients with early RA than through conventional radiography, and 3.4 times more in patients with established RA (246,273). Using second generation probes (i.e., transducers) with high spatial resolution and multiplanar studies, minimal disruptions of the bones may be detected and therefore, microerosions that are not able to grasp in the standard projections of hand for x-ray can be visualized (247). Since the earliest erosive manifestations in patients with RA are located, in most cases, in the second MCP joint, the US study of this anatomical region should be performed routinely in these patients (247). This study as well as the fifth MTP would ensure the best combination of sensitivity, specificity, and suitable time on the US examination (205).

Cartilage. In healthy subjects, the articular cartilage of the metacarpal head appears as a subtle hypoanechoic band bounded by clear margins and homogeneous hyperechoic bands. Normal thickness of articular cartilage of metacarpal heads is between 0.2-0.4 mm. In 85% of RA patients a loss of definition of the articular cartilage (i.e., irregular margins, increased echogenicity, and internal thinning) is observed (273).

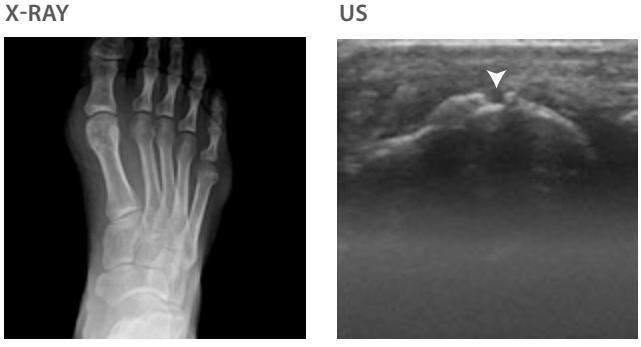

Figure 20. A: shows X-ray of a non-erosive left foot; B: US shows an erosion on the fifth metatarsal head.

Cysts, bursae, ganglions, meniscus injury, solid masses, abnormal ligament, tendon injuries, and tenosynovitis can also be observed by this technique.

OMERACT-7 and US. In 2005, the OMERACT group at its seventh session (276) made of the major alterations that can be evaluated by US; these are presented in Table 14, along with the update from 2009 (277). Results demonstrated good intermachine reliability among multiple examiners, good applicability of the scoring system for the hand on other joints (including shoulder), and helped dispel the myth that US is too subjective.

US diagnostic in RA. Through MRI every erosion detected by US that was not detected by X-ray was identified (278). US has demonstrated a high inter-observer correlation between rheumatologists and radiologists when performing US of the small joints of the hand in patients with established RA. Correlations include evaluating erosions (91%), synovitis (86%), joint effusion (79%), and Doppler signal (87)% (279). In a study in which the three methods were compared at the MTP joint, it was found that the US is more sensitive, accurate, and specific than clinical examination and X-ray with a high sensitivity and specificity (i.e., 79% and 97% respectively) for the detection of erosions taking the MRI with contrast as gold standard (280). Some authors have performed measurements in healthy adults through articular US, describing standard reference values in terms of extent and localization of anatomical structures to be used as a guide to the pathological findings (281). Scoring systems have been used in research to describe in a semi-quantitative form the presence of synovitis and joint effusion in RA patients (Table 15).

Musculoskeletal Doppler in RA. There are different modalities and utilities such as US Doppler mode, pulsed Doppler, color Doppler, duplex, and power Doppler (282-286) (Table 14).

MAGNETIC RESONANCE IMAGING (MRI)

MRI is a good tool for evaluation of most musculoskeletal disorders. The absence of ionizing radiation, the best anatomic detail, and functional information are some of the most important advantages of MRI. It has a high sensitivity in detecting radiographic abnormalities that are hidden. It can detect active synovitis and bone erosions earlier than conventional X-rays. It is useful to differentiate early cases of arthritis and other diseases with joint manifestations (248). It is important to note that the accuracy of resonance depends on technical factors such as the magnetic field and surface antennas used, and the selection of specific sequences. The timing of the examinations is another important aspect of precise diagnosis, as the findings are dependent on the stage of the disease.

CONCEPT	DEFINITION
Bone erosion	An intra-articular discontinuity of the bone surface that is visible in two perpendicular planes
Synovial fluid	Abnormal hypoechoic or anechoic (relative to subdermal fat, but sometimes may be isoechoic or hyperechoic) intraarticular material that is displaceable and compressible, but does not exhibit Doppler signal
Synovial hypertrophy	Abnormal hypoechoic (relative to subdermal fat, but sometimes may be isoechoic or hyperechoic) intraarticular tissue that is nondisplaceable and poorly compressible and which may exhibit Doppler signal.
Tenosynovitis	Hypoechoic or anechoic thickened tissue with or without fluid within the tendon sheath, which is seen in 2 perpendicular planes and which may exhibit Doppler signal.
Enthesopathy	Abnormally hypoechoic (loss of normal fibrillar architecture) and/or thickened tendon or ligament at its bony attachment (may occasionally contain hyperechoic foci consistent with calcification), seen in 2 perpendicular planes that may exhibit Doppler signal and/or bony changes including enthesophytes, erosions, or irregularity.

Table 14. Definitions of articular pathologic findings by ultrasound OMERACT 7 Consensus. *OMERACT*: Outcome Measures in Rheumatoid Arthritis Clinical Trials.

Structural abnormalities detected by MRI in RA

Synovitis. It is defined as an area in the synovial compartment showing a higher than normal enhancement after the injection of Gd (gadolinium) showing a higher thickness than normal synovium (287). It is correlated with active histologically observed inflammation. These signs are related to three aspects (288,289): increase in volume ("mass"), increase signal intensity following injection of intravenous contrast medium, increase water content, and a combination of the above. Although in MRI the normal synovial membrane is not visible, in RA it can discriminate between effusion, hypervascular pannus, and fibrous pannus (290). After intravenous administration, Gd passes within the interstitial space at a rate dependent on the local capillary permeability and tissue perfusion (287). This substance accumulates in areas of inflammation and can improve detection and differentiation of inflammatory processes from internal liquid. MRI is a more accurate method than clinic for detecting synovitis (287) and it can be a useful and sensitive measure of inflammation in the early stages of the disease, thus improving the accuracy of diagnosis (272).

Erosion. To avoid overestimation, the strict use of its definition is essential. Resonance bone erosion is defined as a marginal lesion with juxtaposed location with typical articular signal intensity in different sequences, visible on two planes with cortical rupture, which must be observed at least on one plane. Erosions are also recognized because they replace fatty marrow (248) and they differentiate from a fluid-filled cyst because erosion takes the contrast (290,291). It has been shown that MRI is superior to identify erosive changes earlier than conventional radiographic studies detect them in patients with early arthritis (272,292). This makes it the method of choice for detecting erosions and it is also more sensitive in monitoring erosive progression.

Bone marrow edema. This phenomenon is reversible; it generally refers to bone edema or osteitis. The exact histological correlation is unknown. It can be alone or around bone erosion. There are frequently detected signs of increased water content in the core compartment (293–295). Bone edema has been shown to be associated with erosive lesion and it is considered to be a precursor of erosion. The effective suppression of synovitis may reverse the pre-erosive changes and subsequent structural damage (296).

Cartilage and destructive changes. The image of the cartilage is more specific than the radiographic visualization of the joint space. Although optimal evaluation of small joints has not been established, it is useful in monitoring and controlling treatment. MRI displays inflammatory and destructive changes of the disease; it is considered to play an important role in the proper monitoring of treatment efficacy. The methods used for its investigation are quantitative (i.e., measured), semiquantitative (i.e., scores), or qualitative (i.e., presence or abscense).

GRADE	FINDINGS
0	No alteration. Absence of anechoic, hyperechoic or hypoechoic structures,
1	Mild effusion or hypertrophy
2	Moderate effusion or hypertrophy
3	Severe effusion or hypertrophy

Table 15. Semiquantitative method for classification of synovitis and joint effusion in rheumatoid arthritis.

Quantitative methods estimate the joint inflammatory activity by measuring and determining the volume of inflamed synovium (i.e., the "inflammatory load") (293–295). Erosion volumes can also be estimated. Unfortunately, quantization of the volumes is time-consuming (294). Semiquantitative scoring methods require less time. Currently, the best-validated scoring system has been developed by the OMERACT. The OMERACT rheumatoid arthritis magnetic resonance image scoring system (RAMRIS) has shown good correlation between the scores and volumes of synovitis and erosions (36), but there is a lack of data on sensitivity study of minimal changes (Table16).

Prognostic value in early arthritis. MRI may have prognostic value because it provides predictors of poor prognosis and outcome in RA. The findings of erosive disease in the wrist can predict subsequent radiographic damage at 2 years. The proportion of synovitis detected by MRI in early RA is predictive of radiographic injury during monitoring (297). In future clinical practice, MRI can acquire an important role in the differential diagnosis of early-unsorted polyarthritis, in monitoring therapeutic response, and in patient prognosis. Clinicians using MRI can make optimal decisions in less time due to the availability of more accurate information (297).

COMPUTED TOMOGRAPHY (CT)

Since was developed of CT in the 1970s, it has been extensively used to image osseous structures. CT provides multiplanar imaging with the benefit of good contrast for cortical and trabecular bone (298). CT viewing perspective obviates projectional superimposition, which can obscure erosions and mimic joint space narrowing on conventional radiograph. However, recent developments in multidetector CT technology and computer-enhanced reformatting of image data have revolutionized this modality and broadened its usage. Images are still generally obtained on the transverse plane, but reformatted images on other planes can now be obtained immediately on advanced workstations, allowing for visualization of structures in any linear or even curvilinear plane(299). However, it has the disadvantage of ionizing radiation (197).

Another strength is the excellent soft tissue resolution in many areas, leading a good definition of bone anatomy (300). CT optimally images bone structures and can easily detect erosions. On CT, erosion is visualized as a local area with decreased density of the cortical joint surface, sometimes including the adjacent subcortical bone. Sclerosis can also be seen with CT and may be indicative of reparative changes following erosion (301). Cortical bone, being very dense, is readily visible, as is the interface with adjacent soft tissues. Thus, these imaging techniques are capable of clearly delineating the borders of erosions and differentiating bone (whether edematous or not) from inflamed synovium (Figure 23). In this way, it is more sensitive than the MRI in the detection of early erosions in the hand (302). Otherwise, this has been increasingly used in patients who cannot tolerate, or have absolute contraindications to MRI (300). Given the advantages of other imaging modalities, the potential of CT in the clinical management and clinical trials of RA patients seems minimal. However, it may be a valuable reference method for validation of bone damage observed on MRI and US (299) (Figure 21).

While plain radiography has traditionally been used as the gold standard for imaging erosions, there are many regions such as the carpus in which complex 3-dimensional anatomy is very inadequately depicted using a 2-dimensional technique. This was recognized when the Sharp score was developed for scoring erosive damage in rheumatoid patients, as some areas of the carpus were excluded altogether because of poor visibility. This problem is circumvented by multidetector helical CT, which offers the benefits of multiplanar capability, similar to MRI, with the enhanced cortical definition intrinsic to plain radiography (298,299). CT is also significantly less expensive than MRI and is quicker to do (300). New technologies have been developed. High resolution CT provides a sensitive method with high reader agreement in assessment of structural bone damage in RA, including joint space width measurements. Moreover, it has better correlation of erosion measures with disease duration (301,303).

TREATMENT

The treatment of RA is directed towards the control of synovitis and the prevention of joint injury. The choice of therapies depends upon several factors, including the severity of disease activity when therapy is initiated and the response of the patient to prior therapeutic interventions. Common principles that guide management strategies and the choice of agents have been derived from an increased understanding of the disease, and from evidence from clinical trials and other studies. These strategies include approaches directed towards achieving remission or low disease activity by more rapid and sustained control of inflammation and by the institution of DMARD therapy early in the disease course. The general principles and treatment strategies that should be applied to the management of RA are reviewed here (198).

GENERAL PRINCIPLES

The overall approach to the treatment of these patients depends upon the timely and judicious use of several types of therapeutic interventions. The appropriate use of these therapies is based upon an understanding of a group of general principles that have been widely accepted by major working groups and by professional organizations of rheumatologists (Figure 22). These principles include:

- Early recognition and diagnosis: Achieving the benefits of early intervention with DMARDs depends upon making the diagnosis of RA as early as possible. The recognition of RA early in the course of inflammatory arthritis, before irreversible injury has occurred, is thus an important element of effective management (304).

A. 'Core set' of basic MRI sequences:
It is suggested that future MRI studies, if they intend to assess inflammatory as well as destructive changes in RA joints, should at least include the following: • Imaging in two planes* with T1-weighted images before and after i.v. gadolinium-contrast** • A T2-weighted fat saturated sequence or, if the latter is not available, a STIR sequence * Imaging in two planes can be acquired by obtaining a 2D sequence in two planes, or a 3D sequence with isometrical volumes in one plane allowing reconstruction in other planes ** i.v. gadolinium injection is probably not essential if destructive changes alone (bone erosions) are considered to be important
B. Definitions of important RA joint pathologies:
• Synovitis: an area in the synovial compartment that shows above-normal post-gadolinium enhancement* of a thickness greater than the width of the normal synovium. * Enhancement (signal intensity increase) is judged by comparison of T1-weighted images obtained before and after i.v. gadolinium-contrast • MRI bone erosion: a sharply marginated bone lesion, with correct juxta-articular localization and typical signal characteristics,* which is visible in two planes with a cortical break seen in at least one plane** * On T1-weighted images: loss of normal low signal intensity of cortical bone and loss of normal high signal intensity of trabecular bone. Quick post-gadolinium enhancement suggests the presence of active, hypervascularised pannus tissue in the erosion ** Other focal bone lesions, including metastases, must obviously be considered, but are generally distinguishable with associated imaging and clinical findings • MRI bone edema: a lesion* within the trabecular bone, with ill-defined margins and signal characteristics consistent with increased water content** * May occur alone or surrounding an erosion or other bone abnormalities ** High signal intensity on T2-weighted fat-saturated and STIR images, and low signal intensity on T1-weighted images
C. Scoring system (The OMERACT 2002 RAMRIS)
• Bone erosions: Each bone (wrists: carpal bones, distal radius, distal ulna, metacarpal bases; MCP joints: metacarpal heads, phalangeal bases) is scored separately The scale is 0–10, based on the proportion of eroded bone compared to the 'assessed bone volume,' judged on all available images: 0, no erosion; 1, 1–10% of bone eroded; 2, 11–20% eroded etc. For long bones, the 'assessed bone volume' is taken from the articular surface (or its best estimated position if absent) to a depth of 1 cm, while in carpal bones it is the whole bone • Bone edema: Each bone is scored separately (as for erosions) The scale is 0–3, based on the proportion of bone with edema, as follows: 0, no oedema; 1, 1–33% of bone oedematous; 2, 34–66% oedematous; 3, 67–100% edematous • Synovitis: It is assessed in three wrist regions (i, the distal radioulnar joint; ii, the radiocarpal joint; iii, the intercarpal and carpo-metacarpal joints) and in each MCP joint. The first carpo-metacarpal joint and the first MCP joint are not scored The scale is 0–3 as follows: 0, normal; 1, mild; 2, moderate; 3, severe, where each grade represents a third of the presumed maximum volume of enhancing tissue in the synovial compartment

Table 16. OMERACT MRI in RA group recommendations of a 'core set' of basic MRI sequences, MRI definitions of important RA joint pathologies and a RA MRI scoring system. *2D*: two-dimensional; *3D*: three-dimensional; i.v.: intravenous; *MCP*: metacarpophalangeal; *MRI*: magnetic resonance imaging; *OMERACT*: Outcome measures in rheumatology clinical trials; *RA*: rheumatoid arthritis; RAMRIS: rheumatoid arthritis magnetic resonance imaging studies; *STIR*: short tau inversion recovery. Adapted from Østergaard *et al*. (288).

- Care by an expert: in the treatment of rheumatic diseases an expert such as a rheumatologist, should participate in the care of patients with inflammatory arthritis who are suspected of having RA and in the ongoing care of patients diagnosed with this condition (149,305).
- Early use of DMARDs for all patients diagnosed with RA: importance of tight control with target of remission or low disease activity, use of anti-inflammatory agents, including NSAIDs and glucocorticoids (GCs), only as adjuncts to therapy (198,306,307).

The application of these principles has resulted in significant improvement in the outcomes of treatment (308). Such improvements may owe even more to the therapeutic strategies that have been adopted than to the development and use of newer and more potent drugs (309).

PHARMACOLOGICAL INTERVENTION

Choices between treatment options are based upon multiple factors, including: level of disease activity, stage of therapy, regulatory restrictions, and patient preferences among others (304,307). It is important to use a combination of the following types of therapies:

- Rapidly acting anti-inflammatory medications, including NSAIDs systemic, and intra-articular GCs, which offer reliable but limited relief of pain, swelling, and stiffness,

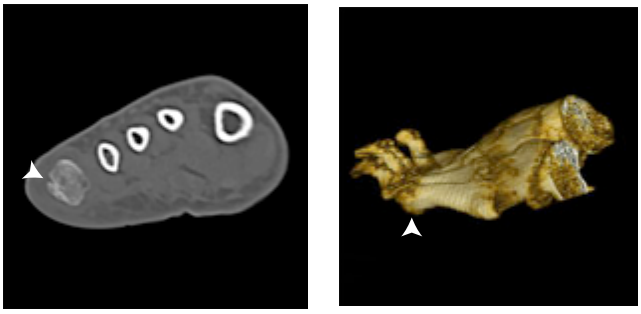

Figure 21. Computed tomography in RA. A: erosions are observed on fifth metatarsal head; B: erosions in the same place on CT-3D.

improving quality of life in the majority of the cases. However, adverse effects are common, and patient education is necessary. Combination of NSAIDs should be avoided (141,205). Selective COX-2 NSAIDs are similar in efficacy to diclofenac or naproxen, and may be of value in patients who cannot tolerate COX-1 NSAIDs. However, many of these medications have been withdrawn from the market due to concerns regarding COX-2 inhibitors and increased of CVD (210).

- DMARDs, which include non-biologic and biologic agents as well as an orally administerd kinase inhibitor, have the potential to reduce or prevent joint damage, and preserve joint integrity and function. The non-biologic DMARDs most frequently used include hydroxychloroquine (HCQ), sulfasalazine (SSZ), MTX, and leflunomide. Biologic DMARDs, produced by recombinant DNA technology, generally target cytokines or their receptors, or are directed towards other cell surface molecules. These include anti-cytokine therapies such as anti-TNF-α (e.g., etanercept, infliximab, adalimumab, golimumab, and certolizumab pegol), IL-1 receptor antagonist (i.e., anakinra), and IL-6 receptor antagonist (i.e., tocilizumab). They also include other biologic response modifiers such as the CTLA-4 blocker, (i.e., abatacept), and the anti-CD20 B cell depleting monoclonal antibody (rituximab). Several kinase inhibitors are in development for use in RA, and one of these, tofacitinib, is available for such clinical use in the USA and is under review for potential approval in Europe. Tofacitinib is an orally administered small molecule DMARD that inhibits cytokine and growth factor signalling through interference with Janus kinases.

All patients diagnosed with RA are started on DMARD therapy as soon as possible. The choice of initial drug therapy depends upon the degree of disease activity. It is necessary to distinguish between those patients with mildly active disease and the majority of patients with more active disease:

- In patients with mildly active RA, initiate anti-inflammatory therapy with a NSAID for rapid symptomatic relief and begin DMARD treatment with either HCQ or SSZ.
- In patients with moderately to severely active RA, initiate anti-inflammatory therapy with either a NSAID or GC, depending upon the degree of disease activity, and generally start DMARD therapy with MTX.
- In patients resistant to initial DMARD therapy (i.e., MTX), treat with a combination of DMARDs (i.e., MTX plus either a TNF-inhibitor or SSZ and HCQ) or, alternatively, switch the patient to a different DMARD of potentially comparable efficacy (e.g. leflunomide or a TNF-inhibitor), while also treating the active inflammation with anti-inflammatory drug therapy.

Concerning to steroid therapy, it should be started initially, with a tapering dose titrated to patient's response and side effects (198,307).

In 2010, the EULAR issued the recommendations for the treatment of RA with synthetic and biological DMARDs and GCs based on evidence from five systematic literature reviews (SLR) performed for these medications, treatment strategies, and economic issues (304). Fifteen recommendations were developed. Recently, these recommendations were updated at the EULAR-2013 meeting (310) based on three SLR and fourteen recommendations were given (Table 17). According to this latest guidance, treatment with DMARDs should be initiated as soon as a diagnosis of RA is made with the goal of reaching a target of remission or lowering disease activity in every patient; as first-line treatment, EULAR recommends rheumatologists to administer MTX or combination therapy of MTX with other conventional synthetic DMARDs. Low-dose GCs should also be considered in combination with DMARDs for up to six months, but should be tapered as soon as clinically feasible. As already stated in the 2010 guideline, by advocating the use of synthetic DMARDs, rather than biologics, as the first-line treatment, this approach avoids the over-treatment of 20-50% of patients with early RA, who will achieve the treatment target with such initial therapy. In addition, it is recommended that patients who have failed to respond to an initial biologic DMARD should receive another biologic DMARD. Patients who have failed to respond to an initial TNF-inhibitor may receive another TNF-inhibitor, or a biologic with an alternative mode of action. If biologic treatment has failed, tofacitinib may be considered where approved, and it is only recommended after at least one biological has failed – in fact, many Task Force members felt it should be used after two biological treatment failures. (310). If a patient has achieved persistent remission, after having tapered GCs, clinicians should consider tapering the biological DMARD, particularly if the treatment is in combination with a conventional synthetic DMARD. In cases of sustained long-term remission, cautious dose-reduction of conventional synthetic DMARDs should be considered (310). The set of recommendations are summarized in the figure 22 and table 17.

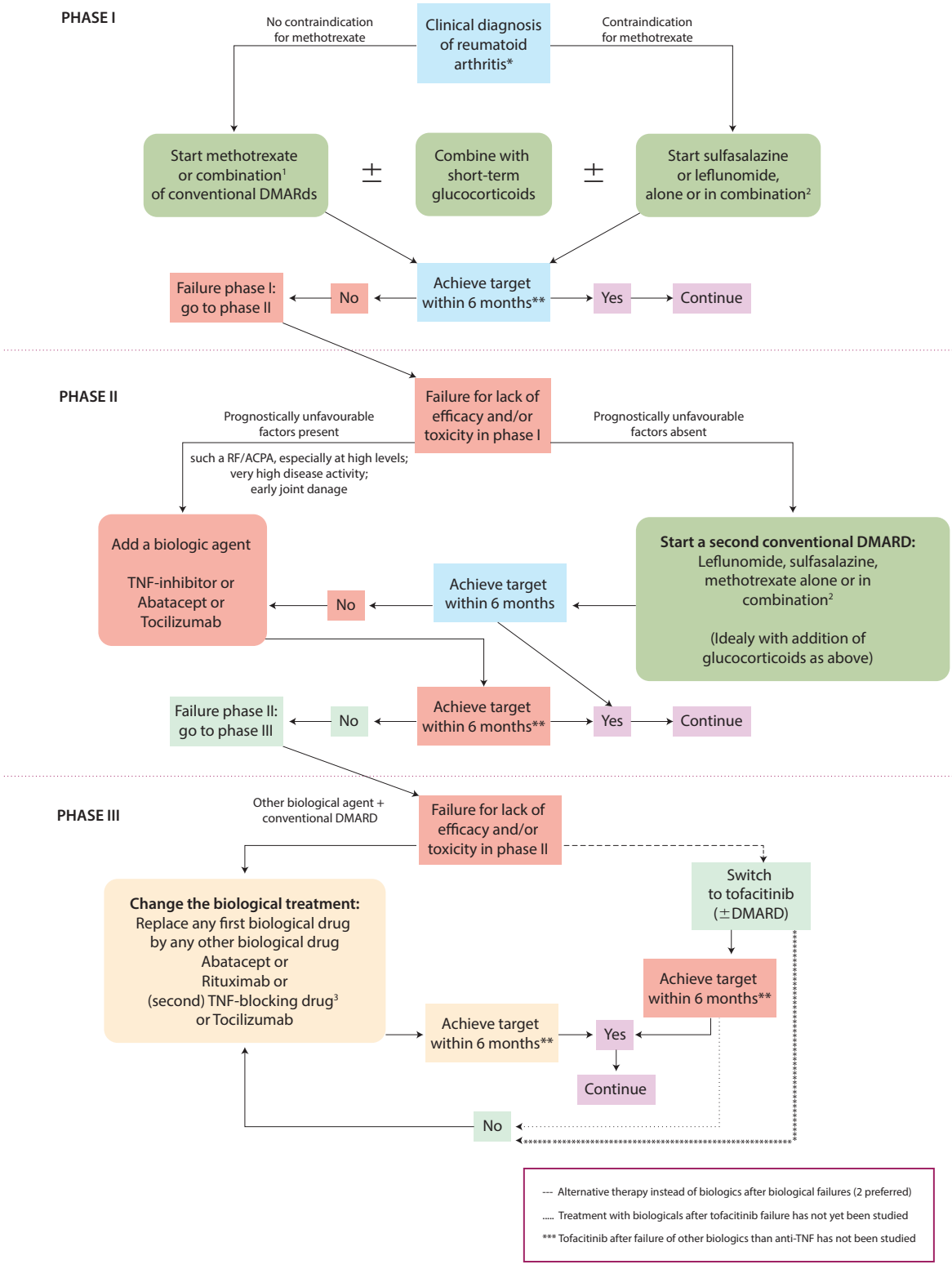

Figure 22. Algorithm based on the EULAR-2010 recommendations, up to date in EULAR meeting 2013.
* 2010 ACR-EULAR classification criteria can support early diagnosis
** The treatment target is remission according to ACR-EULAR definitions or, if remission is unlikely to be achievable, at least low disease activity.
1. The most frequently used combination comprises methotrexate, sulfasalazine and hydroxycloroquine
2. Not many data available for combination without methotrexate
3. Adalimumab, certolizumab, etarnecept, golimumab, infliximab or biosimilar (where approved)
4. Where approved

NON-PHARMACOLOGIC AND PREVENTIVE THERAPIES

Despite advances in pharmacologic therapy for RA, many patients continue to experience some measure of ongoing disease activity and resultant disability. Although measures aimed at identifying early active disease and controlling inflammation are essential, the disease itself and the drugs used for treatment may contribute to increased risks of comorbidities (311). A comprehensive management program for RA includes patient education, exercise and rest, psychosocial interventions, physical and occupational therapy, nutritional and dietary counseling. The management program also includes interventions to reduce the risks of CVD and osteoporosis, and immunizations to decrease the risk of infectious complications in immunosuppressive therapies (312–319).

OVERARCHING PRINCIPLES
Treatment of patients with RA should aim at the best care and must be based on a shared decision between the patient and the rheumatologist Rheumatologists are the specialists who should primarily care for patients with RA RA incurs high individual, societal and medical costs, all of which should be considerd in its management by the treating rheumatologist.
FINAL SET OF 14 RECOMMENDATIONS FOR THE MANAGEMENT OF RA
1. Treatment with DMARDs should be started as soon as the diagnosis of RA is made 2. Treatment should be aimed at reaching a target of remission or low disease activity as soon as possible in every patient. 3. Monitoring should be frequent in active disease (every 1–3 months); if there is no improvement by at most 3 months after treatment start or the target has not been reached by 6 months, therapy should be adjusted. 4. MTX should be part of the first treatment strategy in patients with active RA 5. In case of MTX contraindications (or early intolerance) SSZ or leflunomide should be considered as part of the (first) treatment strategy 6. In DMARD naïve patients, irrespective of the addition of GCs, conventional synthetic DMARD monotherapy or combination of synthetic DMARDs should be applied 7. Low dose GCs should be considered as part of the initial treatment strategy (in combinations with one or more conventional synthetic DMARDs) for up to six months, but should be tapered as rapidly as clinically feasible 8. If the treatment target is not achieved with the first DMARD strategy, in the absence of poor prognostic factors change to another conventional DMARD strategy should be considered; when poor prognosis factors are present, addition of biological DMARD should be considered. 9. In patients responding insufficiently to MTX and/or other conventional synthetic DMARD strategies, with or without GC, biological DMARDs (TNF-Inhibitors, abatacept or tocilizumab, and under certain circumstances, rituximab) should be commenced with MTX 10. Patients who have failed a first biological DMARD should be treated with another biological DMARD 11. Tofacitininb may be considered after biological treatment has failed 12. If a patient is in persistent remission, after having tapered GCs, one can consider tapering biological DMARDs, especially if this treatment is combined with a conventional synthetic DMARD 13. In cases of sustained long-term remission, cautious titration of conventional synthetic DMARD dose could be considered, as a shared decision between patient and physician 14. When adjusting therapy, factors apart from disease activity such as progression of structural damage, comorbidities and safety concerns should be taken into account

Table 17. Recommendations for the management of RA EULAR-2013. *DMARD*: disease-modifying antirheumatic drugs; *EULAR*: european league against rheumatism; *GC*: glucocorticoids; *MTX*: methotrexate; *RA*: rheumatoid arthritis; *SSZ*: sulfasalazine; *TNF*: tumor necrosis factor.

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25 SYSTEMIC LUPUS ERYTHEMATOSUS

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune, multisystemic disease which may affect virtually any organ in the body and encompass a wide spectrum of severity, ranging from relatively mild manifestations (e.g. skin rash or non-erosive arthritis) to seriously disabling or even life threatening complications, such as lupus nephritis, neuropsychiatric disorders and other major organ involvements (1). There is clearly a genetic component to lupus susceptibility. It is generally accepted, however, that environmental or other factors are required to trigger disease development. Lupus can affect almost every organ, most prominently and devastatingly the kidney and the central nervous system (CNS). Its severity in individual patients can range from mild cutaneous involvement to severe organ damage, and its outcome ranges from lasting remission to death.

EPIDEMIOLOGY

The disease affects people worldwide, although the incidence and prevalence varies between countries. The prevalence ranges from 20 to 150 cases per 100,000 persons with the highest prevalence reported in Brazil, and it appears to be increasing as the disease is recognized more readily and survival increases. In the United States, people of African, Hispanic, or Asian ancestry as compared to those of other racial or ethnic groups, tend to have an increased prevalence of SLE and greater involvement of vital organs (2). Overall incidence rates (per 100,000) range from 1.0 in Denmark to 8.7 in Brazil while prevalence rates vary from 28.3 in Denmark to an estimated 149.5 in an analysis of hospitalization data in Pennsylvania, USA that adjusted for the hospitalization rate for SLE (3). Data from several regions in the USA indicate that the incidence of SLE increased 3- to 7-fold between 1950 and 1992 (4). This was probably at least partially attributable to the availability of better diagnostic tests, increased awareness of the disease, and the introduction of standardized diagnostic criteria during this period with some of these advances resulting in increased identification of milder cases. There is little data on the incidence of SLE in Hispanics in the USA or in Latin Americans from Mexico and central and South America. An older study found the rates in Puerto Ricans in New York to be intermediate between those of Europeans and African Americans. A recent study from the Island of Puerto Rico reported a prevalence estimate of 159/105 (277/105 for women) based on an analysis of claims submitted to a health insurance company (5).

There is overwhelming evidence that the probability of surviving 5 years after the diagnosis of SLE increased from <50% in the 1950s to 95% in the most recent studies (6-9). Nonetheless, standardized mortality ratios (SMRs) for SLE patients remain 2-4-fold higher compared to the general population. In the Hopkins Lupus Cohort, survival probabilities were 95%, 91%, 85%, and 78% at 5, 10, 15, and 20 years after diagnosis respectively (7). After the introduction of corticosteroids and, subsequently, of immunosuppressive drugs turned SLE from a rapidly-fatal into a chronic disease, the distribution of the major causes of death began to change. In the late 1970s, it was reported that deaths occurring within 2 years of diagnosis were frequently due to active disease while those taking place after a disease duration of ≥ 5 years were often attributable to vascular diseases. Infections remained a major cause of death throughout the course of the disease. Such a bimodal distribution of the causes of death has since been reported in several large series from the USA, Canada, Denmark, and other European countries, but also from Mexico and Martinique (10).

PHYSIOPATHOLOGY

GENETICS AND EPIGENETICS

SLE occurs when a genetically susceptible individual encounters an environmental trigger, most likely an infective agent, which is responsible for inducing antinuclear antibodies (ANA). After a variable lag of time from the appearance of ANA, deposits of immune material can be found in tissue without concomitant inflammatory lesions (11).

SLE is a multigenic disease. A combination of genome-wide association studies (GWAS) and candidate gene approaches has led to the identification of > 40 robust genetic associations with SLE (12-15). These are genes which induce the transcription of proteins involved in key pathogenic pathways, including apoptosis and clearance of apoptotic material or immune complexes, innate and adaptive immunity functions, and the production of cytokines, chemokines, or adhesion molecules (16).

Both HLA and several non-HLA genes have been found to influence SLE susceptibility (see Chapters 16-18). Highly penetrant mutations such as complete complement fraction (C) 1q, C2, C4A, C4B, and FcγR (Fc fragment of IgG, low affinity receptor) type IIIB deficiency, or mutations in DNA exonuclease named TREX1 (three prime repair exonuclease) account for no more than 1-2% of cases (17). SLE genetic susceptibility is mainly provided by the interplay of fairly common genetic variants, any of which may only slightly increase the disease risk (17). Notably, a remarkable diversity in the genetic background among SLE patients has been observed although the nature of the genes identified so far suggests that patients with SLE have an immune system predisposed to aberrant responsiveness.

Most single-nucleotide polymorphisms (SNPs) associated with SLE fall within noncoding DNA regions of immune response-related genes (18). Some genes have been associated with several autoimmune diseases (e.g., *STAT4* and *PTPN22* with rheumatoid arthritis and diabetes); others appear to specifically increase the risk of SLE. Certain SNPs linked to SLE have been identified for genes whose products may contribute to abnormal T cell function in SLE (CD3-ζ and PP2Ac) (18, 19). A recent large-scale replication study confirmed some of these associations and identified *TNIP1, PRDM1, JAZF1, UHRF1BP1*, and *IL10* as risk loci for SLE (20). Although these findings are promising, the loci identified so far only account for about 15% of the heritability of SLE (21). In addition, an altered copy number of certain genes, such as *C4, FCGR3B*, and *TLR7*, have been linked to disease expression (22-24).

Epigenetics represents a new aspect in the pathogenesis of SLE and refers to changes in gene expression that do not involve changes in the DNA sequence. Epigenetic mechanisms are sensitive to external stimuli, and thus, environmental effects on immune responses can be mediated by changes in epigenetic regulation, leading to stable – but reversible and cell specific – heritable changes in gene expression (25, 26). These modifications in gene expression could explain, at least partly, why no full concordance for SLE is found among homozygotic twins, although it is greater than that found among dizygotic twins or siblings (24–57% vs. 2–5%) (16). The major mechanisms of epigenetics are DNA methylation, histone modifications, and microRNA (miRNA) interference (27), which interact with each other in modulating chromatin architecture and allowing gene transcription or lead to gene silencing. In SLE, abnormalities in both DNA methylation and histone modifications have been reported (26-28) and clues are emerging on the function of miRNA in SLE.

B AND T SIGNALLING ABNORMALITIES

Aberrant immune activation in SLE is crucial, and although failure in tolerance checkpoints is required for the shedding of autoreactive lymphocytes (Figure 1) (29, 30), intrinsic abnormalities in cel signaling are also paramount. Under normal conditions, antigenic stimulation causes clustering of antigen-specific receptors at the cellular membrane (alternatively BCR or TCR) together with their co-receptors and adaptor molecules, and the subsequent recruitment of Src-family kinases that bring about phosphorylation of the ITAM (immune receptor tyrosine-based activation motifs) domains in the cytoplasm (31). Subsequently, kinases of the Syk/ZAP70 family recognize the phosphorylated tyrosine residues of ITAMs and initiate an activator cascade resulting in lymphocyte priming and differentiation. Both T and B cells of SLE patients display multiple signalling abnormalities which may be at least partially due to genetically or epigenetically determined defects (32) and lead to intrinsic hyperactivity and hyper-responsiveness of T and B cells (Figure 2).

DYSREGULATED APOPTOSIS AND DEFECTIVE CLEARANCE OF CELLULAR DEBRIS

Increased exposure of autoantigens due to disturbed apoptosis as well as to defective clearance of cellular debris has been reported (33). This abnormal antigenic availability is thought to play an important role in autoantibody induction since a wide variety of epitopes become accessible for aberrant presentation to the immune system.

NEUTROPHILS AND NETOSIS

Neutrophil extracellular traps (NETs) are web-like structures that are released by neutrophils and optimize microorganism entrapment and killing. They are composed of chromatin, histones, and proteins derived from granules including myeloperoxidases, elastases, matrix metalloproteinases 9, pentraxin 3, and antimicrobial molecules such as cathelcidins (34). NETs are mainly released by activated neutrophils that undergo a novel cell-death mechanism, named NETosis although release from intact cells has also been described (34, 35). Physiologically, NETs represent a means of defence against pathogens that can also control inflammation and

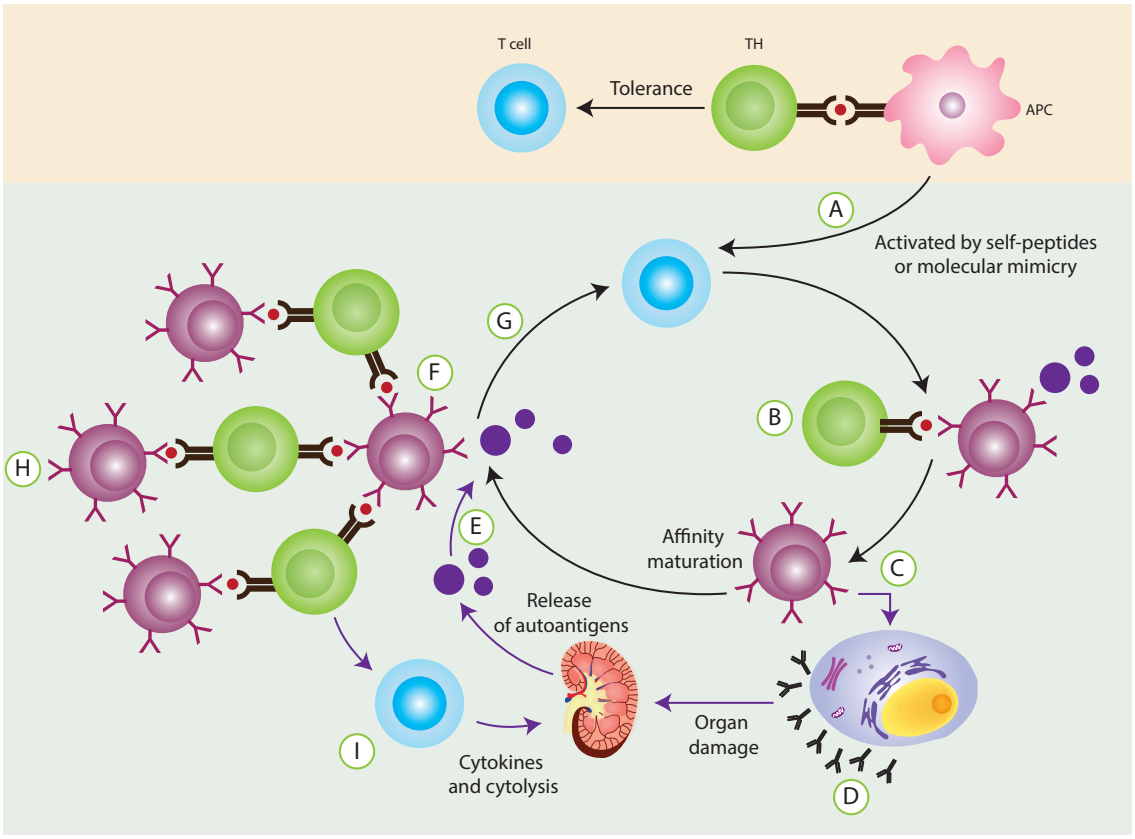

Figure 1. Mechanisms for the induction and amplification of lupus autoimmunity. **A** Although normal T cells exposed to self-antigen in the periphery become tolerized, lupus-prone T cells are sensitive to lower thresholds of activation by agonist or weak-agonist peptides. **B** Once activated, T cells can provide primary stimulation to genetically hyper-responsive B cells. **C** These autoantigen-stimulated B cells undergo somatic hypermutation and affinity maturation. **D** On the synthesis of pathogenic autoantibodies, tissue damage results in the release of self-antigen, **E,F** which is also taken up and presented by specific antigen-presenting B cells in a second round of T Cell activation, **G** therefore leading to a positive-feedback cycle. **H** Autoimmune T- and B-cell responses are diversified, which results in epitope spreading. This continuing and cyclic process of B cell–T cell cognate interaction serves to amplify the ensuing autoimmune processes. **I** Activated T cells can also directly cause tissue pathology by migrating to the target organ and releasing cytokines and by mediating direct cytotoxicity. APC, antigen-presenting cell. Adapted from Shlomchik *et al.* (29).

damage to the surrounding tissues (36); nevertheless, NETs may become a harmful source of autoantigens and promote autoimmunity if not promptly degraded as has been shown to occur in SLE (36). In fact, NETs expose a large amount of dsDNA together with immunostimulatory molecules (37) and can therefore aid autoantibody production.

ANTIBODY FORMATION AND PERPETUATION

To date more than 100 different autoantibodies have been described in SLE (38) with a widely varying frequency, ranging from some antibodies reported only in a few patients to others that are almost always present in SLE. Autoantibodies target a multitude of antigens, mainly nuclear components and other cellular constituents such as phospholipid-associated proteins, cytoplasmatic molecules, endothelial membrane antigens, complement fragments, IFNs, etc. (39).

In SLE, the breakdown of tolerance is often triggered by an infection, mostly due to molecular mimicry, epitope spreading, or bystander activation of immune cells (40), and, conceivably, to NET deposition (34). Of the different pathogens, the Epstein Barr virus (EBV) plays a major role due to its protein repertoire, which includes EBNA-1 (Epstein Barr nuclear antigen 1) and other molecules that may mimic or modify self-antigens, therebyrendering them more immunogenic (41) (See chapter 19). Other reported environmental triggers can also induce an autoimmune response (e.g., drug-induced SLE), mostly by acting at the epigenetic level (42).

AUTOANTIBODIES AND ORGAN DAMAGE

Of SLE autoantibodies, only anti-dsDNA have been shown to correlate with disease activity and specific organ damage (43). Anti-dsDNA antibodies are also included in the American College of Rheumatology (ACR) criteria for the classification

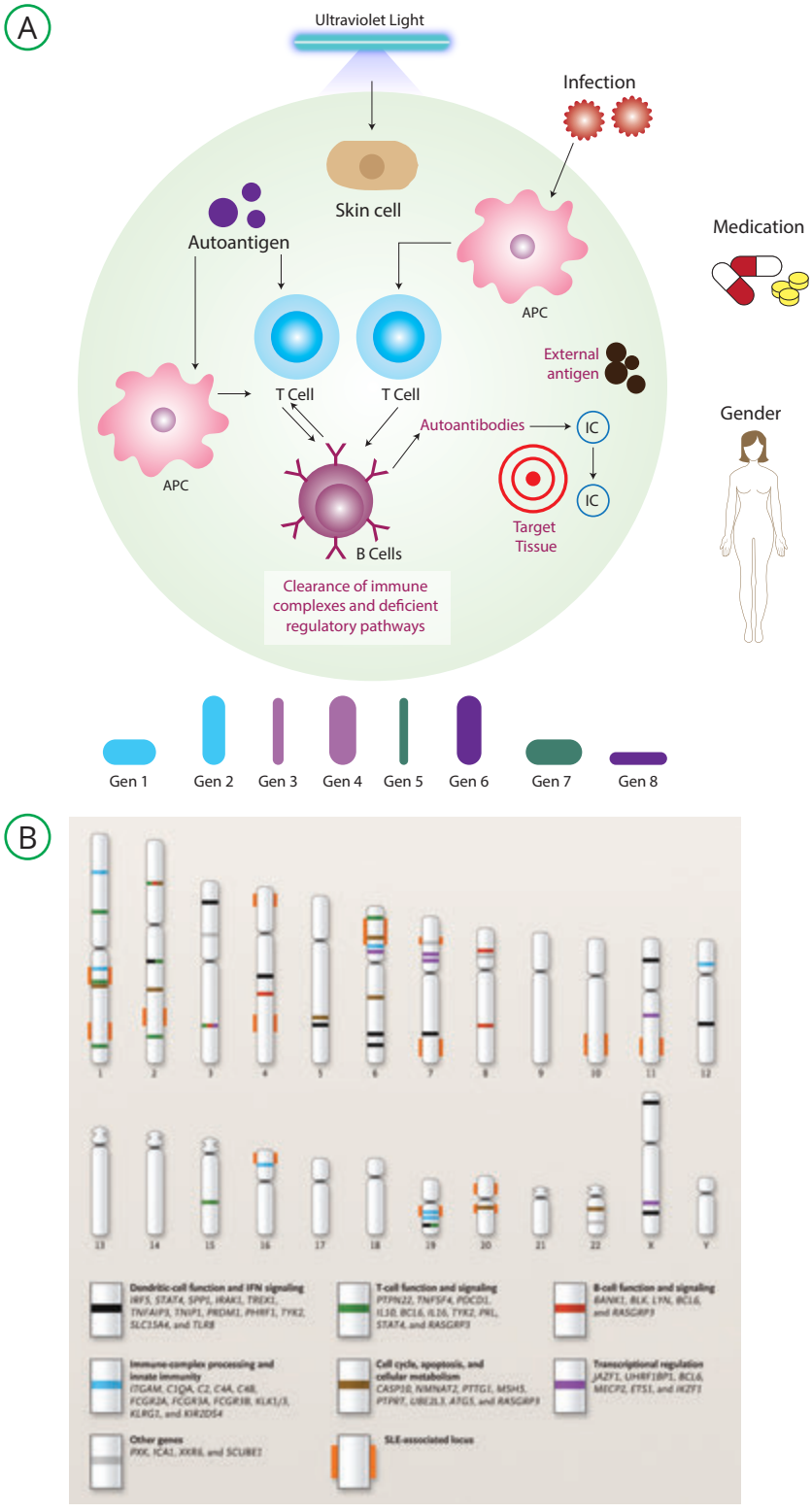

Figure 2. Immunopathogenesis of SLE. The disease is the result of interaction between hereditary and environmental factors over time. Immune abnormalities characterizing the disease are represented in frames to display susceptibility (or protection) genes and possible environmental stimuli that trigger the immune response **(A)**. External factors (i.e., infectious agent) introduce their own antigens to be processed by antigen presenting cells (APC). External stimuli (i.e., UV light) may induce autoantigen translocation, host cell activation or cell death, and thus lead to the release of autoantigens (e.g., U1 RNP, Ro/SS-A), which are processed by APC including B cells. Recognition of antigens, in the context of HLA, by T cells, induces T cell responses, making B cell produce pathogenic autoantibodies, which bind to the (auto) antigen to form immune complexes (IC). Target cells (e.g., glomerulus, endothelial, platelets) subsequently releas more autoantigens and perpetuate the process. Meanwhile, multiple regulatory circuits are ineffective (e.g., IC depuration, T and B regulatory functions). Multiple genes control each of these pathways in different cells **(B).** The approximate locations on the chromosomes of the genes associated with SLE are illustrated. The genes are divided into categories based on the main known function of the gene. Each category is represented by a different color on the 22 autosomal chromosomes and 2 sex chromosomes. An additional category (gray) includes genes that do not belong in these functional groups. Chromosome loci with orange bars on both sides indicate large SLE-associated loci. From Anaya *et al.* (11), and Tsokos (32), with permission.

of SLE together with anti-Sm, antiphospholipid (anti-PL) antibodies, and ANA. ANA may be found in sera of SLE patients many years before overt SLE occurs (44). Prediction based on genetic and serological biomarkers is an encouraging challenge (45). Noteworthy, the presence of an autoantibody by itself does not constitute a diagnosis of SLE. In addition, not all autoantibodies are pathogenic (i.e., with high affinity and specificity, activating the complement). Both pathogenic and non-pathogenic antibodies are known to exist in SLE as well as in other autoimmune conditions. In this respect, ANA may be classified as pathological (i.e., they only occur in people affected with an autoimmune or pre-autoimmune condition, es-

AUTOANTIBODY	PREVALENCE (%)	SENSITIVITY (%)	SPECIFICITY (%)	CORRELATION WITH DISEASE ACTIVITY	ASSOCIATION WITH CLINICAL FEATURES
ANA	95	95	75[a]	No	No
Anti-dsDNA	40-80	70	95	Yes	Initiation of LN
Anti-nucleosome	50-90	73-100	67-84	IgG3 only	Initiation of LN
Anti-C1q	30-50	50 (44-100 in active LN)	72 (70-92 in active LN)	Correlates with nephritic activity	Relapse of LN, hypocomplementemic urticarial vasculitis
Anti-Sm	10-55	26	98	No	NPSLE, mild LN, lung fibrosis, Pericarditis
Anti-ß2GPI IgM and IgG	17-49	29-32	79-86	No	Thrombosis[b], cerebrovascular accidents, NPSLE
Anti-CL IgM and IgG	23-53	Widely variable	66-94	No	Thrombosis[b], recurrent pregnancy loss, focal CNS involvement
LAC	30-40	45-61	66-73	No	Thrombosis[b], recurrent fetal loss, and thrombocytopenia
Anti-ribosomal P protein	13-40	20	100	Yes	NPSLE
Anti-Ro/SSA	24-60	40	Low	Controversial	Photosensitivity, interstitial lung disease, xerophthalmia, neonatal lupus (including congenital heart block), hepatitis, cytopenia and skin rash, prolongation of QT interval

Table 1. Antibodies in SLE and clinical associations. *ANA*: antinuclear antibodies; *anti-dsDNA*: anti-double stranded DNA; *anti-ß2GPI*: anti-ß2 glycoprotein I; *anti-CL*: anticardiolipin; *LAC*: lupus anticoagulant; *LN*: lupus nephritis; *CNS*: central nervous system; *NPSLE*: neuropsychiatric SLE.
[a] Obtained in patients with other connective tissue diseases.
[b] Persistent positivity increases the rate of arterial and venous thrombosis.

pecially SLE) or non-pathological (i.e. ANA do not predict the development of any autoimmune disorder and may also occur in healthy people, e.g. following infection) (46). As a corollary, SLE without ANA is unlikely, whereas ANA without SLE may occur. Moreover, pathological ANA may either be pathogenic, i.e., they trigger disease manifestations by any mechanism (e.g. nephritogenic, interferogenic or even neuropathogenic antibodies) or protective, i.e., they prevent disease by somehow counteracting pathogenic antibodies (e.g., by preventing immune complex formation) or, intriguingly, by hindering antigen antigenicity, meaning they can bind and mask some immunogenic epitopes and thereby carry out a kind of epitope selection which, in turn, prevents the immune cells from being tantalized. The mechanisms by which autoantibodies may harm cells are diverse and not mutually exclusive. They may vary depending on the nature and localization of the target antigens and on the effectiveness of the autoantibody itself, including direct cellular lysis, cell opsonization, immune complex deposition, complement fixation, and subsequent inflammation (43). Table 1 summarizes the most widespread autoantibodies in SLE and their links to the disease course.

FEMALE HORMONES AND SEX

Hormones contribute, through unknown mechanisms, to the increased prevalence of SLE in women (47). The X chromosome may contribute apart from hormones as in castrated female and male mice genetically manipulated to express XX, XO (female), XY, or XXY (male) combinations. the presence of two X chromosomes increases the severity of SLE (48). Among the genes known to contribute to the pathogenesis of SLE is CD40, which is located on chromosome X. Pregnancy may aggravate SLE and, although it is not clear whether rising levels of estradiol or progesterone play a role, a link between pregnancy outcome and the status of the disease at conception has been reported (49); in fact, the levels of these hormones are lower during the second and third trimesters in patients with SLE than in healthy pregnant women (50). Treatment with dehydroepiandrosterone has shown some clinical benefit (51). Pregnancy in patients with SLE presents a clinical challenge that requires the involvement of the relevant specialists.

CLINICAL MANIFESTATIONS

The clinical course of SLE is variable and may be characterized by periods of remissions and of chronic or acute relapses. Patients with SLE are subject to myriad symptoms, complaints, and inflammatory involvement that can affect virtually every organ (52). The most common pattern is a mixture of constitutional complaints with skin, musculoskeletal, mild hematologic, and serologic involvement (53). However, some patients have predominately hematologic, renal, or CNS manifestations. The pattern that dominates during the first few years of illness tends to prevail subsequently (54).

CONSTITUTIONAL SYMPTOMS

Fatigue, fever, and weight loss are typically present at some time during the course of the disease, occurring in 50 to 100 percent of patients. Fatigue is the most common complaint and, occasionally, the most debilitating. It occurs in 80 to 100 percent of patients, and its presence is not clearly correlated with other measures of disease activity (55). Fatigue is strongly associated with diminished exercise tolerance (56). However, fatigue may not be due to active SLE but to one or more of the following: increased work load, depression, unhealthy habits (smoking, fad diets, sedentary living, drug abuse), stress, anaemia, hypothyroidism, some medications (including prednisone and beta-blockers), any inflammatory and/or infectious disease, coexistent fibromyalgia, sleep disturbances, deconditioning, or a perception of poor social support. Fatigue due to SLE may respond to glucocorticoids or antimalarials and, in some studies, to exercise and psychosocial interventions (57, 58).

Weight loss often occurs prior to the diagnosis of SLE. Unintentional weight loss may be due to decreased appetite, the side effects of medications (particularly diuretics or antimalarials), and gastrointestinal disease (e.g., gastroesophageal reflux, abdominal pain, peptic ulcer disease, or pancreatitis).

Fever attributed to active disease is seen in over 50% of patients with SLE (52). Fever may also be caused by infections or drug reactions. The medical history may be helpful in determining the cause of fever. As an example, fever developing while on moderate or high doses of glucocorticoids should lead to a strong suspicion of new infection, particularly if other signs of active lupus have begun to remit. The pattern of fever may be helpful diagnostically. Episodic fever is suggestive of active SLE or infection; in contrast, sustained fever may reflect CNS involvement or an adverse drug effect (59).

1.- SPECIFIC SKIN DISEASE
A. Localized Acute Cutaneous LE (ACLE)
1. Localized ACLE (malar rash, butterfly rash) 2. Inflammatory periorbital edema 3. Mucous membrane lesions 4. Oral and nasopharyngeal ulceration 5. Alopecia (fractured frontal hair, scarring and nonscarring alopecia)
B. Generalized ACLE
1. Disseminated maculopapular eruption ± butterfly rash 2. Photosensitive lupus erythematosus 3. Toxic epidermal necrolysis-like eruption (apoptotic panepidermal necrolysis) 4. Bullous SLE (epidermolysis bullosa acquisita)
C. Subacute Cutaneous LE (SCLE)
1. Annular SCLE 2. Papulosquamous SCLE (psoriasiform, pityriasiform, and photosensitive LE)
D. Chronic Cutaneous LE (CCLE)
1. Classical discoid LE (localized or generalized) 2. Hypertrophic/verrucous discoid LE 3. Lupus profundus/lupus panniculitis 4. Mucosal discoid LE (oral or conjunctival) 5. Lupus tumidius (tumid LE, urticarial plaque of LE) 6. Chilblains LE 7. Lichenoid discoid LE (LE/lichen planus overlap) 8. Palmar-plantar DLE

Table 2. Skin lesions associated with systemic lupus erythematosus.

JOINT INVOLVEMENT

Joint symptoms occur in > 90% of patients at some time during the disease course and are often the earliest manifestation (59). Arthralgia is more often encountered than arthritis. Arthritis occurs in around 70% of patients and tends to be migratory and symmetrical. Only a few joints are usually affected, especially those of the hands. The arthritis is moderately painful and is rarely deforming. When this occurs (i.e., Jaccoud's arthropathy), rheumatoid factor may be present and renal involvement is rarely observed (11).

MUCOCUTANEOUS

The skin and/or mucous membranes are involved at some point in > 80% of patients with SLE (60, 61). There is great variability and diversity in the type of involvement, ranging from the classic butterfly rash to fixed lesions that may be associated with scarring and atrophy (also referred to as discoid lupus erythematosus). In addition, cutaneous bullae, oral and nasopharyngeal ulcers, scarring and non-scarring alopecia, and skin changes resulting from vasculitis may occur in patients with SLE. Photosensitivity is a common theme for lesions characterized by an interface dermatitis and for the tumid lesions characterized by dermal mucin and lymphohistiocytic perivascular and peri-appendageal infiltrate. This includes discoid LE lesions and lesions of subacute cutaneous lupus erythematosus, acute cutaneous LE, and tumid LE. In some chronic forms, the rash can be disfiguring and may require aggressive therapy to minimize scarring and dyspigmentation (62).

Inflammatory periorbital edema is uncommon in patients with SLE, which is useful as a distinguishing feature from dermatomyositis. Patients with localized discoid LE, hypertrophic LE, LE panniculitis, and lupus tumidus tend to have skin disease only; however, progression to systemic disease is possible (63). Table 2 summarizes the main skin lesions in SLE.

RAYNAUD PHENOMENON

Cold- or emotion-induced color changes in the digits of the hands and/or feet (Raynaud phenomenon) are frequent problems and may antedate other features of SLE. Self-reported skin color changes consistent with Raynaud phenomenon occurred in 16 to 40 percent of patients in two large series (51).

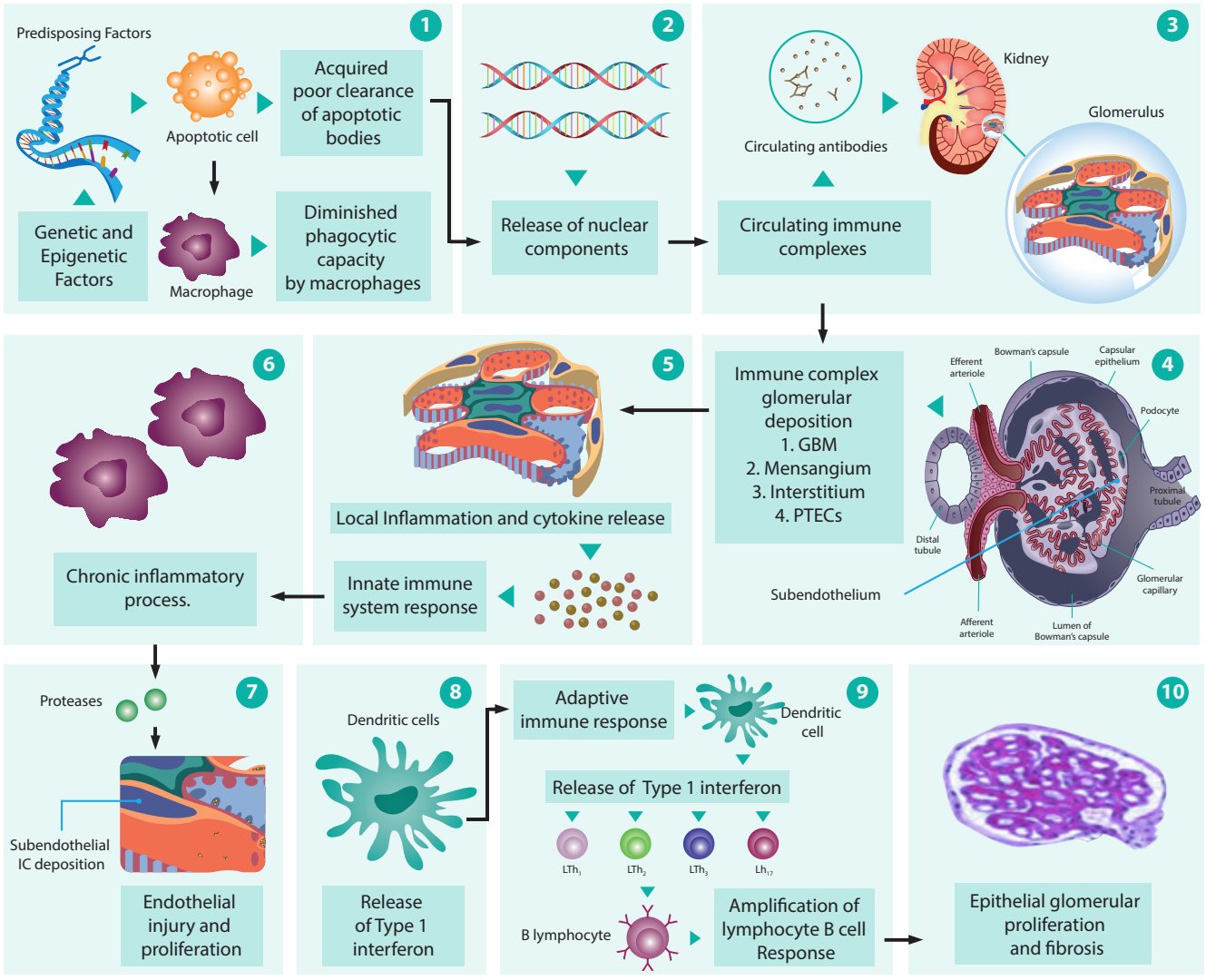

Figure 3. Pathology of lupus nephritis. There is an imbalance between cytokine homeostasis and immune complex (IC) deposition. In predisposing susceptible individuals who develop SLE, there is an acquired poor clearance of apoptotic bodies and a diminished phagocytic capacity by macrophages [1]. Early formation of immune complexes (ICs) include antinucleosomes, anti-double-stranded DNA (anti-dsDNA), DNA extractable nuclear antigen antibodies (ENAs), antibodies against C1q complex of the complement system, free DNA, antiribonucleoproteins (anti-RNP), and histones as byproducts of inefficient phagocytosis of apoptotic bodies [2]. Circulating ICs are deposited initially at the glomerular base membrane (GBM), mesangium, and interstitial tissue within the proximal tubular epithelial cells (PTECs) [3] and [4]. The deposited ICs initiate the release of proinflammatory cytokines and chemokines such as monocyte chemoattractant protein 1 (MCP-1), interleukins 1 and 6 (IL-1, IL-6), and adhesion molecules (CAMs) establishing a chronic inflammatory process [5]. The resulting overload of the mesangial phagocytic system (innate immune system) leads to deposits of subendothelial ICs becoming an easy target for monocyte migration and infiltration and generating endothelial injury and proliferation [6] and [7]. In turn, the adaptive immune system is activated secondary to the presence of ICs and dendritic cells (DCs) [8], which subsequently trigger release of type 1 interferon and induce maturation and activation of infiltrating T cells. This activation leads to sequential amplification of T helper 2 lymphocytes (Th2), T helper 1 (Th1), and T helper 17 (Th17) [9]. Each of these amplifies lymphocyte B cell response and further activates macrophages, generating a second general response, which increases recruitment of effector cells that can no longer be modulated by regulatory T cells and resulting in the end in epithelial glomerular proliferation and fibrosis [10]. From De Zubiria Salgado & Herrera-Diaz (65), with permission.

RENAL

Lupus nephritis (LN) is common in SLE. An abnormal urinalysis with or without elevated plasma creatinine levels is present in a large proportion of patients at the diagnosis of LN, and may eventually develop in up to 75 percent of patients with a diagnosis of SLE. The most frequently observed abnormality in patients with LN is proteinuria (58). There are several types of renal disease in SLE, mostly immune complex-mediated glomerular disease, which are usually differentiated by renal biopsy. In addition, renal diseases unrelated to lupus may be seen (64). The pattern of glomerular injury seen in SLE (and in other immune com-

plex-mediated glomerular diseases) is primarily related to the site of formation of the immune deposits, which are mainly due to anti-double stranded DNA antibodies (anti-dsDNA, or anti-DNA) directed against nucleosomes (i.e., double stranded DNA wound around a histone octamer) (Figure 3) (65). The higher incidence of LN in patients with SLE in the United States as compared with Europe may in part reflect racial and ethnic differences. The incidence of LN is higher in blacks (34 to 51 percent), Hispanics (31 to 43 percent), and Asians (33 to 55 percent) than in whites (14 to 23 percent). Blacks and Hispanics also tend to present with more-severe underlying histopathology, higher serum creatinine levels, and more proteinuria than whites (66, 67). In addition, Blacks, Hispanics, and those living in poverty have a worse prognosis than whites and people with a higher socioeconomic status.

Patients with SLE should undergo testing for renal involvement at regular intervals, including a urinalysis with examination of the urinary sediment, an estimate of urine protein excretion (usually a spot urine protein-to-creatinine ratio), and a serum creatinine and estimated glomerular filtration rate (67). Elevated anti-DNA titers and low complement (C3 and C4) levels often indicate active lupus, particularly LN. The frequency of testing depends upon whether or not the patient has a previous history of renal involvement.

A kidney biopsy should be done on all patients with SLE who develop evidence of renal involvement in order to establish the diagnosis and the class of LN. Determining the

Class I	**Minimal mesangial lupus nephritis**
	Normal glomeruli by light microscopy, but mesangial immune deposits by immunofluorescence
Class II	**Mesangial proliferative lupus nephritis**
	Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits
	May be a few isolated subepithelial or subendothelial deposits visible by immunofluorescence or electron microscopy, but not by light microscopy
Class III	**Focal lupus nephritis[a]**
	Active or inactive focal, segmental or global endo- or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations
Class III (A)	Active lesions: focal proliferative lupus nephritis
Class III (A/C)	Active and chronic lesions: focal proliferative and sclerosing lupus nephritis
Class III (C)	Chronic inactive lesions with glomerular scars: focal sclerosing lupus nephritis
Class IV	**Diffuse lupus nephritis[b]**
	Active or inactive diffuse, segmental or global endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental (IV-S) lupus nephritis when ≥50% of the involved glomeruli have segmental lesions, and diffuse global (IV-G) lupus nephritis when ≥50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation
Class IV-S (A)	Active lesions: diffuse segmental proliferative lupus nephritis
Class IV-G (A)	Active lesions: diffuse global proliferative lupus nephritis
Class IV-S (A/C)	Active and chronic lesions: diffuse segmental proliferative and sclerosing lupus nephritis
	Active and chronic lesions: diffuse global proliferative and sclerosing lupus nephritis
Class IV-S (C)	Chronic inactive lesions with scars: diffuse segmental sclerosing lupus nephritis
Class IV-G (C)	Chronic inactive lesions with scars: diffuse global sclerosing lupus nephritis
Class V	**Membranous lupus nephritis**
	Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations
	Class V lupus nephritis may occur in combination with class III or IV in which case both will be diagnosed
	Class V lupus nephritis show advanced sclerosis
Class VI	**Advanced sclerosis lupus nephritis**
	≥90% of glomeruli globally sclerosed without residual activity

Table 3. International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis. [a] Indicate the proportion of glomeruli with active and with sclerotic lesions. [b] Indicate the proportion of glomeruli with fibrinoid necrosis and/or cellular crescents. Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.

class of LN is important for the following reasons: 1) treatment is guided by the histological subtype (i.e., the International Society of Nephrology/Renal Pathology Society or World Health Organization class, the degree of activity and chronicity) and by complicating lesions such as interstitial nephritis and thrombotic microangiopathy, and 2) the clinical presentation may not accurately reflect the severity of the histological findings. For example, proliferative lupus may be present even if the patient has minimal proteinuria and normal serum creatinine.

Most patients with LN have an immune complex-mediated glomerular disease. Over the last two decades, there have been several attempts by different societies, particularly the World Health Organization (WHO), to classify the different glomerulopathies associated with SLE. Based upon clinical and pathologic correlations, an LN classification system was developed by a group of renal pathologists, nephrologists, and rheumatologists in 2004 (the International Society of Nephrology, or ISN, classification) (68) (Table 3).

Some patients with renal disease have a histological pattern of injury that is indistinguishable from LN, but have no extra-renal symptoms, signs, or laboratory abnormalities suggestive of SLE (69).

GASTROINTESTINAL TRACT

The gastrointestinal tract is often involved, mostly from medication side effects than from active SLE. Examples of the former include gastritis and even peptic ulcers secondary to the use of nonsteroidal antiinflammatory drugs (NSAIDs) alone or in combination with glucocorticoids. On the other hand, SLE vasculitis can lead to pancreatitis, peritonitis, and colitis. Symptoms of esophageal irritation or reflux may occur. Nonspecific abdominal pain is frequent. Liver involvement in lupus is rare, and a presentation with liver abnormalities and a positive ANA test is more consistent with chronic active hepatitis, as part of polyautoimmunity (i.e., "lupoid hepatitis") (70).

PULMONARY

Pleurisy, pleural effusion, pneumonitis, interstitial lung disease, pulmonary hypertension, and alveolar hemorrhage can all occur in SLE. The risk of thromboembolic involvement is increased in patients with antiphospholipid antibodies or lupus anticoagulant. The presence of dyspnea, episodic pleuritic chest pain, and a progressive decrease in lung volume in the absence of interstitial fibrosis or significant pleural disease suggests shrinking lung syndrome. Pulmonary function tests are often significantly abnormal with restrictive abnormalities, prior to complaints of dyspnea (71).

CARDIOVASCULAR

There are a variety of cardiovascular manifestations in SLE. Pericarditis is relatively common. Verrucous (Libman-Sacks) endocarditis is usually clinically silent, but may lead to valvular insufficiency and may be a source of emboli. Myocarditis is uncommon but may be severe. Patients with SLE have an increased risk of coronary artery disease (72) (see Chapter 38). Accelerated atherosclerosis with coronary heart disease (CHD) is a significant cause of morbidity and premature death in SLE patients. The greatest increase in relative risk (55-fold greater) is among young women, who otherwise have a low risk of CHD (73).

NEUROLOGIC

Neurologic and psychiatric symptoms are reported to occur in 10 to 80 % of patients either prior to the diagnosis of SLE or during the disease course (74, 75). The wide range in reported prevalence reflects, in part, the use of different criteria for neuropsychiatric disease. The American College of Rheumatology (ACR) has formulated case definitions, reporting standards, and diagnostic testing recommendations for 19 neuropsychiatric SLE syndromes. Neurological complications include cognitive defects, organic brain syndromes, delirium, psychosis, seizures, headache, and/or peripheral neuropathies. Other, less common problems are movement disorders, cranial neuropathies, myelitis, and meningitis. Psychosis, which may be due to SLE or to glucocorticoid treatment, is one of several psychiatric manifestations of SLE. Others include depression, anxiety, and mania. Thromboembolic events, often in association with antiphospholipid antibodies or with lupus anticoagulant, may occur in around 20 percent of patients with SLE (76). Arterial thromboemboli may cause focal neurological problems, such as stroke or seizures, and/or more diffuse cognitive defects.

The term lupus cerebritis refers to the neuropsychiatric manifestations of lupus that appear to have an organic basis, rather than a specific pathophysiological mechanism. Assaying for specific autoantibodies can help to make the distinction between organic and functional causes of some neuropsychiatric symptoms. Some investigators believe that a strong association exists between the occurrence of neuropsychiatric symptoms and the presence of antineuronal and other antibodies (77). Anti-ribosomal P antibodies have been associated with lupus psychosis and depression by some authors (77), but other authors have not confirmed this association. Some data suggest that cognitive defects may be associated with the presence of elevated levels of antineuronal antibodies, antiphospholipid antibodies, or antibodies to N-methyl-D-aspartate (NMDA) receptors (78).

OPHTHALMOLOGIC

The eye is frequently involved in SLE, with the most common manifestation being keratoconjunctivitis sicca. Note that, not all patients with Sicca symptoms have Sjögren's syndrome (see Chapter 28). Uncommon or rare ophthalmologic manifestations of SLE include: cotton wool exudates due to retinal vasculitis, episcleritis or scleritis, and anterior uveitis (iritis, iridocyclitis).

HEMATOLOGIC

Cytopenias and thrombophilia, an increased propensity to develop thromboembolic disease, may be features of SLE. Patients with SLE frequently develop abnormalities in one or more of the three blood cell lines. Leukopenia is common. While diagnostically useful, it is usually not symptomatic unless severe (i.e., <2000/mm3). White blood counts of <4500/mm3 have been noted in 43 to 66 percent of patients. Many patients have mild anemia, which is most often due to the anemia of chronic disease. Hemolytic anemia is rare but can be very severe. Other patients may have hemolytic anemia, which is more severe and requires immediate therapy. Thrombocytopenia is also frequently seen. However, bleeding usually occurs only with platelet counts < 25,000/mm3. Acute thrombocytopenia is usually associated with active disease. However, some patients have chronic thrombocytopenia, which does not require therapy unless there is evidence of bleeding.

Pancytopenia may result either from peripheral destruction of red cells, leukocytes, and platelets together or from bone marrow failure. Thus, in patients with pancytopenia, bone marrow examination is the most important diagnostic test. There are a number of potential causes to consider such as drugs, other diseases, and bone marrow abnormalities.

An uncommon cause is macrophage activation syndrome. Lymphadenopathy occurs in approximately 50 % of patients with SLE most frequently at disease onset or in association with an exacerbation. Splenomegaly may also be present, particularly during active disease. A lymph node biopsy may be warranted when the degree of lymphadenopathy is out of proportion to lupus activity. Other causes include infection or a lymphoproliferative disorder such as non-Hodgkin lymphoma or angioimmunoblastic T cell lymphoma.

Some patients with SLE, particularly those with antiphospholipid antibodies or with severe nephrotic syndrome, have an increased risk of thromboembolic disease which, in nephrotic syndrome, may manifest as renal vein thrombosis, venous thromboembolism, or arterial disease.

CLASSIFICATION AND DIAGNOSIS

The diagnosis of SLE is straightforward in a patient who presents with several compatible clinical features and who has supportive laboratory studies. However, SLE can also cause isolated cytopenias or single organ involvement (e.g., nephritis or pericarditis) or may first manifest as an incidental laboratory finding, such as a biological false positive test for syphilis. Such patients may subsequently develop the characteristic multisystem features of SLE over a period of months or years.

LABORATORY TESTING

Laboratory tests that may provide diagnostically useful information when SLE is suspected include: complete blood count and differential, comprehensive metabolic profile, creatine kinase, erythrocyte sedimentation rate and/or C reactive protein, urinalysis, and 24-hour urine collection for calculation of creatinine clearance and for quantification of proteinuria or protein/creatinine ratios.

Autoantibody testing is also indicated. Autoantibodies routinely assayed are: ANA, antibodies to double stranded DNA (dsDNA), anti extractable nuclear antigen antibodies (ENAs, i.e., anti-RNP, anti-Sm, anti-Ro and anti-La antibodies), and antiphospholipid antibodies (i.e., lupus antiacoagulant, anticardiolipin antibodies, and anti-beta 2-glycoprotein I antibodies).

Measurement of serum complement levels C3 and C4 may also be helpful, since hypocomplementemia is a frequent finding in active SLE and it is associated with activity of disease.

Most clinicians rely upon the diagnostic criteria for lupus that were developed by the ACR (79) (Table 4). These criteria were developed for the classification of SLE patients

CRITERION	DEFINITION
Malar rash	A rash on the cheeks and nose, often in the shape of a butterfly
Discoid rash	A rash that appears as red, raised, disk-shaped patches
Photosensitivity	A reaction to sunlight that causes a rash to appear or get worse
Oral ulcers	Sores in the mouth
Arthritis	Joint pain and swelling of two or more joints
Serositis	Inflammation of the lining around the lungs (pleuritis) or inflammation of the lining around the heart causes chest pain, which is worse with deep breathing (pericarditis)
Kidney disorders	Persistent protein or cellular cast in the urine
Neurologic disorders	Seizures or psychosis
Blood disorders	Anaemia (low red-cell count), leukopenia (low white cell count), or thrombocytopenia (low platelet count)
Immunologic disorders	Post-test for anti-double-stranded DNA, anti-Sm or antiphospholipid antibodies
Abnormal antinuclear antibodies	Positive antinuclear-antibody test

Table 4. American College of Rheumatology criteria for the diagnosis of systemic lupus erythematosus.

when SLE was compared with other rheumatic diseases for study purposes.

In an effort to address these weaknesses, a consensus group of experts on SLE, the Systemic Lupus International Collaborating Clinics (SLICC), has proposed revised criteria for SLE (80), which require either that a patient satisfy at least 4 of 17 criteria, including at least 1 of the 11 clinical criteria and one of the six immunologic criteria, or that the patient has biopsy-proven nephritis compatible with SLE in the presence of ANA or anti-dsDNA antibodies. These criteria were developed as classification criteria, which are most applicable to use for clinical and epidemiologic research, and the SLICC group has proposed that they could serve as alternative classification criteria for use in SLE clinical care and research. They have not been evaluated for use in diagnosis.

DISEASE ACTIVITY AND SEVERITY

An effective therapeutic regimen first requires confirmation of the diagnosis and accurate determination of both disease activity and severity. Disease activity usually refers to the degree of inflammation while the degree of severity depends on the level of organ dysfunction and the organ's relative importance. The degree of irreversible organ dysfunction is known as the "damage index" (81).

A number of research protocols on disease activity or damage measures or indices, including the Systemic Lupus Erythematosus (SLE) Disease Activity Index (SLEDAI), the Safety of Estrogens in Lupus Erythematosus: National Assessment-SLEDAI (SELENA-SLEDAI), the Systemic Lupus Activity Measure (SLAM), the British Isles Lupus Assessment Group (BILAG), the European Consensus Lupus Activity Measurement (ECLAM), etc., have been designed in an attempt to better monitor disease activity and assess trial outcomes. They all use a combination of the history, examination, and laboratory data; these protocols may have general applicability to clinical practice if simplified. The particular outcome measure used in a trial, even for organ-specific disease such as LN, can influence apparent trial outcomes (82).

TREATMENT

Although the pattern and severity of organ involvement determines specific drug therapy, a number of general issues are applicable to every patient with SLE. Patients should also be informed of the availability of resource information available from the treating center and from lupus support organizations.

GENERAL CONSIDERATIONS

Avoid exposure to direct or reflected sunlight and other sources of ultraviolet (UV) light (e.g., fluorescent and halogen lights). Use sunscreens, preferably those that block both UV-A and UV-B, with a high skin protection factor (SPF). A sunscreen with a SPF of 55 or greater is suggested. Limited data exist concerning the effect of dietary modification in SLE. Patients with hyperlipidemia should be encouraged to eat a low-fat diet. Inactivity produced by acute illness causes a rapid loss of muscle mass, bone demineralization, and loss of stamina resulting in a sense of fatigue. This can usually be treated with isometric and graded exercise. Cigarette smoking may increase the risk of developing SLE (83) and smokers in general have more active disease (84). Patients should be counselled not to smoke or to quit smoking and should be provided with help to do so. Hidroxychloroquine is less effective in smokers (85).

ORGAN INVOLVEMENT

A number of medications are commonly used in the treatment of SLE, including (NSAIDs), antimalarials (primarily hydroxychloroquine), glucocorticoids, and immunosuppressive agents (including cyclophosphamide, cyclosporine, tacrolimus, leflunomide, methotrexate, azathioprine, mycophenolate, and belimumab). Patient compliance with recommended treatment is, as expected, associated with better outcomes than noncompliance.

Topical therapies are often useful for cutaneous manifestations of lupus and reduce the risk of side effects associated with the systemic use of NSAIDs, glucocorticoids or immunosuppressants. NSAIDs are generally effective for musculoskeletal complaints, fever, headaches, and mild serositis. Naproxen may have greater relative cardiovascular safety than other NSAIDs. Celecoxib has been used in SLE patients (86). Antimalarials are most useful for skin manifestations and musculoskeletal complaints. In addition, in long-term studies, the use of antimalarials such as hydroxychloroquine prevented major damage to the kidneys and CNS (87). Their use may also reduce the risk of disease flares, though this is less clear for renal and CNS manifestations.

Systemic glucocorticoids (e.g., high doses of 1 to 2 mg/kg/day of prednisone or equivalent or intermittent intravenous pulses of methylprednisolone, 10-15 mg/Kg/d for three days) used alone or in combination with immunosuppressive agents are generally reserved for patients with significant organ involvement, particularly renal and CNS disease. There is a paucity of data to support the use of intravenous pulses versus daily oral glucocorticoids (88). Patients with organ-threatening disease (e.g., cardiopulmonary, hepatic, renal, hemolytic anaemia, immune thrombocytopenia) are usually given the above-mentioned oral doses, whereas non-organ-threatening disease (e.g., cutaneous, musculoskeletal, constitutional) patients usually respond to 5 to 15 mg of prednisone (or equivalent) daily until a glucocorticoid-sparing agent or antimalarial can take effect.

Immunosuppressive medications other than glucocorticoids (e.g., methotrexate, cyclophosphamide, azathioprine, mycophenolate, or rituximab) (89) are generally reserved for patients with significant organ involvement and/or for patients who respond inadequately to glucocorticoids.

Immunosuppressive agents such as mycophenolate, azathioprine, or cyclophosphamide are given with glucocor-

ticoids to patients with more than mild LN, and cyclophosphamide is given to those with alveolar hemorrhage, systemic vasculitis, and to most patients with significant CNS involvement. Lower doses of glucocorticoids (e.g., ≤10 mg/day of prednisone) may be used for symptomatic relief of severe arthralgia, arthritis, or serositis while awaiting a therapeutic effect from other medications.

Belimumab is a fully-human monoclonal antibody that inhibits the biological activity of the soluble form of a B cell survival factor, B-lymphocyte stimulator or BLyS [also known as B-cell activating factor belonging to the tumor necrosis factor (TNF) family (BAFF)]. Belimumab is a new therapy for the treatment of patients with active SLE who are receiving standard therapy, such as NSAIDs, glucocorticoids, or antimalarials, and/or immunosuppressives (90). However, it has not been adequately studied in patients with severe active LN or with CNS lupus or in patients who have previously used rituximab or who have recently used intravenous cyclophosphamide (90).

HEMATOPOIETIC STEM CELL TRANSPLANTATION

The proposed mechanism of action of hematopoietic stem cell transplantation is that it provides a period free from memory T Cell influence during which maturation of new lymphocyte progenitors can occur without recruitment to anti-self activity (91). Autologous stem cell transplantation remains complex, costly and, despite improvements in treatment-related mortality, risky. Additional studies, including direct comparison with more conventional treatment approaches in randomized controlled trials, are needed before any recommendation can be made regarding the role of stem cell transplantation in the treatment of SLE. The use of allogenic stem cells is an interesting alternative for which there is insufficient data to assess efficacy or safety. (91).

IMMUNIZATIONS

Patients should be advised to receive appropriate immunizations prior to the initiation of immunosuppressive therapies. It had previously been thought that immunization could exacerbate SLE. However, the influenza and pneumococcal vaccines are safe, but the resulting antibody titers are somewhat lower in patients with SLE than in controls (91). The use of glucocorticoids such as prednisone, or other immunosuppressive agents may contribute to the blunted antibody response. In contrast, it is inadvisable to immunize potentially-immunosuppressed patients (including those treated with glucocorticoids alone at doses equivalent to ≥20 mg/day of prednisone for more than two weeks) with live vaccines (e.g., measles, mumps, rubella, polio, and varicella) (92). While the issue of efficacy of hepatitis B (HepB) vaccination has not been completely resolved, the risks posed by this vaccination in SLE patients can be regarded as minimal (93).

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26 ANTIPHOSPHOLIPID SYNDROME

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INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder defined by the persistent presence of antiphospholipid antibodies (aPL) in plasma of patients with vascular thrombosis and/or pregnancy morbidity. APL are formally detected by functional coagulation assay (the so called lupus anticoagulant LAC) and/or by solid phase assays: anti-cardiolipin (aCL) or anti-β2glycoprotein I (anti-β2GPI) antibody tests.

The clinical features and laboratory manifestations associated with aPL have broadened considerably since the first description of APS in 1983 (1) and now include thrombocytopenia, hemolytic anemia, cardiac valve disease, pulmonary hypertension, microangiopathic nephropathy, skin ulcers, livedoreticularis, refractory migraine, cognitive dysfunction, and atherosclerosis (2).

Classification criteria for APS were developed at the 1998 antiphospholipid antibodies congress (8th APLA) and have recently been updated (3). APS is defined by the presence of at least one of the clinical criteria and one of the laboratory criteria. The criteria define patients with "primary" or isolated APS (PAPS) as those with the diagnosis in the absence of any underlying autoimmune disease, and patients with "secondary" APS (SAPS) as those with another autoimmune disorder associated, aPL and the clinical manifestations of the syndrome (3).

A variant of APS characterized by acute thrombotic microangiopathy with subsequent multiorgan failure and high mortality has also been identified: the so-called catastrophic APS (CAPS). (4) Probable and microangiopathic APS are additional disease subsets that have been suggested but not yet formally accepted (4).

There have been recent changes in the management and diagnosis of APS and in current opinions on its pathogenesis. The aim of this chapter is to provide an update on recent insights into the epidemiology, pathophysiology, diagnosis, and treatment of APS.

EPIDEMIOLOGY

A cumulative, retrospective, literature analysis indicates that approximately 30% to 40% of patients with aPL have a history of thrombosis and that 30% of the events are of the arterial bed (5). Cerebral circulation is the most commonly affected arterial region while the coronary arteries and other arterial regions are less frequently reported (6). A meta-analysis of case-control, cross-sectional, and retrospective-prospective studies of the association between LAC, IgG/IgM aCL, and arterial thrombosis reported a strong association with LAC (odds ratio up to 10) while a much-weaker association was found for aCL. In general, only the aCL IgG isotype and moderate/high antibody titers (i.e. >40 GPL units) were significantly associated with arterial thrombosis (first cerebral stroke or myocardial infarction) (7). Arterial events are also the most frequent recurrences, even in already diagnosed and treated APS patients, as reported in the 5-year follow-up on the European series. Specifically, strokes (2.4% of the total cohort) and transient ischemic attacks (2.3%) were the most frequent recurrent manifestations. In addition, myocardial infarction (18.9%) and stroke (13.2%) were also causes of mortality in the same series (8).

Venous thrombosis (VT), usually deep vein thrombosis (DVT), is the most common clinical manifestation of APS and occurs in more than 30% of the patients (9). The frequency of aPL in venous thrombosis is reported to range from 5.2% to 30% for any aPL, 0.6-16% for LAC, and 4-24% for aCL (10). Prospective studies of the general population have shown that aPL are predictive of a first DVT, recurrent thromboembolism, and death (11). Elevated anti-β2GPI IgG significantly reduces the OR for venous thromboembolism (VTE)

by about five-fold (OR: 5.2, 95%CI: 1.5-18) and displays a stronger risk association for VTE than elevated aCL IgG (12).

Pregnancy complications have been observed in aPL-positive women (10-19%) (9). A significantly increased prevalence of IgG anti-β2GPI was found in pregnancies complicated by preeclampsia–eclampsia (up to 10%). LAC has been associated with late recurrent fetal loss (OR: 7.79, 95%CI 2.30–26.45). This association was stronger than that for any other aPL. When all titers were combined, IgG aCL were associated with both early (OR: 3.56, 95%CI: 1.48–8.59) and late, recurrent fetal loss (OR: 3.57, 95%CI: 2.26–5.65) (13).

APS can occur in association with other systemic autoimmune diseases, especially systemic lupus erythematosus (SLE). The prevalence of aPL in patients with SLE ranges from 12% to 44% for aCL, 15% to 34% for LAC, and 10% to 19% for anti-β2GPI. Longitudinal studies show that APS may develop in 50 to 70% of patients with both SLE and aPL after 20 years of follow-up (9, 14, 15).

APL can be found in apparently healthy control subjects with a prevalence ranging from 1 to 5% for both aCL and LAC although at low titers in most cases (10). An increased prevalence of antibodies detectable by all assays has been reported with ageing (16).

Since an association between aPL and syphilis was first described, many other viral, bacterial, and parasitic infections have been shown to induce aPL. The most common infections associated with aPL include hepatitis C virus, human immunodeficiency virus (HIV), cytomegalovirus, varicella-zoster, Epstein–Barr virus, adenovirus, and parvovirus B19 with a prevalence of up to 49% in HIV infections. In most infections, the antibodies found were β2GPI independent and thus did not interfer with anticoagulation, but there are some exceptions. With respect to bacterial infections, aCL are often present in leprosy (42.7%), where they frequently display anti-β2GPI activity (44.8%), and in syphilis infections (8 to 67%). Antiphospholipid antibodies associated with infections are usually transient and followed by APS clinical manifestations only in exceptional cases (17).

Active vaccination may induce the production of autoantibodies, including aPL. However, reports are anecdotal and the antibodies usually transient, at low titers, and with no relationship to clinical manifestations. In other cases, a cause-effect relationship between infections and the development of CAPS or thrombotic events in APS patients has been suggested (18).

APL can be found in a high percentage of children without any underlying disorder with an estimated frequency that ranges from 3 to 28% for aCL and 3 to 7% for anti-β2GPI. The reason for this frequent occurrence in comparison to adults is suggested to be the frequent infectious processes that occur during childhood (19).

PATHOPHYSIOLOGY OF THROMBOSIS IN APS

Multiple molecular pathways are implicated in the pathogenesis of APS. The mechanisms of thrombosis production in patients with APS are not completely clear. However, the interaction between aPL and cells involved in the regulation of hemostasis is one of the mechanisms responsible for the thrombophilic state in APS. Current evidence suggests that inhibition of physiological anticoagulant pathways, impairment of fibrinolysis, and promotion of clot formation result in thrombus formation in APS (20).

Despite the persistent presence of aPL in the circulation, thrombotic events in patients with aPL only occur occasionally, suggesting that the presence of aPL is necessary but not sufficient for clot formation in vivo. The 'two-hit hypothesis' has been proposed in which aPL (first hit) can only exert their prothrombotic influence in the presence of another thrombophilic condition (second hit). This 'two-hit hypothesis' was demonstrated in an animal model of APS in which the injection of aPL in rats only resulted in increased thrombus formation when rats were pre-treated with lipopolysaccharide (LPS), but not when they were injected with pure buffer (21).

The major antigen structures recognized by aPL in patients with APS are phospholipid-binding proteins, β2GPI and prothrombin, which are expressed on the membranes of different cell types. The antibody forms a complex with the corresponding antigen which leads to cell perturbation, activation of cell signaling pathways, transcription of pro-coagulant substances, adhesion molecules and, subsequently, thrombus formation. Table 1 shows aPL-mediated pathogenic mechanisms. aPL are reported to have heterogeneous interactions with endothelial cells, monocytes, and platelets (22). Antibodies directed against plasma protein ß2GPI are considered the most clinically significant as several studies have shown this aPL is strongly related to thrombosis. ß2GPI is a plasma protein that consists of five domains. Recent studies have unveiled domain I of ß2GPI as an integral component of the pathogenicity of this protein (23). The interaction of ß2GPI with phospholipids induces major conformational changes in this protein which expose hidden epitopes within domain I. Point mutations within domain I were shown to abrogate binding of anti-ß2GPI (24, 25). It was confirmed that anti-ß2GPI with specificity to domain I increases susceptibility to venous thrombosis (24).

Nitric oxide (NO) produced by eNOS facilitates the prevention of thrombosis. The activation of eNOS is pertinent in preventing thrombosis as it inhibits platelet aggregation and down regulates adhesion molecules involved in clot formation. It is postulated that aPL may interfere in the activation of eNOS and subsequent NO production (26).

A number of findings suggest that the antiphospholipid syndrome is characterized by greater oxidative stress. Paraoxonase activity, which accounts for the antioxidant properties of high-density lipoprotein cholesterol [preventing oxidation of low-density lipoprotein (LDL) cholesterol], is significantly lower in people with the syndrome (27, 28), whereas levels of 8-epi-prostaglandin F2, a biomarker of lipid peroxidation, are elevated (29).

Plasma levels of ß2GPI–oxidized LDL complexes are elevated in patients with APS as compared to healthy controls (30). Oxidative stress plays a direct role in the structure and function of ß2GPI in patients with APS (31).

aPL-MEDIATED THROMBOSIS
Interference with the components of the coagulation cascade
Protein C pathway
Protein S pathway
Contact activation pathway
ß2GPI-thrombin interaction
Cell interaction
Induction of pro-inflammatory phenotype on endothelial cells
Induction of pro-coagulant activity on endothelial cells and monocytes
Release of membrane-bound microparticles
Pro-coagulant effects on platelets
Disruption of the annexin V shield
Complement activation
aPL-MEDIATED FETAL LOSS
Intraplacental thrombosis
Inflammation
Inhibition of syncitium-trophoblast differentiation
Disruption of the annexin V shield
Complement activation

Table 1. Antiphospholipid antibody (aPL)-mediated pathogenic mechanisms.

PATHOPHYSIOLOGY OF PREGNANCY MORBIDITY IN APS

The pathogenesis of obstetric APS is not fully known although studies have suggested the involvement of complement, ß2GPI, and annexin V. Some observational studies of women with obstetric APS events have shown cases with no evidence of placental thrombosis, infarctions, or vasculopathy which suggests that APS should be redefined as an inflammatory disorder (32).

Murine models strongly suggest the involvement of complement activation in the pathophysiology of pregnancy morbidity in patients with APS. Preliminary data from recent reports indicate that the histology of placental specimens from patients with APS shows evidence of complement activation compared to control placental specimens. However, complement deposition can be detected in both abortive specimens and placentas at term without a clear relationship with either pregnancy outcome, or therapy (33). Although extensive prospective analyses are needed to demonstrate definitive results regarding the involvement of complement in APS-related pregnancy morbidity, the potential role of complement in aPL-mediated clinical manifestations should not be neglected. In addition to causing acute local inflammation, complement components are able to modulate the functions of pro-coagulant cells (monocyte, endothelial cells) and decidual or trophoblastic cells (34).

The ß2GPI-dependent aPL mechanism of action is thought to involve recognition of the antigen on placental tissues, growth inhibition, and differentiation of trophoblasts, resulting in defective placentation (20). TIFI, a cytomegalovirus-derived synthetic peptide was shown to inhibit binding of ß2GPI-dependent aPL and, therefore, expression at a placental level and trophoblast modulation mediated by autoantibodies (35).

Rand *et al.* (36, 37) shed new light on the role of annexin A5, a potent anticoagulant protein that is expressed on the apical membranes of placental villous syncytiotrophoblasts at the interface between the fetus and placenta, endothelial cells, and platelets (36, 37). The protein plays a thrombomodulatory role by shielding phospholipidbilayers and blocking their availability for coagulation reactions. Competitive displacement by aPL IgG-ß2GPI immune complexes interferes with annexin A5 binding which leads to accelerated coagulation and contributes to pregnancy loss and thrombogenic effects. Hydroxychloroquine (HCQ) reduces the exposure of thrombogenic phospholipids by inhibiting and reversing aPL IgG-ß2GPI complex formation and promoting the formation of annexin V secondary patches in areas of disrupted aPL IgG-ß2GPI immune complexes.

CLINICAL MANIFESTATIONS

Thromboses are one of the hallmarks of APS, and venous thrombosis, or embolism, is the most frequent manifestation (2). However, in contrast to thromboses associated with congenital thrombophilias, those associated with APS may also occur in any vascular bed. In the arterial bed, the CNS is most generally affected (2), usually in the form of stroke or transient ischemic attacks. APL have also been associated with venous sinus thrombosis, myelopathy, chorea, migraine, and epilepsy (38), aCL have been linked to cognitive impairment in patients with SLE (39). Similarly, mild cognitive dysfunction has been recorded in more than 40% of patients with APS along with a strong association with cerebral white matter lesions (40). Multiple sclerosis-like CNS lesions and compatible clinical presentations have been noted in a subset of patients with APS (41).

aPL are associated with cardiac valvular disease. The mitral valve is most frequently affected followed by the aortic valve (42). Regurgitation is more common than stenosis, and many patients remain asymptomatic for years. Acute coronary syndromes are much less prevalent than cerebrovascular disease (2). Renal involvement in APS was first described in 1992 (43). Thrombotic microangiopathy is the most characteristic finding but fibrous intimal hyperplasia, focal cortical atrophy, and arterial occlusions have also been described (44). Hypertension with proteinuria (often subnephrotic) and renal failure are typical presentations of APS nephropathy (43, 44). Renal artery stenosis can also present as refractory hypertension (45).

Other clinical features associated with aPL are, in order of frequency, thrombocytopenia, hemolytic anemia, skin ulcers, avascular bone necrosis, and adrenal insufficiency (2). Livedo reticularis is present in about a quarter of the patients with APS and is a physical sign that should lead to clinical suspicion of APS in the appropriate clinical context. Moreover, livedo reticularis may be a marker for patients at high risk for arterial thrombosis (46).

The most severe and, fortunately, infrequent form of APS is CAPS. There is usually a triggering factor that induces widespread small-vessel thrombosis with multiorgan failure and a mortality rate in the short-term of more than 50% (47).

Obstetric complications are the other hallmark of APS with the most common manifestation being recurrent miscarriage, which is usually defined as three or more consecutive miscarriages before the mid-second trimester and with most losses occurring before the 10th week of gestation. Other obstetric features of APS are one or more fetal deaths occurring at or beyond the 10th week of gestation, severe preeclampsia, or placental insufficiency prompting delivery at more than 34 weeks gestation (2, 48). In a retrospective study, women with obstetric APS were at high-risk of subsequent thrombotic complications (49).

CLASSIFICATION AND DIAGNOSIS

The current guidelines for the classification of APS rely upon clinical and laboratory criteria. They were designed to restrict confirmed cases rather than include doubtful ones. For diagnostic purposes, they may not always be applied. There are several situations that may not meet criteria and require a management decision including thrombocytopenia, hemolytic anemia, and the decision to do a skin, kidney, or liver biopsy as well as the cases of when there is a typical clinical picture with no laboratory aPL detected, the so-called seronegative APS (SNAPS). The clinical classification criteria include the presence of vascular and/or obstetric complications. Clinical manifestations are often nonspecific to APS and highlight the importance of including the laboratory definition in the classification criteria (50). The 2006 Sydney APLA Congress consensus concluded that persistent positivity of LAC and/or aCL antibodies, or anti-ß2GPI on two separate occasions, 12 weeks apart, would be required for the revised classification criteria of APS (Table 2). Additionally, the consensus recommended the use of a clotting assay for the detection of LAC and an ELISA technique for the detection of aCL and anti-ß2GPI (3). The serological criteria that define APS are fraught with limitations largely due to the lack of standardization in the above named techniques. This results in significant inter-assay and intra-assay variation and potentially over/under diagnosis of APS (50). Patients misdiagnosed with APS may be at imminent risk of bleeding due to unnecessary thrombolysis (51). In view of this, lupus anticoagulant detection guidelines were revised and published to address this issue. (52) Lakos *et al.* (53) recently formulated long-awaited consensus recommendations for the detection of aCL and anti-ß2GPI.

The international consensus criteria were originally designed for scientific clinical studies and were never intended for diagnostic use. Consequently, there remains a need for firm diagnostic criteria for routine clinical use, which may differ from these. Gardiner *et al*, reported on serological criteria in a cohort of patients diagnosed to have APS based on a comprehensive methodological approach, which included testing for LA as well as IgG, IgMaCL, and antiß2GPI. They found that LA, aCL, and antiab2GPI testing are all required for the accurate diagnosis of APS and that low-titer antibodies should be included in the diagnosis of obstetric APS (54).

A recent study by Otomo *et al*. (55) formulated a score to quantify the risk of thrombosis/obstetric events in a cohort of patients with various autoimmune diseases. A score was given weighted on the relative risk of clinical manifestations of APS for each aPL assay. The assays were totaled to give the overall complete aPL-score. Otomo *et al*. (55) state that combining assays will compensate for the lack of standardization of single assays. High aPL-scores strongly correlated with reported thrombotic or obstetric events in this cohort, and thus have potential to be used as a marker for the probability of having APS. The score has a predictive value for new onset or recurrence of thrombosis in autoimmune disease. This creates an avenue for its use in management of APS as treatment can be modified based on a patient's score. The complete score incorporates the phosphatidylserine-dependent antiprothrombin antibody (aPS/PT) assay, which was not formerly part of the conventional serological criteria and thuse limits its use clinically. A partial-aPL score was formulated which incorporated conventional assays. There was a correlation between higher scores and history of thrombosis/obstetric events although the association was not as strong as the complete score.

Conventional laboratory criteria may not allow proper detection of subsets of patients with what is currently called seronegative APS (SNAPS), who are persistently negative for routine assays detecting LAC, aCL, and anti-ß2GP1 antibodies but harbor clinical manifestations suggestive of APS (56). Routine antibody screening tests for the diagnosis of APS fail to identify cases of seropositive APS as other antibodies, e.g., the IgA isotype aCL and anti-ß2GP1, anti-phosphatidylserine, prothrombin, phosphatidylethanolamine,

CLINICAL CRITERIA
Vascular thrombosis
>1 clinical episodes of arterial, venous, or small vessel thrombosis in any tissue or organ confirmed by objective validated criteria through imaging or histopathology in the absence of significant evidence of inflammation in the vessel wall.
Pregnancy morbidity
≥1 unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, or; ≥1 premature births of a morphologically normal neonate before the 34th week of gestation owing to eclampsia, severe pre-eclampsia, or placental insufficiency, or; ≥3 unexplained consecutive spontaneous abortions before the 10th week of gestation (maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded);
Laboratory Criteria
Lupus anticoagulant present in plasma on ≥ 2 occasions at least 12 weeks apart detected on the basis of the guidelines of the International Society on Thrombosis and Hemostasis. IgG and/or IgM anticardiolipin antibodies present in medium or high titers, in serum or plasma, on ≥ 2 occasions at least 12 weeks apart measured by a standardized ELISA. IgG and/or IgM antiß2 glycoprotein I antibodies present in titer >99th percentile, in serum or plasma, on ≥ 2 occasions at least 12 weeks apart measured by a standardized ELISA.

Table 2. Revised classification criteria for the antiphospholipid syndrome. (3). Antiphospholipid syndrome is present if at least one of the clinical criteria and one of the laboratory criteria are met. ELISA, enzyme-linked immunosorbent assay.

annexin V, and vimentin/cardiolipin complex do not have standardized assays and are not part of the classification criteria (57). Vimentin/cardiolipin antibodies may have a potential diagnostic and pathogenic role in patients with clinical manifestations of APS but do not conform to the conventional serological criteria (57).

CATASTROPHIC ANTIPHOSPHOLIPID SYNDROME

CAPS was first described by Asherson in 1992 (58) and is characterized by multiple vascular occlusive events presenting over a short period of time in aPL-positive patients. It is an uncommon presentation that occurs in <1% of APS patients and often after a triggering factor such as anticoagulation withdrawal, surgery, minor surgical procedures, or infections. The mortality rate is around 50% and treatment includes corticosteroids, anticoagulation, intravenous immunoglobulin (IVIG), rituximab, eculizumab, and plasma exchange (59). The etiopathogenesis of catastrophic APS remains incompletely understood. Several mechanisms have been suggested such as molecular mimicry, infections, and activation of endothelium in the microvasculature and microvascular occlusions. (60) Kitchens *et al*. (61) have suggested that the vascular occlusions are themselves responsible for the continuum of thrombosis. Clots increasingly generate thrombin, fibrinolysis is impaired by the excess of plasminogen activator inhibitor type-1 (PAI-1), and there is consumption of the natural anticoagulant proteins such as protein C and antithrombin. These multiple small vessel occlusions cause extensive tissue necrosis which results in a systemic inflammatory response syndrome (SIRS) with excessive cytokine release from affected and necrotic tissues (62). Pro-inflammatory cytokines, several products of the activated complement system (e.g., C3b, iC3b, and C5a), and aPL themselves have each been shown to activate endothelial cells, provide a stimulatory signal, and up-regulate adhesion molecules and tissue factor. These molecules can also act on leukocytes and platelets to increase their adhesion to vascular endothelium and promote microthrombosis and the local release of toxic mediators including proteases and oxygen-derived free radicals. The interaction between all these cells in the presence of aPL leads to the diffuse microvasculopathy that characterizes CAPS and results in multi-organ failure (60, 62, 63). The most common known trigger for CAPS is infection. Less common causes are anticoagulation withdrawal or low international normalized ratio (INR), medications (e.g., oral contraceptive), obstetric complications, neoplasia, SLE flares, trauma, and surgery. Nevertheless, in almost half of the cases, no obvious precipitating factors have been identified, and CAPS can often occur in patients without any previous thrombotic history (59). The clinical manifestations of CAPS depend on the organs affected, the thrombotic events, and the extent of the thrombosis together with manifestations of SIRS. In contrast to classic APS, single venous or arterial medium-to-large blood vessel occlusions are uncommon in patients with CAPS. Multiple organ dysfunction and failure, as a consequence of thrombotic microangiopathy, are responsible for the majority of clinical features. However, large venous or arterial thrombosis can also occur in about one-fifth of patients.

The diagnosis of CAPS can be challenging due to the acute onset of thrombosis at multiple levels with simultaneous dysfunction of different organs. Patient survival very much depends on an early diagnosis and treatment. Preliminary CAPS

classification criteria (Table 3) were proposed and agreed on at the 2002, APLA Congress in Taormina (Sicily) during the 10th International Congress on aPL. Although these criteria are accepted for classification purposes, they might also be used as a guide to a more consistent diagnostic approach (64). An update of the diagnostic algorithm for CAPS was recently proposed (65) and discussed by the Task Force on Catastrophic Antiphospholipid Syndrome (APS) and Non-criteria APS Manifestations at the 13th International Congress on Antiphospholipid Syndrome, held in Galveston (Texas) in 2011 (66). This approach represents a step forward in the diagnosis of CAPS but must be validated versus other thrombotic microangiopathies.

To diagnose CAPS, there should be clinical evidence of multiple organ involvement over a short period of time, histopathological evidence of multiple small vessel occlusions, and laboratory confirmation of aPL, usually in high titers. The positivity of aPL should also be confirmed later when the acute clinical situation is resolved. aPL may also be positive in sepsis and other critical situations, which share several clinical features with CAPS. A differential diagnosis should be made with sepsis, thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS), acute disseminated intravascular coagulation (DIC) and hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome.

TREATMENT

APS is characterized by recurrent thrombotic events that have not been properly managed. Thus anti-thrombotic medication is necessary for long-term management to reduce thrombotic risk or pregnancy morbidity. Choosing the type of pharmacological treatment and the intensity and duration of anticoagulation depends on the clinical type, co-morbidities, severity of the APS, and the risk of bleeding. Change of life habits that are known to increase the risk of thrombotic events has to be stressed in addition to avoiding estrogens and cigarette smoking. When present, the active control of elevated serum LDL-cholesterol and triglycerides, arterial hypertension, and blood sugar, is recommended.

Patients with APS may be evaluated in an outpatient setting. In patient evaluation is required if the patient presents with a significant clinical event. Patients with CAPS require intense observation and treatment, often in the intensive care unit.

VENOUS THROMBOEMBOLISM

Venous thromboembolism is the most common initial clinical manifestation in APS and occurs in 32% of patients who meet consensus conference diagnostic criteria (67).

Initial treatment consists of unfractionated or low molecular-weight heparin for at least 5 days overlapped with warfarin therapy (68).

The use of warfarin with an international normalized ratio (INR) of 2.0- 3.0 reduces the risk of recurrent venous thrombosis by 80% to 90% irrespective of the presence of aPL (69). For long-term treatment of venous thromboembolism, retrospective case series have suggested that high-intensity warfarin (INR 3.0) is more effective than either aspirin or warfarin administered with an INR < 3.0 (70, 71).

Some studies have found that high-intensity warfarin is better than moderate-intensity warfarin for the prevention of recurrent thrombosis. However, a significant excess of minor bleeding was evident in patients who were given high-intensity warfarin (72).

ARTERIAL THROMBOEMBOLISM

Arterial events in APS most commonly involve the cerebral circulation with stroke being the initial clinical manifestation in 13% and a transient ischemic attack in 7% of patients. (73) The association between APS and other arterial thrombosis, including myocardial infarction, is less certain.

Warfarin and aspirin appear to be equivalent for the prevention of thromboembolic complications in patients with a first ischemic stroke and aPL. Patients with a first ischemic stroke and a single positive antiphospholipid antibody test result who do not have another indication for

1. Evidence of involvement of 3 organs, systems, and/or tissues 2. Development of manifestations simultaneously or in less than 1 week 3. Confirmation by histopathology of small vessel occlusion in at least 1 organ/tissue 4. Laboratory confirmation of the presence of aPL (LAC and/or aCL and/or anti-2GPI antibodies)
Definite CAPS • All 4 criteria Probable CAPS • All 4 criteria, except for involvement of only 2 organs, system, and/or tissues • All 4 criteria, except for the absence of laboratory confirmation at least 6 weeks apart associated with the early death of a patient never tested for aPL before onset of CAPS) • 1, 2, and 4 • 1, 3, and 4, and the development of a third event in > 1 week but <1 month despite anticoagulation treatment

Table 3. International classification criteria for CAPS.

anticoagulation may be treated with aspirin (325 mg/d) or moderate-intensity warfarin (INR, 1.4 - 2.8) (73). Aspirin is likely to be preferred because of its ease of use and lack of need for laboratory monitoring.

It is known that aPL may persist in the serum of APS patients for long periods of time, but thrombotic events occur only occasionally. It has been suggested that aPL ('first hit') raises the thrombophilic threshold (i.e., induces a prothrombotic/proinflammmatory phenotype in endothelial cells), but that clotting only takes place in the presence of a 'second hit' or triggering event (i.e., an infection, a surgical procedure, use of estrogens, prolonged immobilization, etc.) (74).

In general, treatment regimens for APS must be individualized based on the patient's current clinical status, presence of co-morbidities, and history of thrombotic events. Asymptomatic individuals in whom blood test findings are positive do not require specific treatment in addition to avoidance of known risk factors.

PROPHYLACTIC THERAPY

- Eliminate other risk factors such as oral contraceptives, smoking, hypertension, hyperhomocysteinemia, or hyperlipidemia.
- Low-dose aspirin is usually used. Clopidogrel may be useful in patients allergic to aspirin.
- In patients with SLE, consider HQC, which may have intrinsic antithrombotic properties.
- Consider the use of statins, especially in patients with hyperlipidemia.

INITIAL THERAPIES

Heparin. The initial approach to thrombosis in APS is identical to that of many other thromboses. For acute thrombotic events, the first therapy is heparin. Low molecular weight heparin (LMWH) has replaced unfractionated heparin as the standard of care for most thrombotic events.

Full dose LMWH (1mg/Kg twice daily) is usually given simultaneously with warfarin and is overlapped with warfarin for a minimum of four to five days until the International Normalized Ratio (INR) has been within the therapeutic range (2.0 to 3.0) for two consecutive days (72).

Some characteristics of heparin:

- The antithrombotic effects include potentiating the anti-thrombin effects of antithrombin and other endogenous antithrombin effectors, increasing the levels of factor Xa inhibitor, and inhibiting platelet aggregation.
- Heparin may also bind to aPLs and render them inactive (75).
- Heparin may also block tissue factor-mediated placental bed immunopathology (76, 77).

Low molecular weight heparin. Several LMWH products are now available for clinical use. Dosing requirements are individualized for each product (78). The advantages of LMWH over unfractionated heparin are reviewed separately.

Unfractionated heparin. Unfractionated heparin is preferred to LMWH in certain circumstances. The major potential advantage of unfractionated heparin over LMWH is in the setting of hemorrhage (a rare complication of the APS). Unfractionated heparin can be reversed quickly with protamine while LMWH is not completely reversible with this approach. The major condition in which hemorrhage is due to APS is when antibodies to prothrombin are present.

Warfarin. Following stabilization of the patient, warfarin is begun. Warfarin is the standard of care for the chronic management of patients with APS who are not pregnant. INR should be maintained between 2.0 and 3.0 (79). However, aPL may create problems in monitoring the INR. A monotonous diet with only slight variations in the amount of vitamin K intake, intensification of monitoring when a different medication has to be used, and above all, patient education on the importance of close monitoring are crucial for the APS management to succeed.

ANTIPLATELET AGENTS

Aspirin. Aspirin is of minimal or no benefit for the prevention of thrombotic APS manifestations in patients who have experienced previous events according to retrospective series (80). However, some studies suggest that aspirin (81 mg/day) reduces the risk of thrombosis in aPL- positive patients (81). In addition to its antiplatelet effects, low dose aspirin (ASA) (50 to 100 mg) enhances leukocyte-derived interleukin-3 production, which stimulates normal trophoblast growth and hormone expression (82).

Retrospective and prospective observational studies and controlled trials of aspirin for the prevention of thrombotic events in people with aPL with no history of arterial or venous thromboembolism have had disparate results (82, 83). The Antiphospholipid Antibody Acetylsalicylic Acid (APLASA) study consisted of two separate investigations involving patients who were asymptomatic but persistently aPL-positive (84). The conclusions of the APLASA trial were:

- Asymptomatic individuals who are persistently positive for aPL have a low annual incidence of acute thrombosis.
- These individuals do not benefit from low-dose aspirin.
- Thrombotic events in this population are unlikely in the absence of additional risk factors for thrombosis.

Clopidogrel. It has anecdotally been reported to be helpful in patients with APS and may be useful in those allergic to aspirin. Its use is not advised for the treatment of APS (85).

CURRENT TREATMENT OF THROMBOSIS

Treatments in APS are directed at modulating the final event or second hit. Treatments that modulate the early effects of

aPL on target cells – that is monocytes or endothelial cells (first hit) – would be more beneficial and potentially less harmful than current treatments.

The current antithrombotic approach to aPL-positive patients may be replaced by an immunomodulatory approach in the future as our understanding of the mechanisms of aPL-mediated thrombosis improves. Understanding the molecular mechanisms triggered by aPL and identifying biomarkers released as a consequence of cell activation may help us design new ways to treat clinical manifestations in APS.

The main target recognized by aPL binds to endothelial cells and monocytes through its fifth domain. aPL/anti-β2GPI antibodies then bind to domain I of β2GPI, and upon clustering and formation of complexes, they trigger cell activation (86-88).

Therefore, blocking the binding of aPL or inhibiting the binding of β2GPI to target cells may be the most specific approach to ameliorate their pathogenic effects without interrupting any important physiologic mechanisms. Recently, Ioannou *et al*. demonstrated that the soluble recombinant domain I of β2GPI abrogates, in a dose-dependent fashion, the in vitro and in vivo effects of anti-β2GPI antibodies. This underscores the possibility of utilizing decoy peptides that are part of β2GPI to abrogate the binding of pathogenic aPL to target cells in the treatment of patients with APS. Nevertheless, human studies are needed to establish the safety and efficacy of such a treatment (89, 90).

GPIIBIIIA INHIBITORS

aPL-enhanced thrombosis in vivo can be abrogated by infusions of a GPIIb/IIIa antagonist monoclonal antibody. Recently, it has been reported that heterozygosity for platelet glycoproteinsIa/IIa and IIb/IIIa increase arterial thrombosis in patients with APS (91). These data indicate that GPIIb/IIIa antagonists or platelet membrane glycoprotein IIb/IIIa receptor inhibitors may prove to be useful in the treatment of an acute thrombotic event, particularly an arterial event, in patients with APS. In addition, the combination of GPIIb/IIIa antagonists and an ADP receptor antagonist, e.g., ticlopidine, is an attractive therapeutic strategy. It provides fast and continuous platelet inhibition since pre-stimulation of platelets by agonists leads to the exposure of phosphatidylserine on the outer membrane of the cell. As a result, it produces an anti-β2GPI/β2GPI complex on the exposed phosphatidylserine before interacting with a specific platelet receptor to potentiate activation (92-94).

HYDROXYCHLOROQUINE (HCQ)

HCQ inhibits the aPL-induced expression of platelet GPIIb/IIIa receptor (platelet activation) dose-dependently and also reverses the binding of aPL–β2GPI complexes to phospholipidbilayers (95). In SLE patients, those receiving HCQ experienced fewer thrombotic events and results from the Baltimore Lupus Cohort showed a decreased risk of arterial thrombosis (95). HCQ could be used in patients with APS and thrombosis as a second-line agent together with anticoagulation therapy. We still do not have a study result for a consistent recommendation for HCQ in APS although, in SLE, it is known to reduce the thrombotic risk, including during pregnancy.

RITUXIMAB (RTX)

RTX has been shown to be a good treatment for life-threatening CAPS in a few patients and case reports suggest it may be successful in patients with aPL, autoimmune-mediated thrombocytopenia, and hemolytic anemia. Statkute *et al*. demonstrated normalization of aCL antibody titer after autologous hematopoietic stem-cell transplantation in patients with APS secondary to SLE (97-100). Recently, an uncontrolled and nonrandomized pilot study suggested that the safety of rituximab in aPL-positive patients with non-criteria manifestations of APS is consistent with the safety profile of rituximab. Despite causing no substantial change in aPL profiles, rituximab may be effective in controlling some but not all non-criteria manifestations of APS (101).

OBSTETRIC CONSIDERATIONS

Prophylaxis is recommended for prenatal and postpartum women with APS with no history of thrombosis and full anticoagulation for those with a history of thrombosis.

The administration of low-dose ASA alone for the prevention of fetal loss in women with APS has been associated with an increased frequency of successful pregnancy outcome in some studies (102) but was no better than supportive care in others (103).

- Low-dose ASA can be stopped any time after 36 weeks of gestation and, ideally, should be stopped 7 to 10 days before delivery as some studies have reported a slight increase in mostly minor perioperative bleeding with continuation of the drug (104). In women with a past history of serious arterial thrombotic complications such as stroke or myocardial infarction, the potential benefit of continuing ASA through labor and delivery outweighs the small risk of incisional bleeding. Aspirin is not usually stopped in these patients. Use of low-dose ASA has not been associated with either premature closure of the ductusarteriosusor or an increase in significant postpartum events.
- Patients with pregnancy loss receive prophylactic subcutaneous heparin (preferably LMWH) and low-dose aspirin. Therapy is withheld at the time of delivery, restarted after delivery, and continued for at least for 6-12 weeks postpartum.
- Warfarin (Coumadin) is contraindicated in pregnancy, mainly between 6 and 9 weeks. In a few centers, it is used between 15 and 35 weeks, before and after LMWH.

- Patients with a history of thrombosis receive therapeutic doses of heparin during pregnancy combined with low-dose aspirin. Long-term anticoagulation is then continued postpartum.
- Corticosteroids have not been shown to be effective for patients with primary APS and have been shown to increase maternal morbidity and fetal prematurity rates.
- Breastfeeding women may use heparin, low-dose aspirin, and warfarin.

CAPS

- These patients are generally very ill, often with active SLE.
- Treatment with intensive anticoagulation, plasma exchange, and corticosteroids appears beneficial, but no controlled trials have been done. Intravenous immunoglobulin may be of some benefit and cyclophosphamide may be considered in selected cases, especially in SLE-associated CAPS.
- Reports of successful use of rituximab, mainly in hematological complications.

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27 SYSTEMIC AUTOIMMUNE DISEASES AND PREGNANCY

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INTRODUCTION

The female immune system must be actively adapted during pregnancy in order to maintain its defensive capacity and, at the same time, protect the fetus from immunological rejection. This profound immunological change may influence, in various ways, women with an autoimmune disease who are conceiving. One such way is the further development of the disease in the postpartum period. Another way is the aggravation of a pre-existing condition. Additionally, pregnancy losses can be a serious problem in many women with autoimmune diseases. In the chapter, we will review the potential pregnancy complications in the most representative autoimmune diseases.

ANTIPHOSPHOLIPID SYNDROME

The relationship between pregnancy loss and antiphospholipid syndrome (APS) was recognized many years ago. Although many pathogenic mechanisms for the association of pregnancy loss and APS have been described, the exact physiopathology is not fully understood. History of previous pregnancy loss or thrombosis, besides the presence of other autoimmune diseases such as systemic lupus erythematosus (SLE), makes it possible to differentiate several groups of patients, in which the initial treatment may differ. Although establishing pregnancy outcome prognosis in this entity is difficult, pregnancy loss in APS is considered a treatable cause for pregnancy loss. The close interaction between the multidisciplinary health care team and the patient and her family is extremely important for better outcomes (1).

The prevalence of APS during pregnancy is variable based on the population studied and this criteria used for antiphospholipid antibody (aPL) detection. The aPLs can be found in normal pregnancies but their prevalence is limited. Lupus anticoagulant (LA) was found in 0.2% and anticardiolipin antibodies (aCL) in 2% of normal pregnancies (2).

Between 7 and 25% of miscarriages that cannot be explained by other causes are secondary to the presence of aPLs (1). The prevalence of aCL varies from 4.6% to 50.7% with a mean of 15.5% and the prevalence of LA ranges from 0 to 14% with a mean of 8.3%; however, in women with late pregnancy loss (after 20 weeks) the prevalence can be as high as 30% (2). This wide range in the percentages can be explained by the diversity of the population studied and the lack of standardization of aPL detection assays.

It is important to understand that the serologic tests do not closely correlate with one another (3). A patient may have high-titer aCL or anti-beta-2-glycoprotein I antibodies (aβ2GPI) or LA with or without the other autoantibodies. Some authors argue that 'triple positivity' (i.e. all three antibodies are positive) carry the worst prognosis (4,5) while others argue that LA alone worsens the prognosis independent of aCL or aβ2GP1 titer (6). Most experts agree that coexisting predisposing factors such as smoking, diabetes, hypertension, obesity, or renal insufficiency worsen prognosis in individual patients.

The presence of aPLs during pregnancy leads to all sorts of placental dysfunction during all three trimesters and may lead to intrauterine growth restriction, preeclampsia, placental insufficiency, and miscarriages (7-10).

CLASSIFICATION CRITERIA FOR PREGNANCY LOSS IN APS

The current classification criteria (Sidney, 2006) (11) are a revision of the original Sapporo Criteria. APS is considered as present if at least one of the clinical criteria and one of the laboratory criteria that follow are met. The clinical criteria are defined by history or presence of vascular thrombosis (documented by appropriate imaging studies or histopathology) or pregnancy morbidity as below:

1. One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, or
2. One or more premature births of a morphologically normal neonate before the 34th week of gestation because of eclampsia, severe preeclampsia, or recognized features of placental insufficiency, or
3. Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.

The laboratory criteria are the presence of LA in plasma, medium or high titers of aCL of the IgG or IgM isotypes (>40 GPL or MPL, or > the 99th percentile), or aβ2GPI of the IgG or IgM isotypes (in titer > the 99th percentile). All positive tests should be confirmed at least twelve weeks apart.

MECHANISMS OF PREGNANCY LOSS IN APS

Studies with animal mouse models have shown a direct causal relationship between of aPL and pregnancy loss. Inoculation of serum of highly aPL positive women in normal female mice induces early pregnancy termination (12) and active immunization with pathogenic monoclonal aCL induces clinical manifestation of APS in BALB/c mouse (13).

It is well established that aPLs require a cofactor(s) to bind to phospholipids, e.g., apolipoprotein H, also known as β2GPI. β 2GPI is a highly glycosylated glycoprotein with five "sushi-like" domains that interacts with the membrane phospholipids through its domain 5, which is rich in lysine. The binding of aPL to β2GPI forms a complex and increases the affinity to membrane phospholipid (14). The functional role of β2GPI is not fully described but it is known that its deficiency does not seem to be related to the disease. When the aPL-β2GPI complex binds to cellular membranes including trophoblast, it leads to damage and activation of cytokines such as interleukine 3, which is important for the embryonic implantation process (15).

The three well recognized pathogenic mechanisms of aPL in pregnancy are thrombosis, imbalance between prostacyclin and thromboxane, and alteration of trophoblast adhesion molecules (16). The hypercoagulable state induced by aPL is the cause of thrombosis, placental infarction, and spiral artery vasculopathy, which leads to intrauterine growth restriction, signs of fetal hypoxia such as abnormal flow in umbilical artery, and fetal loss.

Placental spiral artery vasculopathy diminishes the flow of maternal blood to intravillous space and interfers with gas and nutrient exchange. This utero-placental insufficiency can result in intrauterine growth restriction and pregnancy loss.

In order to differentiate the placental pathology of aPL from the characteristic of SLE, Magid *et al.* analyzed 40 placentas and did not find any correlation of SLE activity with miscarriage, intrauterine growth restriction, or premature birth (17) although more recent data showed that the disease activity in SLE could be a predictor of pregnancy outcome (18). The placental findings in SLE patients were compatible with hypoxia-ischemia, vasculopathy, thrombosis, and chronic villitis with placental weight reduction. When placentas of APS patients were analyzed, large areas of infarct were noticed (17).

There are different hypotheses on how aPL can induce a hypercoagulable state:

1. aPL can distort eicosanoid equilibrium thus decreasing prostacyclin production and increasing thromboxane (potent vasoconstrictor and enhancer of platelet aggregation) production from endothelium cells (19,20).

2. There is cross reactivity between aPL and glycosaminoglycans (important molecules similar to heparin that can act as auto anticoagulant) that produces a hypercoagulable state by inhibiting natural anticoagulants (21).

3. aPL can inhibit the anticoagulant effect of protein C and S (22).

4. The reduction in annexin V (normal placental anticoagulant protein) produced by the competition between aPL and annexin V to bind with phospholipids, can lead to hypercoagulability in the placenta (23).

5. aPL may activate the complement system which will eading to an inflammatory process and eventually thrombosis (24).

Besides the prothrombotic mechanisms described, there is also a direct vascular damage caused by aPL in the trophoblast (25). Studies with animal models showed that aPL can act directly on the trophoblast and thus interfer with the differentiation and maturation, produce direct damage through apoptosis, inhibit syncytium formation, diminish the production of chorionic gonadotropin, and alter implantation during early pregnancy (26,27).

Infection is also a possible trigger of pathogenic aPL production. Experimental studies have shown that immunization with Haemophilus influenza and Neisseria gonorrhea can trigger antibodies against β2GPI (28). The inoculation of these antibodies in normal pregnant mice resulted in clinical manifestation of APS including pregnancy loss, thrombocytopenia and elevated activated thromboplastin time. In humans, varicella infection was associated with APS (29).

The mechanism for pregnancy loss in APS is heterogeneous, complex, and, so far, not completely understood. Future studies focused on complement activation, new antigens, genetic predisposition, and the exact thrombotic domain for aβ2GPI will help as better understand the pathological features of APS (30,31).

In summary, it is widely accepted that aPL causes recurrent embryonic loss, intrauterine growth restriction, fetal distress, severe pre-eclampsia, and pregnancy loss, some of which are

due to utero-placental insufficiency caused by multiple infarcts, thrombosis and spiral artery vasculopathy. The main targets are platelets, endothelium cells, inhibition of natural anticoagulants, complement activation and fibrinolytic pathway (24,32).

CLINICAL FINDINGS

Clinical manifestations of APS are numerous and of a widely variable spectrum ranging from single, deep venous thrombosis (DVT) or multi organ thrombosis, which affects only pregnancy or is associated with a big array of autoimmune phenomena. Several systemic findings can be explained by vasculopathy and small vessel occlusion due to platelet aggregation and subsequent thrombosis, which may trigger venous thrombosis, arterial thrombosis, pulmonary embolization, transient cerebral ischemia, renal infarction and other conditions. The patient may present exclusively vascular thrombosis, only obstetric morbidity or a mixture of the two forms of presentation of the disease. The presence of aPL is associated with an elevated incidence of first trimester abortion, fetal loss, placental dysfunction, and pre-eclampsia. Frequently the placenta is characterized as being abnormally small and light, and histopathology shows thrombosis and infarction.

Considering first trimester losses, 86% of miscarriages in patients with APS occur after embryonic cardiac activity is seen, but only 43% of miscarriages in patients without APS occur after this finding. This data suggests that the mechanism of pregnancy loss is different in patients with APS, as was shown earlier.

There is some theoretical suggestion that aPLs may have a role in infertility. They may affect implantation, placentation, and early embryonic development as the antibody binds to β2GP1 and is responsible for breakdown of the phospholipid adhesion molecules between different elements of trophoblast (33), but studies failed to show this association. For the time being, there is no evidence to suggest that routine screening should be done on patients with infertility, and therapy is not justified (34).

LABORATORY FINDINGS

The detection of aPL is achieved by either coagulation assays (LA) or ELISA (aβ2GPI and aCL). The lack of standardization and wide variation between assays done worldwide are barriers for the designing and reliability of clinical trials (35). A task force with the experts in the field was established with the goal for achieving better quality and standardization of assays (36,37).

LUPUS ANTICOAGULANT

LA assay is more specific but less sensitive when compared to aCL. It correlates better with thrombosis (especially arterial) and is the best predictor for adverse pregnancy outcome (6,38,39).

Documentation of an LA requires a four-step process: 1) Demonstration of a prolonged phospholipid-dependent coagulation-screening test such as activated Partial Thromboplastin Time or dilute Russell Viper Venom Time (aPTT or dRVVT); 2) failure to correct the prolonged screening test by mixing the patient's plasma with normal platelet poor plasma, demonstrating the presence of an inhibitor; 3) shortening or correcting the prolonged screening test by the addition of excess phospholipid, demonstrating phospholipid dependency; and 4) exclusion of other inhibitors (40).

ANTICARDIOLIPIN ANTIBODIES

aCL is detected by ELISA which allows the identification of isotype and quantification of the titer. In order for the aCL to bind its antigen, the presence of a cofactor is necessary: the β2GPI. Consequently, there are two clusters of aCL: β2GPI-dependent and β2GPI independent. It was shown that the β2GPI dependent is the one associated with thrombosis and the β2GPI independent can be found in the setting of infection and normally is not associated with the clinical manifestations of APS (41). The isotype that best correlates with thrombosis is IgG. Multiple studies showed a weak correlation between IgM and thrombosis or failed to demonstrate one (42). Although IgA is not part of the classification criteria, many authors argue that it is an independent risk factor for thrombosis (43).

ANTI B2-GLYCOPROTEIN I ANTIBODIES

Several studies have shown that the aβ2GPI is the type of antibody that better correlates with the presence of LA and APS clinical manifestations is IgG. This diagnostic test is performed by ELISA and was previously done in patients who exhibited clinical manifestations of APS but were negative for both aCL and LA. Nowadays its wide availability allows it to be performed more frequently.

ANTIPROTHROMBIN ANTIBODIES

Although not part of the classification criteria, antiprothrombin antibodies (aPT) have been largely studied. Early reports showed no association between aPT and pregnancy morbidity, defined by two or more pregnancy losses before week 13 of pregnancy and/or one fetal death (44). A retrospective study showed that IgG aPT had predictive value of a 4.5 fold increased risk for early pregnancy loss in patients with APS, risk that was higher when compared to that of aCL (45).

Additionally, several other antibodies directed against negatively charged phospholipids such as phophatidylserine, phophatidylinositol, and phosphatidic acid or neutral charged phosphatidylethanolamine were described. Their clinical use remains very limited and is mostly done when patients present with classical clinical findings of APS but all laboratory data is negative.

In APS patients, another set of antibodies were described (e.g., anti-annexin V antibody, anti-protein C antibody, anti-protein S antibody, and anti-thrombomodulin antibody, anti-β2GPI domain 1 and domain 4/5, and anti-prothrombin-phosphatidylserine), but their clinical relevance remain uncertain.

TREATMENT

The treatment of obstetric APS should include a multidisciplinary team of specialists such as high-risk obstetricians, hematologists, and rheumatologists with expertise in the management of the syndrome and its possible complications. In order to attain the best pregnancy outcome, it is essential that patients use the medication correctly and have very close follow up during pregnancy. Pregnancy planning is ideal although not always achieved.

Patients must be informed that there is an increased risk of serious complications (eg, hypertension/preeclampsia, prematurity, or thrombosis) that can take place during pregnancy and the puerperium even if the proper treatment is chosen. Pregnancy should be discouraged in all women with important pulmonary hypertension because of the high risk of maternal death and should be postponed in the setting of uncontrolled hypertension or recent thrombotic events, especially stroke (34).

The management of obstetric APS is based on few well conducted studies and is still controversial. Therefore, it is important to try to personalize the management based on the patient as there is no strict protocol available supported by clinical trials. In addition, the heterogeneity and the existence of clinically different subgroups of APS make protocols hard to use. However, for educational purposes, we can divide APS during pregnancy into the following groups:

1. Positive aPL patients with no history of thrombosis, pregnancy loss, or concomitant autoimmune disease.
2. Patients with APS and a history of 3 or more early pregnancy losses.
3. Patients with APS and a history of fetal loss (after 10 weeks) or premature birth before the 34th week due to severe pre-eclampsia or placental insufficiency
4. Patients with APS and previous thrombosis.
5. Patients with SLE and positive aPL.

Group 1 - Positive aPL patients with no history of thrombosis, pregnancy loss, or concomitant autoimmune disease: In this group of patients, aPL was detected for some reason but they have never experienced any episode of thrombosis or pregnancy morbidity. It is recommended a close follow-up searching for signs of placental insufficiency, fetal distress and pre-eclampsia. The use of low-dose aspirin in this group is not supported by literature but widely prescribed by physicians (46).

Group 2 - Patients with APS and a history of 3 or more early pregnancy losses: Treatment with aspirin and heparin in prophylactic doses seems to increase considerably the rate of live births in patients with recurrent miscarriage, as was shown by most clinical trials (47,48) and a Cochrane systematic review (49). However, monotherapy with aspirin also had a high success rate in several observational studies and may be a therapeutic option in this group of women (34). The incidence of late complications in pregnancy including preeclampsia, intrauterine growth restriction, and preterm birth, remains high (50).

Group 3 - Patients with APS and a history of fetal loss (after 10 weeks) or premature birth before the 34th week due to severe pre-eclampsia or placental insufficiency: This group has a limited number of well-designed studies and few patients included, so there is no optimal treatment yet. Most authors recommend aspirin in combination with prophylactic doses of heparin, and both unfractioned and low molecular weight heparin are effective (34).

Group 4 - Patients with APS and previous thrombosis: All women with APS and previous thrombosis should maintain antithrombotic treatment during the entire pregnancy as well in the postpartum period considering the high rate of recurrent thrombosis (51). The gold standard treatment regimen is widely agreed to be combined treatment with low-dose aspirin and full anticoagulant doses of heparin (52). Warfarin can be used during pregnancy as an alternative to heparin after organogenesis is complete, so it must be avoided from 6 to 12 weeks (53,54).

Group 5 - Patients with SLE and positive aPL: Evidence in the literature indicates that antimalarials have antithrombotic effects on lupus patients (46). Currently, hydroxychloroquine (HCQ) and low-dose aspirin are recommended in primary thromboprophylaxis for SLE patients with positive LA or isolated and persistent aCL at medium or high titers. Postpartum prophylactic low molecular weight heparin use is advised in asymptomatic women with aPLs and lupus (34). HCQ use during pregnancy in patients with lupus also reduces the number of flares and hypertensive disorders (55). HCQ is safe for use during pregnancy as well as in breastfeeding. In the others, the management of APS (obstetric or thrombosis) will be the same as previously discussed for non-SLE pregnancy.

Intravenous immunoglobulin (IVIG) has been tested in trials with patients with APS that had unsuccessful pregnancies after treatment with heparin and aspirin due to considering its reduction in aCL levels. In comparison to low molecular weight heparin and aspirin, IVIG offered no advantage (56-58). Given the high-cost of IVIG and the lack of positive clinical trials, it should be reserved for accepted indications in pregnancy, such as thrombocytopenia. Prednisone was found to increase pregnancy morbidity in several studies when used for primary APS (46).

In patients with APS, ultrasonographic examination and umbilical artery Doppler velocimetry measurement should be done every 3 to 4 weeks starting at 18 to 20 weeks' gestation to assess fetal growth, amniotic fluid volume, and fetal

well-being. Restricted fetal growth may reflect uteroplacental failure in patients with APS (34).

POSTPARTUM

The postpartum period is actually the greatest risk period for a woman in terms of thrombosis. Patients on prophylactic heparin during pregnancy should maintain the treatment for six weeks after delivery (50,59). Those patients with APS and prior thrombosis or stroke should maintain anticoagulation for life and switch heparin for warfarin, which is minimally secreted into the breast milk and thus is safe to use during breastfeeding, as well as heparin and aspirin (34). In patients with prior fetal death or recurrent pregnancy loss, the optimal management after pregnancy is still controversial (60). Options include no treatment or daily treatment with low-dose aspirin. It is important to avoid estrogens at all costs and to control additional thrombotic risk factors and triggering events (53).

FUTURE INSIGHTS

The standardization of laboratory assays continues to be one of the most important aspects for APS research development. Considering the obstetric manifestations of the syndrome, trials comparing treatments do not have full agreement among experts, and new studies should be designed for the development of appropriate protocols. HCQ, an antimalarial used in SLE, has been shown to decrease thrombosis rate in SLE patients (46) and can dissociate aPL IgG–β2GPI complexes and reduce the amount of aPL IgG that binds to phospholipid bilayers trapping annexin A5, a potent natural anticoagulant with high affinity for anionic phospholipids (61). The use of HCQ in APS is being currently investigated. New oral anticoagulants have been developed recently and evaluated for the prevention and treatment of thromboembolic diseases. Apixaban and rivaroxaban are specific inhibitors of factor Xa while dabigatran inhibits factor IIa. The studies of their efficacy and safety in APS (but not in pregnancy) is under way. New insights regarding the pathogenesis of the disease will certainly trigger the development of new drugs and modalities of treatment.

SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is a chronic, multisystem autoimmune disease that predominantly occurs in women of childbearing age. The risk of obstetric complications in pregnant patients with lupus is significant. SLE increases the risk of spontaneous abortion, intrauterine fetal death, preeclampsia, intrauterine growth retardation, and preterm birth. In addition, pregnancy may be associated with flares of the disease requiring immunosuppressive therapy. Therefore, SLE pregnancies are considered high risk. Maternal health and fetal development should be monitored frequently during pregnancy. If possible, delivery should occur in a controlled setting. Therefore, pregnant women with SLE should be followed by an obstetrician who is knowledgeable in high-risk pregnancies. Fortunately, because of the medical advances, most pregnancies end in a success (62).

The prevalence of SLE is 14.6-50.8 cases per 100,000 inhabitants. The incidence of lupus is much higher in women than in men. During the childbearing years, the female-to-male ratio is about 12:1. Evidence suggests that SLE is more common in African American and Hispanic groups than in Caucasians.

In the United States, there are an estimated number of 4500 pregnancies in women with SLE each year and women with SLE have complicated pregnancies: one third will result in a ceasarean section, 33% will have preterm birth, and over 20% will be complicated by preeclampsia (63).

Regarding fetal losses, studies suggest that pregnancy losses may be decreasing in recent years. In 1960 to 1965, the mean rate of fetal loss was 43 percent. In 2000 to 2003, the rate was 17 percent (64). However, in a multiethnic population with SLE in North America, the rate of fetal losses and stillbirths was reported in percent in 2008 (65); maybe the fetal loss rate is related to co-morbidity of the patients and activity before pregnancy. So, the risk of fetal loss is higher in women with hypertension, active lupus (66), or lupus nephritis (67-69) and in those with hypocomplementemia, elevated levels of anti-DNA antibodies, aPL, or thrombocytopenia (70-71). Additional research is needed to confirm this correlation in lupus pregnancy. Several factors may predict fetal death such as lupus disease activity, active lupus nephritis, and the presence of aPL (65).

INTERACTION OF PREGNANCY AND SYSTEMIC LUPUS ERYTHEMATOSUS

Pregnancy induces dramatic immune and neuroendocrine abnormalities in the maternal body in order to protect the fetus from immunologic attack by the mother (72). Instead of a general immunosuppression that would weaken the mother's defence against infection, a modulation of composition and function of immune-competent cells and immune-modulatory molecules takes place in the maternal system during pregnancy (73). The fetus promotes tolerance to paternal antigens by migration of fetal cells and cell-free fetal DNA into maternal circulation during normal pregnancy. Fetal cells remain in the mother for decades, and create a state of microchimerism in the mother (74).

After delivery, the maternal body adjusts again to a non-pregnant state, which is not simply a return to the condition before conception, but takes place still under the influence of the immune activation at parturition (105). Furthermore, lactation affects immune functions, which may modulate activity of autoimmune diseases. The profound immunologic adaptions necessary for maternal tolerance toward the fetus in pregnancy and the immunological reset to a non-pregnant state thereafter, do influence maternal autoimmune rheumatic diseases in several ways.

Pregnancy induces substantial changes in hormone levels starting with hormones produced by the corpus luteum and the trophoblast followed by complex alterations initiated by the hypothalamicpituitary-adrenal (HPA) axis. Hormones have powerful effects on blood cells and regulate their proliferation, distribution, and function (76). The activity of immuncompetent cells is regulated by cytokines and chemokines with T helper cells as key effectors. Cytokines are important mediators acting in concert with other factors to support successful pregnancy. In 1993, Tom Wegmann proposed the concept of successful pregnancy as a Th2 phenomenon (77) which suppressed CD4+ T helper 1 type cells (Th1).

Besides Th1 and Th2 cells, there is a third subset of CD4+T helper cells, so called Th17 cells, that activate the immune system. The Th17 cells produce several cytokines, of which the most important are cytokines of the interleukin-17 (IL-17) family (68). IL-17 has a proinflammatory effect and drives inflammation also by inducing other proinflammatory cytokines (78). Presence of proinflammatory cytokines drives development of Th17 cells whereas a milieu of tolerance promotes development of Treg (79). Th17 cells are increased at inflammation sites and in the circulation in RA, SLE, and AS (80). Increased numbers of Th17 cells are also found in pregnancy pathology, e,eg., preeclampsia and recurrent pregnancy loss (81).

SLE is characterized by a loss of tolerance both in the T cell and B cell compartment, resulting in B cell hyperreactivity with pathogenic autoantibody formation. One important factor that has emerged is the response of lupus activity to sex steroid hormones (82). Estrogens enhance antibody production, T-helper type 2 immune responses, and B-cell immunity. At high concentrations as achieved in pregnancy, estrogens as well as gestagens stimulate the secretion of IL-4, IL10, TGF-β, and IFN-γ while suppressing production of TNFα at the same time (83).

A prospective study of pregnant lupus patients measured serum levels of sex steroid hormones as well as several cytokines (84). Estradiol and progesterone were significantly lower in the second and most of the third trimester of pregnancy in lupus patients compared to healthy controls. The failure to produce high concentrations of estradiol and progesterone could be due to impaired function of the placenta which, in turn, might also be involved in the increased rate of fetal loss observed in SLE. Furthermore, reduced serum levels of sex steroid hormones could also influence the secretion of cytokines as shown in the same study (84). In SLE patients, IL-6 serum levels remained low and did not increase in the third trimester of pregnancy as was observed in healthy controls. The absence of a rise in IL-6 is interesting in the context of cytokine function: IL-6 is necessary for T cell help for B cells (85). IL-10 did rise progressively during pregnancy in healthy women (84). In contrast, IL-10 levels were significantly higher already at conception in SLE patients and remained elevated throughout pregnancy and postpartum in SLE patients. IL-10 was originally thought to be a Th2 cytokine but is a pleiotropic cytokine with both immune stimulatory and immune suppressive functions that place it outside of the Th1-Th2 paradigm (85). The persistently high levels of IL-10 indicate a constitucional overproduction of IL-10 in SLE resulting in continuous B cell stimulation. No significant differences between SLE patients and controls were found in either sTNFR I or II levels or profiles before and during pregnancy. sTNFR I levels were significantly higher during pregnancy and postpartum in SLE patients with active disease compared to healthy controls (84). Studies of regulatory T cells in SLE patients have shown reduced numbers in active lupus and impaired suppressive function of Treg (86). A pilot study indicates that there is an imbalance between Treg and number of Th17 in pregnant lupus patients (87).

On the other hand, high prolactin (PRL) levels, in turn, seem to be associated with active Systemic Lupus Erythematosus (SLE) during pregnancy. PRL is capable of influencing immune responses and it is a cytokine. Hyperprolactinemia (HPRL) has been found in 20–30% of SLE patients and it seems to be associated with clinical activity during pregnancy (88).

LUPUS FLARES DURING PREGNANCY

An important aspect of pregnancy in lupus patients is the risk of the occurrence of disease flares. It is not simple to quantify the incidence of such complications because many clinical studies were done using individual definitions of flare. In recent times, many efforts were made to create a "pregnancy-version" of existing activity indexes such as ECLAM, SLEDAI, SLAM, and LAI in order to make studies more comparable (89). The literature reports discordant results from prospective, controlled observational studies: some showed that women are at increased risk of lupus flares when pregnant while other studies reached the opposite conclusion, finding a flare rate unchanged as compared to non-pregnant SLE patients (89). Timing of relapse is also variable among studies (89). A disease flare can occur at any time, but there may be a trend towards flares in the third trimester. Since the timing of flares is unpredictable, regular follow-up is indicated throughout pregnancy and post-partum. A point on which everyone seems to agree, is that the risk of flare depends on the level of maternal disease activity in the 6-12 months before conception. It is increased in women with repeated flares before conception (89) who discontinue useful medications (in particular HCQ) (90) and, in particular, in women with active glomerulonephritis at the time of conception (91). Poor control of disease activity before pregnancy may also have detrimental effects on pregnancy outcome. It has been observed that disease activity for the 6 months before the conception was associated with an increase in the rate of pregnancy loss (92). Patients with the combination of high clinical activity of SLE and either low complement or positive anti-dsDNA had the highest rate of pregnancy loss and preterm birth (93). Hence, the importante of a careful evaluation of the maternal condition before and during pregnancy. As a matter of fact, patients who started a pregnancy in a stable remission period and who continued medications experienced few

flares, mostly mild and generally well managed with a temporary increase in prednisone dose (90).

Another important task for clinicians is to distinguishing lupus activity from physiological changes of pregnancy and from other pregnancy complications that may mimic, in particular, a renal flare. Life-threatening conditions such as pre-eclampsia and HELLP syndrome (haemolysis, elevated liver enzymes, and low platelets) may develop early in pregnancy (around the 20th week) and should be promptly recognized in order to prevent rapid progression.

MATERNAL OUTCOME

Organ involvement and pregnancy

Patients with a high degree of irreversible organ damage are more likely to suffer complications or worsening of the previous damage during and after pregnancy (90).

Lupus Nephritis (LN) is a major manifestation of SLE and, therefore, it is a common situation today to have a pregnancy in SLE women with a biopsy-proven diagnosis of renal disease. SLE patients with active lupus nephritis are at higher risk for pregnancy complications than SLE patients without renal disease, and should be advised against pregnancy until a renal remission of at least 6 months duration, better 12-18 according to most recent recommendations (94), has been achieved. Women with quiescent disease (proteinuria <500 mg/day and inactive urinary sediment) and unaffected renal function are at reasonably low risk during pregnancy but should be tightly monitored. In normal pregnancy, the glomerular filtration rate increases by 30-50% and creatinine clearance rises to over 100 ml/min, causing a decrease in serum creatinine. Tubular reabsorption of protein is decreased during pregnancy, that's why an increase in the normal amount of proteinuria to 150-180 mg/24 h is possible. On the other hand, a new onset of proteinuria >300 mg/24 h can be considered as pathological in pregnant patients without proteinuria at baseline. Variable results have been reported on the pregnancy outcome in SLE women with a pre-existing LN. The rate of successful pregnancies varied between 65% and 92% whereas the incidence of flares ranged from 8 to 30% (91-97). A recent systematic review and meta-analysis of pregnancy outcome (98) demonstrated a significant association between active LN and both the onset of maternal hypertension during pregnancy and the rate of premature birth. History of nephritis was also associated with pre-eclampsia. Another important question to be addressed is whether pregnancy would deteriorate renal function permanently. Different findings were reported, but it seems that permanent damage is more likely to occur in those patients that already have a severe impairment of renal function at conception (91-92,99-100).

Apart from LN, different organ involvement may be negatively influenced by pregnancy. Patients with restrictive pulmonary disease may worsen during pregnancy due to thoracic compression by the growing uterus. Likewise, women with cardiac disease may be at risk of heart failure due to volume overload caused by the normal increase in circulating volume (101). Pregnancy should be considered absolutely contraindicated in women with symptomatic pulmonary hypertension, which carries a higher than 30% maternal mortality during late pregnancy and the puerperium (102).

Morbidity and mortality

A recent study done in the United States on pregnancy related admissions (information derived from discharge codes) showed that women with SLE may be at increased risk of serious medical complications and mortality in comparison to non-SLE pregnant women (103). At the time of conception, SLE patients had more co-morbid conditions than healthy coetaneous. SLE patients, in particular suffered from pre-gestational diabetes, arterial hypertension, pulmonary hypertension, renal failure, and thrombophilia much more frequently than healthy women (103). Moreover, SLE patients tend to become pregnant at an older age in comparison to the general population. Even when adjusted for the increased age, the risk of maternal complications in women with SLE remains higher than in healthy pregnant. In particular, it was estimated a 2-4-fold increase in the rate of caesarean section, pre-eclampsia and eclampsia, especially in women taking high-dose prednisone, with preexisting hypertension and/or renal insufficiency (103). In a different cohort (PROMISSE study), 15% of SLE patients developed pre-eclampsia, rising to 22% if there were also positive aPL (104). The risk for sepsis and pneumonia was found to be greatly increased due to both disease-related immune dysregulation and immunosuppressive therapy (103). Also hematological complications requiring transfusion, e.g., post-partum haemorrhage, antepartum bleeding, anemia at delivery, and thrombocytopenia, were more common in lupus patients (104). The risk of both venous thromboembolism and stroke was 6,5- fold higher compared to healthy pregnant and also the maternal mortality rate was increased, with an excess risk estimated at 20 times higher than in general population (103). These data, collected using the discharge diagnosis, may be not comparable with those derived by tertiary referral centers in which a careful multidisciplinary management of pregnant women with SLE allows a better prognosis for both maternal and fetal outcome. However, these data underline the potential risk for an increased maternal morbidity and mortality and suggest the need for a high level of vigilance during SLE pregnancy.

PREGNANCY AND NEONATAL OUTCOME

Pregnancy outcome

Despite major improvement in the last decades, the risk of obstetric and neonatal complications in SLE pregnancy is greater than in general population. It has been estimated that women with SLE have fewer live births compared to the general population, in particular, those with high disease activity (105). Maternal lupus activity and the presence of concomitant APS were found to be associated with major

obstetrical complications (92,105) being estimated that about 20% of pregnancies in women with SLE end with a fetal wastage (106). The rate of preterm birth (delivery before 37 weeks gestation) is increased in SLE patients. The incidence, according to recent studies, seems to vary between 23 and 28% (107). Preterm birth is usually spontaneous, mainly due to the Premature Rupture of Membranes (PPROM), but there is also an important percentage of cases in which delivery is induced to protect the health of the mother and/or of the baby (onset of fetal distress or pre-eclampsia) (108). Risk factors for preterm delivery include disease activity prior to and during pregnancy (including serological activity, i.e., high titre anti-DNA antibodies, low serum complement levels) (93), higher prednisone dose, hypertension (92), and thyroid disease (109). Particular attention must be placed at babies born before 28 weeks gestation because they are at highest risk of neonatal death and for both long-term medical complications and cognitive impairment (110). Regarding the birth of babies with low weight (<2500 g) or small for gestational age (SGA: birth weight less than the 10th percentile for gestational age), these conditions are more common in SLE pregnancies, ranging from 6 to 35% (107). This finding is not surprising because placental insufficiency, which is frequent in lupus pregnancies (111), leads to an intrauterine growth restriction (IUGR) and to the birth of growth-restricted infants.

Current standard of care in SLE pregnancy includes Doppler studies of uterine arteries and the umbilical artery, which are helpful to assess placental function and to prevent the occurrence of complications such as pre-eclampsia and fetal distress (90). Uterine Doppler studies are useful as a screening test starting from the 20th week and being the 24th week is the best moment for the evaluation. At this time, the test has a high negative predictive value, which means that a normal result will be rarely associated with obstetric complications. The positive predictive value is low, which means that an increased resistance of uterine arteries could suggest an adverse outcome, but the risk is poorly quantifiable. Differently, umbilical Doppler ultrasound gives a more accurate definition of the placental function, showing various degree of impairment such as increased resistance, absent diastolic flow, or even reverse diastolic flow, which is a clear sign of placental insufficiency and fetal distress (112). Many of the previously cited fetal complications may be related to the presence of aPL (this topic will be discussed in a separate paragraph).

On the other hand, women with SLE are at high risk of adverse pregnancy outcomes. However, those who have a perinatal death in their first pregnancy can expect a live birth in a subsequent pregnancy (113).

Neonatal lupus erythematosus

Neonatal lupus erythematosus (NLE) refers to a clinical spectrum of cutaneous, cardiac, and systemic abnormalities observed in newborn infants whose mothers have autoantibodies against Ro/SSA and La/SSB. The condition is rare and usually benign and self-limited but sometimes may be associated with serious sequelae. This is a passive transfer of anti-Ro/SSA and/or anti-La/SSB antibodies that occurs in some babies of mothers with autoimmune disease (114).

These autoantibodies may cause damage to the developing tissue and increase the risk of bearing infants with NLE. Approximately 98% of affected infants have maternal transfer of autoantibodies against Ro/SSA, La/SSB, and, less commonly, U1-RNP. However, only 1-2% of mothers with these autoantibodies have neonates with NLE regardless of whether the mothers are symptomatic or not (114).

The diagnosis is usually established based on the clinical features and the demonstration of neonatal Lupus-associated antibodies in the serum of the mother or the affected infant. The most common clinical manifestations of NLE are, in decreasing order of frequency: dermatological, cardiac, and hepatic abnormalities. Some infants may also have hematological, neurological, or splenic abnormalities. The most serious complication in the neonate is complete heart block, which occurs in approximately 2 percent of such pregnancies. Isolated skin rash occurs in a similar percentage (114-116).

LABORATORY STUDIES

At the first visit after or when pregnancy is confirmed, the following assessments are recommended:

- Physical examination, including blood pressure evaluation
- Renal function tests, including determination of the glomerular filtration rate, urinalysis, and tests of the urine protein–to–urine creatinine ratio
- Complete blood count (CBC)
- Test for anti-Ro/SSA and anti-La/SSB antibodies
- LA and aCL studies
- Anti–double-stranded DNA (anti-dsDNA) test
- Complement (CH50 or C3 and C4) tests

During the first two trimesters, a monthly platelet count or CBC is recommended. The following evaluations are recommended at the end of each trimester of pregnancy:

- Determination of the glomerular filtration rate and measurement of the urine protein–to–urine creatinine ratio
- aCL measurement
- Complement (CH50 or C3 and C4) test
- Anti-dsDNA antibody study

Flares of SLE are likely to be associated with hypocomplementemia and increased titers of anti-DNA antibodies. In comparison, complement levels are usually (but not always) increased in patients with preeclampsia (63,67,117).

TREATMENT

None of the medications used in the treatment of SLE is absolutely safe during pregnancy. Hence, whether to use med-

ications should be decided after careful assessment of the risks and benefits in consultation with the patient. During the first trimester, most of the drugs should be avoided. Although, breastfeeding is feasible for most women with SLE, some medications may enter breast milk. Therefore, immunosuppressive agents are contraindicated, and long-acting non-steroidal anti-inflammatory drugs (NSAIDs) are inadvisable. Short-acting NSAIDs, antimalarials, azathioprine, low-dose prednisone, warfarin, and heparin seem to be safe.

Renal involvement

Patients with a significant flare of LN should be treated with high-dose prednisone and antihypertensive medication (e.g., hydralazine, methyldopa, and calcium channel blockers but not angiotensin converting enzyme inhibitors or some beta blockers).

There is little if any experience with pulse methylprednisolone in pregnancy, and its effects on the fetus are unknown. Cyclophosphamide is not safe during pregnancy, but azathioprine can be used cautiously. In addition, the fetus should be delivered as soon as possible (118-129).

Patients with renal disease should be monitored jointly by a nephrologist and by an obstetrician familiar with the effects of renal disease on pregnancy. General principles of management include the following:

- Increased frequency of prenatal visits; these should occur every two weeks until the third trimester and then weekly.
- Early detection and treatment of asymptomatic bacteriuria.
- Serial monitoring (at least monthly) of maternal renal function.
- Close monitoring for the development of preeclampsia.
- Fetal surveillance with ultrasound and fetal heart rate monitoring to assess fetal growth and well-being.
- Aggressive treatment of maternal hypertension. Preterm intervention may be necessary in the presence of deteriorating renal function, severe preeclampsia, fetal growth restriction, or nonreassuring fetal testing (e.g., fetal distress). In most women, elective delivery is indicated if labor has not occurred by the estimated date of confinement.

Neonatal lupus erythematosus

Maternal use of HCQ may be associated with reduced rates of cardiac manifestations in the newborn, including congenital heart block and isolated cardiomyopathy, and maternal HCQ use is also associated with a decreased risk of recurrence of cardiac neonatal lupus in subsequent pregnancies (131). However, antimalarial agents have potential toxicity and such a slow onset of action that their use in the treatment of this transient condition is probably not indicated. Systemic corticosteroids and immunosuppressive agents are generally not indicated in the treatment of NLE. However, infants with severe hepatic and hematological involvement may require treatment with systemic corticosteroids, intravenous immunoglobulin, and/or immunosuppressive agents (114).

CONTRACEPTION, FERTILITY AND ASSISTED REPRODUCTION

Contraception for SLE/APS patients goes beyond the simple need of avoiding unwanted pregnancies. Several conditions may require an effective contraception: early stage of the disease, very active disease, severe organ involvement or damage, and use of embryotoxic/fetotoxic drugs. Therefore, contraceptive counseling is essential in the rheumatological practice, but in "real life" most women do not receive any information about this issue (132). A common misconception among women with SLE is that they "cannot use birth control," since the "classical" estrogen-containing pill is generally contraindicated. The message should be that women with SLE can be considered good candidates for many contraceptive methods, including hormonal contraceptives, and the most suitable one should be chosen individually (133).

The three main types of contraceptives are: barrier methods, intrauterine device (IUD), and hormonal method. Barrier methods are an effective, cheap method of preventing pregnancy and sexually transmitted disease. However the unintended pregnancy rate remains high–around 17% for condom and diaphragm. The IUD is available in a non medicated or medicated form (with progesterone). With typical use, the rate of unplanned pregnancy is low (around 2%). Complications could be irregular bleeding alter placement, the risk of expulsion of the device (5% falling out over the 5 year life of the device) and the risk of infection after insertion that can lead to pelvic inflammatory disease (PID). Current IUD devices are actually safer also in high risk groups (134), but the infectious risk should be anyway monitored overtime. It could be preferable to use IUD in patients with a single sexual partner and a mild treatment (no immunosuppressive drugs, prednisone lower than 10 mg per day). The use of estrogen-containing oral contraceptives (OC) has been greatly discouraged because of initial reports of SLE flare due to the hormonal treatment and subsequently supported by growing experimental evidence of the role of estrogens in the pathogenesis of SLE (135). However, two recent randomized clinical trials (136,137) supported the safety of low dose combined OC in a well-defined population of stable SLE patients with inactive or stable active disease in regard to the risk of SLE flare. On the other hand, the presence of aPL remains a major contraindication to combined OC due to the increased risk of thrombosis. Progestin-only preparations (daily oral pill, depot medroxyprogesterone, subcutaneous implants) do not appear to increase immune activity and are not associated with increased rate of flares nor does the dose of progestin increase the risk for thrombosis. A recent large study in SLE patients showed good gynecological tolerability (low rate of discontinuation

for breakthrough bleeding or hypoestrogenia) (138). A major concern about the use of progesterone can be the effect on bone health. However, the reduction of bone mineral density has been shown to be reversible after discontinuation of treatment (139).

Women affected by SLE have an overall fertility rate similar to that of the normal obstetric population with a mean family size of 2 live births (140). However, there are a few conditions in which fertility may be impaired (141). Patients with chronic renal failure may have reduced fertility (142). Most of the immunosuppressive drugs do not influence fertility, with the exception of CYC (143). The risk of infertility is related to the cumulative dose of the drug and to the age of the patient, being "older" women with a lower ovarian reserve at higher risk for premature ovarian failure. Protection of ovarian function can be provided by treatment with gonadotropin-releasing hormone analogues (144). Women should be informed that NSAIDs may inhibit ovulation, therefore they should stop them at day 8 of the menstrual cycle when they want to conceive (145). Whatever is the cause of infertility, related or not to the disease, patients with SLE/APS may ask for medical assisted reproductive techniques (ARTs). The most used technique is IVF-ET (in Vitro fertilization and embryo transfer), which requires ovarian stimulation for oocyte pick-up. Ovarian stimulation is actually what may create concern in SLE/APS women for several reasons. These are base on theory basis and on small series reports (146): 1) high dose estrogens may induce a disease flare; 2) the enhanced hormonal milieu may increase the risk of thrombosis, especially in those women with aPL; 3) these complications may become lifethreatening in the case of ovarian hyperstimulation syndrome; 4) there could be a trend toward a worse prognosis for both pregnancy rate and live-birth rate after ARTs. Antithrombotic prophylaxis should be carried out on women with aPL with special attention for those who had a prior thrombosis (147).

RHEUMATOID ARTHRITIS

It has been established that women, especially those at reproductive age, are more susceptible to rheumatoid arthritis (RA) than men. On the other hand, it was thought that the beginning of RA would be delayed by pregnancy and the disease risk would be reduced during gestation. Ever since, various studies have show disease remission in about 75% of patients with RA who got pregnant during the active state of the disease while it may relapse in the postpartum period in 90% of the patients (148-150). In addition to steroid hormone action, immunological changes and the increased galactosylation of IgG seem to play important roles in RA improvement during gestation (151). In 1999, Barret published the largest prospective study with 140 women and showed that most of them presented an increased level of pain and articular edema from 1 to 6 months after delivery. Endogenous hormone influence on RA is reinforced by the fact that the use of oral contraceptive pills plays a protective role in RA incidence. Another explanation for the improvement of the disease activity during pregnancy would be the enhanced immunological tolerance observed during this period. It is believed that immunological changes related to pregnancy are due to maternal exposure to fetal antigens that have a paternal origin (152). Nelson and colleagues noticed a higher number of class II HLA incompatibilities between the mother and the child in women who presented reduced disease activity during pregnancy (153). In addition, the higher Th2 response which occurs during pregnancy also seems to influence the disease activity level, improving RA and worsening SLE (154).

There is no scientific evidence relating parity to the risk of presenting the disease or its severity (155). In patients with RA, there have been reports of higher incidence of premature membrane rupture, longer hospital stay after delivery, and more cesarean births than general population (149), but the results are conflicting considering the incidence of pre-eclampsia in this group of patients (149, 155).

Although most of the patients have the disease in remission during pregnancy, a small number of patients with RA may have disease activity in this period. RA flare in pregnant woman may be treated with low doses of prednisone, HCQ, sulfasalazine and even anti-TNFs, if necessary (156).

Anti-TNF agents such as infliximab, adalimumab, and etarnacept have received a B classification from the FDA (156) meaning they do not appear to cause fetal anomalies in animals and there are no adequate and well-controlled studies in pregnant women. A recent survey with members of the American College of Rheumatology (ACR) has shown that most of them agree that treatment should not be started during pregnancy, but most specialists would not interrupt treatment with biological agents, which had been started before pregnancy.

Current data on the use of anti-TNF agents on pregnant human females is encouraging. A cohort of 30 women with RA exposed to adalimumab during pregnancy has yielded 90% live births, prematurity of birth in 11% of cases and two congenital anomalies (microcephaly and non descending testicles), which was similar to the control group without drug exposure (157). The results from a pregnancy-reporting database with an even larger number of patients that used infliximab have not shown an increased risk of pregnancy loss or congenital anomalies (157). Because of the in utero transfer of these drugs, a few authors recommend their discontinuation at the beginning of the third trimester to avoid immunosuppression in the newborn (157). The CTL4 receptor blocker, abatacept, as well as rituximab and tocilizumab must not be initiated during gestation and should be discontinued due to their limited experience with pregnant women (151).

Treatment with methotrexate (MTX), prescribed alone or in combination with biological therapies, must be discontinued for at least 3 months before conception or switched to azathioprine in patients planning conception due to elevated risk of fetal malformations. If the patient becomes pregnant while using MTX, high doses of folic acid (>10mg/d)

must be administered although the severe cases of teratogenicity may result in spontaneous abortion. Restarting immunosuppressive therapy with MTX shortly after delivery decreases the risk of postpartum RA flare, but MTX is also excreted in breast milk and should not be used while breastfeeding (158).

Considering patients using leflunomide, its active metabolite undergoes extensive enterohepatic circulation and may persist in the body for up to 2 years. It should be discontinued for this period prior to conception or cholestyramine wash-out should be prescribed (155). If the patient becomes pregnant while using this medication, cholestyramine should be administered until there is no further detection of leflunomide level in the serum. The OTIS databank shows 9.3% of congenital anomalies are related to the use of leflunomide, which does not differ from the RA control population (13%) but is larger than that of a healthy population (3.5%) (157).

UNDIFFERENTIATED CONNECTIVE TISSUE DISEASE

Undifferentiated connective tissue disease (UCTD) which, in general, affects women before their 40s, is characterized by clinical manifestations of one or more systemic autoimmune diseases but does not fulfill classification criteria for any disease specifically. The UCTD can persist as an undifferentiated picture or may migrate to a defined diagnostic such as SLE, systemic sclerosis, Sjögrens syndrome, RA or other diseases. The differentiation into a defined entity, when it happens, generally takes place in the first two or three years (159). The concerning complications of UCTD that can happen during pregnancy are the development of nephritis related to SLE or myositis probably related to polymyositis. Just as in patients with SLE, conception in a stable phase of the disease and after three years of development yields a better gestational result. Pre-natal follow-up in with a multidisciplinary team is essential with special attention to signs of disease activity along with obstetric and fetal complications (159). HCQ is recommended during pregnancy and can be used when breastfeeding without any problem (160).

SJÖGREN'S SYNDROME

This is an inflammatory autoimmune syndrome that, apart from general and musculoskeletal symptoms, is clinically characterized by the drying of oral and ocular mucosa as proven by objective tests. The presence of anti-Ro/SSA antibodies (70 to 80%) is part of the diagnostic criteria (161) and may even precede clinical manifestations. It is thought that these antibodies when in Sjogren's syndrome may induce neonatal lupus syndrome with a higher frequency rates than in mothers with SLE, and the surveillance approach must be the same as in SLE patients (162). Apart from rheumatic diseases, Sjögren's syndrome may be associated with thyroiditis and autoimmune liver diseases, with an increased risk for the development lymphoproliferative diseases such as lymphomas.

SYSTEMIC SCLEROSIS

Skin involvement remains stable during pregnancy in systemic sclerosis, but it may worsen after birth (163). Raynaud's phenomenon usually gets better because of physiological changes that lead to increase cardiac output. Gastroesophageal reflux disease, which already frequently occurs in disease free pregnant women, generally gets even worse in systemic sclerotic patients and recurrent episodes of vomit may cause Mallory-Weiss syndrome in the already damaged by the disease-related fibrotic esophagus (149). It is extremely important to make the diagnosis right away and act fast to avoid life-threatening bleeding.

The most serious complication in systemic sclerosis pregnant women is renal crisis due to sudden arterial hypertension. Rise in creatinine with no proteinuria in the initial phase of acute kidney injury makes the diagnosis of systemic sclerosis renal crisis likely. Renal crisis is much more common in patients with less than five years of disease with diffuse skin involvement, positive anti-topoisomerase1 and previous exposure to high doses of steroids (149).

Another serious complication is pulmonary hypertension. This complication of systemic sclerosis is associated with 30 to 50% maternal mortality rates and requires stronger vigilance 48 to 72 hours after delivery (149) when physiological changes may lead to cardiovascular instability. Screening should be done before conception and its diagnosis during pregnancy may be an indication of therapeutic abortion once it may endanger the mother's life (149).

Because they have a higher chance of hypertensive disease, women with systemic sclerosis have higher rates of preeclampsia and their hospital stay tends to be longer after delivery than healthy women. Hepatic enzyme elevation and proteinuria with edema are more common in preeclampsia and HELLP syndrome than scleroderma renal crisis (149).

POLYMYOSITIS AND DERMATOMYOSITIS

Polymyositis (PM) is a systemic autoimmune disease characterized by inflammation of the striated musculature. When inflammation of the muscle is associated with characteristic cutaneous manifestations, it is called dermatomyositis (DM). Myositis may have the onset in childhood or in post-menopause period; therefore pregnancies in patients affected with PM or DM are rare and the available data on pregnancy are from case reports or small series. A retrospective study showed higher risk of flares during pregnancy of the cases diagnosed in the childhood, even the ones in remission for years, than the ones diagnosed in adult life (159). The best obstetric result is obtained when the disease is controlled by conception time (164).

If there is disease activity with elevation of muscular enzymes within the first trimester of gestation, the obstetric outcome is worse than if this elevation occurs in the second or the third trimester. The treatment is normally prednisone 1mg/Kg/day until CPK normalization. The association with azathioprine or cyclosporine, in general, is necessary and it has a sparing effect of steroid. In refractory cases, IVIG or plasmapharesis are possible modalities as they are safe during pregnancy (159).

MIXED CONNECTIVE TISSUE DISEASE

Mixed Connective Tissue Disease (MCTD) is characterized by the overlap of clinical characteristics of various systemic autoimmune diseases associated anti-RNP antibodies, which induce a specific speckled pattern of anti nuclear antibody (ANA). The prognosis tends to be better than in SLE and systemic sclerosis and reports of flares with pregnancy are very rare (159).

SYSTEMIC NECROTIZING VASCULITIDES

There are few prospective studies of pregnant women with vasculitis. When pregnancy is planned for a period of remission in women with granulomatosis with angiitis (previously called Wegener's granulomatosis), microscopic poliangiitis (fulfilling the group nominated as ANCA associated vasculitis), and Churg-Strauss syndrome, usually there is no further intercurrence (149). Takayasu's arteritis may have hypertensive complications during pregnancy despite stable disease (165). The risk of flaring is higher when pregnancy occurs with active or recently diagnosed disease.

TAKAYASU'S ARTERITIS

This is described as a granulomatous vaculitis that affects preferably large caliber vessels like the aorta, its branches and pulmonary arteries. Takayasu's affects typically women in fertile age; therefore it is more commonly associated pregnancy than other systemic necrotizing vasculitides. The aortic valve disease and aortic aneurysm resulting from Takayasu's arteritis may be fatal risk factors in case of pregnancy, therefore, in these cases, pregnancy must be discouraged. These patients have a higher frequency of maternal hypertension and preeclampsia than normal controls, in addition to a higher chance of developing cardiac insufficiency, renal insufficiency, and brain hemorrhage. In spite of those possible complications, in general, pregnancy outcome is favorable in these patients once adequately managed by specialists. Fortunately, the disease clinical activity does not seem to flare with pregnancy (165).

Maternal and gestational outcome seems to be influenced by arterial hypertension. Arterial blood pressure monitoring in women with Takayasu's arteritis may be harder in the presence of different central and peripheral blood pressures. In some cases, it is recommended invasive monitoring of blood pressure and elective cesarean delivery should be considered in patients with severe retinopathy, impaired umbilical artery flow, associated preeclampsia or inability of maternal and fetal monitoring (165). If disease treatment is necessary, it is normally prednisone 1mg/kg/day combined with azathioprine with subsequent reduction after improvement. Arterial hypertension must be aggressively treated with methyldopa, calcium channel blockers or hydralazine, while ACE inhibitors must be avoided (165).

Intra-uterine growth restriction was present in most studies evaluating pregnant woman with Takayasu's arteritis and affected up to 50% of pregnancies(166). It was associated with more severe disease or involvement of abdominal aorta and renal artery suggesting that fetal growth restriction was the result of impaired placental blood flow (165). One author described an elevated incidence of preterm birth and pre-eclampsia in this group of patients (167) but this finding was not confirmed by other authors.

GRANULOMATOSIS WITH ANGIITIS

It is a necrotizing vasculitis of small vessels with preference for superior respiratory tract and kidneys and higher incidence in women between 40 and 50 years old. It is normally associated with the presence of anti-neutrophil antibody (ANCA). Reports of pregnancy in patients with granulomatosis with poliangiitis are rare. Thirty eight gestations in patients with were reported: 21 were in remission during pregnancy, 13 were diagnosed during the pregnancy and 4 had active disease when became pregnant. Disease flare seems to occur with a higher frequency in the first trimester or during puerperium and is more severe when the patient becomes pregnant while the disease is still activite (149). As in SLE, differential diagnosis between renal vasculitis and eclampsia is important and the ANCA titer may be helpful (159). A few authores have reported an increased incidence of prematurity and pre-eclampsia (168).

MICROSCOPIC POLYANGIITIS

It is an autoimmune systemic necrotizing vasculitis with preference for small vessels. Just as granulomatosis with polyangiits, it includes with the presence of ANCA but with a different antigen: myeloperoxidase (anti-MPO). There are reports of anti-MPO antibodies trespassing the placenta and inducing mild microscopic polyangiitis-like disease (169). Patients with microscopic polyangiitis can present with pulmonary–renal hemorrhage syndromes, clinically similar to what can be seen in antiglomerular basement membrane disease (168). As mentioned with previous entities, the best pregnancy outcomes are when the patient is currently in remission and has a planed pregnancy and adequate follow up.

CHURG-STRAUSS SYNDROME

In Churg-Strauss syndrome, the systemic involvement of small blood vessels with pulmonary involvement accompa-

nied by hypereosinophilia is remarkable. The involvement of cardiac and respiratory systems is more common in recurrence (149). Asthma is more common, but cardiac involvement may bring irreversible damage (149). Once more, adequate planning is essential. The disease reactivation seem to occur in half the patients that get pregnant while in remission with worsening of asthma, mononeuritis multiplex, and cutaneous lesions. Even though there are reports of prematurity, in most cases, birth occurs at term. The worst obstetric outcomes were from patients that had the onset of the disease during pregnancy. Disease flare is usually treated with prednisone (159).

BEHÇET'S DISEASE

Behçet's disease is systemic inflammatory process characterized by genital and oral ulcerations, in addition to ocular, gastroenterological, thrombotic, and neurologic manifestations. There is some reduction of disease activity in most pregnancies, although exacerbation may occur in one-sixth to one-fourth of patients (149,159). Most common recurrences were worsening of ulcerations in mucosa, arthritis and ocular inflammation (159). Anticoagulation during pregnancy and postpartum should be considered in patients with previous thrombosis (157). Some case reports and retrospective series indicate that the obstetric results are encouraging, but monitoring and fast treatment, when warranted, is crucial (159).

DISEASE MODIFYING ANTI-RHEUMATIC DRUGS AND ITS USE DURING PREGNANCY AND BREASTFEEDING

In the 90s, the FDA recognized flaws in its proposed system of safety information about medication throughout pregnancy and breast-feeding, and started to look for ways to improve it. Several public auditions were organized to obtain information from specialists and scientists. There was an overall agreement that the letter category is extremely simplistic and provides an inaccurate vision of the risks. At the same time, it does not facilitate data update with new information achievement, when those become available. The new proposal is to remove letter category and report the information in three sections:

1. The first is called "fetal risk summary" and must describe what is known about medication effects on the fetus and, when there is any risk, if this one is grounded in studies taken in animals or in humans. In this proposal it must be informed a conclusion based in available data depending on the quantity and quality.

2. Another section called "clinical considerations" must indicate the effects of the use of drugs taken by the mother before knowing about the pregnancy. This section will also have discussion about the risk of disease in the mother or the fetus, information about dosage, and what to do in case of complications.

3. The third section called "data" must describe in better detail the available data on humans and animals utilized in the development of the fetal risk summary. This section will also bring information about any database of exposed individuals, which collects and maintains data on already approved drugs' effects that are prescribed to pregnant women.

These new sections of recommendations about medication use in breast-feeding will inform about the quantity excreted in the breast milk and the potential effects on lactation. Some recently approved drugs will already use the new classification form, while the already established ones will gradually migrate to the new classification.

ASPIRIN AND ACETAMINOPHEN

Aspirin is used during pregnancy and puerperium in low doses only (80 to 100 mg/day), especially in patients with recurrent fetal loss related to APS, and is not associated with increased risk of antenatal complications or neonatal morbidity (11). Higher doses are associated with premature closure of ductus arteriosus and impaired renal function, especially with third trimester exposure (158). Acetaminophen may be used during the entire gestation and breast-feeding in the lowest dosage possible (149).

NON-STEROIDAL ANTIINFLAMATORIES AND COX INHIBITORS

The lowest dose possible should be used for a short time and it is recommended to be completely discontinued after the 32th week, because of the risks of fetal and maternal hemorrhage in addition to fetal renal disfunction, oligodramnia and premature closure of arterial duct(158). Some of them were considered safe by the America Pediatrics Society to be used during breast-feeding (for example ibuprofen, indomethacin, naproxen) (158). Prescription of acetaminophen should be considered before these medications for pain control. The COX-2 inhibitors, like celecoxib, have small data during pregnancy, may influence renal formation and its use should be avoided during pregnancy (151).

ANTIMALARIALS

Antimalarials have been broadly used in rheumatology for many decades. Even though chloroquine and HCQ trespass the placenta, no fetal defects were observed in pregnant women exposed to these substances. Chloroquine is broadly used in malaria treatment during pregnancy. From Johns Hopkins University SLE cohort, Clowse did not demonstrate any associated risk to this medication in pregnancy (169). There are no malformations, ocular involve-

ments or growth retardations described despite extended fetal exposure to HCQ in numerous studies. Chloroquine has smaller data when compared to HCQ, but no long-term sequel was also demonstrated (158). In addition, a randomized double-blinded placebo controlled Brazilian study, did not show any complication related to HCQ (170).

A systematic review that included random and observational clinical studies aimed to examine antimalarials safety in the ocular function of exposed women's children. The authors used the GRADE established criteria to analyze the papers. Twelve studies with a total of 588 born children from mothers treated with chloroquine or HCQ during gestation fulfilled inclusion's criteria. Five studies with a total of 251 exposed fetuses did not report any ocular abnormality (160). Therefore, the current evidence suggests that there is no fetal ocular toxicity with antimalarials. They are secreted in breast-milk, but there was no report of adverse effects in breast-fed children whose mothers used HCQ (158).

CORTICOSTEROIDS

The morbidities related to steroid use are those that might occur with non-pregnant women, including bone avascular necrosis, osteopenia, immunosuppression, hyperglycemia, hypertension, cataract, etc. It may also precipitate pregnancy complications, e.g., gestational diabetes, arterial hypertension and premature rupture of membranes, so the lowest effective dose must be used (ideally not more than 15 mg/day) (149). Short acting agents (prednisone or prednisolone) for disease treatment should be used, which are metabolized by placenta's 11-β-hydroxysteroid and reduce fetal exposure to approximately 10% of maternal dosage (149). Calcium supplementation is recommended (up to 1200mg of calcium carbonate) and vitamin D (up to 400U/day). Patients using chronic steroids must receive stress dose hydrocortisone supplementation (intravenous hydrocortisone 100 mg every 8 h) when emergency surgery is necessary, c-section or prolonged labor (171). Long-term studies have not shown an increased risk of teratogenicity, although a few reports associated first trimester exposure with an increased relative risk of oral clefts with low absolute risk (158). Corticosteroids do not enter breast milk in large quantities and there is no contraindication to breast-feeding in women who are on corticosteroid therapy (171). Women that are breast-feeding and using higher doses should wait 4 hours after taking the pill to breast-feed, reducing the drug concentration in the breast-milk (158).

AZATHIOPRINE

This immunosuppressant and steroid sparing agent is not associated with teratogenicity in humans as the fetal liver is not capable of metabolizing azathioprine into its active form (158) although teratogenicity has been reported in animal studies (151). Azathioprine is generally the immunosuppressive of choice in most centers specialized in high risk pregnancy because of the safety profile and its steroid sparing property. It should be prescribed (up to 200mg/day) when the disease activity is hard to control and/or the patient is already taking 20mg or more of prednisone for more than one month. The treatment with azathioprine is compatible to breast-feeding with no risks for the child (158).

SULFASALAZINE

There is no reason to believe that the safety studied with this drug in inflammatory bowel disease patients is different from RA or other rheumatic diseases. The drug should be continued (up to 2g/day) in patients planning to get pregnant and, in fact, is the first line for patients with inflammatory bowel disease. Studies with a large number of patients did not show any teratogenicity (171). Folate supplementation is necessary preconception and during pregnancy because sulfasalazine is a potent inhibitor of the reduced folate carrier. Although there is some theoretical concern that sulfasalazine may displace bilirubin and cause neonatal jaundice, there have been no reports of kernicterus in infants exposed to sulfasalazine (158)

CYCLOPHOSPHAMIDE

This immunosuppressive agent is contraindicated during pregnancy and patients at childbearing age should be counseled regarding its risks. In most academic centers it is routine to screen with chorionic gonadotropin before every pulse therapy with the agent and if pregnancy is wanted it should be discontinued for at least three months (171). In life threatening situations it has been used in second or third trimesters in the past but nowadays safer agents such as rituximab and mycophenolate mofetil are available.

METHOTREXATE

MTX is an antimetabolite that interferes in purine synthesis. It induces abortion and teratogenesis and, therefore, must not be used in pregnant women. Once again, childbearing women taking the drug should be counseled regarding its risks and in case the patient decides to get pregnant, MTX should be discontinued three months prior and folic acid supplementation should be started (158). If the patient becomes pregnant while on the drug, the recommendation is to use double doses of folic acid (10mg/day) during the whole pregnancy period. MTX is contraindicated during breastfeeding because it is excreted in breast milk and can accumulate in the infant's tissues (151).

LEFLUNOMIDE

This medication is classified as category X by FDA because animal reproduction studies indicate that leflunomide is both embryotoxic and teratogenic, mainly leading to craniofacial, skeletal, and cardiovascular malformations (151).

The experience reported by OTIS included 64 pregnancies exposed to leflunomide, compared to 108 non-exposed patients and failed to show any difference with regards to microcephaly or other embryopathies. It is important to notice that 95% of the study group underwent leflunomide washout with cholestyramine (158). Leflunomide may induce fetal effects similar to methotrexate, but with the possibility of being quickly reversed as soon as pregnancy is diagnosed, with administration of cholestyramine (8g PO, 3 times a day for 11 days, without risks for the pregnancy) until there is no level of serum leflunomide detectable. It is secreted in breast milk and administration is contraindicated during breastfeeding (151).

MYCOPHENOLATE MOFETIL

This agent has been mainly used for the treatment of systemic lupus nephritis as its safety profile is better than cyclophosphamide. It is also used after transplant surgery to avoid graft rejection. Human studies have shown teratogenicity and a specific syndrome characterized by craniofacial malformations of ear, oral cavity and ocular abnormalities. Limb, cardiovascular, renal and nervous system malformations were reported as well. It is currently category D from FDA and is contraindicated in breastfeeding (172)

BIOLOGICALS

These are monoclonal antibodies or fusion receptor blocking proteins that can dramatically interfere with the immune response. Targets are cytokines such as TNF, IL-1, IL-6 and signaling molecules such as CTLA-4, BLyS or CD20. These agents are relatively new and were introduced into the market in the last two decades after many years of study. This is a very active research field and many new drugs are under development and currently being studied. Available data is from series of cases and registry records. The risk of using certain biological agents in pregnancy is not from proven teratogenicity but is due to unknown long-term safety for the fetus. There are no available data with regards to lactation and the use of biologicals, therefore, their use should be avoided during breastfeeding (158).

ANTI-TNF

Etanercept, infliximab, adalimumab, golimumab and certolizumab are considered risk B by the FDA. Although the experience and knowledge with the use of such medications in pregnant women are increasing, there is no definite safety answer for its use. No adverse effects on fetal development have been described in animal studies and sporadic adverse events in humans were reported, but they had insufficient power to determine toxicity or safety (149). In RA patients there seems to be no risk in the preconception period and in the first trimester, as the drug does not cross the placenta in this phase (151). Considering the lack of data, most authors recommend discontinuation of therapy as soon as pregnancy is diagnosed (149,156).

RITUXIMAB, ABATACEPT, TOCILIZUMAB

Rituximab is a monoclonal antibody against CD20, expressed in B lymphocytes. It does cross the placenta and due to the lack of data about side effects with its before conception and during the first trimester, it is recommended that it be discontinued one year before pregnancy. Exposure to rituximab during the second and third trimester seems to cause depletion of fetal B cells (158). The use of rituximab during the pregnancy can be considered in RA with difficult control, SLE nephritis, ANCA associated vasculitis, and serious forms of APS, such as thrombocytopenia, hemolytic anemia and renal microangiopathy.

Tocilizumab is an antibody directed against IL-6 receptor. There is no adequate data about pregnant patients and use of tocilizumab. Animal studies show increased rate of abortion and fetal mortality but the dose used was 100 times higher than used in humans. It should be discontinued three to six months before pregnancy (158).

Abatacept is a fusion protein anti CTLA4 that works by blocking important activation signaling for the T lymphocyte. It does cross the palcenta but studies in animals did not show any sign of teratogenicity. Data in humans is limited and it is recommended to discontinue the drug 10 weeks prior to pregnancy.

None of the three described medications should be used during breastfeeding due to lack of safety data. They are rated category C by the FDA.

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28

SJÖGREN'S SYNDROME

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INTRODUCTION

Autoimmune diseases (ADs) represent a broad spectrum of chronic conditions that may afflict specific target organs or multiple systems and impose a significant burden on quality of life. The etiology of ADs is multifactorial in which a mosaic of factors (i.e., genetic, hormonal, and environmental) work in concert to induce a loss of tolerance and subsecuent tissue damage (1).

Sjögren's syndrome (SS) is an autoimmune epithelitis that affects the exocrine glands, mainly the lachrymal and salivary ones, with a functional impairment that usually presents as persistent dryness of the eyes and mouth (2–5). SS is a progressive disease characterized by a complex pathogenesis requiring a predisposing genetic background and involving immune cell activation and autoantibody production (2). Over the last few years, compelling evidence has suggested a pivotal role of the epithelium in orchestrating the focal lymphocytic infiltration of the exocrine glands which represent the histological hallmark of the disease (6,7). Its clinical spectrum extends from an autoimmune exocrinopathy (sicca syndrome) to a wide range of organ and system involvement known as extra-glandular manifestations (EGM) (8,9).

The first recorded ocular findings in SS were made by Dr. W.B. Hadden in 1888 who noted the association of dry eyes with dry mouth and filamentary keratitis. Hadden introduced the term xerostomia. Johann Mikulicz described the disorder in 1892. Mikulicz's patient was a 42-year-old farmer with bilateral parotid and lacrimal gland enlargement associated with a small round cell infiltrate. At autopsy, the swollen lacrimal, submandibular, and parotid glands were studied histologically by Mikulicz who described mononuclear infiltration of the lacrimal and salivary glands. This was the first description of what we now refer to as SS. Subsequently, the term Mikulicz syndrome was used to refer to swelling of the lacrimal and parotid glands due to a variety of causes (i.e., tuberculosis, other infections, sarcoidosis, and lymphoma) and evidently this is a misnomer. In fact, Mikulicz syndrome, Mikulicz disease, and SS are synonyms. In 1925, Gougerot, a French ophthalmologist, described the association of lacrimal gland hypofunction and filamentary keratitis. In 1933, a Swedish ophthalmologist, Henrik Sjögren, wrote the most comprehensive article on the subject and referred to the dry eye condition as keratoconjunctivitis sicca (KCS). He described clinical and histologic findings in 19 women, 13 of whom had probable rheumatoid arthritis (RA), with dry mouth and dry eyes. In 1936, Duke Elder honored Sjögren by naming the disease SS. In 1953, Morgan and Castleman presented a case study of a patient with SS and rekindled interest in the condition originally known as "Mikulicz's disease." Subsequently, these patients have been termed SS, whereas the term Mikulicz is still occasionally used to refer to the lymphoepithelial islands seen on glandular biopsy (10). The clinical features of the disease just summarized and as we currently recognize it in its florid form were outlined in 1956 by Bloch (11–12).

GEO-EPIDEMIOLOGY AND BURDEN OF THE DISEASE

While sicca complaints in the setting of SS are quite common, a relatively limited number of studies have tried to estimate the population prevalence of this condition. SS is the second most common systemic AD worldwide. The prevalence of SS varies from report to report and changes that were up to as much as 10-fold were reported between countries and even within the same country with a reported range of 0.1 to 4.8% (7). The prevalence varies worldwide with 320 cases in North America and 200-3,000 and 200-600/100,000 individuals for Northern and Southern Europe respectively

(13). This inconsistency may reflect methodological obstacles such as differences in the definition and application of SS diagnostic criteria (i.e., different methods for lachrymal and salivary gland function testing) or dissimilar reporting systems (i.e., community vs. hospital-based reports). However, genetic and environmental factors, which play a crucial role in the pathogenesis of ADs, may clarify some of these geographic and ethnic differences (13). Taking into account the fact that there is paucity of epidemiological data on the prevalence of SS from regions outside of Europe and North America, the existing data suggest that the highest rates of SS are documented in northern Europe while the rates in North America and mainland Europe seem to be comparable and the lowest rates are observed in some parts of Asia (14). Note that, there is no published series of SS cases in African populations.

The disease overwhelmingly affects middle aged women, who are in their fourth and fifth decades of life, but may also affect children, men, and elderly people with a female-to-male ratio as high as 9:1 (3,7). The clinical presentation of SS varies. Onset is insidious. Patients may have difficulty determining when the disease actually began and new symptoms can be easily overlooked or misinterpreted resulting in a delay of several years between onset and diagnosis. Complaints in SS may seem minor compared with those of more serious diseases, but both the complexity of symptoms and chronicity of the disease lead to a decrease in patients' quality of life (15). It has been estimated that up to 50% of SS patients are currently undiagnosed, whereas up to 30% of the patients with other ADs can be diagnosed with polyautoimmunity (i.e., SS and another AD in the same individual) (16–18). The difficulty in diagnosis means that the estimated interval between initial symptoms and diagnosis of the disease is approximately 6 to 10 years (19,20). The cost of health care for patients with SS is double that of the mean for primary care patients and similar to that for patients with RA (21,22).

APPROACH OF PATIENTS WITH SICCA SYNDROME AND DIFFERENTIAL DIAGNOSIS

Establishing a diagnosis of SS is often difficult. Sicca symptoms (dry mouth and eyes) are among the most common oral and ocular complaints seen by general practitioners. They are non-specific, especially in older patients, partly due to age-related atrophy of secreting tissues and partly due to a variety of conditions (e.g., drugs, metabolic alterations, infections). Therefore, the disease is often underdiagnosed or misdiagnosed. Hence, it is important that all suspected cases of SS should be referred to a specialist to confirm the diagnosis (3).

Once the possibility of SS is raised, it is important to rule out other causes of Sicca syndrome. While some of these are defined as SS exclusion criteria, others are considered mimics of SS symptoms such as trauma and scarring. Dryness of the eyes can also be induced by inflammatory conditions such as chronic blepharitis or conjunctivitis, neurological conditions impairing eyelid blinking or lacrimal gland dysfunction and mouth dryness can be caused by diabetes mellitus, hypercalcaemia, psychogenic factors, etc. (16,23,24) (Figure 1). Lately, a new entity that resembles SS has been defined and termed the IgG4-related systemic disease. It is characterized by raised serum levels of IgG4 and infiltration of plasma cells that cause fibrosis and enlargement of the lacrimal and salivary glands as well as other organs. This results in mild xerophthalmia and xerostomia, the presence of autoimmune pancreatitis, allergic rhinitis, and various systemic manifestations. In addition to high levels of IgG4, the absence of the typical sero-reactivity to Ro/SSA and La/SSB antigens also differentiates between SS and IgG4-related diseases (25,26).

The differential diagnosis for parotidomegaly is broad. SS patients have bilateral asymmetric salivary gland enlargement as opposed to unilateral nontender enlargement, which is commonly seen in other conditions. When this enlargement is unilateral, the differential diagnosis includes bacterial infection, chronic sialadenitis, obstruction, and primary neoplasm (e.g., adenoma, lymphoma, adenocarcinoma, and mixed salivary gland tumors). For bilateral compromise, it includes viral infection (e.g., Epstein-Barr virus, mumps, cytomegalovirus, and coxsackie A virus), amyloidosis, granulomatosis diseases (e.g., sarcoidosis, tuberculosis, and leprosy), human immunodeficiency virus, hyperlipidemia, cirrhosis, alcoholism, acromegaly, and anorexia (27).

THE DIAGNOSTIC CHALLENGE OF SS

There is no single disease-specific diagnostic criterion for SS. The diagnosis of SS is a complex task that cannot be readily done. There is no sign, symptom, or test that is unique to this syndrome. The diagnosis of SS is based on the combination of sicca symptoms and the presence of the autoimmune phenomena characterized by the activation of T and/or B cells. In addition, for many years the diagnosis of SS lacked standardization. This was partially overcome by defining sets of criteria for the diagnosis of this disease. There have been 12 sets of classification criteria for SS since 1965 including the widely used 2002 American–European and the Sjögren's International Collaborative Clinical Alliance (SICCA) criteria. The trouble with many of these sets of criteria was their reliance on tests for individual components of the syndrome that may have been outdated and not diagnostically equivalent.

The American-European Diagnostic Criteria were revised in 2002 and altered to allow a higher sensitivity and specificity in testing. At present, the most widely used classification criteria are those revised in 2002 in a joint effort by research groups in Europe and in the USA (American-European Consensus Group [AECG]) (17,28). A positive answer to at least one of the three questions about ocular or oral symptoms included in the AECG criteria has a positive value of 54-77% and a negative answer has a negative predictive value of 94-98% (17). Fullfilment of at least four of these criteria (which must include the histopathological or antibody criteria) has a sensitivity of 93.5% and a specificity of 94% for SS (17).

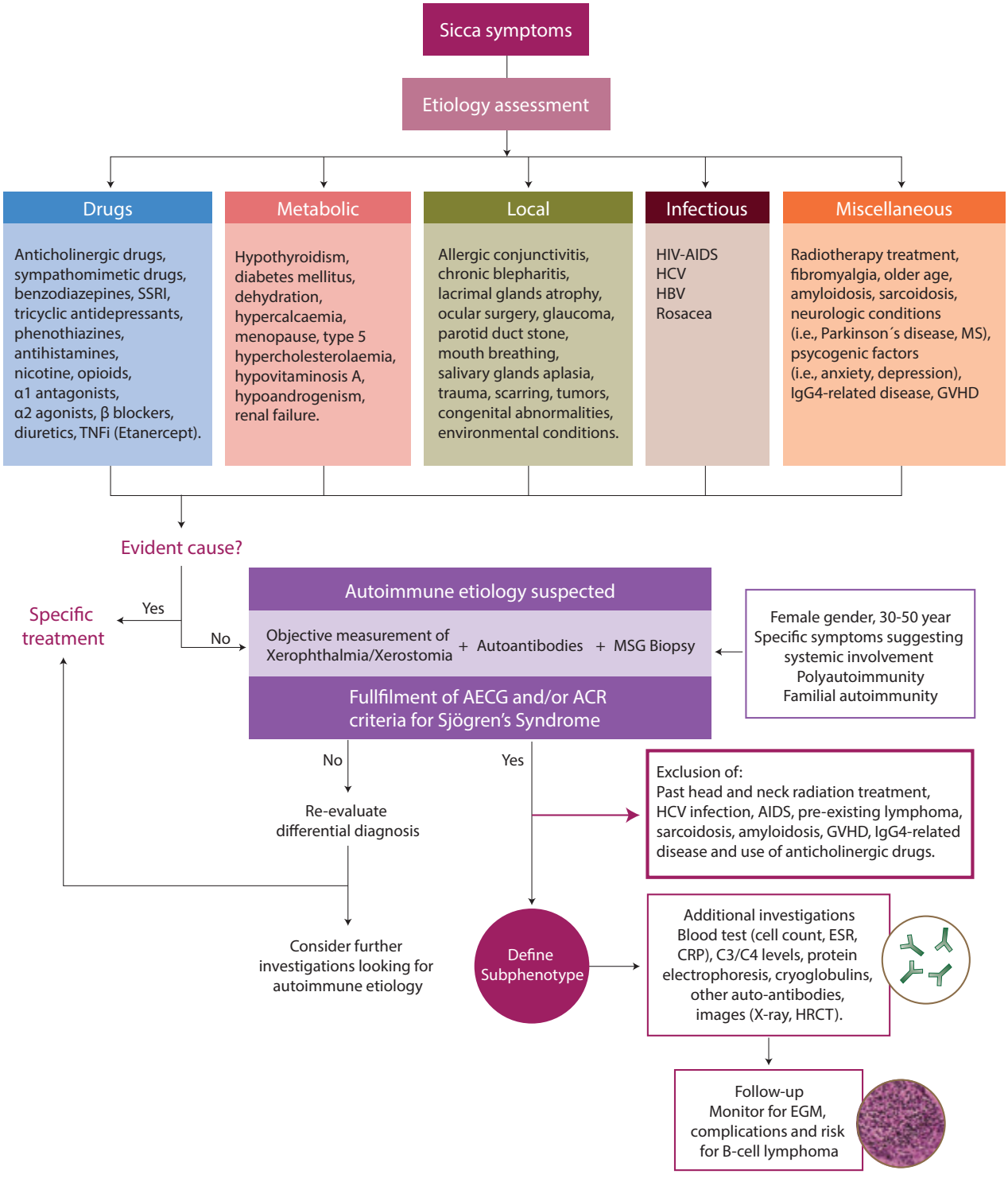


Figure 1 Approach of patient with sicca syndrome and differential diagnosis. *ACR*: American College of Rheumatology; *AECG*: American-European Consensus Group; *AIDS*: acquired immunodeficiency syndrome; *CRP*: C reactive protein; *ESR*: erythrocyte sedimentation rate; *GVHD*: graft-versus host disease; *HBV*: hepatitis B virus; *HCV*: hepatitis C virus; *HIV*: human immunodeficiency virus; *HRCT*: high resolution computed tomography; *MS*: multiple sclerosis; *MSG*: Minor salivary gland; *SSRI*: selective serotonin reuptake inhibitor; *TNFi*: tumor necrosis factor inhibitors.

AECG CLASSIFICATION (17)	ACR CLASSIFICATION (31)
Inclusion criteria	
I. Ocular symptoms. A positive response to at least one of the following questions: 1. Have you had daily, persistent, troublesome dry eyes for more than 3 months? 2. Do you have a recurrent sensation of sand or gravel in the eyes? 3. Do you use tear substitutes more than 3 times a day?	None
II. Oral symptoms. A positive response to at least one of the following questions: 1. Have you had a daily feeling of dry mouth for more than 3 months? 2. Have you had recurrently or persistently swollen salivary glands as an adult? 3. Do you frequently drink liquids to aid in swallowing dry food?	None
III. Ocular signs - that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests: 1. Schirmer I test, performed without anesthesia (≤ 5mm in 5 minutes). 2. Rose Bengal score or other ocular dye score (≥ 4 according to van Bijsterveld's scoring system)	Keratoconjunctivitis sicca with ocular staining score ≥ 3 (assuming that individual is not currently using daily eye drops for glaucoma and has not had corneal surgery or cosmetic eyelid surgery in the last 5 years)
IV. Histopathology: in minor salivary glands (obtained through normal appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score ≥ 1, defined as number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue	Minor salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 focus/4 mm^2
V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests: 1. Unstimulated whole salivary flow (≤ 1.5 ml in 15 min) 2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in major ducts. 3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer	None
VI. Autoantibodies. Presence in the serum of the following autoantibodies: 1. Antibodies to Ro/SSA or La/SSB antigens, or both	Positive serum anti-SSA/Ro and/or anti-SSB/La or (positive rheumatoid factor and ANA titer ≥ 1:320)
Classification rules	
For Primary SS:	For SS:
In patients without any potentially associated disease, primary SS may be defined as follows: a. The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive. b. The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI). c. The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in clinical-epidemiological survey	The classification of SS, which applies to individuals with signs/symptoms that may be suggestive of SS, will be met in patients who have at least 2 of the 3 objective features previously described
For Secondary SS:	
In patients with a potentially associated disease (for instance, another well defined connective tissue disease), the presence of item 1 or item II plus any 2 from among items III, IV and V may be considered as indicative of secondary SS	Eliminated the distinction between primary and secondary forms of SS
Exclusion criteria	
1. Past head and neck radiation treatment 2. Hepatitis C infection 3. Acquired Immunodeficiency Syndrome 4. Pre-existing lymphoma 5. Sarcoidosis 6. Graft-versus host disease 7. Use of anticholinergic drugs (since a time shorter than 4-fold the half life of the drug)	Prior diagnosis of any of the following conditions would exclude participation in SS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests: 1. History of head and neck radiation treatment 2. Hepatitis C infection 3. Acquired Immunodeficiency Syndrome 4. Sarcoidosis 5. Amyloidosis 6. Graft-versus host disease 7. IgG4-related disease

Table 1. Comparison of the Revised American-European Consensus Group (AECG) Classification criteria and the American College of Rheumatology (ACR) Classification criteria for Sjögren's syndrome.

With respect to the data on the performance of the AECG-criteria in clinical practice and in research, there is a general agreement that stringency might represent at the same time both the strong and the weak points in the AECG-criteria when compared to the previous European preliminary criteria. On one hand, stringency guarantees the homogeneity of the patients enrolled in clinical trials or in epidemiological studies and, in some instances, increases the sensitivity and the specificity with regard to SS as an AD. On the other hand, by claiming that any given patient with SS must have either anti-Ro/SSA and/or anti-La/SSB autoantibodies (item VI) or a positive lower lip biopsy (item IV) or both, an invasive technique is made mandatory simply to fulfill classification criteria and only a subgroup of patients might be defined as affected by SS in both clinical practice and the field of research. This could lead to those patients with negative salivary gland biopsy and negative autoantibodies, who have demonstrated, nonetheless, an outcome similar to the AECG patients during the follow-up, being excluded from the diagnosis of SS. There is, therefore, a general feeling that the current 2002 classification criteria do not cover the broad clinical and immunological heterogeneity of SS. Based on this point of view, Manthorpe R (29), also pointed out that the abnormal focus score (item IV) and the presence of anti-SSA and/or anti-SSB autoantibodies (item VI) in serum might not be independent variables (interdependency of classification criteria). Thus, in most cases, positivity of one might be followed by positivity of the other and viceversa which would increase the probability of those patients with a negative biopsy and/or autoantibodies not classifying as affected by SS. Another major criticism of the AECG-criteria is that these criteria apparently did not suggest any clear prognostic significance for the patients for the follow-up. As stated by Ramos-Casals *et al*. (30) the criteria met at diagnosis did not influence the outcome for patients with SS irrespective of the autoantibody pattern or the labial biopsy positivity. This is not particularly surprising considering that none of the six items have individually been associated with the main adverse outcomes in SS (i.e., lymphoma and death). It should be noted that poor prognosis factors such as skin vasculitis, cryoglobulinemia, low levels of C3 complement component, low C4 levels, and monoclonal gammopathy are not included in the 2002 classification criteria (28).

Recently, the SICCA proposed a new expert consensus approach consisting of classification criteria based entirely on objective measures (31). In particular, not only have ocular and oral symptoms been deleted, but the study of salivary gland involvement has also been excluded from the criteria (31). The new criteria for classifying SS have been validated using a rich data set collected over the past decade in the SICCA registry. Despite having well-defined criteria, making a diagnosis is not a simple matter and requires careful consideration. In addition, the cut-off points for the disease criteria are not absolute (32) (Table 1).

FROM COMMON MECHANISMS TO DIVERSE SUBPHENOTYPES IN SS

Sicca phenotype in SS can present either alone or in the context of an underlying AD. Whether or not it is called primary or secondary reflects a taxonomy issue. ADs share several common mechanisms including genetic factors, environmental triggers, pathophysiological abnormalities, and certain subphenotypes that represent the autoimmune tautology (33,34). Polyautoimmunity is defined as the presence of more than one AD in a single patient (9,35,36). When three or more ADs coexist, this condition is called multiple autoimmune syndrome (MAS) (37–39). In addition, ADs cluster within families of patients with SS. This familial aggregation of ADs is further evidence that clinically different autoimmune phenotypes might share common susceptibility gene variants (40).

The fourth-stage-model for the pathophysiology of SS includes the complex interaction of predisposing and stochastic factors, the pre-clinical stage (presence of autoantibodies in serum without symptoms), and the clinical stage with different subphenotypes (Figure 2). This broad spectrum of subphenotypes is based on sociodemographic, clinical, and serological features which define subsets of patients with different outcomes.

THE CLINICAL SPECTRUM OF SS

SS has a wide clinical spectrum which extends from benign local exocrinopathy to systemic disorder that affects parenchymal organs. It may also be associated with lesions due to immune complex hyperproduction and vasculitic involvement (e.g., purpura, peripheral neuropathy, glomerulonephritis). In some patients, SS could evolve to malignat lymphoma (41–43) (Figure 3).

GLANDULAR MANIFESTATIONS

Xerophthalmia (subjective feeling of eye dryness) and xerostomia (subjective feeling of mouth dryness) are the key symptoms of SS, occurring in more than 95% of the patients (3). Patients with xerophthalmia may describe a 'gritty' or 'sandy' feeling in their eyes, and this may be associated with soreness, photosensitivity, ocular fatigue, and reduced visual acuity. Diminished tear secretion may lead to chronic irritation and destruction of conjunctival epithelia (i.e., KCS) which leads to deteriorating vision and an increased risk of recurrent infections (44). KCS usually presents insidiously over a period of several years. Untreated severe dry eye can result in infection of the eyelids, corneal ulceration, and further vascularization, opacification, and perforation that may lead to impairment of vision.

Xerostomia could have a severe impact as patients find eating, speaking, swallowing, and sleeping difficult. Oral symptoms include soreness, adherence of food to the mucosa, dysgeusia, and dysphagia. Oral signs include a lobulated or depapillated red tongue and angular cheilitis (Figure 4). Loss of the protective and antimicrobial properties of saliva may

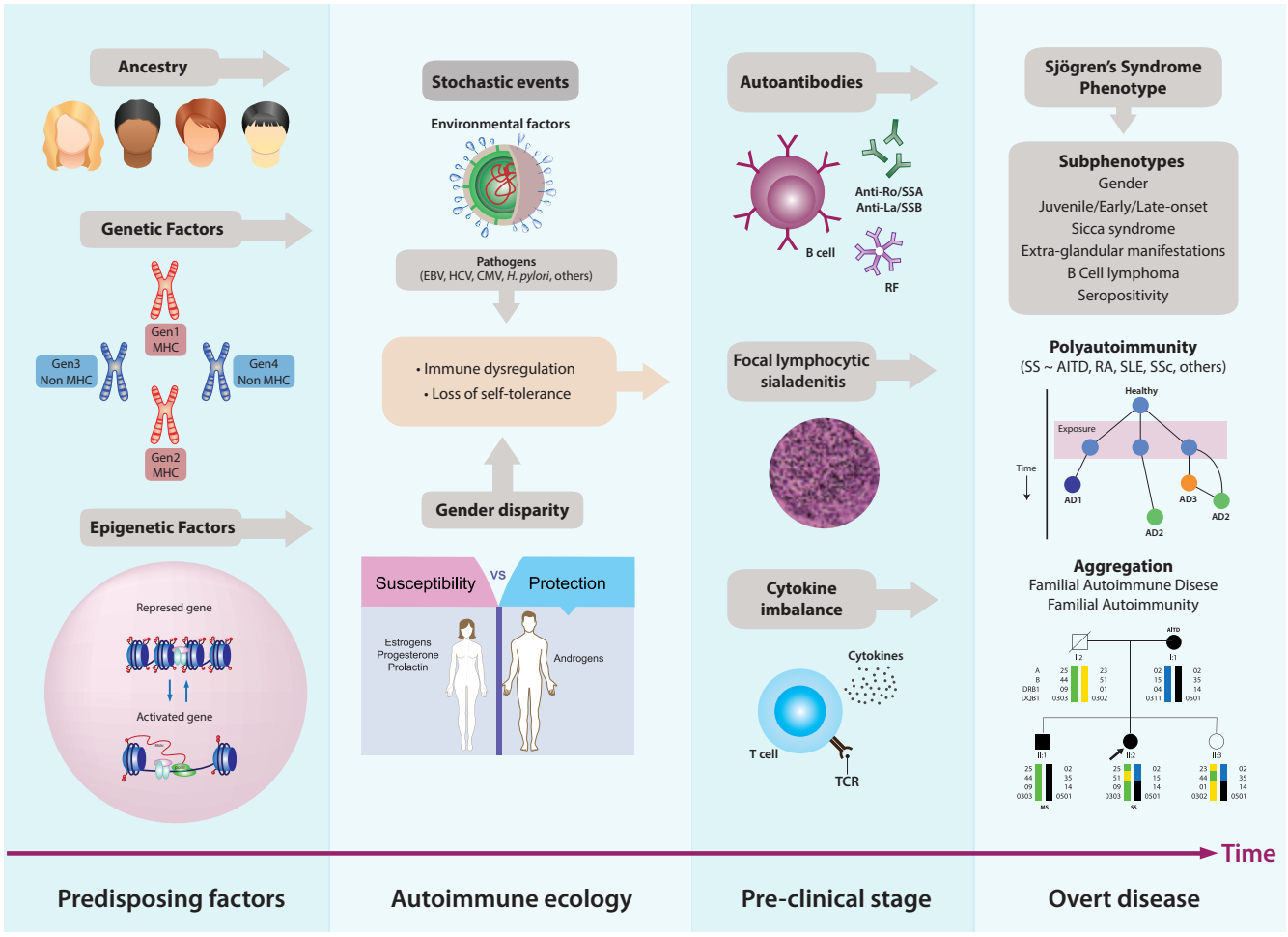


Figure 2 Fourth-stage-model for the pathophysiology of Sjögren's syndrome. The etiology of SS is multifactorial, in which a mosaic of predisposing and stochastic factors play in concert for the induction of loss tolerance and subsecuent organ damage. The pre-clinical stage is characterized by the presence of autoantibodies in serum before the clinical onset, as well as pro-inflammatory markers and lymphocytic infiltrate in salivary glands. The clinical stage has a broad spectrum of subphenotypes which can influence the outcome of patients with Sjögren's syndrome. Note that familial autoimmunity corresponds to the presence of different autoimmune diseases in a nuclear family. *AD*: autoimmune disease; *AITD*: autoimmune thyroid disease; *CMV*: cytomegalovirus; *EBV*: Epstein-Barr virus; *HCV*: hepatitis C virus; *MHC*: major histocompatibility complex; *RA*: rheumatoid arthritis; *RF*: rheumatoid factor; *SLE*: systemic lupus erythematosus; *SS*: Sjögren's syndrome; *SSc*: systemic sclerosis; *TCR*: T Cell receptor.

increase dental caries and predispose patients to periodontal disease (5). This may cause signs such as oral mucosal inflammation, mucosal sloughing, erythematous mucosa, and traumatic ulcers (45). Patients often struggle with social withdrawal, economic burden, and poor nutrition as a result of their difficulty eating (5,19). The prevalence of *Candida albicans* may be as high as 68% in patients with SS (46–48). Nearly 30% of the patients may present episodic inflammatory swelling of the major salivary glands (parotid and submandibular glands). Recurrent salivary gland enlargement may be used to aid in diagnosis (44) (Figure 5).

Skin dryness (xerosis) that causes pruritus affects as many as 55% of the patients with SS (49). Additional sicca symptoms that often coexist with dry eyes and mouth are less common. They include the involvement of other exocrine glands (e.g., respiratory, gastrointestinal, and genital tract) and may display as nasal crusting, epistaxis, recurrent sinusitis, hoarseness, dry throat, chronic non-productive cough (xerotrachea), oesophageal mucosal atrophy, atrophic gastritis, constipation, and in women, dyspareunia.

EXTRA-GLANDULAR MANIFESTATIONS

Clinical suspicion of SS should rise when systemic manifestations accompany the sicca complaints. Specific symptoms suggesting systemic involvement are frequent and may require referral to the appropriate specialist (3).

General symptoms. One of the most frequent symptoms in SS is represented by abnormal fatigue, which is promi-

nent in approximately 70-80% of patients and is often related to work disability (50). Though the instruments for fatigue assessment are still inadequate or lacking, it seems that the physical and somatic rather than mental aspects of fatigue are more severely and frequently affected in SS. Other non-specific symptoms closely associated with fatigue are sleep disturbances, anxiety, and depression with a prevalence of nearly 15%, 20%, and 40%, respectively (51,52). Chronic pain is often associated with polyarthralgia and myalgia, which are reported by more than 50% of these individuals. Some patients, especially children and young adults, may present with low grade, self-limiting fever.

Organ-specific involvement. It is of note that 40-70% of the patients develop systemic involvement before or after the diagnosis of SS (53–56). In addition, this group of patients commonly has circulating anti-Ro/SSA and anti-La/SSB autoantibodies in comparison with sicca-limited disease pa-

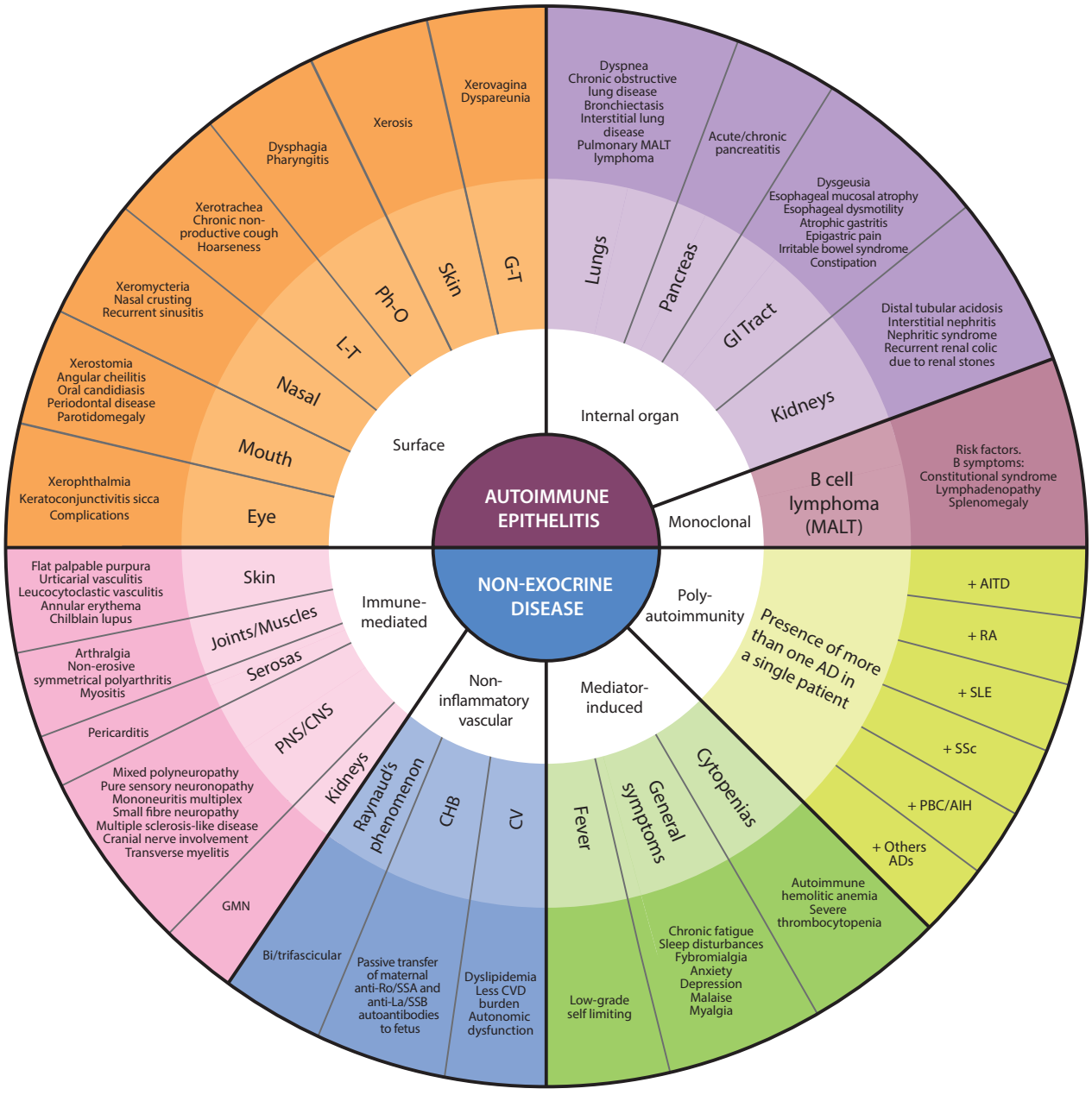


Figure 3 Clinical spectrum of of Sjögren's syndrome. *AD*: autoimmune disease; *AIH*: autoimmune hepatitis; *AITD*: autoimmune thyroid disease; *CHB*: congenital heart block; *CNS*: central nervous system; *CV*: cardiovascular; *GI*: gastrointestinal; *GMN*: glomerulonephritis; *G-T*: genital tract; *L-T*: laryngo-tracheal; *MALT*: mucosal-associated lymphoid tissue; *Ph-O*: pharyngo-oesopheal; *PBC*: primary biliary cirrhosis; *PNS*: peripheral nervous system; *RA*: rheumatoid arthritis; *SLE*: systemic lupus erythematosus; *SS*: Sjögren's syndrome; *SSc*: systemic sclerosis.

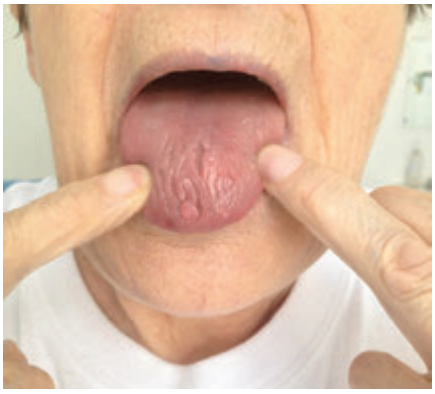


Figure 4 Xerostomia in Sjögren's syndrome. 62-year old woman with long-standing Sjögren's syndrome with extra-glandular manifestations and severe xerostomia with lobulated and depapillated tongue (Courtesy of Dr. Castellanos-de la Hoz).

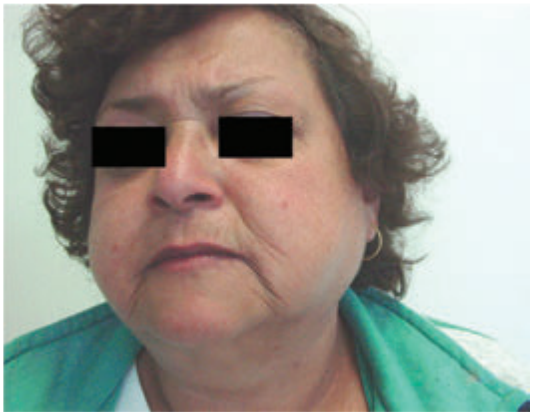


Figure 5 Parotidomegaly in Sjögren's syndrome. 54-year old woman with Sjögren's syndrome and bilateral parotid gland enlargement.

tients. Most EGM, like the exocrine gland involvement, can be considered an expression of the so-called autoimmune epithelitis because the primary target of the autoimmune response is the epithelial component. Nevertheless, in other clinical manifestations, the pathogenesis seems to be completely different as it may involve vasculitis and/or immune complex deposition and complement activation as is the case in skin vasculitis and peripheral neuropathy (57).

Skin. Nearly half of the patients with SS may present cutaneous manifestations consisting of skin xerosis, angular cheilitis, erythema annulare, chilblain lupus, and skin vasculitis (i.e., flat or palpable purpura and urticarial vasculitis) (58). Vasculitis is one of the common cutaneous manifestations of SS and mostly involves small vessels. It is associated with other EGMs and the presence of autoantibodies and cryoglobulines (59). Flat purpura is usually seen in patients with hypergammaglobulinemia, whereas palpable purpura is distinctive for dermal vasculitis, found in 10% of the patients, and is linked to SS-related EGM and lymphoma. Erythema annulare consists of photosensitive erythematous lesions with indurated borders that may be present in 5-10% of SS patients. Intriguingly, patients with SS may exhibit rashes that resemble systemic lupus erythematosus (SLE) skin disease and cannot be discriminated either clinically or histologically from subcutaneous lupus (4,10).

Musculoskeletal. Articular manifestations occurred in 30–60% of SS patients with arthralgia as the most common symptom reported and involving small and large joints equally. In addition, an intermittent symmetrical non-erosive polyarthropathy may be observed in 15-30% of the patients and affects mainly small joints (8). The most frequently affected joints are metacarpophalangeal joints, shoulders, wrists, and metatarsophalangeal joints (53,60,61). Muscular involvement is documented in 27% of SS patients. Likewise, myalgias are frequent while myositis is rarely diagnosed in SS. SS-related myopathy is usually mild and responds well to steroids and immunosuppressive drugs. The presence of significant muscle weakness and dramatic muscle enzyme elevations in SS patient raises the possibility of myositis (62). Hypothyroidism should be also ruled-out.

Lungs. Respiratory manifestations are frequently detected, but they are clinically significant in only 10% of the patients with SS. Respiratory airways are the main target tissue affected. Therefore, in SS, obstructive lung disease is more frequent than interstitial lung disease. One of the clinical symptoms caused by small airway obstruction and interstitial lung disease (5% of cases) corresponds to dyspnea.

The histological pattern of pulmonary lesion changes radically when the population under study has clinically evident lung involvement. In this case, interstitial expansion of the inflammatory process is frequent and may take various forms. Non-specific interstitial pneumonia seems to be the most frequent histological pattern but lymphocytic and usual patterns are also present (63). Notably, these patterns are not clear-cut as they may coexist or evolve in the same individual along with the progress of the disease. In addition, chronic obstructive lung disease and bronchiectasis have been described in nearly 10% of patients (64). Whether interstitial pneumonia appears as a result of more severe bronchiolitis in those patients who finally become symptomatic is not known. It would be rational, however, to consider the possibility that an initial subclinical insult in the form of subepithelial peribronchial inflammation in a selected minor percentage of patients could expand to the interstitial tissues and become clinically apparent. Finally, ongoing lymphocytic proliferation may progress to pulmonary mucosally-associated lymphoid tissue (MALT) lymphoma. This involves approximately 1-2% of SS patients and, as in the case of salivary glands, the main histopathologic pattern is that of low-grade marginal zone lymphoma (6).

Cardiovascular. Published data on the risk of accelerated atherosclerosis in SS are lacking. Although cardiovascular disease represents the leading cause of mortality in several ADs, the burden of the disease in SS is milder compared to RA and SLE. Subclinical atherosclerosis (intima-media thickening of the large vessels) was reported in about 50% of a cohort of young women with SS (65). Note that these vascular changes were not associated with the presence of cardiovascular risk factors or any drug-induced effect which suggests that certain immune system characteristics of the disease trigger early atherosclerosis. However, the presence of early subclinical atherosclerosis does not always correlate with an increased risk of cardiovascular-related death. The prevalence of atherosclerosis is not appreciably increased in patients with SS. Dyslipidemia has been found to be the most significant traditional risk factor linked with cardiovascular disease in these patients (66–69) (See Chapter 38). Consequently, further studies are needed to determine the risk of atherosclerosis and cardiovascular-related mortality among patients with SS (70).

Other cardiovascular involvements in patients with SS are Raynaud's phenomenon, observed in 18 to 37% of patients and being much less severe than in systemic sclerosis (SSc) pericarditis (<5%), and symptoms of autonomic dysfunction (3).

Gastrointestinal. Gastrointestinal manifestations include nausea, dysphagia, or epigastric pains that are frequently due to dryness of the pharynx and esophagus or to esophageal dysmotility and gastritis. The typical histological pattern is chronic atrophic gastritis with lymphoid infiltration. Hyperamylasemia is rather frequent though it is very rarely an expression of acute or chronic pancreatitis (<5%). Liver involvement in patients with ADs is not very common and, when present, it usually follows a benign course. Moreover, even in the case of hepatic injury, it is very difficult to classify the lesion as primary with associated autoimmune manifestations or as an AD with secondary liver involvement. In view of the preservation of hepatic architecture and the benign course of the lesion, hepatic involvement in SS can be mainly characterized as autoimmune cholangitis. This is especially true given the fact that the immunohistochemical findings in autoimmune cholangitis and primary biliary cirrhosis are practically identical (6). Celiac disease is more prevalent in SS patients compared to normal controls, but varies considerably among populations (71).

Renal. The kidneys may be also involved in SS. The major clinicopathological entity is interstitial nephritis, which appears early or may even precede the onset of sicca symptoms (5% of cases) (72). Distal renal acidosis (Type 1) is the most frequent clinical presentation (10%) (73). Glomerulonephritis is extremely rare (<5%). When observed, cryoglobulinemia and SLE should be ruled-out (74). Hypertension, mild proteinuria, and microscopic hematuria are the major signs and, in contrast to interstitial nephritis, tend to present late in the progress of the disease. Renal injury in SS is due to lymphocyte infiltration of the interstitial space rather than the immune complex deposition seen in other ADs such as SLE. Several serum markers correlate with renal injury in SS including hypergammaglobulinemia and high levels of serum β2-microglobulin (75).

Neurological. An accurate prevalence of neurological manifestations is difficult to assess because of the heterogeneity of the series (2%-60%). Both peripheral and central nervous systems may be affected and precede the diagnosis of SS or appear at a later stage. The pathogenic mechanisms responsible for most forms of neurological involvement in SS remain unknown, but vascular, ischemic, and immunological mechanisms have been considered (76).

Peripheral nervous system involvement includes sensory polyneuropathies as the most common manifestations (5-10%) (77). Sensorimotor polyneuropathy and polyradiculopathy, mononeuritis multiplex, autonomic neuropathy (e.g., Adie's pupils and orthostatic hypotension), trigeminal, and other cranial neuropathies are other manifestations of the involvement of the peripheral nervous system in SS. Neuropathy has been linked to a more aggressive disease, the presence of vasculitis, and lymphoma (78).

Central nervous system involvement is much less common with white matter lesions (multiple *sclerosis-like* changes) (<5%), seizures, transverse myelitis (<5%), aseptic meningitis, optic neuritis, cranial nerve involvement (V, VIII and VII) (7%), diffuse encephalopathy, and dementia as reported manifestations (3,79) (Table 2).

Haematological and lymphoproliferative disorders. Patients with SS can develop autoimmune hemolytic anemia (<5%) and severe thrombocytopenia (<5%). SS may be a serious disease with excess mortality caused by hematological cancer (80). B cell lymphoma is the most serious complication of SS and occurs in about 5-10% of the patients. A higher prevalence of lymphoproliferative diseases, mainly malignant lymphoma, can be explained by the B cell hyperactivity characterizing SS (5). Of note, among ADs, SS displays the second highest incidence of malignant lymphoproliferative diseases (81). Patients with SS have a 10-50 times higher risk of lymphoma than healthy individuals, and the largest case series found that 2-9% of the patients with SS develop lymphoma (82–84). Often these tumors involve the salivary glands. In virtually all cases, they are B cell MALTomas (85).

Prospective studies have identified several clinical and immunological risk factors for the co-occurrence of lymphoma and SS (Table 3) (54,86,87). The presence of clonal expansion of B cells in salivary glands has been shown to increase the risk of developing lymphoma, and it has been suggested that patients displaying this feature should be monitored more closely than others. Recent findings seem to show that low Vitamin D levels in the patients with SS could be associated with severe complications such as lymphoma and peripheral neuropathy (88).

PERIPHERAL DISORDERS	CENTRAL DISORDERS
AXONAL POLYNEUROPATHIES Symmetric pure sensory peripheral neuropathy Symmetric sensorimotor peripheral neuropathy	FOCAL Seizures Movement disorders Cerebellar syndrome Optic neuropathies Pseudotumor lesions Motor and sensory loss
Sensory ganglioneuronopathy	MULTIFOCAL DISEASE Cognitive impairment Encephalopathy Dementia Psychiatric abnormalities Aseptic meningoencephalitis
Motor neuropathy	SPINAL CORD DYSFUNCTION Chronic progressive myelopathy Lower motor neuron disease Neurogenic bladder Acute transverse myelitis
Small-fiber neuropathy	Progressive-multiple sclerosis-like syndrome
Multiple mononeuritis	Central nervous system vasculitic involvement
Trigeminal and other cranial nerves neuropathies	
Autonomic neuropathies	
Demyelinating polyradiculoneuropathy	

Table 2. Neurological manifestations in Sjögren's syndrome. Adapted from Tobón *et al*. (76).

Congenital heart block. Anti-Ro/SSA and anti-La/SSB antibodies are directed against autoantigens found in all tissues and can cross the placenta and cause inflammatory lesions of the heart, skin, and other organs of the developing fetus. Pregnant women known to have anti-Ro/La antibodies have a 1 in 20 risk of delivering a baby with heart block. Congenital heart block is the result of the passive transfer of maternal autoantibodies to the fetus in the presence of genetic predisposing factors that allow antibody-mediated cardiac damage. The fetal heart rate should be monitored every 4 weeks from week 12 onwards. There is some evidence to suggest that treating the fetus with dexamethasone administered to the mother may reverse heart block if it is treated in its very early stages. There is thus a strong argument for referring such patients to specialist units equipped for fetal cardiac monitoring. Note that it has also recently been claimed that Vitamin D may be linked to congenital heart block in pregnant women with anti-Ro/SSA and anti-La/SSB autoantibodies (89–91).

Sexual outcomes. Patients with SS can also suffer from gynecological problems more often than healthy women. Vaginal dryness and dyspareunia affect more than half of the patients with a significant difference between them and age-matched normal controls. These problems could lead to a relevant impairment of sexual function in women with SS.

SS AND GENDER

SS overwhelmingly affects middle aged women during their fourth and fifth decades of life but may also affect children, men, and elderly people. Verheul *et al*. studied the effect of gender hormone predominance by doing a gonadectomy on the non-obese diabetic mouse, an accepted SS model (92). This caused a worsening of the disease, and the use of additional estrogen doses did not change the outcome of the disease. This may suggest a stronger protective effect of androgens than a vulnerability because of estrogens. Several studies have

	EXTRA-GLANDULAR MANIFESTATION	B-CELL LYMPHOMA
Clinical	Parotid enlargement Palpable purpura Lymphadenopathy	Persistent parotid enlargement Lymphadenopathy Splenomegaly Palpable purpura Leg ulcers Peripheral nerve involvement Low-grade fever Male gender
Histopathological	Intense salivary gland infiltration	Severe involvement of the exocrine glands Clonal expansion of B cells in salivary glands
Serologic	Hypocomplementaemia (low C4 levels) Mixed monoclonal cryoglobulinemia (type II) Seropositivity (anti-Ro/SSA, anti-La/SSB antibodies, ANA, rheumatoid factor and cryoglobulins)	Hypocomplementaemia (low C4 levels) Cytopenias (i.e., anemia, lymphopenia) Monoclonal gammopathy Mixed monoclonal cryoglobulinemia (type II) Hypogammaglobulinemia Decrease in the serum levels of autoantibodies (i.e., rheumatoid factor) Cross-reactive idiotypes of monoclonal rheumatoid factors Low Vitamin D levels

Table 3. Risk factors for complications in Sjögren´s syndrome. *ANA*: anti-nuclear antibodies.

assessed the influence of gender on SS. Some found some differences with respect to immunological, clinical, and severity features (93–96) (Table 4). Nevertheless, while SS in men generally has characteristics that are similar to those observed in women (15), men with SS are at higher risk for lymphoma and neurological involvement than women. Accordingly, male gender should be considered a risk factor for lymphoma development in the patients with SS (97).

AGE AT ONSET OF DISEASE

Age at onset of disease (AOD) refers to the time period in which a patient experiences the first sign(s) and/or symptom(s) (98). AOD varies among ADs and has been related to prognosis in some of them. Late-onset traits are more sensitive to environmental variation than genetic influence. Anaya *et al*. (99) described the clinical characteristics of juvenile SS. They found that juvenile SS is a common subphenotype, and the main features at onset are parotitis and the presence of rheumatoid factor (RF) and anti-nuclear antibody (ANA) positivity. Sarmiento-Monroy *et al*. (100) recently assessed the influence of AOD on the course and severity of the disease in adult patients through a systematic literature review. Some studies demonstrated certain differences in serological abnormalities and disease manifestations though most of them lacked statistical significance (19,101–108) (Figure 6). Just one study found AOD to be a prognostic factor in SS (i.e., lymphoma) (104). In contrast to other ADs, AOD does not have a critical effect on disease expression and autoimmune response in the patients with SS.

SS AT THE CROSSROAD OF POLYAUTOIMMUNITY

SS has been described in association with a large variety of both organ-specific and systemic ADs including autoimmune thyroid disease (AITD), RA, SLE, SSc, autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), etc. (9,35,36,39) (Table 5, Figure 7). A recent cohort of 410 well-characterized patients with SS (39) showed a 32.6% prevalence of polyautoimmunity with AITD being the most common (21.5%). The prevalence of systemic diseases, e.g., RA and SLE, was around 8%. In a series of 114 patients with SS (109), a range of 13 associated ADs was detected. In all, 38 patients (33%) were diagnosed as having 1 additional AD, 7 (6%) had 2, and 2 (2%) had 3. Once again, the most common autoimmune disorder was AITD (14%).

AUTHOR, COUNTRY [YEAR], REFERENCE	STUDY DESIGN (n, GENDER)	RESULTS	COMMENTS
Anaya *et al*. US, [1995] (97)	Case-control Men (n = 13) Women (n = 26)	No statistical differences in the frequency of EGM or in the presence of autoantibodies were observed between men and women Men patients were more likely to have EGM	SS in men is an uncommon condition with clinical and serological characteristics similar to those observed in women
Brennan *et al*. US, [1999] (96)	Case-control Men (n = 14) Women (n = 18)	Women had significantly higher ANA titers and ESR than men A significant sex difference was also noted in EGM, with more women reporting fatigue compared to men (68 vs 21%, respectively)	Women may have more positive serological findings than men and a higher prevalence of fatigue. No sex differences could be established with other EGM of SS
Cervera *et al*. Spain, [2000] (95)	Cross-sectional Men (n = 19) Women (n = 204)	Men with SS presented a lower prevalence of articular involvement (21% vs. 46%; OR 0.32; 95% CI 0.07-0.97; p = 0.03)	Except for a lower prevalence of articular involvement, there weren't any notable differences in clinical and immunological characteristics between male and female patients with SS
Gondran *et al*. France, [2008] (93)	Cross-sectional Men (N = 42)	A significantly greater percentage of women reported lymphopaenia (26% vs. 8%; p = 0.02) and leucopaenia (18% vs. 3%; p = 0.015) at onset Thrombocytopenia was more common in the male patients (21% vs. 6%, p = 0.001)	Except for haematological presentation, there weren't any notable differences in clinical and immunological characteristics between male and female patients with SS
Horvath *et al*. Hungary, [2008] (94)	Cross-sectional Men (n = 60) Women (n = 432)	RP (30% vs. 7%; p = 0.0001) and AITD (7% vs. 0%) were more frequent in women than in men Arthritis was frequently presented as EGM in both genders, but the ratio was higher in men (68% vs. 42%; p = 0.0002) ANA positivity were most frequent in female than in men (63% vs. 37%; p = 0.0002) and ACPA antibodies were more prevalent in men (15% vs. 4%; p = 0.001)	SS develops also in men with the predominant symptoms of vasculitis or arthritis besides KSC or xerostomy

Table 4. Influence of gender in Sjögren's syndrome subphenotype. *AITD*: autoimmune thyroiditis; *ANA*: antinuclear antibody; *ACPA*: anti-cyclic-citrullinated peptide antibodies; *EGM*: extra-glandular manifestations; *ESR*: erythrocyte sedimentation rate; *KCS*: keratoconjunctivitis sicca; *NS*: no significant; *OR*: Odds Ratio; *RP*: Raynaud's phenomenon; *SS*: Sjögren´s syndrome; *US*: United States.

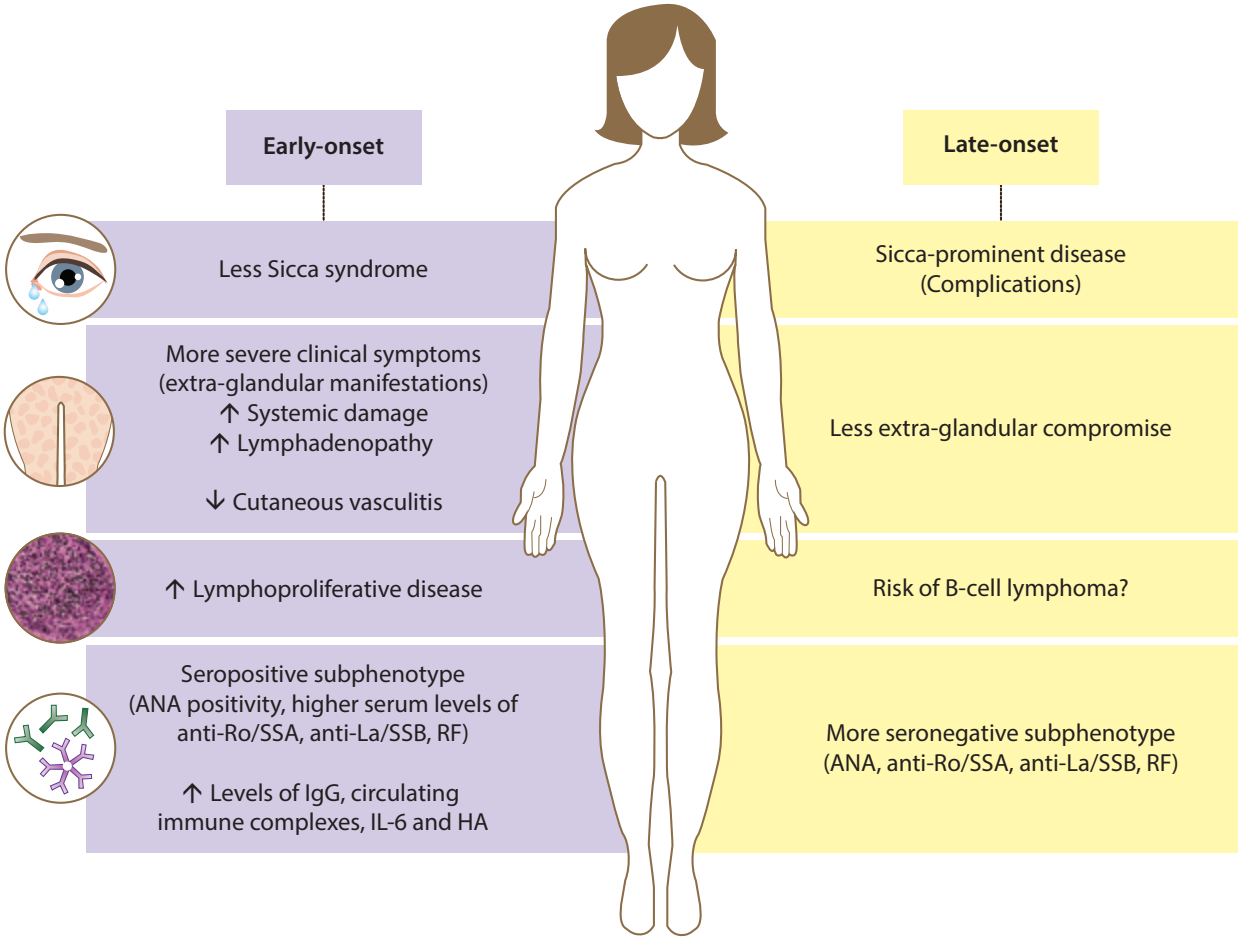


Figure 6 Influence of age at onset in adult Sjögren's syndrome subphenotype. *ANA*: anti-nuclear antibodies; *HA*: hyaluronic acid; *IL-6*: interleukin 6; *KCS*: keratoconjunctivitis sicca; *RF*: rheumatoid factor.

SS and AITD. Endocrine symptoms are documented in SS patients mainly due to concomitant thyroid dysfunction. Approximately 15-30% of the patients with SS develop AITD (primarily, Hashimoto thyroiditis and, to a lesser extend, Graves' disease) and more than 50% of them have subclinical hypothyroidism. This group of patients is usually seropositive for the presence of anti-thyroid peroxidase and anti-thyroglobulin antibodies, which can be used as primary indicators of patients who are prone to developing thyroid disease in the future (110). In addition, the prevalence of SS is 10 times higher in patients with autoimmune thyroiditis. Hence, it is advisable for patients with SS to be screened periodically for thyroid function. The histological picture of Hashimoto thyroiditis per se is highly similar to that of SS. The infiltrate consists of primarily $CD4^+$ T lymphocytes. The thyroid epithelial cells express HLA class II molecules and adhesion molecules while progress to B cell MALT lymphoma is probable. Hashimoto thyroiditis may evolve to lymphoma in 0.5% of patients. Moreover, one-third of the patients with AITD have SS features and one out of 10 ANA positive AITD patients shares the diagnosis of SS (6).

SS and RA. RA is frequently associated with both sicca symptoms and true SS. In a Spanish cohort of RA patients, a cumulative prevalence of polyautoimmunity in SS was described in 17% of the patients with a disease duration of 10 years (111). In a cohort in Austria, the reported prevalence was 22% (112). In a Greek cohort, RA patients with high titers of rheumatoid factor were reported to be more likely to have polyautoimmunity (113). In Finland, a doubled standardized incidence ratio for non-Hodgkin lymphoma in RA patients with polyautoimmunity when compared to RA patients without SS was described (114). In recent studies, the prevalence of sicca symptoms in the patients with RA ranges from 30 to 50% and the percentage of RA patients who fulfill SS classification criteria ranges from 4 to 31% (115).

SS and SLE. SLE is probably the AD most closely related to SS due to the significant overlap in their clinical and immunogenetical expression. Recent studies have described a prevalence of associated SS in SLE patients ranging between 9 and 19% (116–118). The relationship between SS and SLE

CHARACTERISTIC	SJÖGREN´S SYNDROME		
	+ RA	**+ SLE**	**+ SSc**
Overall picture	The prevalence ranged between 3.5 and 31% Many authors consider SS as an extrarticular RA feature, although differences in genetic and immunological pathways involved in disease process have been documented	SLE and SS frequently coexist sharing many clinical and immunological features From the analysis of seven studies which considered a total of 2,611 SLE patients the prevalence of SS was 14.8%	Is characterized by the coexistence of SSc and SS features It is reported in 18.5% of cases among SSc patients
Autoimmune profile	Prevalence of ACPA has been reported in 7.2% of patients with SS who are RF negative and without arthritis Iwamoto *et al*. detected ACPA in 21% of SS patients with arthritis and in 0% of those without arthritis. Notably, ACPA were found in 71.4% of patients classified as having RA-SS and in 6% of SS patients with arthritis but without RA Prevalence of RF is high in both RA and SS, without any difference between SS patients with and without arthritis. Thus, RF is not helpful in differentiating patients with SS from those with RA-SS overlap Patients with SS overlapping with other AD, including RA, are less frequently anti-Ro/SSA and anti-La/SSB antibody positive than patients with only SS (12% vs 82%)	SLE-SS patients had a higher frequency of SS-related immunological markers, such as RF, polyclonal hypergammaglobulinemia, anti-Ro/SSA and anti-La/SSB compared with SLE patients without SS. Anti-La/SSB antibodies are considered the serological markers of this case of polyautoimmunity By contrast, SLE-related antibodies are less frequent in SLE-SS patients	A specific immunologic marker of SSc-SS has not yet been identified Anti-Ro/SSA and anti-La/SSB antibodies are reported in 38.8% and 22.3% of cases, respectively The prevalence of ACA was similar (37.3% vs 36.4%), but anti-Scl70 was lower (13.4% vs 27.6%) in SSc-SS than in SSc patients followed-up in a large German cohort Interestingly, anti-RNA polymerase antibodies, which are highly specific to SSc and predict renal crisis, were infrequently observed in group of patients (2%)
Subphenotype characteristics	Sicca symptoms are reported in 30-90% of RA patients; on the other hand arthralgia-arthritis is reported in 70% of SS patients The prevalence of focal lymphocytic infiltration at lip biopsy was similar between SS and SS with polyautoimmunity, including RA. However, a higher B/T cell ratio and higher expression of CD20 B cells were observed in SS compared with RA-SS patients Differences in the number of joints involved in patients affected with RA, with and without SS, were not found; in addition, the levels of DAS-28 in RA and RA-SS patients were similar (3.35 vs 2.81 respectively) Notably, erosive arthritis was recently reported in 100% of patients with RA-SS Data on other clinical features are very limited Adenopathy, Raynaud's phenomenon, renal and neurologic involvement were not reported by Hernández-Molina; in this study only one case of unspecified vasculitis was observed	The prevalence of sicca syndrome in SLE ranges between 18% and 34%; SS criteria were fulfilled in 9.2–31% of SLE cases. It has been suggested that patients with SLE-SS are characterized by milder SLE-related features and a predominance of SS-related features In a large prospective series recently published by Baer *et al*., skin involvement, such as photosensitivity or malar rash, oral ulcers, arthritis, Raynaud's phenomenon and psychosis were reported more frequently in the SLE-SS group SLE-SS patients are older with a lower risk of developing glomerulonephritis compared with SLE patients. Other authors reported a higher frequency of fatigue and thrombocytopenia in SLE-SS patients	Sicca symptoms are common in scleroderma being observed in 68–83% of cases; however, only 14% of SSc-SS patients fulfill the criteria for SS Lymphocytic infiltration of salivary glands leading to oral dryness is one of the main features in SS; notably, half of the SSc patients have salivary gland fibrosis which also leads to a reduced salivary flow and is associated with more severe disease and higher mortality rate Prevalence of limited and diffuse SSc in SSc-SS was 83.6% and 16.4% of cases, respectively Upper gastrointestinal (88.2%), lung involvement (70.6%) and arthritis (41.2%) are commonly observed, being more prevalent than in non SSc-SS patients Digital ulcers were reported in 11.8% of cases and pulmonary hypertension in 23.6% Skin involvement in limited SSc seems to be less severe when it appears in SSc-SS and incidence of digital ulcer was lower than that reported in SSc patients

Table 5. Sjögren's syndrome at the crossroad of polyautoimmunity. *ACA*: Anti-centromere antibodies; *ACPA*: anti-citrullinated peptide antibodies; *AD*: Autoimmune diseases; *DAS-28*: disease activity score-28; *RA*: rheumatoid arthritis; *RF*: Rheumatoid factor; *SLE*: systemic lupus erythematosus; *SS*: Sjögren's syndrome; *SSc*: Systemic sclerosis; *TNF*: tumor necrosis factor. Adapted from Iaccarino *et al*. (35)

CHARACTERISTIC	SJÖGREN´S SYNDROME		
Prognosis and treatment approach	Joint involvement should be carefully evaluated in SS patients Morning stiffness, rheumatoid nodules and erosive arthritis suggest RA-SS polyautoimmunity, especially in those patients with ACPA positivity. In these patients corticosteroids and immunosuppressants, such a methotrexate, are widely used and recommended Data on the use of anti-TNF agents in patients with RA-SS are debated Very few data are available on the use of anti-TNF agents in RA-SS patients The use of Rituximab should be considered in refractory cases CD20 B cell infiltrate found in lip biopsy of patients with RA-SS, although to a lesser extent compared with SS, suggests a potential effectiveness of Rituximab also in this condition	Very few data are available in the literature on the treatment of SLE-SS patients Hydroxychloroquine, corticosteroids and in more severe cases immunosuppressants have been effectively used, including Rituximab, suggested as an option in refractory cases especially as a steroid-sparing agent	Low dose corticosteroids and hydroxychloroquine are widely used in the treatment of SS and they are not contraindicated in patients with SSc In refractory or severe cases (i.e. patients with lung involvement, diffuse skin involvement or glomerulonephritis), immunosuppressants, such as azathioprine, mycophenolate mofetil or cyclophosphamide have to be considered High dose intravenous immunoglobulin can be an effective alternative option since this has been successfully used in SSc and SS patients and is useful in SSc-SS patients Biological drugs are rarely used in patients with SSc-SS

Table 5. Sjögren's syndrome at the crossroad of polyautoimmunity. *ACA*: Anti-centromere antibodies; *ACPA*: anti-citrullinated peptide antibodies; *AD*: Autoimmune diseases; *DAS-28*: disease activity score-28; *RA*: rheumatoid arthritis; *RF*: Rheumatoid factor; *SLE*: systemic lupus erythematosus; *SS*: Sjögren's syndrome; *SSc*: Systemic sclerosis; *TNF*: tumor necrosis factor. Adapted from Iaccarino *et al*. (35).

Figure 7. Polyautoimmunity in Sjögren's syndrome. *AIH*: autoimmune hepatitis; *AITD*: autoimmune thyroid disease; *MS*: multiple sclerosis; *PBC*: primary biliary cirrhosis; *RA*: rheumatoid arthritis; *SLE*: systemic lupus erythematosus; *SSc*: systemic sclerosis; *SS*: Sjögren's syndrome (For details see Table 5).

has been recently addressed in a meta-analysis (119). In a total of 2,489 SLE patients, the estimated prevalence of SS was 17.8%. SS-SLE patients constitute a subphenotype characterized by milder SLE-related features and a predominance of SS-related features (117,120,121). Note that, the combined disease SLE-SS seems to be characterized by less organ involvement, a more specific autoantibody profile, and a favorable clinical outcome (122).

SS and SSc. Sicca syndrome is also common among the patients with SSc due to fibrotic changes of the salivary glands (123). While recent studies found a 67% to 68% prevalence of sicca symptoms in the patients with SSc, the full SS syndrome was found in 14 to 20% of the patients with SSc (124,125). In original cohorts of SSc patients, the prevalence of polyautoimmunity was reported to be 17% and 29% (125,126). In recent studies involving 133 patients with SSc (124,127), it was found that SS associated with SSc was more often complicated by peripheral neuropathy and additional ADs or autoantibodies that were not typical for either SS or SSc. It was suggested that SS may be protective against SSc-associated pulmonary fibrosis. Limited SSc was predominantly associated with SS in these studies (122).

SS and other ADs. Abnormal liver tests are not uncommon but AIH hepatitis is diagnosed in 1.7-4% of the patients with SS while autoimmune cholangitis (with histological changes similar to stage-I PBC) develops mainly within the 5% to 10% of the patients with antimitochondrial antibodies (128).

AUTOIMMUNE AGGREGATION IN FAMILIES WITH SS

Diverse ADs may coexist in the same individual and in families, which implies a common etiology. The aggregation of ADs among first-degree relatives (FDR) of patients with SS was investigated through a population-based, case-control family study (40). In this study, 101 families of women classified as having SS according to the revised AECG criteria and 124 families of matched controls without AD were enrolled to investigate the presence of ADs. In family cases, 38% had at least one FDR with an AD, versus 22% in control families (OR 2.2; 95% CI 1.2–3.9). An AD was registered for 7.3% of 876 patients' FDR as compared to 3.85% of 857 controls' FDR (OR 1.97; 95% CI 1.28–3.03). The most frequent ADs registered among the SS patients' FDR were AITD, SLE, and RA, which disclosed aggregation. The proband phenotype (i.e., SS) was correlated with AITD, SSc, and all ADs when considered together as a trait. Maternal transmission of the autoimmunity trait was observed in cases but not in controls (129).

AUTOANTIBODIES AS A MARKER OF THE DISEASE

SS is characterized by a strong polyclonal B cell activation leading to chronic hypergammaglobulinemia, higher levels of ß2 microglobulinemia, and concomitant presence of a variety of circulating organ specific and non-specific autoantibodies (85). Autoantibodies are present in the majority of SS cases. Anti-Ro/SSA, anti-La/SSB antibodies, RF, and ANA are strongly indicative of SS although not exclusive. Patients with SS can also develop a broad spectrum of autoantibodies.

Anti-Ro/SSA and anti-La/SSB antibodies

While anti-Ro/SSA antibodies are found in 50–70% of SS patients, anti-La/SSB antibodies, which are considered to be more specific, are detected in 30–60% (17). These autoantibodies are considered the classical hallmark of SS and are included in both the AECG and SICCA classification criteria. These antibodies likely play a pathogenic role in inducing local inflammation and damage and are serological markers of systemic complications.

None of these autoantibodies is highly specific to SS as they are commonly detected in other ADs (e.g., SLE). Nonetheless, they are of great importance for the diagnosis and prognosis of SS. Anti-Ro/SSA and anti-La/SSB antibodies define a subphenotype (i.e., seropositive) with a higher rate of EGM and a more active immunological status when compared with seronegative SS cases. Anti-Ro/La-positive patients with SS may show severe hypergammaglobulinemia, cryoglobulins, and a high risk of developing lymphoma (130). Their presence at time of diagnosis has been reported to be associated with the emergence of recurrent salivary gland enlargement, cutaneous vasculitis, and interstitial lung disease (131,132). In addition, these autoantibodies correlate with early-AOD as well as with its activity and severity. Up to 20% of SS patients will be defined as seronegative and, like other ADs, it seems that negative serology correlates with a milder form of the disease (55). Anti-Ro/SSA and anti-La/SSB antibodies are detected on average 5 years before the appearance of an overt clinical phenotype of SS and thus serve also as predictive markers of the disease (1). Hence, it may be possible to use the presence of these autoantibodies as a predictor of disease severity in newly diagnosed patients (55). In addition, an immunogenetic background is important for autoantibody formation since it has a stronger association with HLA-DR2 and HLA-DR3 haplotypes (130).

Other autoantibodies. Several markers can be detected in the patients with SS as associates or substitutes of anti-Ro/SSA antibodies and are probably involved in the pathogenesis of different aspects of the disease such as anti-α fodrin (133), anti-carbonic anhydrase, and anti-muscarinic receptor antibodies (134,135). Other autoantibodies, e.g., anti-centromere antibodies (ACA) and anti-Ku, may define a more complex disease with overlaping features and a different prognosis (136). ACA are detected in 5-10% of SS cases as an alternative to anti-Ro/La antibodies. SS-ACA positive patients show SSc features (e.g., Raynaud's phenomenon, puffy hands, dysphagia, and teleangectasia) but have a lower rate of pulmonary involvement compared to SSc-ACA positive individuals. These patients show sicca

symptoms not due to glandular fibrosis, as observed in SSc, but due to a high rate of lymphocyte infiltration as well as anti-Ro/La positive SS (137).

Anti-Ki/SL, anti-Ku, and anti-p80 coilin antibodies are more rarely found in SS. Anti-Ki/SL antibodies, originally found in SLE with sicca, have been described in SS patients associated with anti-Ro/SSA or as isolated markers (138). Anti-p80 coilin has been detected in SS or SSc, especially when associated with PBC (139). Moreover, anti-Ku antibodies are usually considered markers of overlaping SSc-myositis or SSc-SLE syndrome (140). They have been detected in SS with features of SSc, cutaneous lupus, and/or myositis.

Recent studies have analyzed the clinical significance of autoantibodies considered characteristic of other ADs in patients with SS (137). The clinical significance of these autoantibodies varies widely with a broad spectrum of prevalence, clinical patterns of disease expression, and evolution to polyautoimmunity. However, the prevalence of these autoantibodies does not correlate with their clinical significance. Antiphospholipid antibodies, anti-neutrophil cytoplasmic antibodies, and cryoglobulins are frequently found in patients with SS with a prevalence ranging between 10 and 20%. Nevertheless, within this subphenotype, polyautoimmunity (e.g., SS-APS or systemic vasculitis-SS) is only detected in around 10% of the cases. In contrast, anti-dsDNA and ACA have a prevalence of only 5 to 10% but are more closely related to other features of SLE and limited SSc respectively, leading to the fulfillment of classification criteria for these diseases in more than 25% of cases. It is important to consider anti-dsDNA antibodies in the immunological follow-up of the patients with SS, especially in those with arthritis or leukopenia. Finally, anti-Sm, anti-RNP, and anti-Scl 70 have a prevalence of less than 5% and are rarely associated with the development of another AD thus suggesting little clinical significance in patients with SS. Hence, autoantibodies should be tested in the patients with SS who have specific clinical and immunological profiles or when specific features suggestive of polyautoimmunity appear during follow-up (9).

Novel autoantibodies. Recent studies identified additional autoantibodies in SS to salivary gland protein 1 (SP-1), carbonicanhydrase 6 (CA6), and parotid secretory protein (PSP). These autoantibodies were present in two animal models for SS and occurred earlier than antibodies to Ro/SSA or La/SSB in the course of the disease. Patients with SS also produced antibodies to SP-1, CA6, and PSP. These antibodies were found in 45% of the patients meeting the criteria for SS who lacked antibodies to Ro/La. Furthermore, in the cases of patients with idiopathic xerostomia and xerophthalmia for less than 2 years, 76% had antibodies to SP-1 and/or CA6 while only 31% had antibodies to Ro/La. Antibodies to SP-1, CA6, and PSP may be useful markers for identifying patients with SS at early stages of the disease or those that lack antibodies to either Ro/La antigens (141).

Subphenotypes based on autoantibodies. A variety of autoantibodies, which may define several subphenotypes of the disease, have been described (Table 6). Most typical among them are the anti- Ro/La ribonucleoprotein complexes described above. Other autoantibodies with clinical implications that are characteristic of SS include ANA, RF, and cryoglobulins, all of which have been associated with EGM and an active immunological profile. Cryoglobulins, in particular, distinguish a subphenotype with worse prognosis as they have been correlated with lymphoma development and are considered a risk factor for SS-related death. Anti-citrullinated protein antibodies are found only in a small percentage of SS patients and have been linked by some authors to the development of articular manifestations (142). Anti-mitochondrial and anti-smooth-muscle a ntibodies, on the other hand, have been associated with a more frequent development of liver involvement in SS and are considered indicative of PBC and AIH respectively. Furthermore, while ACA characterizes a subset of patients with features of SS and SSc, antibodies against carbonic anhydrase have been associated with renal manifestations of SS and renal tubular acidosis in particular. Finally, anti-muscarinic antibodies have been the subject of intense research during the last few years and could account, at least in part, for the salivary gland hypofunction, gastroesophageal symptoms, and the bladder smooth muscle hyperresponsiveness seen in SS. In conclusion, defining the clinical associations of different autoantibody specificities detected in SS is important not only for advancing our understanding of the pathophysiology of the disease but also for improving patient management (143).

PATIENT ASSESSMENT

Ocular and oral involvement should be measured objectively for a proper diagnosis of SS (Figure 8).

XEROPHTHALMIA

Dry eye syndrome is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface itself (144). Ophthalmologists recognize and diagnose dry eye syndrome to prevent or treat ocular surface pathologies. The tests of choice to diagnose dry eye include Schirmer test, ocular surface staining (i.e., fluorescein and lissamine green), and tear break-up time (TBUT) (31). The lissamine green dye test is very sensitive and stains damaged epithelial cells without causing any discomfort to patients (2). The reasons for ocular surface staining use lie in their reproducibility, sensitivity, and in the fact that all of them are quite easy to carry out. This test has the advantage of being simple and cost-effective but has been shown to be difficult to reproduce accurately in mild cases of KCS (44). Rose Bengal stains the conjunctival surface to reveal any breaks in the corneal-epithelial surface, but it has been progressively abandoned because of patient discomfort (48).

Corneal staining with colorants for slit lamp examination to detect conjunctival epithelium destroyed by desiccation is often used. In 2010, eight SICCA ophthalmologists developed a new quantitative ocular grading system (SICCA ocular staining score [OSS]) and analyzed OSS distribution among the SICCA cohort and its association with other phenotypical characteristics of SS. The SICCA cohort includes participants ranging from possible early SS to advanced disease. Procedures include sequenced unanesthetized Schirmer test, TBUT, OSS, and external eye examination by the slit lamp. Using statistical analyses and proportional Venn diagrams, they examined interrelationships between abnormal OSS (>3) and other characteristics of SS (labial salivary gland biopsy with focal lymphocytic sialadenitis and focus score >1 positive anti-SSA antibodies, anti-SSB antibodies, or both). Among 1,208 participants, they found strong associations between abnormal OSS, positive serological results, and positive labial salivary gland focus scores. Analysis of the overlapping relationships of these 3 measures defined a large group of participants who had KCS without other components of SS, which represented a clinical entity distinct from the KCS associated with SS (145).

AUTOANTIBODIES	PROPERTIES	SUBPHENOTYPES	PREVALENCE IN SS
anti-Ro/SSA anti-La/SSB	Disease marker Possible pathogenetic role in neonatal lupus	Younger age Parotidomegaly ↓ Schirmer test/ ↑Rose Bengal stain ↓ Salivary flow Heavier infiltrates and germinal centers in MSG biopsy EGM (e.g., cytopenias) Hypergammaglobulinemia Seropositivity: ANA, RF, cryoglobulins Neonatal lupus-congenital heart block	33–74% (anti-Ro/SSA) 23–52% (anti-La/SSB)
RF	Disease marker	Younger age Positive MSG biopsy EGM Hypergammaglobulinemia Seropositivity: anti-La/Ro, cryoglobulins, ANA Hypocomplementaemia (C3/C4)	36–74%
ANA	Disease marker	Younger age Parotidomegaly EGM (e.g., cytopenias) Hypergammaglobulinemia Seropositivity: RF, anti-La/Ro, APA	59–85%
Cryoglobulins	Disease marker	Younger age Parotidomegaly EGM (e.g., cytopenias) Seropositivity: RF, anti-La/Ro, APA Hypocomplementaemia (C3/C4) Monoclonal gammapathy B-cell Lymphoma Death	9–15%
ACPA	Disease marker	Polyautoimmunity (SS-RA)	3–10%
AMA	Disease marker	Polyautoimmunity (SS-PBC)	1.7–13%
ASMA	Disease marker	Polyautoimmunity (SS-AIH)	30–62%
ACA	Disease marker	Older age Raynaud's phenomenon ↓Hypergammaglobulinemia ↓ anti-Ro/SSA, anti-La/SSB antibodies, ↓RF ↓ Leucopenia	3.7–27%
anti-CA	Possible pathogenetic role	Renal tubular acidosis	12.5–20.8%
anti-M3 receptors	Possible pathogenetic role	Cytopenias ↑ ESSDAI scores	62.2–81.8%

Table 6. Subphenotypes in Sjögren's syndrome based on serological profile. *ACA*: anti-centromere antibodies; *ACPA*: anti-citrullinated peptide antibodies; *AIH*: autoimmune hepatitis; *AMA*: anti-mitochondrial antibodies; *ANA*: anti-nuclear antibodies; *Anti-CA:* anti-carbonic anhydrase antibodies; *Anti-M3 receptors*: anti-muscarinic receptor antibodies; *APA*: antiphospholipid antibodies; *ASMA:* anti-smooth muscle antibodies; *EGM*: extra-glandular manifestations; *ESSDAI*: European Sjögren's Syndrome disease activity index; *MSG*: minor salivary gland; *PBC*: primary biliar cirrhosis; *RA*: rheumatoid arthritis; *RF*: rheumatoid factor. Adapted from Bournia *et al.* (143).

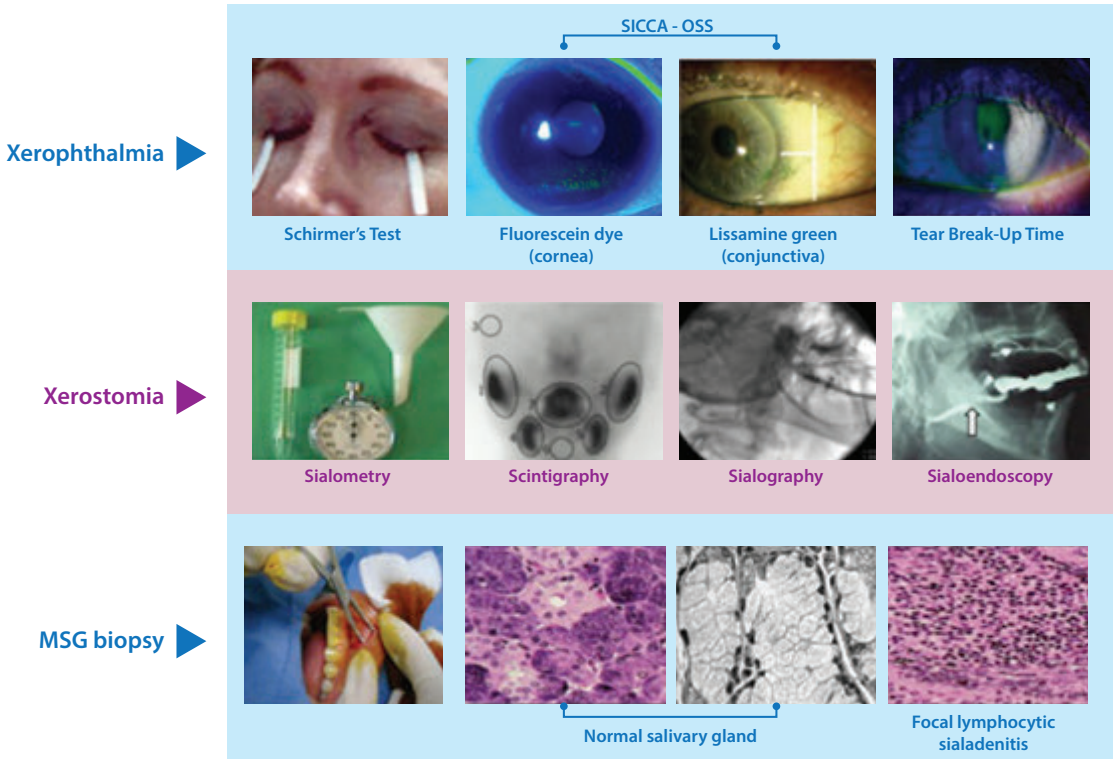


Figure 8. Sicca assessment in Sjögren's syndrome. *MSG*: minor salivary gland; *SICCA-OSS:* Sjögren's international Collaborative Clinica Alliance Ocular Staining Score.

The sequence and time intervals of these ocular tests are of critical importance to their accuracy and reproducibility. The application of a vital dye or any other substance to the ocular surface can alter the tear film and adversely affect subsequent results. Therefore, Schirmer test I (without anesthesia) is done first, followed by fluorescein dye test, tear break up time determination, and the corneal fluorescein staining pattern grading. After fluorescein grading of the cornea, lissamine green dye is applied and the conjunctiva is quickly examined and graded before the dye diffuses or the intensity of staining diminishes. The total time for the complete eye examination is approximately 20 minutes. The Schirmer test I is done before any drops are instilled in the eye. Standardized Schirmer strips are bent at the notch and placed carefully over the lower lid margin as close to the temporal angle of the lids as possible. The patient is instructed to keep her/his eyelids closed during the test. Strips remain in place for five minutes, or until they are completely saturated with tears. After 5 minutes, wetting of the strips is measured using the millimeter scale on each strip. It is generally agreed that a Schirmer I test of 5mm or less in 5 minutes is abnormal, but the variability of the test, even in normal eyes, invalidates any direct comparison between individual patients with KCS and normal controls. Immediately after removing the strips, one drop of 0.5% fluorescein is applied to the conjunctival fornix of each eye, and the participant is told to squeeze the eyelids tightly to remove excess dye. Two minutes after the application of fluorescein dye, the TBUT is measured by the slit lamp. Then, the corneal fluorescein staining and conjunctival lissamine green staining patterns are assessed with a resulting OSS. An OSS higher than 0 is considered to be abnormal and may be a sign of KCS. But scores of 1 or 2 can also represent a late staining artifact if interpretation of the fluorescein corneal staining pattern is delayed beyond 8 minutes. Because this could lead to a high level of misclassification, an abnormal OSS is defined as being a score of 3 or above (145).

XEROSTOMIA

Xerostomia can be objectively assessed by sialometry, scintigraphy, parotid gland sialography, sialoendoscopy, and minor salivary gland biopsy (146). Unstimulated salivary flow measurement has simple technical requirements readily available in general practice and is highly reproducible. The patient is instructed to spit saliva into a graduated test tube every minute. A quantity of <1.5 mL collected over a 15 minute period indicates impaired saliva secretion. This measurement has a sensitivity of 56% and a specificity of 81% for SS (Table 7). Parotid scintigraphy has greater sensitivity (80%) and specificity (86%) and evaluates the degree of involvement of the major salivary glands (17). Severe involvement (grade IV) at diagnosis is prospectively associated with a greater risk of lymphoma and death (147). The

TEST	BASIS AND METHOD	ABNORMAL VALUE	Se/Sp	COMMENT
Schirmer test	Semiquantitative measurement of tear secretion made by tear test strips	≤ 5 mm/5min	Se 42-76% Sp 72-76%	Screening and clinical practice test
Break-up time	Index of tear film stability. A 1% fluorescein solution is instilled in the inferior fornix of both eyes. After blinking, the interval in seconds between the last blink and the first break on tear film is measured	≤ 10 s	Se 82-92% Sp 17-86%	Clinical practice test Slit-lamp needed Age and technique dependent
Whole saliva flow collection	Non-stimulated: saliva collection is performed during 5 min or 15 min by the spitting method	Non-stimulated ≤ 1.5 ml/15min	Se 56-64% Sp 81%	Clinical practice test Affected by age, time of the day and drugs

Table 7. Dignostic performance of objective tests for dry eyes and dry mouth. *Se*: sensitivity; *Sp*: specificity. Adapted from Hernández-Molina and Sánchez-Hernández (233).

role of sialography has become more important in recent years. This test involves the injection of a small amount of contrast medium into the salivary ducts of a single gland, followed by routine X-Ray projections. It can estimate the amount of saliva excreted as well as the presence of obstructions or strictures in the gland ducts.

In addition to standard tests for assessment of salivary gland involvement, other methods have been studied such as magnetic resonance sialography and ultrasonography (148). Cornec *et al*. (149) recently determined the accuracy of salivary gland ultrasonography (SGUS) for diagnosing SS and suggested modification of the AECG classification criteria. Doppler waveform analysis and gland size measurement showed poor diagnostic performance. The results of the ROC curve analysis showed that the highest grade among the 4 glands provided the best diagnostic value. The optimal grade cutoff was 2 (62.8% sensitivity and 95.0% specificity). A weighted score was constructed using scores for the 5 variables selected by logistic regression analysis as follows: (salivary flow×1.5) + (Schirmer test×1.5) + (salivary gland biopsy×3) + (SSA/SSB×4.5) + (SGUS×2). According to ROC curve analysis, a score of ≥5 of 12.5 had 85.7% sensitivity and 94.9% specificity compared to 77.9% sensitivity and 98.7% specificity for the AECG criteria. The addition of SGUS to the AECG criteria increased sensitivity to 87.0% but did not change specificity. It has also been suggested that SGUS may provide useful diagnostic information comparable to that of a biopsy of the minor salivary glands but is less expensive and non-invasive (150,151).

HISTOPATHOLOGICAL ASSESSMENT

A minor salivary gland (MSG) biopsy is usually taken from the lower lip and is considered diagnostic of the oral component of SS if lymphocytic aggregation is observed in the perivascular or periductal areas on histological observation (146). Focal lymphocytic sialadenitis with a focus score >1 (aggregates of >50 lymphocytes/4mm^2) is the histopathological hallmark of the disease. The infiltrate is composed of T and B cells (80%:20%), which produce a plethora of immunologically active products (4). MSG biopsy is highly specific for the diagnosis of SS with a sensitivity of 82% and a specificity of 86% (17) and is indicated principally in seronegative patients (Table 8).

The diagnostic role of the histopathology of MSG has been considered important and is currently considered a gold standard although a recent meta-analysis has shown that the diagnostic usefulness has actually been evaluated in only a few studies (152). A recent study by Stewart *et al*. (153) suggested that the labial MSG can be misdiagnosed. The study compared oral and surgical pathologists and found that agreement among pathologists is poor for judgments of diagnostic status, focus scores, and histological characteristics of biopsy specimens. Histopathological examination of affected labial MSG reveals other characteristic changes consisting of a loss of acinar cells and the relative preservation of ductal cells (19). The end results of the pathological processes in the salivary glands in SS are a reduction in functional acinar tissue, a loss of secretory output, and symptoms of oral dryness (85).

OTHER INVESTIGATIONS

No single laboratory test allows for definitive diagnosis of SS. However, a combination of abnormal test results is frequently observed, including elevated erythrocyte sedimentation rate (ESR) (25% of patients with SS have an ESR >50 mm in the first hour), mild normochromic normocytic anemia, leukopenia, and polyclonal hypergammaglobulinemia (IgG > IgA > IgM) (85). They may help point to a systemic cause of sicca features. Patients with SS demonstrate numerous serological markers of autoimmune reactivity. Other parameters that should be tested for, or ruled out, are complement levels, serum monoclonal band (i.e., protein electrophoresis), and cryoglobulins. Hypocomplementaemia, monoclonal gammopathy, and mixed cryoglobulinaemia are associated with an increased risk of lymphoma and death (82).

The clinical significance of cryoglobulinemia in SS is threefold. First, cryoglobulins have been associated with a higher prevalence of extra-glandular disease. Second, cryoglobulinemia is associated with the development of non-Hodgkin lymphoma. Third, it is also associated with

1. The prepared slide should contain sections from 3 to 5 whole labial minor salivary glands stained with H&E. In discussing these specimens, it is helpful to recall conventional anatomic nomenclature: minor salivary glands are divided into several lobes which are further divided into lobules. These biopsy specimens are best composed of entire glands (each 1 to 2+ mm in diameter), which were surgically separated from their surrounding connective tissue during biopsy
2. Histopathological criteria for minor salivary gland diagnoses in SS assessment: a. ***Within normal limits:*** normal architecture, densely arranged normal acini and scattered or small aggregates of plasma cells with no or few lymphocytes. Scattered plasma cells are normal in all salivary glands and the source of secretory IgA b. ***NSCS:*** the presence of scattered or focal infiltrates of lymphocytes and macrophages that are not immediately adjacent to normal-appearing acini and located in gland lobules, lobes or entire glands that exhibit some combination of mild to moderate acinar atrophy, interstitial fibrosis and duct dilation, often filled with inspissated mucus c. ***FLS:*** the presence of one or more foci containing dense aggregates of 50 or more lymphocytes (most have several hundred or more) that are usually located in perivascular or periductal locations. These foci are adjacent to normal-appearing mucous acini, in lobes or lobules that lack duct dilation and contain no more than a minority proportion of plasma cells. This diagnosis is assigned when these foci are the only inflammation present in a specimen, or the most prominent feature. Focus scores are then calculated (see below) d. ***SCS:*** an advanced stage of NSCS in which interstitial fibrosis, various patterns of chronic inflammation (scattered or focal) and acinar atrophy predominate e. ***Granulomatous inflammation:*** is present when there are clusters of macrophages (CD68 positive), with or without multinucleated giant cells and absent necrosis f. ***MALT (extra nodal marginal zone) lymphoma:*** is diagnosed in minor salivary glands exhibiting diffuse lymphocytic infiltration with loss of intra glandular architecture and composed of sheets of CD20 positive B cells without follicular distribution, scattered CD3 positive T Cells and few if any follicular dendritic (CD21 or CD23 positive) cells
3. Establishing a focus score (only on those specimens diagnosed with FLS): a. ***FS:*** is calculated to give both a semi-quantitative estimation of inflammatory severity and a significance threshold to this pattern of lymphocytic infiltration b. ***Microscope calibration:*** gland area is determined by inserting a 10 x 10 eyepiece grid into one of the eyepieces and calibrating it for that microscope. The calibration needs to be done only once for a given microscope: use a stage micrometer to determine the precise area of each square in the eyepiece grid at low magnification (usually with a 4x objective). That area is then converted arithmetically into a factor that represents the number of squares that exactly equal 1 mm^2 c. ***Area measurement and FS calculation:*** after such one-time calibration, the pathologist measures a specimen's area by counting the number of squares that cover the total glandular area and dividing that number by the factor determined at calibration, giving the specimen area in mm^2. The focus score then comes from dividing the total number of foci in the specimen (as defined in 2c. above) by the total gland area and multiplying the result by 4, giving the focus score as XX foci per 4 mm^2

Table 8 Methods and histopathological diagnostic criteria for assessing labial salivary gland biopsies. *H&E*: Hematoxylin and Eosin stain; *NSCS*: non-specific chronic sialadenitis; *FLS*: Focal lymphocytic sialadenitis; *SCS*: sclerosing chronic sialadenitis; *MALT*: mucosa-associated lymphoid tissue; *FS*: focus score. Adapted from Retzlaff *et al*. (32)

life-threatening vasculitic involvement (59). Fauchais *et al*. found a prognostic value in cryoglobulinemia, which was the exclusive immunological parameter statistically associated with SS related death in a large cohort of patients (122). Hence, SS-associated cryoglobulinemia should be considered potentially life threatening with a high risk of developing systemic vasculitis and lymphoproliferative disorders, or death.

In patients with lung involvement, abnormal pulmonary tests reveal small airway obstruction while a detailed high-resolution computerized tomography detected wall thickening predominantly at the segmental bronchi. The most frequent tomography findings are ground-glass attenuation, bronchiectasis, reticular pattern, and honeycombing (6).

COURSE AND ACTIVITY OF SS

Most of the patients with SS have a stable and mild course of disease. Nevertheless, some patients may suffer from an aggressive disease with serious systemic complications. Several risk factors have been linked to the severity of SS including lower levels of C3 and C4, the presence of vasculitis or cryoglobulins, and an extensive involvement of the salivary glands at diagnosis (154).

To improve our ability to estimate the activity of the disease, the European League Against Rheumatism (EULAR) published two indices. One was EULAR Sjögren's syndrome disease activity index (ESSDAI), which is designed to measure disease activity in patients with systemic complications (155), and the other was EULAR Sjögren's syndrome patient reported index (ESSPRI), which is a patient-based question-

DOMAIN [WEIGHT]	ACTIVITY LEVEL	DESCRIPTION
Constitutional [3] Exclusion of fever of infectious origin and voluntary weight loss.	No = 0	Absence of the following symptoms:
	Low = 1	Mild or intermittent fever (37.5–38.5°C)/night sweats and/or involuntary weight loss of 5–10% of body weight.
	Moderate = 2	Severe fever (>38.5°C)/night sweats and/or involuntary weight loss of >10% of body weight.
Lymphadenopathy [4] Exclusion of infection.	No = 0	Absence of the following features:
	Low = 1	Lymphadenopathy ≥1 cm in any nodal region or ≥2 cm in inguinal region.
	Moderate = 2	Lymphadenopathy ≥2 cm in any nodal region or ≥3 cm in inguinal region and/or splenomegaly (clinically palpable or assessed by imaging).
	High = 3	Current malignant B-cell proliferative disorder.
Glandular [2] Exclusion of stone or infection.	No = 0	Absence of glandular swelling:
	Low = 1	Small glandular swelling with enlarged parotid (≤3 cm), or limited submandibular or lachrymal swelling.
	Moderate = 2	Major glandular swelling with enlarged parotid (>3 cm), or important submandibular or lachrymal swelling.
Articular [2] Exclusion of osteoarthritis.	No = 0	Absence of currently active articular involvement
	Low = 1	Arthralgias in hands, wrists, ankles and feet accompanied by morning stiffness (>30 min).
	Moderate = 2	1–5 (of 28 total count) synovitis.
	High = 3	≥6 (of 28 total count) synovitis.
Cutaneous [3] Rate as 'no activity' stable long-lasting features related to damage.	No = 0	Absence of currently active cutaneous involvement.
	Low = 1	Erythema multiforma.
	Moderate = 2	Limited cutaneous vasculitis, including urticarial vasculitis, or purpura limited to feet and ankle, or subacute cutaneous lupus.
	High = 3	Diffuse cutaneous vasculitis, including urticarial vasculitis, or diffuse purpura, or ulcers related to vasculitis.
Pulmonary [5] Rate as 'no activity' stable long-lasting features related to damage, or respiratory involvement not related to the disease (tobacco use, etc).	No = 0	Absence of currently active pulmonary involvement.
	Low = 1	Persistent cough or bronchial involvement with no radiographic abnormalities on radiography or
		Radiological or HRCT evidence of interstitial lung disease with no breathlessness and normal lung function test.
	Moderate = 2	Moderately active pulmonary involvement, such as interstitial lung disease shown by HRCT with shortness of breath on exercise (NHYA II) or abnormal lung function tests restricted to 70%>DLCO≥40% or 80%>FVC≥60%.
	High = 3	Highly active pulmonary involvement, such as interstitial lung disease shown by HRCT with shortness of breath at rest (NHYA III, IV) or with abnormal lung function tests DLCO<40% or FVC<60%.
Renal [5] Rate as 'no activity' stable long-lasting features related to damage and renal involvement not related to the disease. If biopsy has been performed, please rate activity based on histological features first.	No = 0	Absence of currently active renal involvement with proteinuria <0.5 g/day, no haematuria, no leucocyturia, no acidosis, or long-lasting stable proteinuria due to damage.
	Low = 1	Evidence of mild active renal involvement, limited to tubular acidosis without renal failure or glomerular involvement with proteinuria (between 0.5 and 1 g/day) and without haematuria or renal failure (GFR ≥60 ml/min).
	Moderate = 2	Moderately active renal involvement, such as tubular acidosis with renal failure (GFR <60 ml/min) or glomerular involvement with proteinuria between 1 and 1.5 g/day and without haematuria or renal failure (GFR ≥60 ml/min) or histological evidence of extra-membranous glomerulonephritis or important interstitial lymphoid infiltrate.
	High = 3	Highly active renal involvement, such as glomerular involvement with proteinuria >1.5 g/day or haematuria or renal failure (GFR <60 ml/min), or histological evidence of proliferative glomerulonephritis or cryoglobulinaemia-related renal involvement.

Table 9. The EULAR Sjögren's syndrome disease activity index (ESSDAI): domain and item definitions and weights. *CIDP*: chronic inflammatory demyelinating polyneuropathy; *CK*: creatine kinase; *CNS*: central nervous system; *DLCO*: diffusing CO capacity; *EMG*: electromyogram; *EULAR*: European League Against Rheumatism; *FVC*: forced vital capacity; *GFR*: glomerular filtration rate; *Hb*: haemoglobin; *HRCT*: high-resolution computed tomography; *IgG*: immunoglobulin G; *NCS*: nerve conduction studies; *NHYA*: New York Heart Association classification; *Plt*: platelet; *PNS*: peripheral nervous system. Adapted from Seror *et al*. (155).

DOMAIN [WEIGHT]	ACTIVITY LEVEL	DESCRIPTION
Muscular [6] Exclusion of weakness due to corticosteroids.	No = 0	Absence of currently active muscular involvement.
	Low = 1	Mild active myositis shown by abnormal EMG or biopsy with no weakness and creatine kinase (N<CK≤2N).
	Moderate = 2	Moderately active myositis confi rmed by abnormal EMG or biopsy with weakness (maximal deficit of 4/5), or elevated creatine kinase (2N<CK≤4N).
	High = 3	Highly active myositis shown by abnormal EMG or biopsy with weakness (deficit ≤3/5) or elevated creatine kinase (>4N).
PNS [5] Rate as 'no activity' stable long-lasting features related to damage or PNS involvement not related to the disease	No = 0	Absence of currently active PNS involvement.
	Low = 1	Mild active peripheral nervous system involvement, such as pure sensory axonal polyneuropathy shown by NCS or trigeminal (V) neuralgia
	Moderate = 2	Moderate = 2 Moderately active peripheral nervous system involvement shown by NCS, such as axonal sensorimotor neuropathy with maximal motor defi cit of 4/5, pure sensory neuropathy with presence of cryoglobulinamic vasculitis, ganglionopathy with symptoms restricted to mild/moderate ataxia, inflammatory demyelinating polyneuropathy (CIDP) with mild functional impairment (maximal motor defi cit of 4/5 or mild ataxia) Or cranial nerve involvement of peripheral origin (except trigeminal (V) neralgia)
	High = 3	Highly active PNS involvement shown by NCS, such as axonal sensorimotor neuropathy with motor déficit ≤3/5, peripheral nerve involvement due to vasculitis (mononeuritis multiplex, etc), severe ataxia due to ganglionopathy, infl ammatory demyelinating polyneuropathy (CIDP) with severe functional impairment: motor defi cit ≤3/5 or severe ataxia
CNS [5] Rate as 'no activity' stable long-lasting features related to damage or CNS involvement not related to the disease	No = 0	Absence of currently active CNS involvement.
	Low = 1	Moderately active CNS features, such as cranial nerve involvement of central origin, optic neuritis or multiple sclerosis-like syndrome with symptoms restricted to pure sensory impairment or confi rmed cognitive impairment.
	High = 3	Highly active CNS features, such as cerebral vasculitis with cerebrovascular accident or transient ischaemic attack, seizures, transverse myelitis, lymphocytic meningitis, multiple sclerosis-like syndrome with motor deficit.
Haematological [2] For anaemia, neutropenia and thrombopenia, only autoimmune cytopenia must be considered Exclusion of vitamin or iron defi ciency, drug-induced cytopenia	No = 0	Absence of auto-immune cytopenia.
	Low = 1	Cytopenia of auto-immune origin with neutropenia (1000<neutrophils<1500/mm^3) and/or anaemia (10<haemoglobin<12 g/dl) and/or thrombocytopenia (100000<platelets<150000/mm^3) Or lymphopenia (500<lymphocytes<1000/mm^3).
	Moderate = 2	Cytopenia of auto-immune origin with neutropenia (500≤neutrophils≤1000/mm^3) and/or anaemia (8≤haemoglobin≤10 g/dl) and/or thrombocytopenia (50000≤platelets≤100000/mm^3) Or lymphopenia (≤500/mm^3)
	High = 3	Cytopenia of auto-immune origin with neutropenia (neutrophils <500/mm^3) and/or or anaemia (haemoglobin <8 g/dl) and/or thrombocytopenia (platelets <50000/mm^3)
Biological [1]	No = 0	Absence of any of the following biological features
	Low = 1	Clonal component and/or hypocomplementaemia (low C4 or C3 or CH50) and/or hypergammaglobulinaemia or high IgG level between 16 and 20 g/l
	Moderate = 2	Presence of cryoglobulinaemia and/or hypergammaglobulinaemia or high IgG level >20 g/l and/or recent onset hypogammaglobulinaemia or recent decrease of IgG level (<5 g/l)

Table 9. The EULAR Sjögren's syndrome disease activity index (ESSDAI): domain and item definitions and weights. *CIDP*: chronic infl ammatory demyelinating polyneuropathy; *CK*: creatine kinase; *CNS*: central nervous system; *DLCO*: diffusing CO capacity; *EMG*: electromyogram; *EULAR*: European League Against Rheumatism; *FVC*: forced vital capacity; *GFR*: glomerular filtration rate; *Hb*: haemoglobin; *HRCT*: high-resolution computed tomography; *IgG*: immunoglobulin G; *NCS*: nerve conduction studies; *NHYA*: New York Heart Association classification; *Plt*: platelet; *PNS*: peripheral nervous system. Adapted from Seror *et al*. (155).

naire that assesses patients' symptoms in SS (156). The ESSDAI consists of 12 organ-specific domains contributing to disease activity. Each domain is classified into three or four levels based on their severity (Table 9). The ESSPRI consists of the main SS symptoms: dryness, pain, and somatic and mental fatigue, which are ranked on a single 0–10 numerical scale based on the patient's own report. These criteria and activity indices improve our ability to do comparable clinical studies but mainly highlight the fact that treatment of a patient with SS is tailored to his/her clinical manifestations, activity, and severity of disease (157).

FOLLOW-UP AND MONITORING

The periodicity for follow-up depends on the specific subphenotype (i.e., Sicca syndrome, EGM, organ damage). As mentioned, SS has a relatively stable course and immune profile (158). In fact, there is a spontaneous improvement in symp-

toms in approximately 12% of patients indicating that not all disease is progressive (136). This data contrasts with that of other studies. Fauchais *et al*. found that 26% of 445 SS patients exhibit new systemic manifestations during follow-up. These complications could appear 20 years after the initial SS diagnosis which suggests that SS patients must be carefully monitored although they do not present EGM for a long period. In addition, systemic complications could appear during SS evolution despite the disappearance of the initial active immunological profile. Hence, due to the risk of developing EGM, follow-up is justified for all patients with SS. Differences among studies are mainly due to ascertainment bias. Internists and rheumatologists are called to see more severe patients.

Patients should be routinely asked about cutaneous lesions, night sweats, and weight loss. Routine physical examination should include evaluation of the mouth and eyes to exclude local complications and an examination for peripheral lymphadenopathy and enlargement of parotid and submandibular glands, the liver, and the spleen. Refer patients with persistently high fever or night sweats, unexplained weight loss, polyadenopathies, or increased or persistent (>1 month) parotid swelling to a haematologist to rule out haematological neoplasia. Undiagnosed involvement of other organs may delay appropriate therapy and result, in some cases, in end stage organ failure (renal failure, pulmonary fibrosis, progressive neurological disease) and death. Advise fertile women with anti-Ro/La antibodies about the risk of congenital heart block, which has a frequency of <5% and may cause fetal death or the need for a pacemaker in the newborn. Yearly laboratory tests should include full blood count, ESR, renal and liver function tests as well as serum protein electrophoresis as part of the basic investigation for complications. Immunological tests are not necessary in routine follow-up with two exceptions, patients with markers associated with a poor prognosis and clinical suspicion of polyautoimmunity (9).

PROGNOSIS

Apart from a notably higher incidence of malignant non-Hodgkin lymphoma (NHL), SS patients typically show only modest or clinically insignificant deterioration in specific organ-related symptomatology and function. This, together with the low frequency of systemic effects, is likely to provide an explanation for why mortality rates are only slightly higher in patients with SS in comparison to the remainder of the population. This slight rise in mortality rates in SS patients is mainly because of the increased incidence of lymphoproliferative disease. These largely unaffected mortality rates also contrast with those of other ADs such as RA, SSc, and SLE, which are higher mainly owing to CVD (65). Comorbidities are able to influence the prognosis of primary disease. The larger the number of comorbidities, the greater the risk of patient hospitalization and mortality. Comorbidities might be associated with SS itself (i.e., nephritis, interstitial lung disease, and peripheral neuropathy) or with its treatment (such as immunosuppressive therapy). Unfortunately, no published data on the incidence of these comorbidities and their mortality risk among SS patients exists (70).

PHYSIOPATHOLOGY

Combinations of genetic, epigenetic, environmental, and hormonal factors that ultimately induce loss of immune tolerance, immune dysregulation, and tissue damage, are thought to cause the disease (1) (Figure 9).

GENETICS OF SS

The vital role of genetic components in SS was initially recognised by studies demonstrating higher concordance rates among monozygotic twins as well as familial aggregation (159). In addition, a high incidence of other ADs such as SLE, RA, AITD, psoriasis, and multiple sclerosis is noticed among SS patients (i.e., polyautoimmunity) and their family members (i.e., family autoimmunity) and thus pointed to several genes incriminated in the pathogenesis of SS (40). The genetics of SS is complex and involves HLA and non-HLA genes. The polymorphic major histocompatibility complex genes are the best documented genetic risk factors for the development of ADs. HLA-DRB1*0301-DQB1*0201-DQA1*0501 haplotype is the strongest risk factors for SS and for anti-Ro/La response (160).

Polymorphisms of non-HLA genes involved in the interferon α (IFNα) pathway, e.g., interferon regulatory factor 5 (*IRF5*), and signal transducer and activator of transcription 4 (STAT4) were found to be associated with susceptibility to SS (161). The *IRF5* gene encodes a transcription factor that is important for the production of type I interferon and induction of an pro-inflammatory state. STAT4 is a signal transduction molecule that induces T lymphocyte differentiation into Th1 subtype, and mutant STAT4 was related to several ADs including SS (162). A recent genome-wide association study (GWAS) disclosed *STAT4, IL12A, HLA, BLK, CXCR5* and *IRF5* as the strongest genes associated with disease (163). Noteworthy, among non-HLA genes, *IRF5* has also been confirmed in other ADs such as RA and SLE (164). Another study has revealed a correlation between polymorphisms in *IRF5* (CGGGG indel, SNP rs10488631) and STAT4 (SNP rs7582694) and SS development in a Swedish and Norwegian cohort (161). Significant elevation of gene expression for IFN-α-inducible protein 27 (IFI27) was also reported in a small study of SS patients further supporting the role of IFN in the pathogenesis of SS (165). These findings indicate that a genetic susceptibility conferring a greater IFN response to different innate stimuli could promote disease progression (166). Other non-HLA genes encoding for the cytokines interleukin (IL) 6 and 10 were linked to SS. Hulkkonen *et al*. (167) documented a significant correlation between IL-6 levels and severity of SS.

Genetic, genomic, and proteomic studies are rapidly evolving and are expected to continue enhancing our understanding of SS etiology and pathogenesis. The next stage of SS genetic research will be to do unbiased genome-wide association scans and large-scale replication studies in

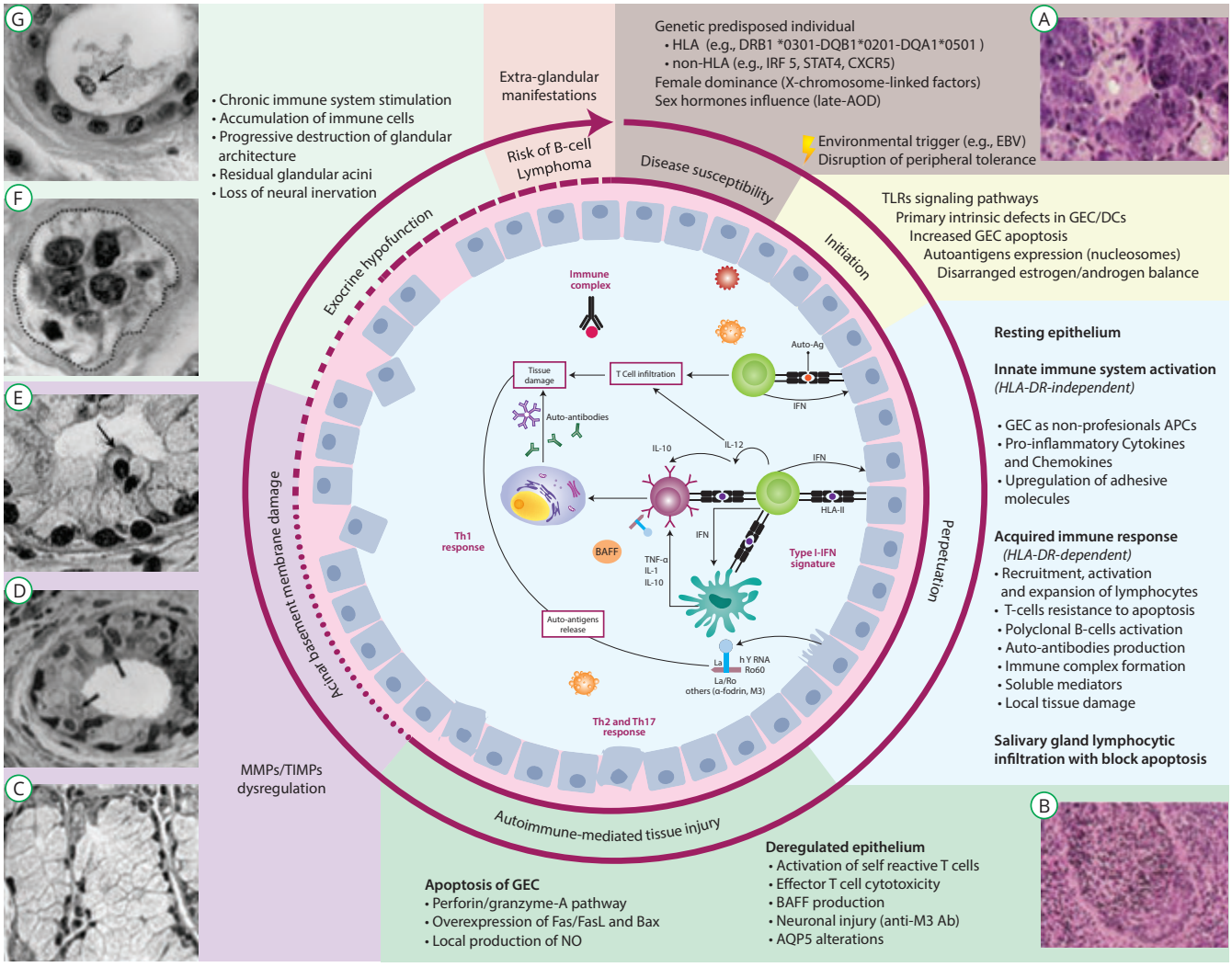


Figure 9. Sequence of physiopathological mechanisms in Sjögren's syndrome development. The pathogenetic mechanisms of this autoimmune epithelitis have not been fully elucidated. In genetically predisposed individuals, a trigger factor (most probably an epitheliotropic virus), activates the exocrine glands' epithelium. This leads to the recruitment, activation and expansion of lymphocytes. Immunologically-activated or apoptotic glandular epithelial cells that expose autoantigens might drive autoimmune-mediated tissue injury. Alterations in several immune mediators, such as up-regulation of type I interferon-regulated genes, abnormal expression of B-cell-activating factor and activation of the interleukin-23–type 17 T-helper cell pathway, might play a role. Extension of the pathological process that affects the exocrine glands into periepithelial and extraepithelial tissue can cause a considerable percentage of patients to exhibit systemic findings. Histologically normal MSG biopsy is shown in frame A; in comparison, a MSG biopsy from a SS patient is depicted in frame B, which shows lymphocytic infiltrates in the central region of the lobule. Photomicrographs of the MSG biopsies: Goicovich *et al.* (207). Frame C is a normal salivary gland biopsy. Frames D, E, F and G are from a patient with SS. Frames F and G show high endothelial venules containing red blood cells and lymphocytes migrating into the gland. *Ab*: antibodies; *AOD*: age at onset of disease; *APCs*: antigen-presenting cells; *AQP5*: aquaporin 5; *BAFF*: B cell-activating factor; *CXCR5*: C-X-C chemokine receptor type 5; *DCs*: dendritic cells; *EBV*: Epstein-Barr virus; *GEC*: glandular epithelial cells; *IRF*: interferon regulatory factor; *MMPs*: matrix metalloproteinases; *NO*: nitric oxide; *STAT4*: signal transducer and activator of transcription 4; *TIMPs*: tissue inhibitors of metalloproteinases; *TLRs*: Toll-like receptors.

much larger patient cohorts. Initial efforts are underway. These studies require extensive collaboration and contribution from clinicians and researchers since no single group will be able to independently recruit the large number of well-characterized cases needed. Studies on this scale are expected to elicit many new associated loci and pathways not previously evaluated. Whole genome sequencing is now becoming feasible and the cost will likely be at a sufficiently low level to make this technique feasible on a broad scale in the next 5 or so years. This will be an important phase in SS genetic studies as many rare variants have yet to be explored, and GWA scans are typically designed to evaluate more common variants. Transcriptome sequencing and expanded proteomics studies will also provide important insight. Integration of these rich datasets in the coming years, along with detailed clinical information, will undoubtedly be informative for dissecting the complex etiology and disease mechanisms in SS (168).

AUTOIMMUNE ECOLOGY IN SS

The final factor that determines the date of emergence of an AD is most probably an environmental trigger, which is generally of infectious origin (169). In the interplay between infectious agents and autoimmunity, it was found that while the same infectious agent [e.g. Epstein-Barr Virus (EBV)] may be involved in inducing many ADs, the same AD may be caused by various agents (e.g., EBV, cytomegalovirus, *Helicobacter pylori,* etc.) (170). Furthermore, a certain infectious agent may determine why, out of a group of individuals with the 'proper' genetic background, one individual will develop an AD while the others do not and what the clinical manifestations and severity will be.

Environmental factors such as glandular viral infection could prompt epithelial cells to activate the HLA-independent innate immune system through toll-like receptors (TLRs). TLRs recognize conserved molecular patterns shared by large groups of microorganisms and products of apoptosis and induce signaling pathways that lead to the production of inflammatory cytokines and up-regulation of co-stimulatory and adhesion molecules (171). Although a number of infectious agents such as EBV, human T-lymphotropic virus type 1, and hepatitis C virus have been implicated in the pathogenesis of SS, their association with SS appears weak (172). Despite the lack of clarity in the association between SS and viral infections, high incidence of EBV reactivation in SS patients has been reported. This reactivation could contribute to the initiation or perpetuation of an immune response in target organs (173). EBV is a common virus that infects both salivary epithelial cells and B cells. Nonetheless, it remains to be clarified exactly how reactivation of EBV is induced in lesions of patients with SS and which specific molecular mechanisms are involved in the process of viral reactivation. Non confirmed evidence that the coxsackievirus might infect MSG cells was reported (174). Variations in the genetic polymorphisms of virus receptors and HLA in patients as well as differences in the prevalence of enteroviral infections and methodological approaches could explain the observed discrepancies among studies. Other factors related to the autoimmune ecology such as socioeconomic factors and toxins deserve to be further investigated (see Chapter 14).

HORMONAL FACTORS AND APOPTOSIS

The female dominance and the late-AOD (40 to 50-years of age) in SS can be explained by the regulatory role of sex hormones. A link between gender, genetic susceptibility, and SS was documented by Dillon *et al*. (16). In this study, the prevalence of SS was 4 times higher among females carrying the triple X chromosome (47XXX) compared to normal females (46XX) and thus implies that 'X chromosomes dosage' is responsible for the female bias of SS. Estrogens seem to specifically protect secretory glandular acinar cells against apoptosis, whereas lack of estrogens during menopause and climacterium specifically leads to increased apoptosis of the exocrine secretory cells. Male gonads produce testosterone and convert it in exocrine glands to dihydrotesterosterone, which is anti-apoptotic and protects against acinar cell apoptosis. Estrogen-deficient women need to produce dehydroepiandrosterone in the adrenal glands and convert it to dihydrotesterosterone in exocrine glands in a complex and branching reaction network in which individual enzymatic reactions are catalyzed in forward and backward directions by a myriad of different isoforms of steroidogenic enzymes. Tailoring dihydrotesterosterone in peripheral tissues is much more complex and vulnerable in women than in men. In SS the intracrine steroidogenic enzyme machinery is deranged. These endo-/intracrine changes impair acinar remodeling due to impaired integrin α1β1 and integrin α2β1 expression. Therefore, the intercalated duct progenitor cells are unable to migrate to the acinar space in order to differentiate to secretory acinar cells upon contact with laminin-111 and laminin-211 specifically found in the acinar basement membrane. The disarranged endo-/intracrine estrogen/androgen balance induces acinar cells to release microparticles and apoptotic bodies and to undergo apoptotis and/or anoikis. Membrane particles contain potential autoantigens recognized by T- and B cell receptors and danger-associated molecular patterns recognized by TLRs. In membrane particles (or carrier-complexes) antigen/adjuvant complexes could stimulate professional antigen capturing, processing, and presenting cells which can initiate auto-inflammatory and autoimmune cascades, break the self-tolerance, and finally lead to SS (175).

AUTO-ANTIGEN EXPRESSION

The chronic immune system stimulation is thought to play a central role in the pathogenesis of the disorder as illustrated by several indices of immunological hyperactivity including various autoantibodies, in particular anti-Ro/SSA and anti-La/SSB. Anti-Ro/SSA antibodies recognize a macromolecular complex which consists of 60 kDa and 52 kDa Ro proteins and short cytoplasmic RNA molecules. The 60 kDa and 52 kDa Ro antigens are encoded by different genes and are completely different in aminoacid sequence, epitopes, and biological role within the cell. The 60 kDa Ro is directly bound to the RNA of the Ro complex and seems to have a role in DNA replication. In contrast, 52 kDa Ro does not contain an RNA-binding sequence but could be considered part of the 'Ro protein' through the link with 60 kDa Ro. It seems to play a role in ubiquitination and modulation of the innate immune system though regulation of proinflammatory cytokines and IFN-related factors (176,177). Interest in anti-52 kDa Ro has risen since there are new insights into the mechanisms of intracellular immunity mediated by these autoantibodies when they penetrate cells (178). Circulating levels of anti-Ro/La do not correlate with disease activity. Nevertheless, the IgA anti-Ro/SSA titer seems to be associated with the rate of lymphocyte glandular infiltration. In addition, the expression of La and 60 kDa Ro antigens in ductal glandular cells could represent a trigger for inducing and maintaining a local inflammation and tissue-specific immune response. Moreover, a strong correlation was found between circulating autoantibodies and Ro/La producing cells

in salivary glands (179). Anti-Ro and anti-La autoantibodies from SS sera, but not healthy IgG, can activate caspase 3 and determine apoptosis in human salivary gland cells *in vitro* (180). In addition, anti-Ro/SSA antibodies stimulate the production of proinflammatory cytokines (e.g., IL-6 and IL-8) by healthy human salivary gland epithelial cells (181). Therefore, these autoantibodies seem to have a pathogenic role for the impairment of the secretory function in the salivary glands.

CENTRAL ROLE OF EPITHELIUM

Epithelium plays a key role in the regulation of the local inflammatory responses in the MSG tissues of patients with SS. The up-regulation of adhesion molecules and the production of chemokines and cytokines are critical to the initiation and perpetuation of SS pathogenesis. The two together promote the migration of lymphocytes and dendritic cells (DCs) into the glands (70). Nevertheless, the fact that endothelial cells in the salivary glands of SS patients express adhesion molecules and are morphologically similar to high endothelial venules that might draw lymphocytes and DCs into the glands, their role in mediating the local immune response has not been clearly defined. In contrast, evidence exists that epithelial cells in SS lesions are active participants in the induction and perpetuation of the inflammatory process (182). They are though to be non-professional antigen-presenting cells. Their ability to show enhanced expression of CD40 and adhesion molecules as well as to produce lymphoid chemokines, cytokines, and B cell-activating factor (BAFF) indicates their potential role in the accumulation of DCs and T and B cells in the inflamed salivary glands and in the formation of lymphoid tissue (183). The fact that the activation status of epithelial cells derived from patients with SS remains unaltered *in vitro* for a long period of time prompts the hypothesis that they become activated intrinsically. After their migration to the glands, lymphocytes interact with epithelial cells. The latter are further activated by proinflammatory cytokines such as IL-1β, IFN-γ, and tumor necrosis factor (TNF) produced by neighboring T cells and DCs (184). SS salivary acinar epithelial cells express elevated apoptosis regulator BaX and undergo apoptosis, which causes dysregulation of the hosting organ. In contrast, infiltrating lymphocytes express elevated apoptosis regulator Bcl2 and are resistant to apoptosis, which prolongs their survival. Furthermore, through apoptosis and the formation of membrane-bound vesicles known as exosomes, epithelial cells present intracellular auto-antigens, a process that contributes to the breakdown of immune tolerance (185). Ro/SSA and La/SSB are translocated from the nucleus to apoptotic blebs where they trigger the production, by infiltrating B cells, of autoantibodies against these autoantigens. Given that BAFF can be secreted by human salivary gland epithelial cells following stimulation by type I IFN and that viral infection directly induces BAFF secretion by epithelial cells, it has been suggested that epithelial cells might not only express and present autoantigens but also concomitantly activate B cells by the local secretion of BAFF (186) (Table 10).

ROLE OF DCS

Plasmacytoid DCs were detected in human SS salivary glands by immunohistochemistry, indicating that the persistence of type I IFN signature could be related to an inflammatory

IMMUNE-RELATED PROCESSES/ RESPONSES	FEATURES OF SALIVARY GLAND EPITHELIAL CELLS	
Innate immunity	Toll-like receptors	TLR-1, TLR-2, TLR-3, TLR-4, TLR-7, TLR-9 CD91
Immune-cell homing	Adhesion	ICAM-1/CD54, VCAM/CD106, E-selectin
T Cell activation	Antigen presentation	MHC class-I (HLA-ABC) MHC class-II (HLA-DR, HLA-DP, HLA-DQ)
	Costimulatory	B7-1 (CD80), B7-2 (CD86) PD-L1 CD40
B-cell survival, maturation and differentiation		BAFF CD40
Expansion/perpetuation/ organization of infiltrates	Cytokines T Cell attracting/ germinal-center- forming chemokines	IL-1, IL-6, TNF-α, IFNs, IL-18 (pro-active), BAFF, adiponectin CCL3/MIP-1a, CCL4/MIP-1b, IL-8, CCL5/RANTES, CCL20/LARC, STCP-1/MDC, CXCL-9/Mig, CXCL-10/IP-10, CXCL12/SDF-1, CXCL13/BCA-1, CXCR3, CCL17/TARC, CCL19/ELC CCL21/SLC/TCA
Exposure of intracellular autoantigens	Apoptotic cell death Exosome secretion	Fas, FasL

Table 10. Sjögren's syndrome salivary gland epithelial cells are immune-modulatory players. *BAFF*: B cell-activating factor; *ICAM*: intercellular adhesion molecule; *IFN*: interferon; *IL*: interleukin; *MHC*: major histocompatibility complex; *RANTES*: regulated on activation normal T cell expressed and secreted; *TLR*: toll like receptor; *TNF*: tumor necrosis factor; *VCAM*: Vascular Cell Adhesion Molecule. Adapted from Tzioufas *et al*. (166).

cycle that is associated with an inappropriate maturation of DCs, stimulation of autoreactive T cells, production of autoantibodies, and increased production of endogenous IFN-α (187). The early appearance of DCs in the salivary glands and the production of type I IFNs by these cells could be important as they can cause abnormal retention and subsequent activation of lymphocytes in these tissues, which will also result in higher metalloproteinase activity (188).

SS AS AN INTERFERONOPATHY

One consistent characteristic of SS and SS-like diseases in mouse models is the strong upregulated expression of both the type I (α/β) and type II (γ) IFNs. In addition, global transcriptome studies have identified a variety of IFN-stimulated gene (ISG) transcripts differentially expressed in tissues of SS patients and mouse models exhibiting SS-like disease. Analyses of these transcriptome databases indicate that the sets of differentially expressed genes are highly restricted and suggests that there is a unique specificity in ISGs activated (or suppressed) during development and onset of disease. As a result, these observations have led to both SS and SS-like diseases being designated as 'interferon-signature' diseases or interferonopathies, which is one of the shared characteristics among ADs (see Chapter 14). While SS and SS-like diseases may be designated as such, very little effort has been made to determine what an interferon-signature might signify relative to autoinflammation and whether it might point directly to an underlying etiopathological mechanism and consecuently to a particular treatment (187,189,190).

CYTOKINE NETWORK IN SS

On examination of cytokine mRNA expression in the salivary glands of SS patients and animal models of the disease, an increase in proinflammatory cytokines, including IL-1β, IL-6, IL-l7, IL-10, IFN-γ, and TNF, was noted (191). While cytokines produced by type 2 T-helper (Th) cells (Th2 cytokines) have been found to dominate the early phase of SS, Th1 cytokines are associated with a later stage of the disease (6). Higher serum levels of IL-17 in SS patients, along with higher levels of Th17 cells and related cytokines, are dominant in salivary glands and strongly correlate with the histological focus score. Although compensatory mechanisms of Treg1 cells against Th17 cell expansion seems to occur in early and moderate infiltrations, in advanced lesions, Treg1 cells fail to control immune-mediated tissue injury (192).

Th1/Th2 and Be1/Be2. Classically, Th cells are divided into two main subsets which control the polarization of the immune response. Th1 cells produce IFN-γ, IL-2, and lymphotoxin and are involved in cellular immunity. Th2 cells produce IL-4, IL-5, and IL-13 and play a major role in B cell activation and humoral responses. The Th1/Th2 balance is altered in most ADs (see Chapter 9). Several groups have studied the expression of type-1 and type-2 cytokines in labial salivary glands of patients with SS, and the results conflict. However, recent evidence has shown that B cells are not strictly controlled by Th cells. B cells may regulate the levels of Th1 and Th2 cells (see Chapters 5 and 6). Indeed, B effector (Be) cells have been described: while Be1 cells produce IFN-γ and IL-2 and promote the expansion of Th1 cells, Be2 cells secrete IL-4 and favor the development of Th2 cells. Thus, the shift from a predominantly Th1 to a predominantly Th2 cytokine pattern may be under B cell control as immunopathological lesions progress during the course of SS (193).

IL-6/IL-17 system. IL-6 levels are increased in the serum and in the target tissues of patients with various ADs, including SS. This cytokine participates in the maturation of B cells and probably plays a role in the rupture of tolerance leading to autoimmunity. Indeed, under the influence of IL-6, B cells express the RAG1 and RAG2 enzymes which are instrumental in the rearrangement of immunoglobulin V region genes during B cell proliferation within GC. In an IL-6 up-regulation situation, B cells may aberrantly re-express RAG enzymes outside secondary lymphoid organs and modify their specificity, possibly for an auto-antigen. IL-6 also plays a major role in the development of Th17 cells, a proinflammatory T cell subset which has the capability of secreting cytokines such as IL-17, TNF-α, or IL-22 (193) (see Chapter 9).

DYSREGULATION OF B CELLS

As the lymphoid infiltration in the salivary glands is established, CD4+ T cells and DCs produce B cell targeted cytokines and other survival factors locally including BAFF and APRIL (a proliferation inducing ligand, also known as TNF ligand superfamily member 13) (184). B cell dysregulation in SS patients can be demonstrated by the presence of circulating immune complexes, hypergammaglobulinemia, alterations in subpopulations of peripheral B cells, oligoclonal B cell expansion, and a well described greater risk of developing NHL (84).

The role of BAFF and APRIL. BAFF has pleiotropic effects that are as numerous as they are varied. The real effect of each form (spliced, glycosylated, membrane bound, soluble, homotrimerized, heterotrimerized, multimerized) has not been well described yet. Consequently, there are conflicting results regarding the serum concentrations of BAFF or its functional effect in literature (194).

Two cytokines and their receptors have been demonstrated to be key in B cell homeostasis: BAFF, which rescues B cells from apoptosis, and APRIL, which participates in B cell activation. APRIL and BAFF have two receptors in common: the B cell maturation antigen and the transmembrane activator calcium modulator and cyclophilin ligand interactor. In addition, BAFF binds specifically to BAFF receptor 3. These receptors are mainly expressed on B cells. BAFF is produced by all sorts of macrophages and dendritic cells and from epithelial cells and activated T lymphocytes. Its mRNA has also been detected in myeloid cells, bone marrow-derived

stromal cells, astrocytes, and fibroblast-like synoviocytes in response to pro-inflammatory cytokines. BAFF can be bound to the membrane of producing cells and detected as polymers of different molecular weights in its soluble form. This diversity may explain the difficulty in assessing BAFF levels. BAFF is critical for B cells to survive on the periphery. It is also involved in B cell selection by promoting the resistance of transitional B cells to apoptosis. BAFF transgenic mice manifest B cell hyperplasia in their exocrine glands and develop SLE and SS-like disease (193).

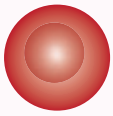
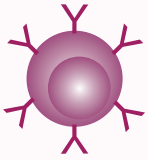
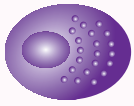
BAFF is overexpressed in SS as in other systemic ADs, and its level may correlate with the titer of autoantibodies. There is good evidence that local production of BAFF contributes to the deleterious effects of activated B cells by raising their expression of CD19 molecules as well as ensuring survival of B cell aggregates and autoantibody isotype switching outside and inside GCs. This process is sustained by the aberrant expression of BAFF by B lymphocytes infiltrating the salivary glands. After B cell depletion therapy in SS patients, B cell repopulation in the blood and in the salivary glands is modulated by BAFF. Thus, this cytokine is crucial at several levels in the pathogenesis of SS and may be targeted by future treatments (193). BAFF is an important mediator in the neogenesis of germinal centers in SS patients. The fact that BAFF can be secreted not only by salivary epithelial cells but also directly by B cells highlights the important effect of this factor in the initiation and perpetuation of B cell dysregulation in SS (195). The reduced level of apoptosis among BAFF-expressing cells in the salivary glands from SS patients may potentially lead to abundant BAFF expression thereby amplifying B cell signaling and promoting the regional proliferation of B cells and their differentiation into autoantibody producing plasma cells (196). Plasma levels of BAFF in SS patients are strongly associated with autoantibody titers including those of RF and anti-Ro/SSA (197). In the salivary glands of patients with SS, the combination of apoptotic bodies from epithelial cells and anti-Ro/SSA antibodies could induce the production of IFN-α by infiltrating cells. This, in turn, could induce BAFF expression by epithelial cells which would lead to the stimulation of autoreactive B cells (186). High levels of infiltration by macrophages have been related to type II cryoglobulinemia, parenchymal-organ involvement, glandular inflammation and, most importantly, NHL thus suggesting the potential involvement of these cells in disease severity and NHL development (198). Together, these data suggest that the sustained survival of autoreactive B cells, production of autoantibodies, or later development of NHL could be attributed to local BAFF expression (199).

IMMUNOPATHOLOGY

Both T and B cells actively and interdependently participate in the disease. In fact, the composition of the local round cell infiltrates varies according to lesion severity with T cells predominating in mild lesions and B cells in severe. While T regulatory cells have been found to prevail in intermediate lesions, low incidence of MSG infiltrating Tregs correlates with parotid gland enlargement and C4-hypocomplementemia, which are adverse indicators for lymphoma development (54). Infiltrating macrophages also increase with lesion severity and high incidence has also been associated with lymphoma development (198). Despite the fact that Th1 responses are thought to predominate in SS autoimmune lesions, evidence suggests that Th2 are the leading cytokines in mild lesions while Th17 responses have been shown to correlate with lesion severity (200,201). The occurrence of IL-12, IL-18, and BAFF in the inflammatory lesions of SS needs special attention. The expression of IL-12 and IL-18 has been negatively and positively correlated with parotid gland enlargement and C4-hypocomplementemia respectively. This suggests that IL-12 is a positive, whereas IL-18 is an adverse histopathologic prognostic factor for the development of lymphoma. BAFF over-expression in the infiltrating mononuclear and epithelial cells of the MSG tissues of SS patients has been directly linked to lymphomagenesis since it has been implicated in the expansion of autoreactive B lymphocytes, altered B cell differentiation and distribution, formation of ectopic germinal centers, and the lymphoma transformation that occurs in SS patients (202). The association between intense salivary gland inflammation and the occurrence of EGM such as Raynaud's phenomenon, vasculitis, lymph node/spleen enlargement, and leucopenia indicate that the local immune responses in SS are linked to the systemic manifestations of the disease (203).

Even though the major immunohistopathological features of the MSG autoimmune lesions have been resolved, several issues need to be delineated. These include the evolution of the autoimmune lesions and the lymphocytic populations that comprise them over time, the factors implicated in the activation of infiltrating cells, and the malignant transformation in MSG lesions as well as the manner in which the predominance of certain types of immune responses could affect the severity of the disease (204).

The infiltrate composition in SS differs based on lesion severity; however, these differences are not well-defined. Christodoulou *et al*. (205) investigated the differential distribution of the major infiltrating mononuclear cell (MNC) types in SS-lesions. They found that while T cells, $CD4^+$-Tcells and Tregs, B lymphocytes, MΦ, and iDCs were significantly different in MSG tissues with mild, intermediate, or severe inflammatory lesions, $CD8^+$-T cell, fDC, and NK cell incidence did not correlate with lesion severity. T cell, $CD4^+$-T cell, T/B cell ratio, and iDC incidence correlated negatively, whereas B cell and macrophage incidence correlated positively with the degree of infiltration and biopsy focus score. Tregs predominated in intermediate lesions. Multivariate analysis revealed several associations between the incidence of each infiltrating MNC-type and disease manifestations, which suggests an involvement of local immune responses in systemic disease features (Figure 10). These findings support the hypothesis that the distribution of infiltrating MNCs at the SS-lesions varies based on lesion severity and correlates with disease manifestations. The significance of this differential distribution and the underlying aetiopathogenic factors needs to be elucidated.

TYPE OF INFILTRATING MNCs		MILD	INTERMEDIATE	SEVERE	DISEASE MANIFESTATIONS
	$CD3^+$-T	+++	++	+	SG enlargement, RF +
	$CD4^+$-T	+++	++	+	Arthritis, RF +
	$CD8^+$-T	++	+	+++	-
	$CD4^+$/ $CD8^+$-T	++	+++	+	-
	Treg	+	+++	++	EGM, Serum C4 levels
	B cell	+	++	+++	-
	MΦ	+	++	+++	Lymphoma, Cryoglobulinemia
	iDC	+++	++	+	Hypergammaglobulinemia
	fDC	+++	+	++	
	NK	+	++	+++	Serum C4 levels

MSG INFILTRATION GRADE

Figure 10. Characteristics of the minor salivary gland infiltrates in SS. Distribution of the infiltrating-cell-types at MSG tissues that belong to the three SS-subgroups, as these classified by the grade of the inflammatory lesion. *EGM*: extra-glandular manifestations; *fDC*: follicular dendritic cells; *iDC*: interdigitating dendritic cells; *MΦ*: macrophages; *MNCs*: mononuclear cells; *NK*: natural killer cells; *RF*: rheumatoid factor; *SG*: salivary gland; *Th*: T helper cell; *Treg*: T regulatory cells. Adapted from Christodoulou *et al*. (205).

Important changes in acinar and ductal morphology and function, together with pronounced extracellular matrix (ECM) remodeling, are detectable in the MSG of SS patients. The classical symptoms of this disease, xerostomia, and KCS, have been related to the presence of autoantibodies and cytokines and to progressive denervation of the MSG. Physiological remodeling of ECM components depends on a balanced expression of proteolytic enzymes and their inhibitors such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) respectively (206). Some pathological processes are associated with excessive or insufficient ECM degradation, thereby highlighting the importance of ECM for the maintenance of epithelial architecture and function. The basal lamina plays an important role in this maintenance function since it lies on the interface between epithelial cell plasma membranes and the connective tissue. The ECM is also responsible for transmitting signals to cells via cell surface receptors, which include integrins and proteoglycans. Loss of adhesive interactions between epithelial cells and their microenvironment modifies gene expression and may lead to cell death. Such increased MMP activity is directly related to greater damage to parenchymal glands but is most likely not associated with the presence of infiltrating cells. The expression of MMPs is induced by cytokines, e.g., IL-1α, IL-6, TNF-α, and INF-γ. Studies of MSG from patients with SS showed that these cytokines are expressed by infiltrating mononuclear cells and by

acinar and ductal cells. Thus, MMPs could be induced in acinar and ductal cells by autocrine and paracrine pathways (207).

Another suggested mechanism of injury to the salivary glands is not that of direct glandular inflammation but rather of an immune-mediated neuronal injury. The innervation of the salivary glands is done by parasympathetic nerves which secrete acetylcholine to activate the M3 muscarinic receptors on salivary gland epithelial cells. Damage to muscarinic receptors by autoantibodies, or other soluble factors, may precipitate the exocrinopathy (208). Furthermore, the secretion of saliva from epithelial cells is dependent on water channel proteins such as aquaporins which are located on the apical surface of the cells. Aquaporin 5 isoform was found to be located on the basolateral membrane rather than on the apical membrane of acinar cells in SS patients (209).

B cell transformation and NHL. Patients with SS display severe B cell abnormalities compared to what has been observed in other ADs such as SLE with an increased concentration of BAFF serum levels (210). The numbers of $CD19^{+}CD27^{-}$ naïve B cells are decreased and the numbers of $CD19^{+}CD27^{+}$ memory B cells are increased in patients with active SLE. This contrasts with SS patients, who display a considerable reduction in the number of peripheral $CD27^{+}$ memory B cells as a result of their migration from peripheral blood to the inflamed salivary glands (211). The lymphoid population acquired secondary to the autoimmune process (known as the MALT component) surrounds and infiltrates the salivary ducts and represents the substrate from which B cell lymphomatous proliferation occurs (212). The transition of reactive lymphoepithelial sialadenitis from monoclonality to monoclonal lymphoma is generally considered to represent a multistep process yet is poorly understood. Speculation exists that chronic stimulation by exoantigens or autoantigens plays an essential role in the development of these tumors by driving the proliferation of specific B cells and by increasing the frequency of their transformation (213). In view of the intense modification of immunoglobulin genes during immune responses, a number of critical transforming events such as the inactivation of tumor suppressor genes (i.e., p53 mutations) or chromosomal rearrangements might result from the intense cell activity occurring within ectopic germinal centers in SS (214). Accordingly, SS patients who have a high risk of developing NHL exhibit splenomegaly, lymphadenopathy, type II cryoglobulinemia, and parotid swelling, all of which are indicators of extensive lymphoproliferation (215). In addition, the occurrence of type II cryoglobulinemia is thought to represent the transition from polyclonal B cell hyperactivity, a hallmark of SS, to monoclonal expansion of B cells. This produces an IgMk immunoglobulin with RF activity and suggests that some SS derived NHL originate from precursors that bear a functional autoreactive B cell receptor (216). The persistence of self-reactive, RF-expressing B cells in SS might reflect abnormal antigen selection, altered stimulation, and compromised censoring all mechanisms that magnify the risk of malignant B cell transformation (217).

TREATMENT

Education, prevention, substitution, stimulation (sicca syndrome), and immunointervention (organ involvement) are the steps to follow when treating patients with SS (Table 11). While symptomatic treatment with saliva substitutes and eye drops are effective in the relief of sicca syndrome complaints, immunomodulatory and immunosuppressive agents are used in patients with severe EGM and should be tailored to the specific organ involved (218–220). The objective of 'disease-modifying' drugs is to restore the deregulated immunological pathways that are responsible for disease process. Nevertheless, none have been shown to be disease modifying. Vitamin D supplementation may be an additional tool for optimization of SS treatment. Therapies could concentrate on four basic areas. First, methods to improve lubrication of local manifestations of dryness involving the eye and mouth. Second, recognition of associated problems of xeropthalmia and xerostomia, e.g., oral yeast infections, ocular blepharitis, and gastrotracheal reflux. Third, recognition and treatment of EGM including vasculitis and lymphoproliferative features. Fourth, assessment of and therapy for fatigue and vague cognitive symptoms that are not clearly the result of a systemic autoimmune process.

SICCA SYNDROME TREATMENT

Symptomatic treatment not only has beneficial effects on oral and ocular dryness, but it can also prevent complications of sicca syndrome (Figure 11).

Xerophthalmia. Once a diagnosis is confirmed, management of dry eye depends on the cause and severity of the condition. New treatment approaches are designed to modify the underlying disease process. Every associated condition must be treated. Therapy should normalize the tear film, decrease ocular surface inflammation, stimulate epithelial healing, improve neural feedback, decrease lacrimal gland inflammation, and improve its function. Therapy should be focused on protecting the ocular surface, alleviating the signs and symptoms of dry eye, and, most importantly, breaking the vicious cycle leading to chronic inflammation and thus improve the quality of life of patients (2).

If frequent use of artificial tears is inadequate or impractical, punctal occlusion is the treatment of choice. It is a highly effective method for maximizing the preservation of tears. This technique involves sealing the lacrimal puncta through which the tears normally drain away to the nose (90% of drainage occurs through the inferior punctum). Several different types of punctual plugs are available and plugs (called intra-cannicular plugs) that do not protrude into the corneal surface seem to be preferred. Local infections and even pyoderma-like reactions have been reported around the plugs. Some ophthalmologists begin with preliminary temporary plugs to ensure that punctal occlusion does not result in excess tear accumulation. Temporary plugs

EDUCATION ⇔ PREVENTION		SUBSTITUTION ⇔ STIMULATION		IMMUNOINTERVENTION
Non-pharmacologic measures		**Sicca syndrome**		**EGM**
Patients should be properly informed about the nature of their illness Alcohol, smoking and coffee should be avoided Avoidance of aggravating drugs (diuretics, beta blockers, tricyclic antidepressants, antihistamines) Regular sipping of water Home fluoride regimes tailored to individual patient Monitoring and reinforcement of oral hygiene instruction Written information Patient groups	Environmental irritants such as smoke, wind, air conditioning and low humidity should be avoided Meticulous oral hygiene. Diagnosis and treatment of mucosal candidiasis Diagnosis and treatment of comorbidity (i.e., depression, hypothyroidism) Suspect malignancy (clinical and paraclinical risk factors) Ophthalmologist and dentist valoration	Preservative-free teardrops, ocular lubricating ointments and autologous serum eye drops Punctual occlusion Saliva substitutes (mucin, caboxyme-thycellulose, hydroxyl-methilcellulose). Nose, ear and vaginal lubrication	Local Mechanical or gustatory stimulation (i.e., sugar-free chewing gum, citric juices, sugarless candies, electrostimulation)	Corticosteroids Hydroxycloroquine Methotrexate Azathioprine Leflunomide Mycophenolate mofetil Cyclophosphamide Others NSAIDs Analgesics
			Systemic Muscarinic agonists: Pilocarpine* (Salagen®) Cevimeline* (Evoxac®) Novel secretagogues	Biologic therapies Anti-CD20/22 mAbs Anti-BAFF/BlyS mAbs Anti-IFN-α mAbs Anti-TNFα mAbs
		Topical treatment NSAIDs, Corticosteroids Ciclosporin A*, Tracolimus, Pimecrolimus Transdermal testosterone		Other treatments DHEA B12 supplementation Thalidomide

Table 11. Treatment overview of Sjögren's syndrome. *BAFF/BlyS*: B cell-activating factor; *DHEA*: dehydroepiandrosterone; *EGM*: extra-glandular manifestations; *mAbs*: monoclonal antibodies; *NSAIDs*: non-steroidal anti-inflammatory drugs; *TNF*: tumor necrosis factor. *FDA-approved drugs for the treatment of dry eye.

often do not adequately block the puncta. Thus, failure to improve comfort with these temporary devices does not preclude the use of permanent punctal occlusion. Also, temporary plugs might be used to avoid a permanent change in patients who might regain near-normal lacrimal function with appropriate therapy. The availability of intra-cannicular plugs (that do not protrude into the ocular surface) has the added advantage that they can be removed nonsurgically. When indicated, laser or handheld thermal cautery can be used for a permanent closure. It is important to realize that punctal occlusion is a tear preservation strategy. As a result, it is of little benefit, unless supplemented with artificial lubricants for those with minimal to no-tear production (10).

Xerostomia. One of the most important consequences of oral dryness is the loss of teeth. It is extremely important that patients with SS regularly floss their teeth after meals, receive regular professional dental hygiene treatments including fluoride treatments at frequent intervals such as every 3 months, and recognize the role of dietary factors with respect to the correlation between sucrose intake and caries. Although frequently grouped together, it is important to consider dental caries as distinct from periodontal disease. The loss of teeth in patients with SS results from a combination of low oral pH that facilitates loss of dental calcium and the alterations of oral flora that lead to accelerated decay (10). A novel treatment for xerostomy is electrical stimulation of the lingual nerve which alleviates symptoms although salivary flow is not increased (221). Other invasive methods for enlarging salivary ducts and improving flow such as parotid sialoendoscopy with thorough rinsing and Stenson's duct dilation may be considered in certain patients (222).

Xerosis. Local treatment for cutaneous symptoms of SS focuses on dry skin. Treatment of dry skin in SS is similar to managing xerosis in other conditions. The patient should moisturize with a nonfragranced cream moisturizer once or twice a day. Moisturizing is done immediately after bathing or showering while the skin is still damp to prevent further evaporation from the skin. Sometimes, in cases of extreme dryness, an ointment is suggested for its barrier and protective properties (such as petrolatum jelly or petroleum jelly-based ointments). If this is used, then it should be applied to damp skin as the ointment itself does not contain water. Excess greasiness can be blotted with a towel. Sometimes a moisturizing cream with hydroxy acid, or urea, can add extra moisture, but in cases of cracks in the skin, it will sting and irritate. Excessive, long, hot showers or baths should be avoided in addition to heavily fragranced cleansers. The usual recommendation is to cleanse with a moisturizing, unfragranced soap, or a soap-free cleanser in liquid or bar form. If the xerosis leads to pruitis, then safe antipruritic topical treatments are recommended. Over-the-counter lotions containing menthol, camphor, 2% lidocaine, or pramoxine are easily available. Oral antihistamines should be used with caution because of their anticholinergic effects. Sometimes, topical corticosteroids are used for pruitis, but their use should be limited because of long-term side effects such as skin

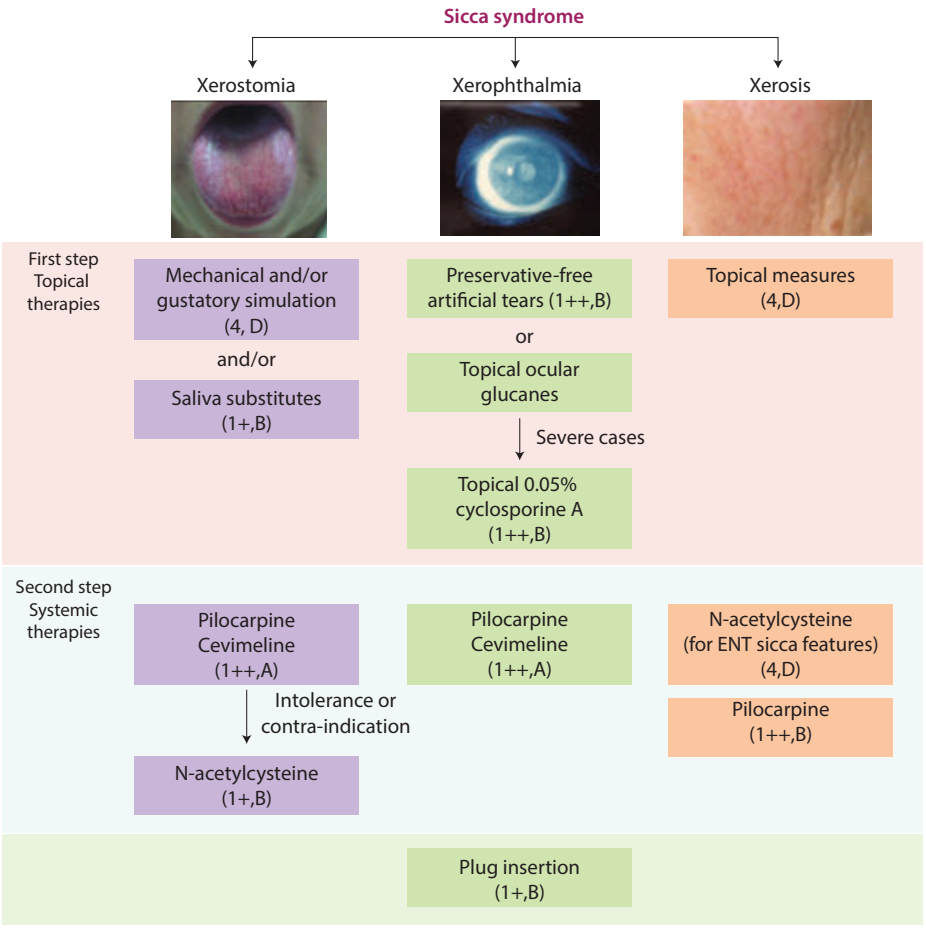


Figure 11. Therapeutic algorithm for the treatment of sicca features in Sjögren's syndrome. Level of evidence (1–4; recommendations based on evidence from studies that predominantly included patients with sicca syndrome or patients with SS are denoted by + and ++, respectively) and strength of recommendation (A–D) are shown in parentheses for each therapy according to the grading recommendations of Harbour and Miller. The first step in the management of sicca symptoms always consists of topical therapies; systemic therapies represent the second step, if topical interventions are not effective. Patients with acceptable salivary flow outputs might have poor tolerance of saliva substitutes owing to the 'sticky' feeling they can cause; in these patients, mechanical and/or gustatory stimulation (sugar-free candies and chewing gum) might be a useful first-line therapy before intervention with muscarinic receptor agonists (pilocarpine or cevimeline) is considered. Although a high level of evidence supports the use of certain topical ocular therapies, these agents have a grade B recommendation because studies were overwhelmingly performed in patients with keratoconjunctivitis sicca, with only a variable proportion of patients with SS included. Limited evidence is available for the treatment of sicca features other than those affecting the eyes and mouth in primary SS. Lacrimal punctal occlusion using plugs or thermal cautery can be useful in refractory and/or severe cases. *ENT*: ear, nose and throat; *SS*: Sjögren's syndrome. Adapted from Ramos-Casals *et al*. (219), with permission.

atrophy, tachyphylaxis, and absorption. In the case of inflammatory skin findings, local treatment with potent topical steroids can augment systemic treatments (223).

SECRETAGOGUES

For patients who have residual salivary gland function, stimulation of saliva flow with a secretagogue is the treatment of choice and is, at present, the most efficacious means of preventing long-term oral complications. Two muscarinic acetylcholine receptor agonists (pilocarpine and cevimeline) are licensed for the treatment of sicca symptoms in SS. These agents stimulate the muscarinic acetylcholine receptors M1 and M3 present on salivary glands to improve secretory function (219).

Pilocarpine. To date, eleven studies have analyzed the use of the muscarinic acetylcholine receptor agonist pilocarpine in patients with SS. In the two largest RCTs, pilocarpine doses of 5 mg and 7.5 mg every 6 h resulted in markedly improved oral, ocular, nasal, vaginal, and skin dryness and salivary flow rates. Uncontrolled studies have also reported a substantial therapeutic benefit from this drug based on the findings of ocular tests and reduction of candidiasis. Data from RCTs have revealed a high frequency of adverse events associated with pilocarpine use including sweating, increased urinary

frequency, and flushing (observed in 43%, 10% and 10% of patients respectively). In a dose-adjustment RCT, 23% of the patients were switched from the 7.5 mg to the 5 mg regimen after 6 weeks of treatment which suggests that the lower dose is better tolerated (219,224).

Cevimeline. The efficacy of another muscarinic acetylcholine receptor agonist, cevimeline, has been assessed in eight studies that enrolled patients with SS. Three RCTs have compared dosages ranging between 15 mg and 60 mg taken three times daily, and the best results –including considerable improvements in dry mouth and dry eyes, salivary flow rates, and ocular test results– were achieved with a dose of 30 mg. A crossover trial also found a marked reduction in candidiasis, dental plaque burden, and gingival bleeding. In the two largest trials of cevimeline in SS, both the 30 mg and 60 mg regimens were associated with higher frequencies of nausea (RR 1.68 and 2.77 respectively) and sweating (RR 2.16 and 3.00 respectively) in comparison to a placebo and rigors were more common with the 60 mg dose than with the placebo (RR 1.92) (219,225).

GENERAL SYMPTOMS

No clear benefit from hydroxychloroquine for general symptoms (muscle and joint pain, fatigue) was reported by controlled and prospective studies (all had small sample sizes). Its use is only supported by retrospective studies. Even when general symptoms are severe, the off-label use of biological agents to treat them is not warranted at present (220).

ORGAN-SPECIFIC TREATMENT

Systemic therapy should be tailored to the organ affected and the severity. All the drugs currently used in the treatment of ADs have also been administered to patients with SS in order to improve sicca symptoms and modify the immune inflammatory pathways involved in disease progression. Unfortunately, evidence supporting the use of these agents is limited. The decision on how to treat the patient depends on the clinical manifestations presented, their severity, and individual considerations. Immunosuppressant agents, e.g., ciclosporin A, azathioprine, methotrexate, mycophenolic acid, and leflunomide are all used empirically in SS. Indeed, only a few studies that had a low number of patients and used a short term follow-up (6 months) have been published. Therefore, their conclusions have a low level of evidence (Table 12). A benefit with respect to sicca symptoms without a significant improvement in objective tests has been reported by some. These drugs are currently used in the treatment of EGM and tailored to organ-specific involvement (219) (Figure 12).

THERAPY	AUTHOR, YEAR, (REFERENCE)	PATIENTS (WOMEN)	STUDY DESIGN	MEDICATIONS USED	DURATION (RANGE)
Glucocorticosteroids	Izumi *et al*., 1998 (234) Miyawaki *et al*., 1999 (235) Fox *et al*., 1993 (236) Pijpe *et al*., 2007 (237) Ramos-Casals *et al*., 2007	161 (151)	1 RCT 3 prospective 1 case-control	Prednisone (variable doses) Prednisolone (variable doses)	2-136 months
Antimalarials	Kruize *et al*., 1993 (238) Tishler *et al*., 1999 (239) Rihl *et al*., 2009 (240) Fox *et al*., 1998 (241) Fox *et al*., 1996 (242) Cankaya *et al*., 2010 (243) Yavuz *et al*., 2011 (244)	179 (178)	1 RCT 3 prospective 2 case-control 1 retrospective	Hydroxychloroquine (variable doses)	5-24 months
Methotrexate	Skopouli *et al*., 1996 (245)	17 (17)	1 prospective	Methotrexate 0.2 mg per kg weekly (10–15 mg per week)	12 months
Leflunomide	van Woerkom *et al*., 2007 (246)	15 (15)	1 prospective	Leflunomide 20 mg per day	6 months
Mycophenolic acid	Willeke *et al*., 2007 (247)	11 (11)	1 prospective	Increasing dose (from 360 to 1,440 mg per day)	6 months
Azathioprine	Price *et al*., 1998 (248)	25 (23)	1 RCT	Azathioprine 1 mg per kg daily	6 months
D-Penicillamine	ter Borg *et al*., 2002 (249)	19 (15)	1 prospective	D-penicilamine 250 mg per day	6 months

Table 12 Studies of immunomodulatory agents use in patients with Sjögren's syndrome. *RCT*: randomized controlled trial. Adapted from Ramos-Casals *et al*. (219).

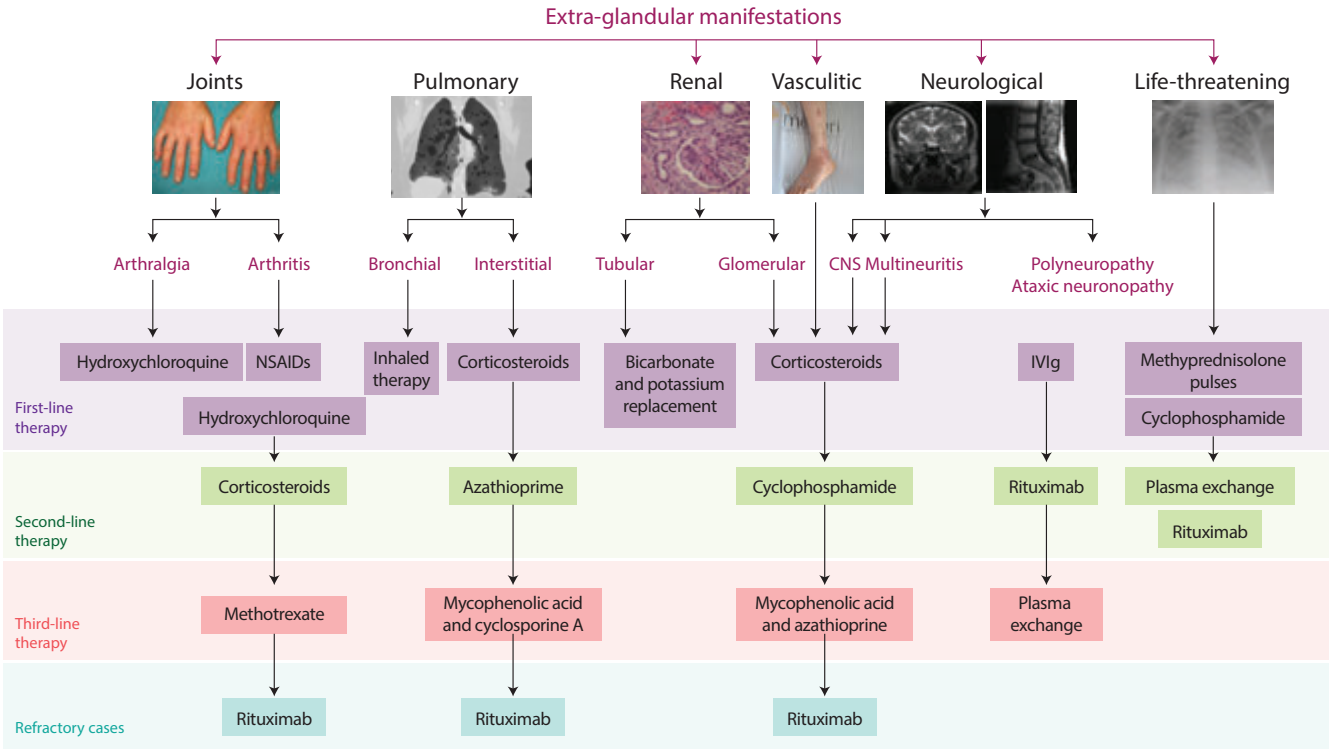


Figure 12 Therapeutic algorithm for treatment of extra-glandular manifestations in Sjögren's syndrome. Proposed therapeutic algorithm for treatment of the main extraglandular manifestations of SS. Available data for treatment of extraglandular SS symptoms come from nonanalytical studies, such as retrospective series or case reports (evidence level 3, on a scale of 1–4) representing the lowest strength of recommendation (grade D, rated from A–D) according to the grading recommendations of Harbour and Miller. The sole exception is a RCT using rituximab, which showed a reduction of the number of reported extraglandular manifestations compared with placebo (though the number of patients was too small to provide a specific recommendation). Corticosteroids should be used at the minimum dose and length of time necessary. Classification of drugs as first-line, second-line and third-line therapies is based on the number of case reports that have shown them to be effective and the authors' experience. No data are available on immunosuppressive maintenance therapy; thus, regimens similar to those used in other autoimmune diseases (systemic lupus erythematosus, vasculitis) are recommended. *CNS*: central nervous system; *IVIg*: intravenous immunoglobulin; *SS*: Sjögren's syndrome. Adapted from Ramos-Casals *et al*. (219), with permission.

BIOLOGICAL THERAPY

In the biological therapy era, novel therapeutic agents are being used to treat SS. Initial enthusiasm over the anti-TNF-α agents proved to be premature, and they have not been found to be effective in SS (226,227). Recent research interest has focused on biological agents that are directed against the underlying autoimmune inflammatory process (i.e., B cells, IFN type I, IL-6).

Suppression of B-lymphocytes may be considered for this AD. Although the marked inflammatory infiltrate in the affected glands includes a high percentage of T cells, there is evidence that B cells are an important pathogenic factor in SS. This includes the increased number of circulating B cells and B cell activation manifested by hypergammaglobulinaemia and production of autoantibodies (228). Hence, recent trials have used specific monoclonal antibodies directed not only against B cell antigens, especially anti-CD20 (Rituximab), but also against CD22 (Epratuzimab). Of these, the best experience has been gained with Rituximab (229). The cytokine B-lymphocyte stimulator (BlyS) appears to be antiapoptotic for B cells and stimulates their production of antibodies, so it may be another target for therapy. Nevertheless, large-scale clinical trials are still needed. With Rituximab, several placebo-controlled trials to date have had different outcome measures with none achieving significant improvement in the primary outcome measure at the trial conclusion. However, there has been evidence of some benefit either at intermediate time points or for some of the secondary measures. In a recent open label study of Belimumab (anti-BlyS) 19 out of 30 patients reached the primary endpoint (32). In the end, establishing a positive diagnosis and treating patients in whom the symptoms are driven by an ongoing autoimmune process is the best way to realize the promise of biological therapies down the road.

Other candidates for immune modulation in SS are the INF pathways (230). However, while there is evidence about the activation of type I IFN pathways and the mechanisms of its increase in SS, the means for directly suppressing it are still in the investigative stages. Although INF and IL-6 seem to play crucial roles in the pathogenesis of SS, at this point no data are available on the effectiveness of therapy directed at these cytokines. Future studies on the role of the Tocilizumab agent or novel reagents directed at IFN are needed.

LIFE-THREATENING SITUATIONS

Treatment of severe, life-threatening involvement has rarely been detailed, and at present, there are only a few retrospective studies and isolated case reports. However, this scanty evidence, taken together with expert review, suggests that methylprednisolone and cyclophosphamide pulses, possibly together with plasma exchanges, should be used in patients with rapidly progressing extraglandular features (glomerulonephritis, neuropathy, interstitial lung disease, or myelitis) or with severe systemic vasculitis. Rituximab is increasingly used in life-threatening situations and cases of B cell lymphoma (220).

In summary, treatment of SS is generally symptomatic with most patients only requiring treatment for dryness. Adequate explanation is essential. Many subjects, for example, may not realize that their central heating or air conditioning creates a drying environment or that a windy day is likely to make their eyes dryer. Simple measures such as humidifiers, sips of water, chewing gum, and simple replacement tears will be adequate for the majority of subjects. The rest should be told of the wide range of artificial fluids available and encouraged to try several different formulations. Treatment of other manifestations of SS has been influenced by our treatment of other ADs. The most serious (and fortunately rare) complications such as vasculitis and neurological disease require immunosuppression (10).

TRANSLATIONAL RESEARCH IN SS

New light has been shed on SS pathogenesis, diagnosis, and treatment. Advances of novel, high throughput genomic and proteomic technologies have appeared as new potential tools for generating pathogenetic, diagnostic, and prognostic biomarkers for SS. Genomic and proteomic technologies are widely considered to be complementary in their potential scientific application: the former explore gene expression profiling and the latter are a large-scale study of the proteins expressed by the genome. Both high throughput gene expression assays and mass spectrometric proteomics have provided promising results. This is particularly true in the case of in utilizing saliva to explore biomarkers for diagnostic purposes. Joint efforts are also under way in the search for ways to improve knowledge about the pathogenesis of the disease and to identify new therapeutic targets in SS (231).

PERSPECTIVES

SS is rather far from being considered a simple disease of 'dry mouth and dry eyes'. Research on SS is extremely active and aims at improving the classification of patients through more objective criteria by probing deeper into the etiology and the complex pathogenesis. At present, individual efforts that look at single diagnostic parameters have not led to findings that can be translated into a clinical setting. This is partly because complex diseases like SS might require a more systemic multidimensional approach that can efficiently capture the most relevant factors associated with this syndrome. Currently, the available data retrieved from genomic and proteomic studies represent a challenging starting point for a more thorough characterization of biomarkers that reliably describe the different pathophysiological aspects of SS, help to establish the diagnosis, and predict the outcome of the disease.

In the near future, the ultimate goal of the integration of genomic and proteomic studies should be the development of novel, targeted therapies for SS which might replace the currently adopted treatments that are mainly focused on symptomatic relief. A step forward in this direction is represented by gene therapies which offer the possibility to re-engineer the glands and thus restore the function of damaged salivary glands. SS is a challenging and intriguing disorder as the underlying pathophysiological mechanisms remain partially obscure, disease activity is challenging to evaluate, and no specific treatments are known to be effective. Over the last few years, emerging biotechnologies have shown great complementary potential for characterizing different subphenotypes of SS. These appear to offer promising tools to meet the challenge of the discovery of new, accurate biomarkers for SS from a novel perspective and, ultimately, to adopt new personalized therapeutic strategies for SS (232).

ABBREVIATION LIST

- **ACA:** anti-centromere antibodies
- **ACPA:** anti-citrullinated peptide antibodies
- **ACR:** American College of Rheumatology
- **ADs:** autoimmune diseases
- **AECG:** American-European Consensus Group
- **AIDS:** acquired immunodeficiency syndrome
- **AIH:** autoimmune hepatitis
- **AITD:** autoimmune thyroid disease
- **ANA:** anti-nuclear antibodies
- **AOD:** age at onset of disease
- **APRIL:** A proliferation-inducing ligand
- **BAFF:** B cell-activating factor
- **BlyS:** B-lymphocyte stimulator
- **CMV:** Cytomegalovirus;
- **CRP:** C reactive protein
- **DCs:** dendritic cells
- **EBV:** Epstein-Barr virus
- **ECM:** extracellular matrix
- **EGM:** extra-glandular manifestations
- **ESR:** erythrocyte sedimentation rate
- **ESSDAI:** EULAR Sjögren's syndrome disease activity index
- **ESSPRI:** EULAR Sjögren's syndrome patient reported index
- **EULAR:** European League Against Rheumatism
- **FDR:** first-degree relatives
- **GEC:** glandular epithelial cells
- **GMN:** glomerulonephritis
- **GVHD:** graft-versus-host disease
- **GWAS:** genome-wide association study
- **HBV:** Hepatitis B virus
- **HCV:** Hepatitis C virus
- **HIV:** human immunodeficiency virus;
- **HRCT:** high resolution computed tomography
- **IFN:** interferon
- **IL:** interleukin
- **IRF:** interferon regulatory factor
- **KCS:** keratoconjunctivitis sicca
- **MΦ:** macrophage
- **MALT:** mucosally-associated lymphoid tissue
- **MAS:** multiple autoimmune syndrome
- **MHC:** major histocompatibility complex
- **MMPs:** matrix metalloproteinases
- **MNC:** mononuclear cell
- **MS:** multiple sclerosis
- **MSG:** minor salivary gland
- **NHL:** non-Hodgkin lymphoma
- **NK:** natural killer cells
- **OSS:** ocular staining score
- **PBC:** primary biliary cirrhosis
- **RA:** rheumatoid arthritis
- **RF:** rheumatoid factor
- **RP:** Raynaud's phenomenon
- **SGUS:** salivary gland ultrasonography
- **SICCA:** Sjögren's International Collaborative Clinical Alliance
- **SLE:** systemic lupus erythematosus
- **SS:** Sjögren's syndrome
- **SSc:** systemic sclerosis
- **STAT4:** signal transducer and activator of transcription 4
- **TBUT:** tear break-up time
- **TCR:** T cell receptor
- **TIMPs:** tissue inhibitors of metalloproteinases
- **TLR:** toll-like receptor
- **TNF:** tumor necrosis factor
- **Treg:** T regulatory cells

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29

AUTOIMMUNE DIABETES MELLITUS (TYPE 1A)

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INTRODUCTION

Autoimmune diabetes mellitus or T1DM is an organ-specific autoimmune disease that affects the insulin-producing pancreatic beta cells, after an inflammatory process leads to a chronic deficiency of insulin in genetically susceptible individuals (1). The clinical manifestation (i.e., hyperglycemia) represents the final stage of insulitis (i.e., inflammation in pancreatic islets). At the time of diagnosis, only 10 to 20% of the insulin-producing beta cells continue to function (2). To date, there are no preventive or immunosuppressive therapies that can prevent damage or disease manifestations. However, our increasing knowledge of pathophysiology and immunogenetics has important therapeutic implications for measures aimed at prevention. New approaches for immune therapy such as anti-CD3 antibodies have shown success in modulating the natural history of the disease without the need for chronic immunosuppression. In the near future, it is likely that combination approaches will be needed to bring about lasting remission of the disease (3).

In 1997, the Committee of Experts of the American Diabetes Association recommended dividing T1DM into type 1A diabetes (i.e., immune-mediated) and type 1B (i.e., other forms of diabetes with severe insulin deficiency but without proof of autoimmune etiology or also known as idiopathic) (1) (Table 1). The best method to diagnose T1DM is to demonstrate the presence of autoantibodies directed against antigens present in the pancreatic islets detected by highly specific methods (4). However, with the current methods for detecting these autoantibodies, the autoimmune component cannot be demonstrated in a subgroup of children (around 10% of cases) with T1DM (5-7). One explanation for this finding is that the autoantibody titer decreases as the immune and pathological process progresses from the pre-diabetic period to the clinical phase (7).

Table 2 presents the main parameters for the differential diagnosis of T1DM. Type 1A, despite being an autoimmune disease, has some features that differentiate it from other autoimmune diseases (ADs). This pathology is presented on a one to one ratio (male: female) unlike other AD widely described in this book, e.g., systemic lupus erythematosus and rheumatoid arthritis, that present a high female predominance.

EPIDEMIOLOGY

The incidence is increasing worldwide while it is also presenting at younger ages (8). The disease has always been considered a childhood disease, but recent epidemiological studies have suggested that the incidence is comparable in adults (9). There is a huge difference in the incidence according to the population study. A child in Finland is 40 times more likely to develop the disease than a child in Japan and 100 times more likely than a child in Zunyi (China) (8). The incidence of the disease has been estimated at 0.1/100,000 population per year in countries like China and Venezuela and as high as 36.8/100,000 per year in Sardinia and 36.5/100,000 in Finland (10). This represents a greater than 350-fold variation in incidence for 100 different populations worldwide (10). The EURODIAB study, which involved records in 44 countries from Europe, showed an annual increase in incidence that was between 3 and 4% (11). The largest increase was seen in children between 0 and 4 years. The disease is diagnosed more in winter months in countries with high incidence, suggesting an environmental component at least as a trigger (10).

T1DM is associated with the presence of other autoimmune diseases, mainly autoimmune thyroid disease (AITD) and celiac disease (CD) (12). The Belgian Diabetes Registry showed a prevalence of thyroid autoantibodies in 22% of

I. Type 1 diabetes mellitus* (β-cell destruction, usually leading to absolute insulin deficiency) A. Immune-mediated B. Idiopathic
II. Type 2 diabetes (may range from predominantly insulin resistant with relative insulin deficiency to a predominantly secretory defect with insulin resistance)
III. Specific types of diabetes Including genetic disorders of the function of the pancreatic beta cell (i.e. MODY), diseases of the exocrine pancreas (e.g., pancreatitis, hemochromatosis), drug-induced or toxic (e.g., pentamidine, glucocorticoids, Vacor), infections, endocrinopathies (acromegaly, Cushing syndrome)
IV. GDM

Table 1. Etiological classification of diabetes mellitus. *GDM*: gestational diabetes mellitus; *MODY*: maturity-onset diabetes of the young. Adapted from The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1).

CLASSIFICATION	AUTOANTIBODIES	GENETICS	COMMENTS
Diabetes mellitus type 1A	Positive >90%*	HLA 30-50% DR3 and DR4 HLA 90% DR3 or DR4 HLA <3% DQB1*0602	90% non-Hispanic, white children 50% black children 50% Latin American children
Diabetes mellitus type 1B#	Negative	Unknown	Rare in whites
Diabetes mellitus type 2	Negative	Unknown	If positive autoantibodies LADA diagnosis is feasible, with similar HLA to type 1A
Other forms of diabetes	Negative	MODY mutations, other syndromes	

Table 2. Differential diagnosis of T1DM. *LADA*: latent autoimmune diabetes adult; *MODY*: mature-onset diabetes of the young; * Other markers of autoimmunity such as autoantibodies directed against components of the islets but not routinely determined in the laboratory may be present; # idiopatic T1DM. Adapted from Eisenbarth *et al*. (88).

the patients with T1DM. About 1 of 10 patients presented anti-transglutaminase IgA antibodies and more than half of these patients had CD demonstrated in intestinal biopsy specimens. At least 1 of 50 patients with T1DM had anti-21 hydroxylase antibodies, and approximately 25% of these patients progress to the clinical presentation of Addison's disease (Table 3). There is also a familial aggregation (FA) of AD in families of T1DM patients (Table 4).

GENETICS

Different alleles or genetic variants are associated, both as a risk or protective factor, with T1DM development. The concordance of T1DM is approximately 50% in monozygotic twins, and the risk of developing the disease in a first degree relative is about 6% (13). Comparing this last incidence with the presentation of the disease in the general population (0.4%), the risk of having T1DM is 15 times higher in a sibling of a patient with T1DM (Table 5). Although the risk of developing the disease is much higher in relatives of patients with T1DM, it is important to note that the majority (over 85%) of individuals who develop the disease do not have a first degree relative with this pathology. This high frequency of sporadic cases is mainly because almost 40% of individuals in the general population are carriers of high-risk HLA alleles. Different susceptibility genes have been determinants in the pathology as defined by both association and linkage studies. Thus, at least 60 loci have been identified and contribute to the risk of developing the disease. Table 6 summarizes the single-nucleotide polymorphism (SNP) associated with T1DM (14).

The major genetic determinant for the pathology is the major histocompatibility complex (MHC) on the short arm of chromosome 6 (called DDM1 site). Over 90% of people who develop T1DM carry either the DR3, the DR4 marker, or both, compared to only 40% of normal controls possessing these HLA (15). DR3-DR4 heterozygosity is higher in children who develop the disease before 5 years of age (50%) and lower in adults who develop the disease (20-30%) compared to only 2.4% in the U.S. population. Table 7 presents the haplotypes of HLA class II DR and DQ that are associated with both susceptibility and protection from the disease. A significant correlation associated with susceptibility or resistance to disease has been described in the amino acid at the position of HLA-DQβ on the chain. If an aspartic acid residue at position 57 occupies both alleles of the chain, there is little likelihood that diabetes will occur. On the other hand, its absence is an important marker of susceptibility. Individuals not carrying the aspartic acid on both alleles have a relative risk (RR) of 10^7 of developing the

DISEASE	PREVALENCE OF AUTOANTIBODIES	DISEASE PREVALENCE (%)
Thyroiditis or Graves' disease	25 % (antiperoxidase or anti-thyroglobulin)	4
Celiac disease	2 % (transglutaminase) (89)	6
Addison's disease	1.5 % (21-Hydroxylase) (90)	0.5
Pernicious anemia	21 % (parietal cell) (91)	2.6

Table 3. AD associated with T1DM. The prevalence of antibodies is determined in individuals with type 1A diabetes mellitus with or without clinical symptoms or signs of pathology. Prevalence is determined by clinical findings (signs and symptoms) and laboratory testing. Adapted from Eisenbarth *et al.* (88).

AUTOIMMUNE DISEASE	NUMBER OF ADIN FDR OF PATIENTS			NUMBER OF ADIN FDR OF CONTROLS		
	All	F	M	All	F	M
Megaloblastic anemia	1	1	0	0	0	0
Systemic lupus erythematosus	1	0	1	0	0	0
Vitiligo	1	0	1	3	0	3
Autoimmune diabetes mellitus	8	7	1	0*	0	0
Autoimmune hypothyroidism	15	7	8	6†	4	2
Number of ADin FDR	26/312‡ 8.3%	15/312 4.8%	11/312 3.5%	9/362§ 2.4%	4/362 ¥ 1.1%	5/362 1.3%

Table 4. Familial aggregation of other AD in first-degree relatives of patients with type 1A diabetes mellitus. These results are derived from a study done by Anaya *et al.* with 98 cases and 113 controls. CI: confidence interval; FDR: first degree relatives; F: female; M: male; OR: odds ratio; T1DM: type 1 diabetes mellistus; *comparing the frequency of T1DM in FDR of cases and controls, a statistically significant difference was obtained. OR: 20.24, 95% CI = 1.16-352.3, p = 0.002; † comparing the frequency of autoimmune hypothyroidism in FDR in cases and controls. OR: 3, 95% CI = 1.15-7.82, p = 0.02; ‡ 26 AD were observed in 25 FDR. There was a FDR who had two AD. No FDR of controls suffered more than one AD; § comparing the frequency of AD in FDR of cases and controls. OR: 3.56, 95% CI = 1.64-7.32, p = 0.0008; ¥ comparing the frequency of AD in FDR female cases and controls. OR: 4.63, 95% CI = 1.52-14.1, p = 0.0041.

INDIVIDUAL WITH DIABETES MELLITUS	% CHILDHOOD DIABETES MELLITUS (ANNUAL INCIDENCE)	ANTI-ISLET AUTOANTIBODY	COMMENTS
U.S. general Population	0.3% (15-25/100,000)	3% single antibody; 0.3% multiple antibodies	Japanese incidence 1/100,000; incidence increasing in United States and many European countries; Colorado now 25/100,000.
Offspring	1%	4.1%	
Sibling	3.2%; 6% lifetime	7.4%	
Dizygotic twin	6%	10%	
Mother	2%	5%	Lower risk than offspring of father with diabetes mellitus.
Father	4.6%	6.5%	
Both parents	10%?		
Monozygotic twin	50%, but incidence varies with age of index twin.	50%	In Japan, 40% of risk.

Table 5. Risk of developing T1DM. T1DM: type 1 diabetes mellitus. Adapted from Eisenbarth *et al.* (88).

GENE/REGION	CHROMOSOME	POSITION (MB)	SNP
PREVIOUSLY REPORTED			
PGM1	1	63.9	rs2269241
PTPN22	1	114.2	rs2476601
RGS1	1	190.8	rs2816316
IL10	1	205	rs3024505
Intergenic, IL1 8RAP	2	12.6	rs1534422
Intergenic	2	24.5	rs2165738
IFIH 1	2	162.9	rs3747517
CTL4	2	204.4	rs231727
Intergenic	4	25.7	rs10517086
HLA	6	32.7	rs9272346
BACH2	6	91	rs11755527
C6orf173	6	126.7	rs9388489
SKAP2	7	26.9	rs7804356
COBL	7	51	rs4948088
GLIS3	9	4.3	rs7020673
IL2RA	10	6.1	rs12251307
PRKCQ	10	6.4	rs947474
RNLS	10	90	rs10509540
INS	11	2.1	rs689
CD69	12	9.8	rs4763879
12q3	12	54.8	rs2292239;rs1701704
SH2B3	12	111	rs17696736
ZFP36L1	14	68.3	rs1465788
Intergenic	14	97.6	rs4900384
CTSH	15	77	rs3825932
CLEC16A	16	11.1	rs12708716
ORF;PRM3;TNP2	16	11.3	rs416603
UMOD	16	20.3	rs12444268
IL27	16	28.4	rs4788084
CTRB2	16	73.8	rs7202877
DNAH2	17	7.6	rs16956936
GSDMB, ORMDL3	17	35.3	rs2290400
SMARCE1	17	36	rs7221109
PTPN2	18	12.8	rs2542151
PRKD2	19	51.9	rs425105
SIRPG	20	1.6	rs2281808
Ubash3a	21	42.7	rs876498
LOC729980	22	28.9	rs5753037
C1QTNF6	22	35.9	rs229541
GAB3	x	153.6	rs2664170
RECENTLY REPORTED			
EFR3B	2	25.3	rs478222
AFF3	2	100.2	rs9653442
SLC11A1	2	219.3	rs3731865
CCR5	3	46.3	rs1592410
IL2	4	123.3	rs2069763

GENE/REGION	CHROMOSOME	POSITION (MB)	SNP
RECENTLY REPORTED			
CAPSL	5	35.9	rs6897932
LOC729653	6	29.5	rs1592410
HLA region	6	31.3	rs3094663
AGER	6	32.1	rs9469089
TNFAIP3	6	138	rs10499194
TAGAP	6	159.4	rs1738074
6q27 region	6	170.4	rs924043
IKZF1	7	50.5	rs10272724
CUX2	12	111.4	rs1265564
LMO7	13	76	rs539514
DLK1	14	100.4	rs941576
RASGRP1	15	36.7	rs17574546
CCR7	17	38.8	rs7221109
Trp53	17	7.6	n/a
FHOD3	18	34.2	rs2644261
CD226	18	67.5	rs763361
PRKD2	19	47.2	rs425105
FUT2	19	49.2	rs601338

Table 6. Genetic variants associated with T1DM. *T1DM*: type 1 diabetes mellitus; *SNP*: single-nucleotide polymorphism. Adapted from Morahan *et al*. (14).

DRB1	DQA1	DQB1
HIGH RISK		
0401, 0403, 0405	0301	0302 (DQ8)
0301	0501	0201 (DQ2)
MODERATE RISK		
0801	0401	0402
0404	0301	0302
0101	0101	0501
0901	0301	0303
MODERATE PROTECTION		
0403	0301	0302
0701	0201	0201
1101	0501	0301
STRONG PROTECTION		
1501	0102	0602 (DQ6)
1401	0101	0503
0701	0201	0303

Table 7. Diabetes Risk of Representative DR and DQ Haplotypes. Adapted from Eisenbarth *et al*. (88).

disease. More recently, it has been shown that the presence of the amino acid arginine at position 52 of the DQα chain confers an increased risk for the disease. This risk is additive with the increase conferred by the lack of aspartic acid at position 57 of the DQβ chain. It is believed that the absence of this last amino acid at position 57 and the presence of arginine at position 52 of the DQα chain allow an autoantigen to engage in the HLA antigen presentation site manager to be recognized by the T-cell receptor (TCR) (15). Another site of genetic susceptibility called IDDM2 has been identified. It corresponds to the insulin gene located on chromosome 11. This gene contributes to about 10% of the FA of disease (16). This locus corresponds to a polymorphic region which maps to a variable number of nucleotide repeats (VNTR). Studies have shown that the different sizes of VNTRs of the insulin gene are associated with the risk of T1DM. The longer form of the VNTR (> 100 repeats or class III) is associated with protection from disease (17). This finding has been explained by improved immunological tolerance to a larger insulin gene with a higher level of thymic expression and with the consequent tolerance. There are other genes associated with monogenic forms of polyendocrinopathy [AIRE gene (autoimmune regulator associated with type 1 autoimmune polyendocrine syndrome) and XPID gene], which includes within its manifestations the presence of diabetes.

PTPN22, a gene encoding for a lymphoid-specific phosphatase that influences TCR signaling has been identified for T1DM risk (18). Polymorphisms in the *CTLA4* (IDDM 12)

are apparently associated with the development of Graves' disease and autoimmune diabetes but not in all populations (19). A locus associated with the IL-2 receptor also has a statistical association (20). In summary, many genetic loci have been identified as a risk for developing diabetes. Of these, the HLA typing at birth is being used in some populations to define the risk of the disease depending on the relationship of the individual with an index case of diabetes. For example, siblings of patients with T1DM and the genotype DR3-DQ2/DR4-DQ8 have a risk of developing diabetes that exceeds 50% (21).

ENVIRONMENTAL ASPECTS

Several features suggest that T1DM has a significant environmental component. The rapid increase in the incidence together with the variability found between genetically similar populations (e.g., double incidence in the United Kingdom compared to France) suggest that an environmental agent may trigger the autoimmune process in different countries. Two hypotheses could explain the recent increase in the incidence. The first one is that an infectious agent such as a virus in the general population is increasing and causing infectious processes (22). The most common appearance of the disease in winter months and some epidemic flares of T1DM suggest that certain viruses such as rotavirus (23) and Coxsackie virus (24) and some dietetic aspects may influence the risk of developing T1DM. Multiple associations have been described with various environmental agents and viruses. However, despite more than four decades of research in this area, the only consistent association of T1DM with a viral agent is congenital rubella infection (25). Children affected with this type of diabetes usually present high-risk HLA alleles and high prevalence of AITD (26,27). The exact mechanism by means of which congenital rubella infection increases the risk of T1DM is unknown. In addition, associations with a particular viral agent have been based on antibodies or viral antigen detection at the time of diagnosis. Because clinical manifestations appear after a long-standing immunological process, it is difficult to identify a pathological relationship between these infectious agents and the disease. The finding of viral particles at the diagnosis may be explained by a higher insulin requirement in the course of an undercurrent infection with the presence of the infectious agent being an incidental finding.

Another environmental agent implicated in the development of the pathology has been early feeding with cow's milk [bovine serum albumin (BSA)]. This hypothesis was based on retrospective studies in which this factor was suggested (28). However, several prospective studies did not validate these data. For instance, a study done in Denver, Colorado, in which children were assessed at birth, showed no evidence of disease association with bovine milk feeding, enteric viral infections, or vaccination history (29).

Some studies provided evidence that early (<3 months) introduction of cereals may increase the development of islet autoimmunity (30). Vitamin D and ω-3 fatty acids, which can influence the immune function, have also been associated with the risk of diabetes (31). Another environmental factor involved lately is the toxins derived from Streptomyces bacteria found in soil, which can colonize foods like vegetables. In mice models, these toxins can cause damage to the pancreatic cells (32). Vaccination has been implicated as a trigger for diabetes in genetically susceptible children. However, different studies have not confirmed this possibility (33, 34).

The second scenario, called "hygiene hypothesis" suggests that environmental factors may inhibit the development of autoimmunity. The environment for children is cleaner today, which may lead to defective immuno-regulatory mechanisms that result in a Th2 mediated-response pattern (such as asthma) or diseases characterized by a Th1 pattern such as T1DM (35,36). Other epidemiological observations suggest the environmental component of the pathology. The non-twin brothers and dizygotic twins share 50% of their genetic material unlike identical twins who share the entire genome. However, the concordance of the disease is higher in dizygotic twins than non-twin siblings which suggests an environmental component because the former are generally exposed to the same environmental stimuli (i.e., same food, infections, etc.).

AUTOANTIBODIES

As previously described, over 90% of the patients with new onset T1DM possess at least one autoantibody against components of the pancreatic islets. A significant variety of specific antibodies against constituents of pancreatic beta cells have been identified. They include insulin, the isoform of glutamic acid decarboxylase 65 and 67 (GAD 65 and GAD 67 respectively) and the IA-2 secretory protein, which has a domain like-tyrosine phosphatase. These autoantibodies are markers of the autoimmune process rather than the direct effectors of damage. GAD antibodies (GADA) can be detected in 50 to 80% of Caucasian patients with newly diagnosed diabetes (37, 38). It has been also demonstrated that these autoantibodies appear several years before clinical manifestations and diagnosis, and tend to persist in the serum of patients for many years (39). Anti-IA-2 autoantibody (IA-2 Ab) prevalence ranges from 55 to 75% and has a tendency to be higher in patients with new-onset diabetes and early onset (i.e., preschool) (40, 41). These autoantibodies are found mainly in patients who are under 15 years of age and are, therefore, more useful in the study of young patients with diabetes (42). A new auto-antigen, called ZnT8 has been described. ZnT8 is a member of the large cation efflux family (10 mammalian homologues, almost 100 family members) of which at least 7 are expressed in pancreatic islets. Autoantibodies against ZnT8 are found in 60–80% of new-onset T1DM compared to <2% of healthy controls, <3% of type 2 diabetic patients, and up to 30% of patients with other autoimmune disorders with a T1DM association (43). ZnT8 have been found in 26% of T1DM subjects classi-

fied as autoantibody-negative on the basis of existing GADA, IA-2 Ab, and anti-islet antibodies markers (43). The insulin autoantibodies are the only specific autoantibodies of beta cell autoimmunity and are found primarily in children younger than 5 years old (44). However, a negative result for these autoantibodies does not exclude the diagnosis of T1DM. The presence of autoantibodies in relatives of patients with T1DM or in healthy individuals has a significant positive predictive value for disease development.

PATHOGENESIS

The main specific damage in this disease is the selective destruction of beta cells in the pancreas with the presence of inflammatory infiltrates or insulitis (Figure 1). Several studies have determined that the presence of autoantibodies in children with T1DM from birth suggest the future development of the disease (45). However, the role of autoantibodies in the pathogenesis of T1DM has not been fully elucidated. In fact, they seem to be an epiphenomenon secondary to autoimmune destruction of β cells mediated mainly by cell immunity mechanisms. In support of these findings, one case report showed the development of the pathology in a patient with X-linked agammaglobulinemia which suggests that autoantibodies are not required for the progression of the pathology (46). Generally speaking, the disease is considered a T-cell mediated disease. Histological and immuno-phenotypical evaluation confirms insulitis with an infiltrate composed of CD4 and CD8 T lymphocytes, B lymphocytes, and macrophages thereby suggesting a role for this group of cells in the beta cell destruction (47) (Figure 2). Although the main cells involved in the T1DM pathogenesis are the CD4 and CD8 T cells, another population of T cells, the Th17 cells, has recently been described. Taken together, defective regulatory T cells lose control of Th17 expansion. The abnormalities on Th17 cells are also mediated by the secretion of IL-1b and IL-6 by antigen-presenting cells and macrophages. The pathogenic Th17 cells can cause the imbalance between T effectors and T regulatory cells. The new description of Th17 cells on T1DM is being studied as a potential target to treat the disease.

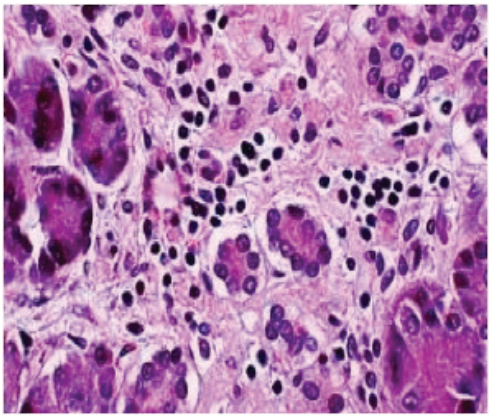

Figure 1. insulitis. inflammatory infiltrates on langerhans' islets of pancreas.

Concerning the T cells as therapeutic targets, previous studies have shown the utility of anti-CD3 antibodies in mice as a therapeutic strategy to prevent the development of the disease. Studies using these drugs in humans are currently being done (48). Figure 3 describes a general model that shows the process of destruction of pancreatic beta cells from inception to clinical presentation. The initial interaction of genes and environmental factors triggers an immune-mediated response with the appearance of different autoantibodies as the first sign of beta cell destruction. This is followed by the loss of first-phase insulin response. The progression to clinical diabetes is triggered by the development of a more aggressive T-cell phenotype, which changes the balance of Th1 and Th2 to produce a more inflammatory background. The expression of Fas–Fas ligand molecules on cytotoxic T cells also promotes the clinical presentation of diabetes. The evaluation of pancreatic islets during insulitis suggests that Fas-mediated apoptosis occurs and provides a possible mechanism of beta cell destruction (49).

More recently, an increasing importance is being given to some subgroups of regulatory T cells which have shown the ability to control the development of pathology in both NOD mice and BioBreeding rats (BB) (50,51). Three main groups of T cells have been described in the pathophysiology: Th2 cells, which appear after administration of soluble beta cell autoantigens; T regulatory cells characterized by the expression of CD4, CD25, and the transcription factor FoxP3; and natural killer cells, which probably appear spontaneously during ontogeny. A large number of T cell clones that react to insulin or to other antigens has been described. There is doubt about whether any given autoantigen is the main target of autoimmune recognition although some studies have provided evidence for a central role for T-cell autoimmunity directed against the insulin (52). The role of several proinflammatory and anti-inflammatory cytokines is being studied as potential therapeutic targets. A large number of cytokines are involved in the differentiation and activation of T cells that contribute to the pathogenesis of T1DM. Thus, the utility of blocking cytokines, e.g., IFN-γ receptor, IL-2, or IL-12, has been demonstrated. Prevention has also been shown in animals with blocking proinflammatory cytokines such as IL-1, IL-6, or TNF-α. The uses of cytokines with regulatory properties such as IL-4, IL-10, and IL-13 have shown utility in delaying the progression of the pathology (50).

In summary, the immune-mediated beta cell destruction begins when macrophages and dendritic cells present beta cell to naïve CD4 T cells through the MHC. Through cytokine signaling, CD4 T cells are activated, which in turn activates CD8 T cells directly responsible for causing beta cell death. Beta-cell destruction results in the release of additional intracellular antigens and allows antigen-presenting cells further

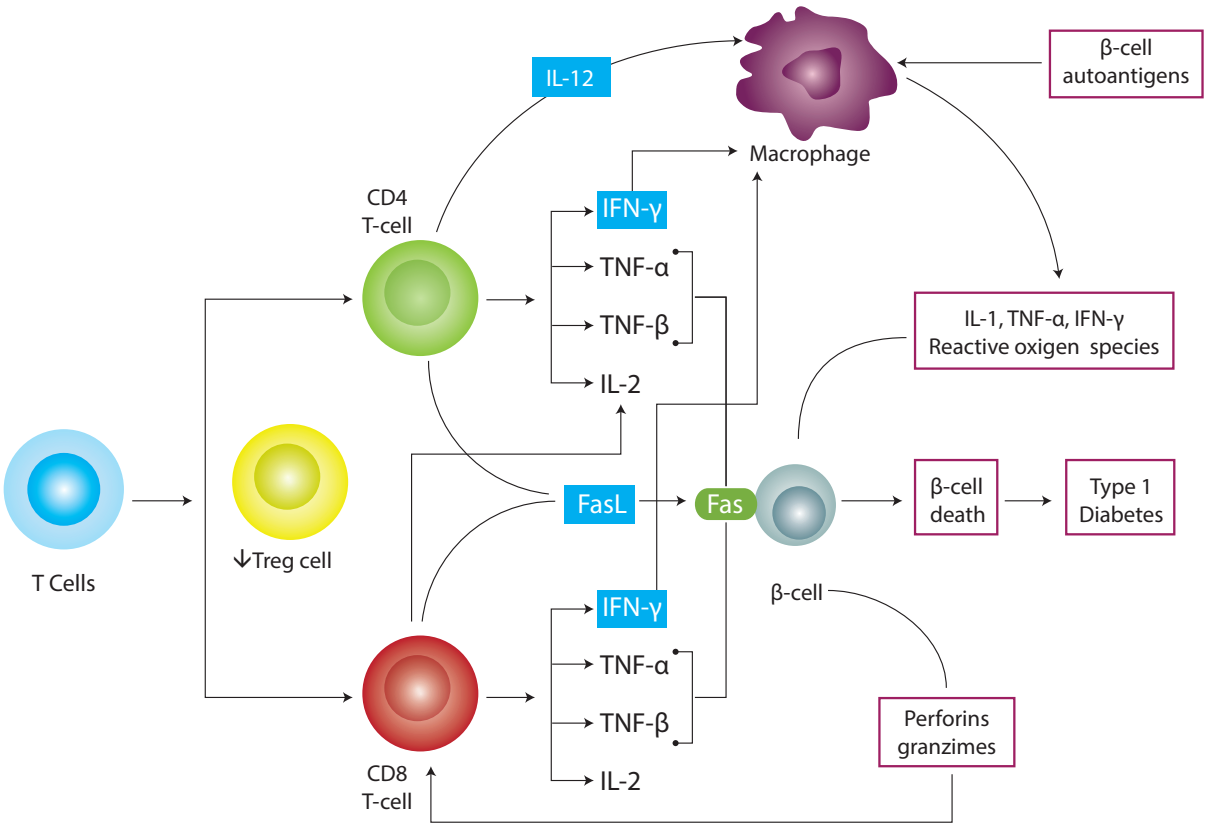

Figure 2. Autoimmune diabetes pathogenesis. Immune-mediated β-cell destruction begins when macrophages and dendritic cells present β-cell to naïve CD4 T cells through the MHC. Through cytokine signaling, CD4 T cells are activated, which in turn activates CD8 T cells directly responsible for causing β-cell death. Beta-cell destruction results in the release of additional intracellular antigens and allows antigen-presenting cells further access to typically sequestered auto-antigens. This activation leads to activation of additional autoreactive T cells through epitope spreading. Recently, another population of T cells, the Th17 cells, has been described. The pathogenic Th17 cells can cause the imbalance between T effectors and T regulatory cells.

access to typically sequestered auto-antigens. This activation leads to activation of additional autoreactive T cells through epitope spreading. In contrast, the presence of autoantibodies does not appear to be directly pathogenic to beta cells although they are useful markers for T1DM risk and prediction as they indicate autoreactive T cell activation.

CLINICAL PRESENTATION

The peak of disease presentation is around puberty. The symptoms and signs are associated with the presence of hyperglycemia and the effects result from an imbalance in fluid and electrolytes. These symptoms usually include polyuria, polydipsia, polyphagia, and weight loss. Because some infectious diseases can precipitate the initial presentation, symptoms of infection like fever, sore throat, cough, or dysuria, etc. may be present. The onset of symptoms may be of short evolution, especially when it manifests as ketoacidosis, although it may be insidious. When the clinical presentation begins with ketoacidosis, other manifestations such as abdominal pain, nausea, and vomiting occur in addition to disturbances in the mental state, from a slight alteration to a deep coma.

LABORATORY FINDINGS

The plasma glucose levels at the time of diagnosis are elevated with variable ranges (generally greater than 300 mg/dl). If clinical presentation is not complicated, fluids and electrolytes parameters may be completely normal. Moreover, if ketoacidosis is present, acidosis, and dehydration are present. In these patients, serum sodium is usually at the lower limit of normal, or is low and reflects the osmotic effect of hyperglycemia. Despite significant loss of potassium in urine and total body potassium deficiency, the presence of acidosis usually leads to a high concentration of serum potassium at the time of presentation. Serum bicarbonate levels are usually low (less than 15 mEq/L.) Ketone body elevation is also presented. In conjunction with the increase in serum glucose, the increases in ureic nitrogen invariably increase the serum osmolality, often to greater than 300 mmol/kg.

PREDICTION OF DISEASE

The prolonged prodromal phase preceding the onset of symptoms suggests it can be predicted and studies could

be designed seek a way to prevent T1DM development. The development of T1DM in relatives of patients can now be predicted with relative certainty by determining the number of autoantibodies against pancreatic islets. Antibodies specific for pancreatic islet cells, insulin, 65 Kd GAD (GAD-65), and IA-2 protein are predictive markers for T1DM. Their positive predictive value (PPV) is 43%, 55%, 42%, and 29% respectively. The risk of first-degree relatives of patients with T1DM developing the disease grows progressively with the duration of follow-up and with the number of positive autoantibodies and is of 2%, 25%, and 70%, with one, two, and three or four positive Abs respectively (53,54). Antibodies against the zinc transporter ZnT8 have recently been described as useful in prediction (55, 56). A study published by Mrena *et al*. (57) shows interesting models for predicting T1DM in siblings of affected children based on autoantibody, genetic, and sociodemographic variables. The study analyzed 701 siblings of affected children. During the 15-year observation period, a total of 47 siblings (6.7%) developed T1DM. Of 47 patients, 38 initially tested positive for at least one diabetes-associated autoantibody. The risk of developing T1ADM was associated with younger age at the first sampling, HLA DR-conferred disease susceptibility, the number of initially detectable diabetes-associated autoantibodies, and the number of affected family members. Based on the Cox regression model, the authors calculated an individual prognostic risk, and a cutoff index of 0.25 separated progressors from non-progressors. Thus, at present, we have an advanced capability to predict T1DM, especially in high risk populations.

Other studies have assessed T1DM-specific antibodies in the general population (58). In this study, 12 of 4,502 children (median age 14 years) had more than one T1DM-associated autoantibody, and six developed the disease over an 8-year follow-up. Thus, the presence of two antibodies was over 99.5% specific and the PPV was 50%. In another study, Kupila *et al*. (59) did a population-based, birth-to-age-four screening study that combined both HLA typing and autoantibodies in 31,526 children in Finland. Using this strategy, they were able to identify 75% of those developing T1DM. However, the costs of this strategy are expensive. In this case, it would probably be better to do the immunological screening only on those subjects with increased risk based on HLA typing. In the DAISY study, which evaluated high-

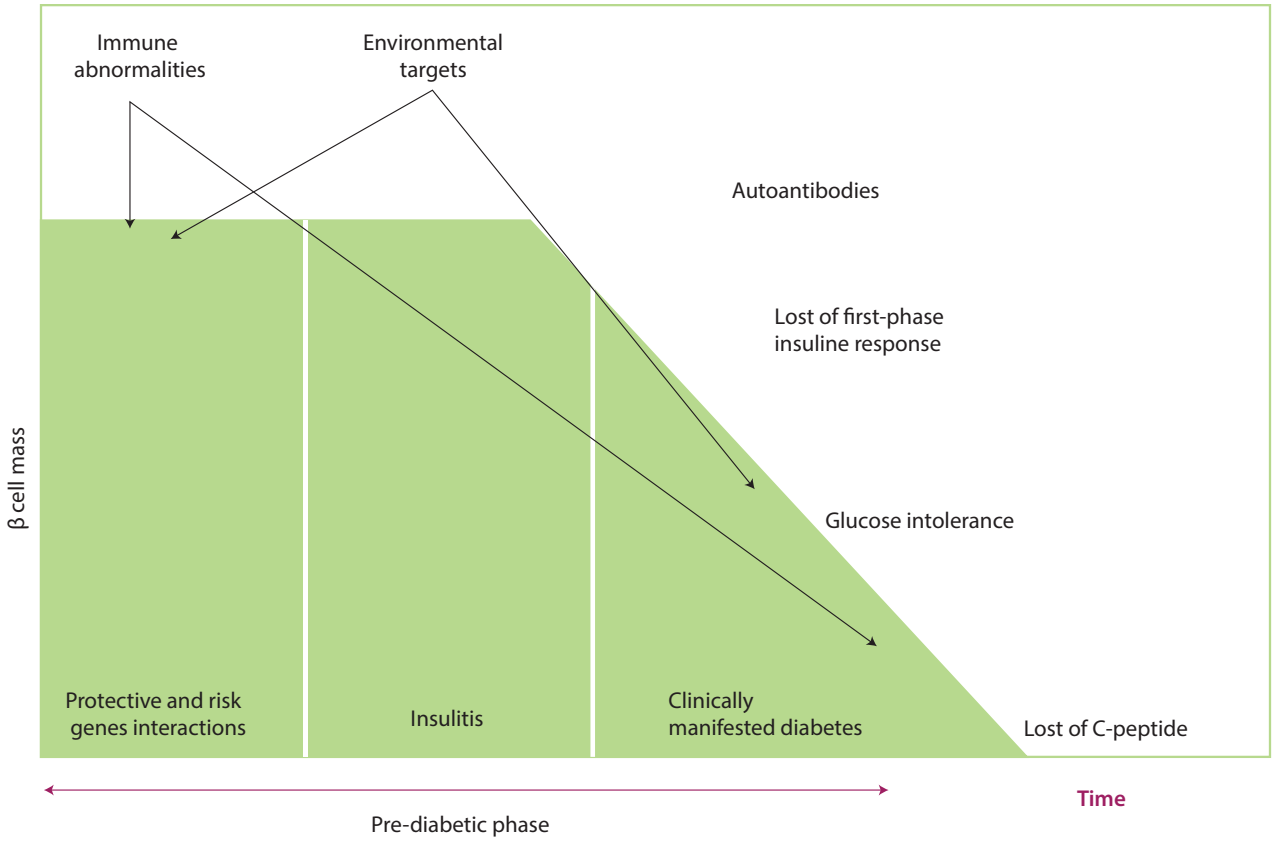

Figure 3. Natural history of Type 1 Diabetes. This graphic model shows the process of destruction of pancreatic β cells from inception to clinical presentation. The initial interaction of genes and environmental factors triggers an immune-mediated response with the appearance of different autoantibodies as the first sign of β-cell destruction. This is eventually followed by the loss of first-phase insulin response. The progression to clinical diabetes is triggered by the development of a more aggressive T cell phenotype which changes the balance of Th1 and Th2.

risk children with positive autoantibodies (without intervention), there was a lower incidence of ketoacidosis at onset of the disease, improved glycosylated hemoglobin levels at baseline, and fewer hospitalizations due to its closer clinical monitoring (60). This study suggests that despite not being able to take preventive measures in high-risk patients, closer clinical follow-up may encourage better clinical outcomes until effective preventive measures become available. The use of autoantibodies has spread to subdivide patients previously classified as type 2 diabetics. The results of the UK Prospective Diabetes (UKPDS) indicate that at least 30% of young patients with "type 2 diabetes" presented an autoimmune process and that, over the course of three years, these patients progressed to insulin-dependance (61). This subgroup of patients has been called latent autoimmune diabetes in adults (LADA). In summary, prediction in T1DM has been widely studied. With the analysis of genetic susceptibility factors in first-degree relatives of patients and several autoantibody tests, one can predict the development of the disease. The main goal after predicting T1DM is to introduce preventive actions to delay or prevent the development of disease (62) (Figure 4).

IMMUNE THERAPY IN TYPE 1 DIABETES

Immune therapy for T1DM is approached at three stages: primary prevention, secondary prevention, and treatment. Primary prevention involves the immunological tolerance to islet tolerance in individuals with increased risk of T1DM. Secondary prevention can be done with nonantigen-specific or antigen-specific approaches in genetically susceptible individuals who had developed islet autoantibodies. Immune therapy treatment at onset of T1DM can involve both nonautoantigen-specific and autoantigen-specific therapies in order to reduce insulin requirements and complications. The most common primary outcome in clinical studies and trials is the preservation of C-peptide levels. Figure 3 shows the points of primary prevention, secondary prevention, and treatment based on the physiopathology model.

PREVENTION TARGETS AND IMMUNE THERAPY INTERVENTIONS

To date, no treatment has been shown to prevent human T1DM. More than 100 different treatments can prevent illness in NOD mice (63). These targets and treatments are summarized in Table 8.

Two major studies have been carried out to study prevention of T1DM. In the United States, the study of the prevention of diabetes (DPT-1) was launched in 1994 in order to determine whether a regimen based on the antigen (i.e., insulin), both oral and parenteral, would prevent or delay the development of diabetes in families with high or moderate risk. These treatments generally did not delay disease development. The European study of intervention with nicotinamide (ENDIT) also found no difference in protection from disease when participants were assigned to either placebo or oral nicotinamide.

Early studies of therapies used to prevent the destruction of pancreatic βeta cells were based on immunosuppressive therapies. Studies with cyclosporine indicated that while this drug was administered there was a prevention of further loss of beta cells and better metabolic function (64-66). However, if this therapy were initiated after the development of clinical diabetes, its benefits were minimal and transient. The inability of therapy to "cure" diabetes and the high toxic effects resulting from this (particularly nephrotoxicity and increased risk of malignancy) has caused its clinical use to be dismissed for this purpose. Other immunosuppressive therapies such as prednisolone or azathioprine have shown relatively little effect (67, 68). Studies using methotrexate have not demonstrated efficacy (69).

To date, although diabetes is mediated by the immune system, it is not treated with immunomodulating agents or current suppressors. Because of this, studies focused on preventing the development of T1DM are necessary and many of them are already in progress. Phase 2 and Phase 3 studies, involving treatment of subjects with new-onset T1DM, were initiated with the modified anti-CD3. They showed preservation of C-peptide response as a measure

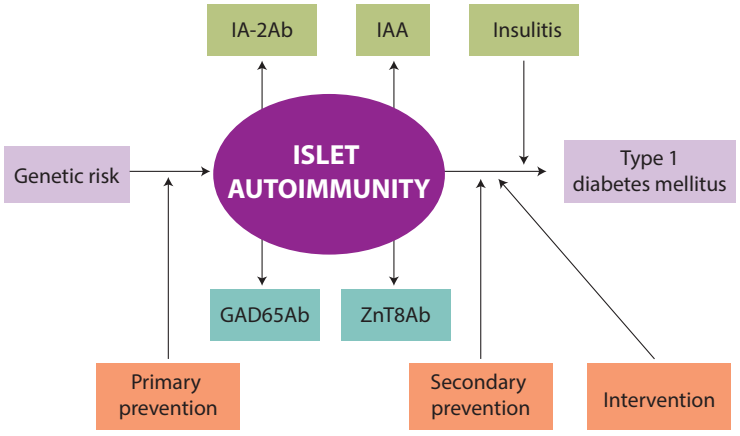

Figure 4. Representation of pathogenesis of T1DM indicating the points for primary prevention, secondary prevention, and treatment.

NAME	FEATURES	PREVENTION	COMMENTS
SPONTANEOUS			
NOD Mice: polygenic	Genetic basis similar to human HLA-dependent. More than 15 different genes increase susceptibility. Unlike human, in this model more females are affected than males. Insulin antibodies are detected before the development of clinical disease.	Over 100 therapies prevent the development of the disease in NOD mice. These include nicotinamide, vaccination with insulin or GAD treatment.	This Is the most studied animal model of the disease
BB Rats (BioBreeding rat) oligogenic	Susceptible rats have an autosomal recessive mutation that leads to a severe lymphopenia. There is also a genetic component to HLA alleles, mainly RT1-U. However, the number of genes involved is lower than in NOD mice.	Prevention is more difficult to achieve than in NOD mice. For example insulin intervention can prevent pathology but requires higher doses that may cause hypoglycemia.	First animal model described.
Rats Long-Evans Tokushima Lean (LETL)	Similar to BB rats. It has the allele RT1-U and oligogenic inheritance.		
INDUCED			
-	Strains of mice with diabetes induced by drugs such as streptozotocin.	-	High dose induces the disease quickly. Low dose induces a chronic installation.
-	Polyinosinic acid copolymers and polycytidylic induce diabetes in strains with mutations RT1-U.	-	This copolymer induces production of IFN-α.

Table 8. Autoimmune diabetes animal models. *GAD*: glutamic acid decarboxilase; *IFN*: interferon; *NOD*: non-obese diabetic mouse; *T1DM*: type 1 diabetes mellitus.

of insulin production, a decrease in exogenous insulin use, and improved glycemic control following a 12- to 14-day of modified anti-CD3 (e.g., teplizumab) infusion in patients diagnosed with T1DM within the previous 6 weeks. However, one Phase 3 trial failed to find the same benefits (70-72). As mentioned previously, at least 100 therapies have been shown be effective in prevention or disease treatment in NOD mice. These therapies are summarized in Table 9 (50)

VACCINATION

In animal models, multiple therapies have been used to prevent the development of diabetes. The immunological vaccination is a striking strategy mainly because of its specificity and low risk compared to the conventional immunosuppression. The basic concept is the induction of lymphocytes that recognize a given antigen of the islets. Once this recognition has occurred, they generate the production of cytokines that suppress autoimmunity and tissue destruction (73, 74). Th2 T cells produce more Interleukin 4 (IL-4) than IFN-γ or IL-2 (produced by Th1 cells) and decrease cell-mediated destruction. Induction of the protective immune response may depend on the route of administration of the given antigen (e.g., oral tolerance) or the use of modified antigens. For example, oral or subcutaneous insulin prevents the development of diabetes in NOD mice (75, 76). However, no intact insulin is required to produce this response (77).

TREATMENT

Insulin remains the mainstay of treatment for patients with T1DM. The Diabetes Control and Complications Trial (DCCT) demonstrated the importance of strict metabolic control in order to delay and prevent the chronic complications of diabetes (78). However, the risk of inducing hypoglycemia during treatment is still a major constraint on achieving adequate metabolic control. The introduction of rapidly absorbed insulin analogues reduced the variability in the absorption and made its application possible even during meals (79). In the last few years, new insulin analogues with a behavior that is more similar to basal insulin secretion without the presence of peaks have been introduced to the market. The available insulin can be divided on a pharmacokinetic basis into three broad categories: rapid-acting, intermediate, and long-acting (80). In Tables 10 and 11 the main goals of insulin therapy, derived from intensive therapy and the presentations of the drug available, are summarized.

An alternative method of delivering insulin is by an external mechanical pump. The pump delivers insulin as a pre-programmed basal infusion in addition to patient-directed boluses given before meals or snacks or in response to elevations in the blood glucose concentration outside the desired range. The insulin pump should be used only by candidates who are strongly motivated to improve glucose control and willing to work with their health care provider in assuming substantial responsibility for their day-to-day care (81).

1 T-cell depletion or Sequestration 1.1 Depletion Anti-CD3 Anti-CD4 Anti-CD8 Anti-CD44 Anti-CD45RA Anti-CD45RB Anti-Thy l.2 Antilymphocyte globulin Neonatal thymectomy 1.2 T cell- Sequestration Anti-CD43 Anti-VLA-l Anti-VLA-4 VLA-4/Ig fusion protein Anti-CD62L	7 Pharmacologically active cytokines 7.1 IL-4 7.2 IL-10 7.3 IL-13 7.4 IL-3 7.5 G-CSF 7.6 Lymphotoxin 7.7 IL-11 7.8 IL-1α 7.9 TNF-α	11 Cell therapy 11.1 Islet or segmental pancreas transplantation (+ immunosuppression) Syngeneic Allogeneic (+ immunosuppression) 11.2 Intrathymic islet transplantation 11.3 Bone marrow transplantation Allogeneic Syngeneic 11.4 Dendritic cells 11.5 Natural killer T cells 11.6 CD4 cell lines Polyclonal Anti-lag7 11.7 Allogeneic cells Macrophages Spleen cells
2 Blockade of T-cell activation 2.1 Chemical immunosuppressants Cyclosporin A FK-506 Azathioprine Rapamycin Deoxyspergualin 2.2 γ Irradiation	8 Tolerance to soluble β-cell autoantigens 8.1 Insulin Oral Oral + IL-10 Intranasal Subcutaneous Native protein B chain Inactive analogue DNA vaccination Gene-transfer delivery (proinsulin gene) Cholera-toxin conjugate 8.2 Glutamic acid decarboxylase (GAD) Oral Intranasal Subcutaneous Intrathymic DNA vaccination Anti-GAD antibody 8.3 Heat shock protein 60 (hsp60) Subcutaneous or intraperitoneal Protein P277 peptide Gene-transfer delivery 8.4 Pancreatic extracts (oral)	12 Inhibition of β-cell lesion 12.1 Nicotinamide 12.2 Antioxidants Vitamin E [146] Probucol analog Probucol + deflazacort Aminoguanidine 12.3 Anti-inflammatory agents Pentoxifylline Rolipram
3 Targeting of T-cell receptors 3.1 TCRαβ antibody 3.2 CD3 antibody 3.3 Vβ8 antibody 3.4 T-cell vaccination Polyclonal activated T cells Glutaraldehyde-treated T cells Activated T cells Vβ8 T cells Anti-hsp60 T-cell clone 3.5 Blocking peptides		13 Miscellaneous 13.1 Immunomodulators Linomide Ling-zhi-8 D-Glucan Multi-functional protein 14 Ciamexon Cholera toxin B Vanadate Vitamin D3 analogue 13.2 Hormones and related proteins Androgens IGF-I 13.3 Immunomanipulation Natural antibodies Lupus idiotype Lipopolysaccharide 13.4 Diet Casein hydrolysate 13.5 Other Sulfatide Bee venom Kampo formulation Silica Ganglioside Antiasialo GM-1 antibody Hyaluronidase Concanavalin A
4. Targeting of MHC molecules 4.1 Anti-class-I 4.2 Anti-class-II 4.3 MHC transgenic mice Class I I-A I-E		

Table 9. Immunotherapeutic agents or other treatments used in NOD mice. CD45RA(B): CD45 receptor A(B); CDXXL: CDXX ligand; CFA: complete Freund's adjuvant; *GAD*: glutamic acid decarboxylase; *G-CSF*: granulocyte-colony-stimulating factor; *ICAM-1*: intercellular adhesion molecule-1; IFN: interferon; *MHC*: major histocompatibility complex; *TCR*: T cell receptor; *V*: variable region of immunoglobulin; *VLA*: very late antigen. Adapted from Bach *et al*. (55).

5 Targeting of co-stimulation and adhesion molecules 5.1 Co-stimulation molecules Anti-CD28 CTLA-4–Ig fusion protein Anti-B7.2 Anti-CD40L 5.2 Adhesion molecules Anti-ICAM-1 Soluble ICAM-1 Recombinant protein Gene therapy Anti-Mac Anti-LFA-I	9 Stimulation of regulatory T cells 9.1 Pathogens Bacteria Mycobacteria Mycobacterium bovis M. avium Complete Freund's adjuvant Lactobacillus casei Streptococcal extract Klebsiella extract Escherichia coli (+ oral insulin) Viruses Mouse hepatitis virus Lactate dehydrogenase virus Lymphocytic choriomeningitis virus Parasites Filariae Schistosomes 9.2 Stimulation of innate immunity α-Galactosylceramide 9.3 Nondepleting anti-T-cell antibodies Anti-CD3 Anti-CD4 Superantigens	
6 Cytokine blockade 6.1 IFN-γ Anti-IFN-γ IFN-γR/IgG1 fusion protein 6.2 IL-2 Anti-IL-2R IL-2R/Ig fusion protein IL-2 diphtheria-toxin protein 6.3 IL-12 Anti-IL-12 IL-12 antagonist (p40)2 6.4 IFN-α (oral) 6.5 IL-1 IL-1 antibody IL-1 antagonist 6.6 IL-6 6.7 Lymphotoxin receptor	10 Gene therapy 10.1 β-cell antigens DNA vaccination GAD immunoglobulin 10.2 IL-4 Retrovirus (T-cell transfection) Biolistic Adenovirus IL-4/IgG1 fusion protein 10.3 IL-10 T-cell transfection Local Systemic 10.4 ICAM-1 10.5 IFN-γR/IgGl fusion protein 10.6 TGF-β 10.7 Calcitonin	

Table 9 continuation. Immunotherapeutic agents or other treatments used in NOD mice. CD45RA(B): CD45 receptor A(B); CDXXL: CDXX ligand; CFA: complete Freund's adjuvant; *GAD*: glutamic acid decarboxylase; *G-CSF*: granulocyte-colony-stimulating factor; *ICAM-1*: intercellular adhesion molecule-1; IFN: interferon; *MHC*: major histocompatibility complex; *TCR*: T cell receptor; *V*: variable region of immunoglobulin; *VLA*: very late antigen. Adapted from Bach *et al*. (55).

The use of metformin added to insulin is increased in patients with T1DM. Some studies have suggested that metformin could be beneficial for T1DM patients who are overweight and are receiving high doses of insulin or have a HbA1c over 8% (82). The coexistence of insulin resistance in patients with T1DM is an area of current interest, which was previously only applicable in type 2 diabetes. Pancreatic islet transplantation combined with appropriate immunosuppressive therapy may eventually cure diabetes. This transplant is considered for the limited but important group of patients with recurrent episodes of severe hypoglycemia unresponsive to proper medical management (83). The inability to control autoimmunity and alloimmunity, added to the lack of donor organs, limits the further application of islet transplantation. Another strategy added to pancreatic islet transplantation is bone marrow transplantation. Both allogeneic and syngeneic transplantation can be useful in the control or prevention of pathology, probably through immunoregulatory cytokine production and production of regulatory mechanisms that outweigh effector mechanisms (84, 85).

Gene therapy can be used many ways to prevent or cure diabetes. Gene therapy can be done based on insulin or other therapeutic strategies. Furthermore, gene therapy has been done on the immune system in several lines of research. A possible approach is the development or overexpression of cytokines or receptors to the pharmacological effects of the endogenous molecule. Among them, there have been experiments with cytokine release in the pancreatic islets or systemically. Several vectors have been used (both viral and non-viral) for this type of research, in which the use of IL-4, the fusion protein IL-4-Ig, IL-10, IFN-γ-recep-

PARAMETER	NORMAL	ADA	ACE
Premeal PG (mg/dl)	<100 (mean ~90)	70-130	<110
Postprandial PG (mg/dl)	<140	<180	<140
HbA1c (%)	4-6	<7	≤6.5

Table 10. Glycemic targets. *ACE*: American College of Endocrinology; *ADA*: American Diabetes Association; *HbA1c*: glycosylated hemoglobin; *PG*: plasma glucose concentration. Adapted from Eisenbarth *et al*. (88).

PREPARATION	ONSET (HR)	PEAK (HR)	DURATION (HR)
RAPID-ACTING			
Regular	0.5-1	2-4	6-8
Lispro	0.25	1	3-4
Aspart	0.25	1	3-4
Glulisine	0.25	1	3-4
INTERMEDIATE-ACTING			
NPH	1-3	6-8	12-16
LONG-ACTING			
Glargine	1	NA	11-24
Detemir	1	3-9	6-23

Table 11. Pharmacokinetic properties of insulin preparations. *NPH*: neutral protamine Hagedorn. Adapted from Eisenbarth *et al*. (88).

tor, and TGF-β protected mice from developing the disease (86). Numerous clinical trials using antigen-specific strategies and immune-modifying drugs have been published although most have proven to be toxic or have failed to provide long-term -cell protection. Strategies under consideration include infusion of several types of stem cells, dendritic cells, and regulatory T cells either manipulated genetically ex-vivo or non-manipulated. Their use in combination approaches is another therapeutic alternative. Cell-based treatments directed to block the uncontrollable autoimmune response may become a clinical reality in the future for the treatment of patients with T1DM (87).

ABBREVIATIONS

- **AD:** autoimmune diseases
- **AITD:** autoimmune thyroid disease
- **CTLA-4:** cytotoxic T lymphocyte antigen-4
- **FA:** familial aggregation
- **GADA:** glutamic acid decarboxylase antibodies
- **HLA:** human leukocyte antigen
- **IA-2 Ab:** islet antigen-2 antibodies
- **IFN:** interferon
- **IL:** interleukin
- **LADA:** latent autoimmune diabetes in adults
- **MCH:** major histocompatibility complex
- **NOD:** non-obese diabetic mice
- **PPV:** positive predictive value
- **PTPN22:** protein tyrosine phosphatase, nonreceptor 22
- **SNP:** single-nucleotide polymorphism
- **T1DM:** type 1 diabetes mellitus
- **TGFβ:** transforming growth factor β

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30 THYROID DISEASE AND AUTOIMMUNE DISEASES

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INTRODUCTION

Autoimmune thyroid diseases (AITD) are the most prevalent organ-specific autoimmune diseases (ADs) and affect 2 - 5% of the population (1) with great variability between genders (i.e., women 5-15% and men 1-5%) (2). AITD include Graves' Disease (GD) and Hashimoto Thyroiditis (HT), among others. HT and GD are the major causes of hypothyroidism and hyperthyroidism, respectively (3). They reflect the loss of immunological tolerance and share the presence of cell and humoral immune response against antigens from the thyroid gland with reactive infiltration of T cells and B cells, autoantibody generation and, subsequently, the development of clinical manifestations (4, 5).

The lymphocytic infiltration causes tissue damage and alters the function of the thyroid gland. The injury is caused when the autoantibodies and/or sensitized T cells react with the thyroid cells causing the inflammatory reaction and, in some cases, cell lysis (6). Generally, while T lymphocytes are the main cell type infiltrating the gland in HT, a B cell response predominates and determines the presence of GD (7).

As with other ADs, there is a multifactorial etiology with a complex interaction of environmental factors in genetically susceptible individuals (8) (Figure 1). Some of these genes are specific for GD and HT while others are mutual for both diseases, which indicates a genetic predisposition shared in these processes together. Candidate genes include immunoregulators [e.g., human leukocyte antigen (HLA), cytotoxic T lymphocyte antigen-4 (CTLA-4)] and others specific to the thyroid (e.g., TSH receptors, thyroglobulin, etc.). The main environmental factors are smoking, stress, and iodine consumption (6).

AITD is the most hierarchical AD coexisting in the same individual [e.g., polyautoimmunity and multiple autoimmune syndromes (MAS)] (5,9). In fact, rheumatologists may be consulted for clinical manifestations of AITD that mimic several symptoms of other ADs (3).

GENETICS

Genetics play a key role in the pathogenesis of AITD. In fact, a number of immune-related genes have been implicated in genetic susceptibility to AITD. For instance, it is calculated that up to 80% of the susceptibility for the development of GD is secondary to the presence of determined genes (10). These genes include both immunological-synapse genes [e.g., *HLA-DR, CTLA-4, CD40*, and the protein tyrosine phosphatase, nonreceptor type 22 (*PTPN22*) gene] and regulatory T–cell genes (e.g., *FOXP3, CD25*) (3).

The association between HLA alleles and AITD is well known, but the primary etiological variant in this region is still uncertain (1). Thus, the pathological findings for both GD and HT are similar, and are associated with particular *HLA-B* and *HLA-DR*, suggesting that inherited risk factors are important in the development of these entities (11,12). *HLA-DR* locus plays an important role since *HLA-DR3* is present in up to 55% of the patients with GD compared to the 30% prevalence in the general population (4). In fact, it has been discovered that *HLA-DR3* and *HLA-DR5* are linked to HT and provide a greater risk for the disease (4). Moreover, Ban *et al*. (11) identified arginine at position 74 of the *HLA-DRβ1* (DRβ-Arg74) as the critical DR amino acid (a.a) conferring susceptibility to GD, and glutamine at position 74 as a protective a.a. Regarding HT, Menconi *et al*. (13,14) reported similar findings for this disease. They were able to identify the thyroglobulin peptides that could be presented by *HLA-DR* pockets containing arginine at position beta 74 to T cells and, therefore, initiate the autoimmune process (14).

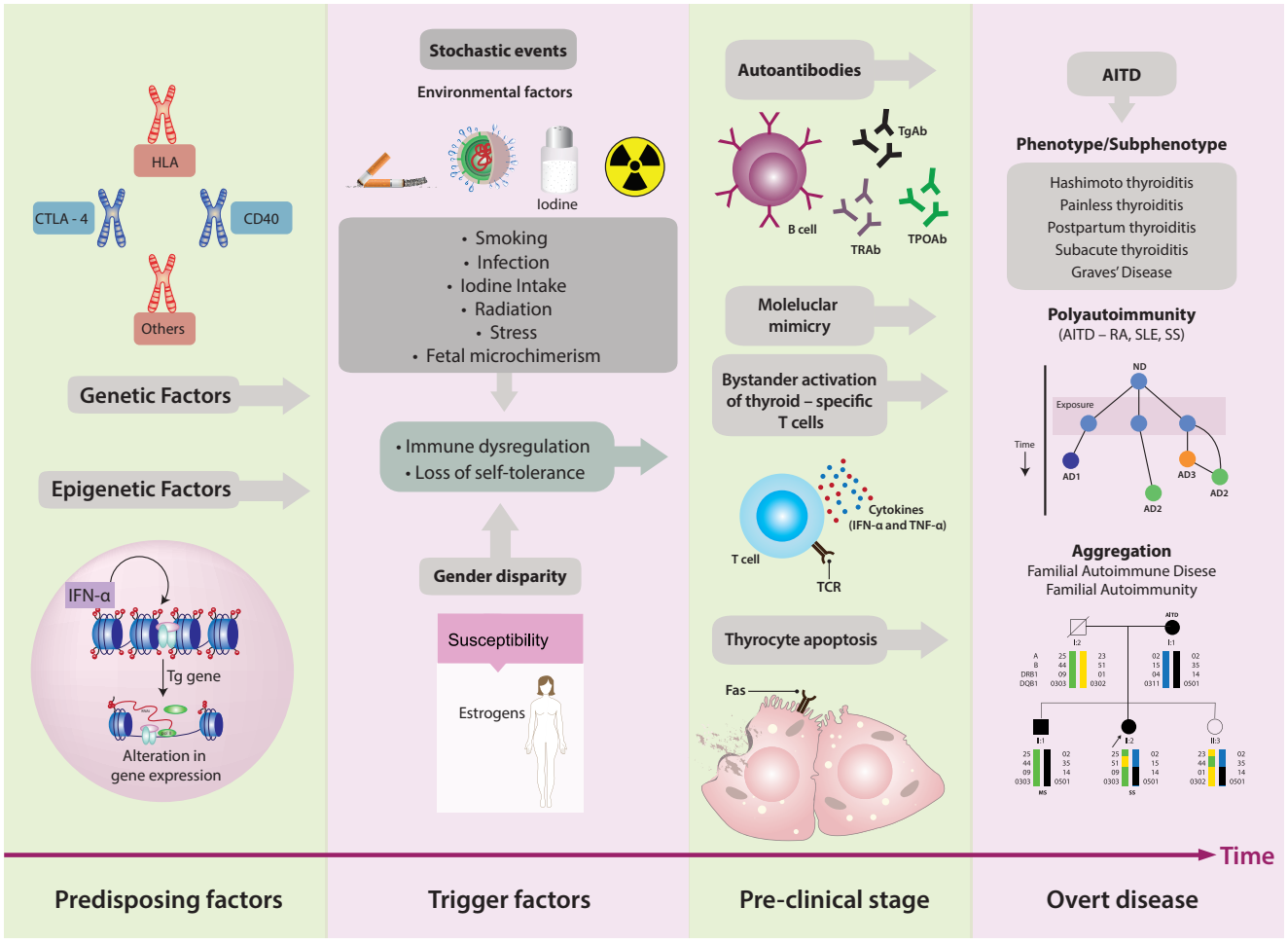

Figure 1. Risk factors for AITD: multifactorial disease. The etiology of AITD is multifactorial, in which a mosaic of predisposing and stochastic factors play in concert for the induction of loss tolerance and subsequent organ damage. The pre-clinical stage is characterized by the presence of autoantibodies in serum before the clinical onset (e.g., TPOAb), and pro-inflammatory markers. The clinical stage has a broad spectrum of subphenotypes (e.g., Hashimoto thyroiditis, Painless, Postpartum and Subacute Thyroiditis, and Graves´ Disease). *AITD*: autoimmune thyroid disease; *CTLA-4*: cytotoxic T lymphocyte antigen-4; *HLA*: human leukocyte antigen; *IFN*: interferon; *RA*: rheumatoid arthritis; *SLE*: systemic lupus erythematosus; *SS*: Sjögren syndrome; *Tg*: thyroglobulin; *TgAb*: thyroglobulin antibodies; *TNF*: tumor necrosis factor; *TPOAb*: thyroperoxidase antibodies; *TRAb*: TSH receptor antibodies. Adapted from Anaya *et al*. (8).

There are, in turn, studies that suggest alleles from *HLA-DQ* might be genetic markers that confer resistance to the development of AITD (15). Likewise, atrophic and subacute thyroiditis are strongly associated with *HLA-B35* in many ethnic groups while painless thyroiditis is associated with *HLA-DR3* (11,12,16).

There are associations with a number of immune-related genes, other than HLA genes, which have also been found in many other ADs and presumably underpin the inherited susceptibility to autoimmunity. Polymorphism in certain alleles of *CTLA-4* predispose to GD and HT (17–21). For instance, in a study of 379 patients with GD in the United Kingdom, 42% had a particular allele (G allele) of the *CTLA-4* gene compared to 32% of the controls (20). This genetic abnormality also stimulates higher levels of thyroid-specific autoantibodies and clinical thyroid disease when interacting with other loci (4,20–22). Currently, both *HLA-DR3* and *CTLA-4* are considered to be the main genes associated with the development of AITD.

CD40 is expressed on thyroid follicular cells and on B cells. Polymorphisms of this gene are associated with a 20-30% increase in translation of *CD40* mRNA transcripts in patients with AITDs (23).

Polymorphisms of the *PTPN22* gene, which encodes a negative regulator of T cell activation, have been associated with AITD and other ADs. A gain of function trait has been identified, but its role in autoimmunity is uncertain (24,25). Finally, abnormalities in the *FOXP3* gene (differentiation of T cells into natural Treg cells) have been associated with juvenile GD (26). Changes in the *CD25* gene region have also been associated with GD (27).

EPIGENETICS

Although the link between susceptibility genes and environmental triggers is clear, the mechanisms by which gene variants interact with environmental factors to cause autoimmunity are still unclear. Recent data suggest that epigenetic mechanisms might underlie genetics. Their effects on gene expression have been broadened to include non-DNA-sequence-encoded effects on gene expression that are mitotically stable (28). Epigenetic modulation of gene expression can occur through alterations in DNA methylation, histone modification patterns (e.g., acetylation, deacetylation, and methylation), and RNA interference through microRNAs. Recently, interferon-a (IFN-α) was shown to induce alterations in thyroglobulin (Tg) gene expression through epigenetic changes in histone modifications (29). Since IFN-α is secreted locally during viral infections, this could be an attractive mechanism by which infections can trigger AITD (30). Another epigenetic phenomenon described in the pathogenesis of AITD is the X chromosome inactivation. The degree of X chromosome inactivation is an important contributor to the increased risk females have of developing AITD, as demonstrated by Yin *et al*. (31).

ENVIRONMENTAL FACTORS

There are a number of environmental factors associated with the occurrence of AITD such as low birth weight, iodine excess, selenium deficiency, use of anovulatories, parity, stress, smoking, allergy, radiation exposure, viral or bacterial infections, and fetal microchimerism (32).

SMOKING

This habit, as well as cessation, are the main risk factor for AITD (e.g., GD) (33). The increased risk in the onset of AITD with cessation of smoking may be useful in monitoring susceptible patients who stop smoking for the myriad health benefits. For example, cessation of smoking may be associated with weight gain, and hypothyroidism should be considered a possible cause. Cigarette smoke contains cyanide, which is metabolized to thiocyanate and can interfere with iodine concentration in the thyroid and in the lactating breast (34).

INFECTION

There is evidence suggesting the involvement of infection in the development of this pathology. However, not only pathogenic but also non-pathogenic microorganisms induce pro-inflammatory or regulatory immune responses within the host (e.g., commensal microbiota) (35). Data regarding the role of B. *burgdorferi* and Y. *enterocolitica* as triggers of thyroid autoimmunity remain inconclusive. Some reports have suggested molecular mimicry that may explain an association between both of these pathogens and AITD. Additionally, significant homologies had been found for 16 *Borrelia spp*. proteins and 19 *Yersinia spp*. proteins. The number of motif copies was found to be greater in the regions of homology of thyroid autoantigens with *Yersinia spp*. than those with *Borrelia spp*., with the most common being *HLA-DR3, DR-4*, and *DR-7* (28,36). Other microorganisms implicated in the pathogenesis of AITD include *H.pylori*, Coxsackie virus, Hepatitis C virus, and retroviruses (28).

IODINE

Although it is essential for normal thyroid function, iodine is one of the most important precipitants of thyroid dysfunction. Thus, while mild iodine deficiency is associated with a lower prevalence of HT, excessive intake is associated with a higher prevalence. Potential mechanisms by which iodine can induce autoimmunity include direct stimulation of immune responses to the thyroid, increased immunogenicity of highly iodinated Tg, and direct toxic effects of iodine on thyrocytes via the generation of reactive oxygen species. However, thyroid autoimmunity associated with iodine might be a transient phenomenom (37-39). Kahaly *et al*. (37) followed a group of patients with endemic goiter that received iodine for 6 months and another group that received T4. High titers of thyroid autoantibodies were found in 19% of the patients receiving iodine. After iodine was withdrawn, antibodies levels decreased significantly, and after a 4-year follow-up, these levels had normalized in four out of the six patients (66%).

RADIATION EXPOSURE

Radiation is perhaps the best characterized environmental exposure linked to effects on the thyroid. The most common thyroid manifestation of radiation is hypofunction due to direct destruction of the gland, but stimulation of thyroid autoantibodies may be another mechanism for both hypothyroidism and hyperthyroidism (28). AITD has been linked to therapeutic medical radiation (40) as well as environmental radiation exposure (41). In a study in which 160 people were occupationally exposed to ionizing radiation, 10% of the subjects met criteria for AITD compared to 3.4% of those without exposure. Subjects with more than five years of exposure were considered to be at higher risk. It will be important in future studies to determine if there is a dose-response relationship to radiation exposure (42).

ENVIRONMENTAL TOXINS

Many environmental pollutants have been shown to be toxic to thyroid cells and promote the onset of AITD (43). For instance, a high prevalence of hypothyroidism was observed in individuals exposed to polybrominated biphenyls with an associated elevation in thyroperoxidase antibodies (TPOAb) and Tg antibodies (TgAb). Bisphenol A, commonly used to manufacture plastic products, may bind to the TSH receptor (TSHR) and act as an antagonist to thriiodothyroinine (T3) thus, inhibiting its transcriptional activity (39,43,44).

MEDICATIONS

Several medications may play a role in the development of AITD. IFN-α, IL-2, lithium, amiodarone, and highly active antiretroviral therapy are the agents most commonly associated with thyroid dysfunction (28,39). For most of these medications, patients at greatest risk of developing AITD are those with previous thyroid autoantibody positivity (28,39,43). Some medications, such as lithium, may not trigger autoimmunity but accelerate the autoimmune process by interfering with thyroid hormone synthesis. Thyroid function testing and measurement of TPOAb should be considered before beginning these medications on patients (32).

STRESS

Various types of stress had been linked to GD. The postulated mechanisms include induction of immune suppression by non-antigen-specific mechanisms, perhaps due to the effects of cortisol or corticotropin-releasing hormone on immune cells followed by immune hyperactivity leading to AITD. A mechanism like that might be operative in postpartum thyroiditis. However, there is currently no evidence linking stress to AITD probably because the triggering event occurred years before thyroid gland damage (28,39,43).

SEX STEROIDS AND PREGNANCY

HT is most common in women than in men, suggesting a role for sex steroids. However, older women may be more likely to have HT than younger women, implying that the presence or absence of estrogen may not be the important factor (45). Another possible explanation for female predominance is skewed X-chromosome inactivation that was found in 34% of female twins with AITD and only 11% of controls (31).

During pregnancy, the transition from immune suppression to release from suppression is associated with the onset of a number of ADs (32). Additionally, there is a marked increase in $CD4^+CD25^+$ regulatory T cells which leads to diminished function of activated T cells and B cells, causing the rebound from this immunosuppression. Pregnancy immunosuppression is associated with a switch to Th2 cells and a shift in cytokine profiles. Likewise, progesterone produced by the placenta affects cytokine profiles across the whole maternal immune system (46).

FETAL MICROCHIMERISM

Fetal cells have been identified within maternal thyroid glands in patients with AITD. Such cells may initiate graft versus host reactions with the thyroid gland and play a significant role in the development of HT (47,48). To date, however, this remains hypothetical.

AUTOANTIBODIES

The TPOAb and TgAb are the most common thyroid autoantibodies present in patients with AITD and are associated with complement - mediated cytotoxicity against thyrocytes (49,50). In fact, the absence of these autoantibodies could exclude a diagnosis of AITD, which means that these tests have high negative predictive value (51). The TSHR antibody (TRAb) is identified in patients with GD (52). There are other less common autoantibodies such as those against the sodium/iodine symporter (NIS) and pendrin, but their clinical utility is limited (53,54) (Table 1 and Figure 2).

These autoantibodies may be present in individuals without clinical or laboratory evidence of thyroid dysfunction (55,56). Presence of antibodies in the absence of disease has been related to a greater risk of developing an AITD

THYROID ANTIBODY	DETECTION METHOD	HT (%)	GD (%)	GENERAL POPULATION (%)
TPOAb	Radioimmunoassay (most sensitive) ELISA Hemagglutination	>90	40 - 70	20
TgAb	ELISA (most sensitive) Hemmagglutination	50-90	20 - 40	10
TRAb	Immunoassays (detects presence and titer) Bioassays (determine activity)			
Stimulating		NA	90	10
Blocking		10	NA	NA
NIS	NA	20	11	NA
Pendrin	NA	97	74	NA

Table 1. Antibodies described in AITD, frequency of presentation and detection method. *ELISA*: enzyme-linked immunosorbent assay; *GD*: Graves' disease; *HT*: Hashimoto thyroiditis; *NA*: not available; *NIS:* sodium/iodine symporter; *TgAb:* thyroglobulin antibodies; *TPOAb*: thyroperoxidase antibodies; *TRAb*: TSH receptor antibodies.

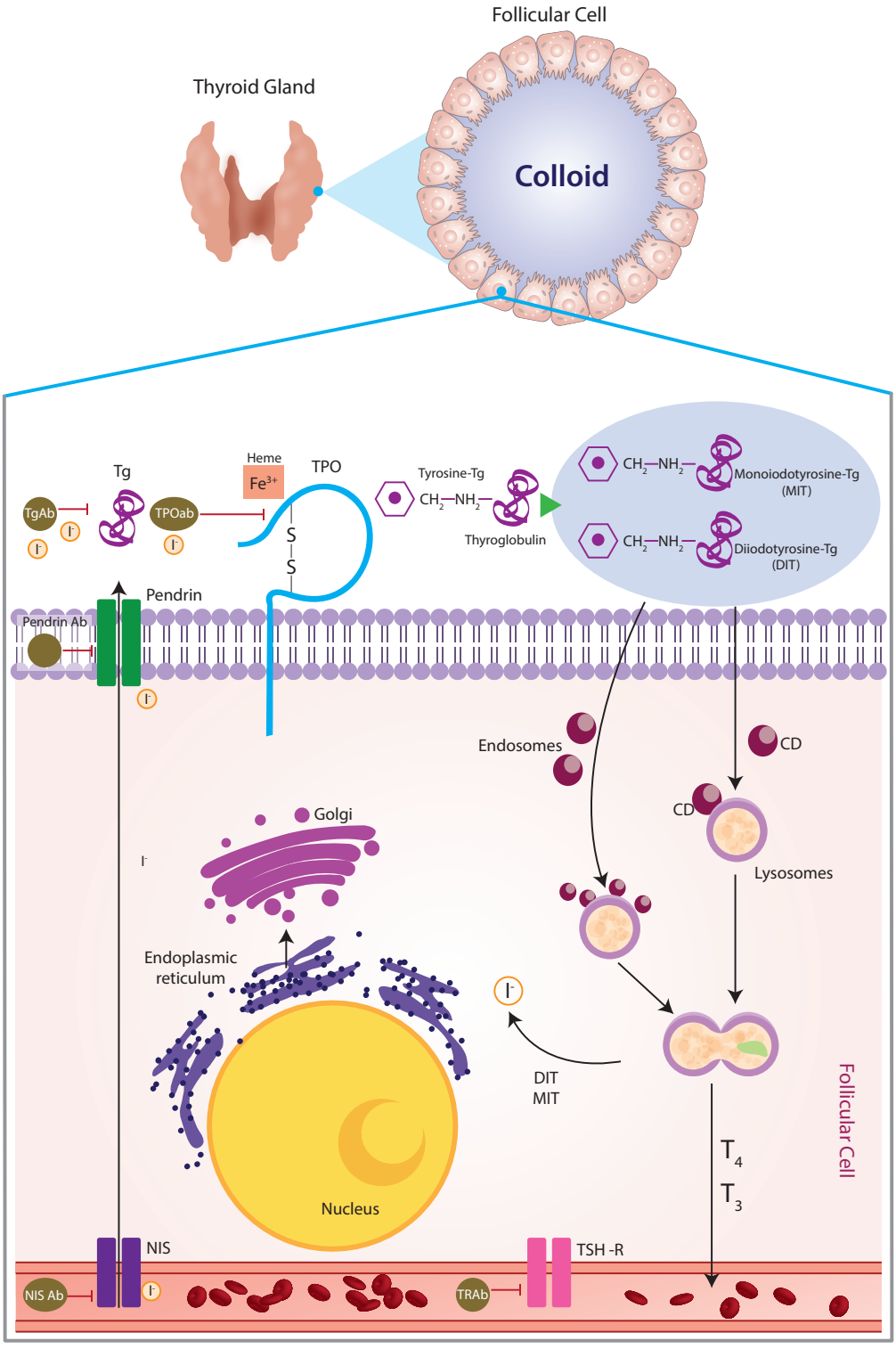

Figure 2. Molecular target for thyroid autoantibodies. (1) Iodine is translocated to the thyrocyte lumen through the NIS located at the basal cell membrane and travel down its electrochemical gradient to the apical surface. (2) Synthesis of Tg in the endoplasmic reticulum and posttranslational modification in Golgi; travels in small vesicles to the apical surface of the thyrocyte. (3) Pendrin protein transports iodine from the apical membrane into the follicular lumen. (4) Thyroid peroxidase catalyzes the organification of inorganic iodine and the coupling of iodinated tyrosines to generate T3 and T4. (5) Tg, retrieved from the colloid by macro or micropinocitosis enters the thyrocyte by means of the endosome – lysosomal pathway. (6) Thyroid hormones leave the lysosomes and T3 and T4 are released into the bloodstream. *CD*: colloid droplet; *DIT*: diiodotyrosine; *MIT*: monoiodotyrosine; *NIS*: sodium/iodine symporter; T3: triiodothyrosine; *T4*: thyroxine; *Tg*: thyroglobulin; *TgAb*: thyroglobulin antibodies; *TPO*: thyroperoxidase; *TPOAb*: thyroperoxidase antibodies; *TSHR*: TSH receptor; *TRAb*: TSHR antibodies. Image modified from Goldman's Cecil Medicine, 24th edition, 2011 Elsevier.

in the future, especially postpartum thyroiditis, as seen in women with recurrent abortion (57). These antibodies has also been linked to an increased probability of thyroid dysfunction secondary to the use of medication that can potentially alter the thyrocyte function (58). The aging process is, likewise, associated with a greater presence of these antibodies. Moreover, these antibodies have been detected in up to 50% of women with a first degree relative with AITD and in 30% of men in the same situation and thus, suggests a dominant inheritance pattern (19).

Even though the recognition of these antibodies does not generate a direct intervention against them, they allow us to predict a clinical outcome or therapeutic response. It is known that the complete ablation of the thyroid tissue results in complete disappearance of these antibodies, in accordance with the theory that the production of these antibodies is triggered by the presence of autoantigens (59). Another clinical utility of these antibodies is related to thyroid ophthalmopathy. Patients that present with this clinical manifestation have higher titers of TRAb and lower levels of TPOAb (60).

THYROGLOBULIN ANTIBODIES

The Tg is the main component of thyroid follicular colloid (51) (Figure 2). It is a large glycoprotein dimer, synthesized by follicular cells, and secreted into the lumen (i.e., colloid) (61). Tg plays an essential role in the storage of iodine and synthesis of the thyroid hormones (51). Normally, no more than 25% of the Tg is iodinated (51). Along with posttranslational modifications (e.g., glycosylation), the extent of the iodination is the most important determinant of Tg immunogenicity. Highly iodinated Tg has been found to be more antigenic (51,62,63).

TgAb are primarily IgG with the main subclasses being IgG2, found in HT, and IgG4, which predominate in GD (64). TgAb is mainly used, together with TPOAb, for the diagnosis of HT. They are found in 50-90% of the patients with HT and less frequently in GD (i.e., 20-40%) (65). TgAb are also present in differentiated thyroid cancer, other ADs, and in up to 20% of the euthyroid population (51,66) (Table 1). Another clinical use of TgAb is in the follow - up of patients with differentiated thyroid cancer post-thyroidectomy and radioactive iodine ablation (51,67).

THYROID PEROXIDASE ANTIBODIES

The TPO is a heme containing oxidoreductase and a membrane-spanning glycoprotein located on the apical surface of thyrocytes (51,68). Its function is to catalyze the iodination of tyrosine residues of Tg to form monoiodotyrosine and diiodotyrosine (Figure 2) (51). The thyroid microsomal antibody was one of the first TPOAb identified (69).

There is circumstantial evidence that TPOAb are responsible for thyroid failure either by inhibiting the TPO or by causing direct damage to the thyrocytes (51). Most of TPOAb are in the IgG1 subclass, which is a complement activator. Moreover, TPOAb can bind through their Fc region to Natural killer cells that, in turn, cause cytotoxic damage to thyrocytes (51,70).

TPOAb are considered diagnostic of AITD (3). They are present in the majority of both HT (i.e., >90%) and GD (i.e., 40-70%) (3). The annual risk for progression to overt hypothyroidism in women with TPOAb is 2.1%, and this is correlated with the titer of the antibody, whether it is weakly, moderately, or strongly positive (23%, 33%, and 53%, respectively) (51). TPOAb also increases the risk of postpartum thyroiditis (discussed below) as the presence of these antibodies during the first trimester is associated with a probability of 30-52% for developing the disease (71). Like TgAb, TPOAb antibodies are found in other ADs and in the general population (51,55,72).

THYROID - STIMULATING HORMONE RECEPTOR ANTIBODIES

The TSHR is a G-protein coupled receptor (73,74) and the main regulator of the thyroid gland. Signaling through the TSHR via the stimulation of cytosolic second messengers promotes the synthesis of thyroid hormones and the growth of the thyroid follicular cell (51).

The natural ligand of the TSHR is the TSH which binds to multiple sites in the extracellular domain (51). However, there are other hormones with the ability to bind to the receptor (e.g., luteinizing hormone and human chorionic gonadotropin) (51). This receptor is expressed predominantly on the surface of thyroid cells. Nevertheless, TSHR mRNA is also found in other tissues, e.g., adipocytes, cardiac muscle cells, pituitary cells, bone cells, and fibroblasts, though its exact function on these cells is not fully understood (75).

TRAb are usually part of the IgG1 subclass (51). The binding site of the TRAb is the region of the receptor to which TSH binds, which consists of a binding pocket-encompassing leucine-rich repeat region (76). They can be functionally classified into three categories: stimulating, blocking, and neutral.

Stimulating autoantibodies. These antibodies were first identified by their prolonged thyroid – stimulating activity when serum from GD patients was transferred into animals. Initially, this finding was called long – acting thyroid stimulators (77). Stimulating antibodies are those that bind to the TSHR and induce conformational changes that activate cytosolic second messengers and promote the synthesis of thyroid hormones and thyroid growth. By mimicking the action of the TSH, these autoantibodies compete with the hormone for the binding site on the receptor. Stimulating antibodies constitutes the hallmark of GD pathophysiology (3,51).

Blocking autoantibodies. These are antibodies that bind to the TSH receptor but do not induce a conformational

change nor promote hormone synthesis. Furthermore, they impede the binding of the TSH to its receptor (3,51). As a consequence, they decrease thyroid function. Blocking antibodies are usually found in patients with HT and in some with GD (51).

Neutral autoantibodies. These antibodies bind to the TSHR but neither induce a conformational change nor block its function. Their pathophysiological or clinical relevance remains unclear (51).

TRAb are useful for the diagnosis of GD as they are present in >95% of the patients (3,51,78). These antibodies are specific but not very sensitive, so a negative result is not conclusive. This sensitivity is due to the fact that TRAb are present in the serum at very low concentrations (51).

Other Antibodies. Antibodies against specific non – thyroid antigens, e.g., antinuclear antibodies (ANA), have been described in patients with AITD. The prevalence of ANA varies from 9-35% and is much higher when TPOAb and TgAb are present (75% and 69%, respectively) (56). Anti-double stranded DNA antibodies are also found in GD and HT in patients without clinical evidence of systemic lupus erythematosus (SLE) (79).

AUTOIMMUNE THYROID DISEASES

The clinical entities found in AITDs are diverse and vary depending on whether a state of hypothyroidism (HT), hyperthyroidism (GD), or both [Painless thyroiditis (PT), Postpartum thyroiditis (PPT), and Subacute thyroiditis (SAT)] predominate in the patient (7) (Table 2).

The term thyroiditis encompasses a varied group of disorders characterized by some form of thyroid inflammation. They include conditions that cause acute illness with severe thyroid pain and others in which there is no clinically evident inflammation, and the illness is manifested primarily by thyroid dysfunction or goiter. PT, PPT, HT, and GD all have an autoimmune basis (6).

HASHIMOTO THYROIDITIS

HT (chronic autoimmune thyroiditis or chronic lymphocytic thyroiditis) is the prototypic example of organ-specific AD and the most common cause of hypothyroidism in iodine sufficient areas of the world (3,80,81). It was first defined in 1912 by surgeon Hakaru Hashimoto who described four women with a condition he initially denominated *struma lymphomatosa* (82).

Epidemiology. Its prevalence is about 1 in 1,000 people and increases with age, affecting up to 40% of elderly women (80,81). Thyroid failure is seen in up to 10% of the population. The incidence of HT is higher in countries where there is excess iodine in the diet, approximately 1.3%, compared to 1% in countries that are iodine sufficient (83). It affects more women than men with a female to male ratio of 18:1 (82). The sibling recurrence risk is >20 (21). There is a peak frequency during the fourth decade, and the mean age of presentation is 35 years (82). The disease clusters in families, sometimes alone and sometimes in combination with GD (see below) (84). Although thyroid lymphoma is a rather rare condition, there is an increased risk for this disease in patients with HT with a RR of 67 (85).

Pathogenesis. HT is characterized by gradual loss of thyroid function, goiter, or both due to autoimmune–mediated destruction of the thyroid gland through apoptosis of the thyrocytes (82). Several mechanisms have been suggested to explain the pathogenesis of HT. These include first, molecular mimicry. Here HT is thought to be caused by an immune reaction against an antigen that shares structural similarities with endogenous proteins (86). Second, there is bystander activation. In this case, a virus reaching the gland or activation of non–specific lymphocytes within the thyroid by virus may cause the release of cytokines which, in turn, activate local thyroid–specific T cells and favor an inflammatory reaction (i.e., thyroiditis) (87). Third is thyroid cell expression of HLA antigens. As previously discussed, thyroid follicular cells in patients with HT express HLA-II. The expression of these molecules can be induced by IFN-γ and other products from activated T cells or by virus directly. Thyroid cells expressing HLA-II, in turn, become non-professional antigen presenting cells (88,89). Fourth is thyroid cell apoptosis. In autoimmune thyroiditis, cytokine (e.g., IL–1) production from antigen presenting cells (APC) and Th1 cells induces the expression of Fas and Fas ligand on thyrocytes, thereby causing self–apoptosis (90).

Clinical Manifestations. The two major clinical forms of HT are goitrous autoimmune thyroiditis and atrophic autoimmune thyroiditis (82). The decrease in circulating thyroid hormones causes a negative effect on the metabolic rate and on multiple organ systems (91). The deposit of glycosaminoglycans secondary to an increase in the synthesis of hyaluronic acid and the decrease in the metabolic rate explains most of the clinical manifestations of patients with hypothyroidism (91).

Clinically, the signs and symptoms of hypothyroidism that characterize HT are shown in the Figure 3. These symptoms are usually insidious and are unrecognized by the patient for a prolonged time (82). The most typical clinical manifestations include:

- Skin: findings on the skin depend on the degree of the hypothyroidism and the ethnicity of the patient (91). Usually there is xeroderma, thickening of the skin, cold intolerance, livedo reticularis, and loss of lateral eyebrows (Queen Anne sign) (82,91). The face is swollen, and the tongue is thickened. Patients complain of hair loss and the nails are frail (82).

AITD	EPIDEMIOLOGY	HISTOPATHOLOGIC FEATURES	CLINICAL MANIFESTATIONS	LABORATORY FINDINGS	US	RaIU	TREATMENT
HT	Most frequent cause of hypothyroidism Prevalence 1/1000 people Peak frequency fourth decade Female to male ratio 18:1	Diffuse lymphocytic infiltrate with lymphoid germinal centers and Hürthle cells	Hypothyroidism	High TSH Low fT4	Enlarged or shrunken gland. Diffusely hipoechogenic If nodule FNAB.	Low uptake	Thyroxine supplementation
PT	1 - 5% of hyperthyroidism Peak frequency 30 - 40yrs Female to male ratio 4:1	Diffuse lymphocytic infiltrate with absence of germinal centers and Hürthle cells	Triphase: 1. Hyperthyroidism 2.Hypothyroidism 3. Recovery to euthyroid	TSH and fT4 depends upon the phase. TPOAb up to 50% CBC without leukocytosis ERS not accelerated CRP negative	Heterogenous, hypoechogenic normal size or slightly enlarged	Low uptake	Hyperthyroidism: BB. Hypothyroidism: thyroxine supplementation
PPT	Prevalence 8 - 11%. Presence of TPOAb during first trimester increases risk	Lymphocyte infiltration with occasional germinal centers and, disruption and collapse of thyroid follicles	20 - 30% triphasic as PT 20 - 40% hyperthyroidism 40 - 50% hypothyroidism	Same as PT TPOAb 60 – 85%	Same as PT	Relative contraindication during breastfeeding	Same as PT. Propranolol is the BB of choice Thyroxine supplementation must be prolonged if breastfeeding or planning pregnancy
SAT	Incidence 3/100.000/year Female to male ratio 4:1 Seasonal incidence during summer Associated with Echovirus and Coxsackie virus A and B	Central core of colloid surrounded by multinucleated giant cells, that progress to form granulomas	Prodrome of non-especific symptoms Triphase as PT Thyroid pain as presenting symptom	TSH and fT4 depends upon phase. CBC with leukocytosis ESR accelerated. CRP elevated TPOAb 25%	Usually enlarged but diffusely echogenic	Low uptake	1st Line: NSAIDs 2nd Line: GCs
GD	Most common cause of hyperthyroidism Incidence 20 - 25/100.000/yr Female to male ratio 10:1 Peak frequency 40 - 60yrs	Hyperthrophy and hyperplasia of thyroid follicular cells. Lymphocytic infiltration and colloid is sparse	Hyperthyroidism Goiter Ophthalmopathy Dermopathy	Low TSH levels High fT4 and fT3	Diffusely enlarged echogenic gland	Increased uptake	BB + ATD. Radioactive Iodine. Total thyroidectomy

Table 2. Main characteristics of autoimmune thyroid diseases. *ATD*: ati-thyroid drugs; BB: beta-blockers; *CBC*: complete blood count; *CRP*: C-reactive protein; *ESR*: erythrocyte sedimentation rate; *FNAB*: fine needle aspiration biopsy; *fT3*: free-fraction triiodothyrosine; *fT4*: free-fraction thyroxine. *GCs*: glucocorticoids; *GD*: Graves' Disease; *HT*: Hashimoto thyroiditis; *NSAIDs*: nonsteroidal antiinflamatories; *PT*: painless thyroiditis; *PPT*: postpartum thyroiditis; RaIU: radioactive iodine uptake in 24hrs; *SAT*: subacute thyroiditis; *TPOAb*: thyroperoxidase antibodies; *US*: ultrasonography.

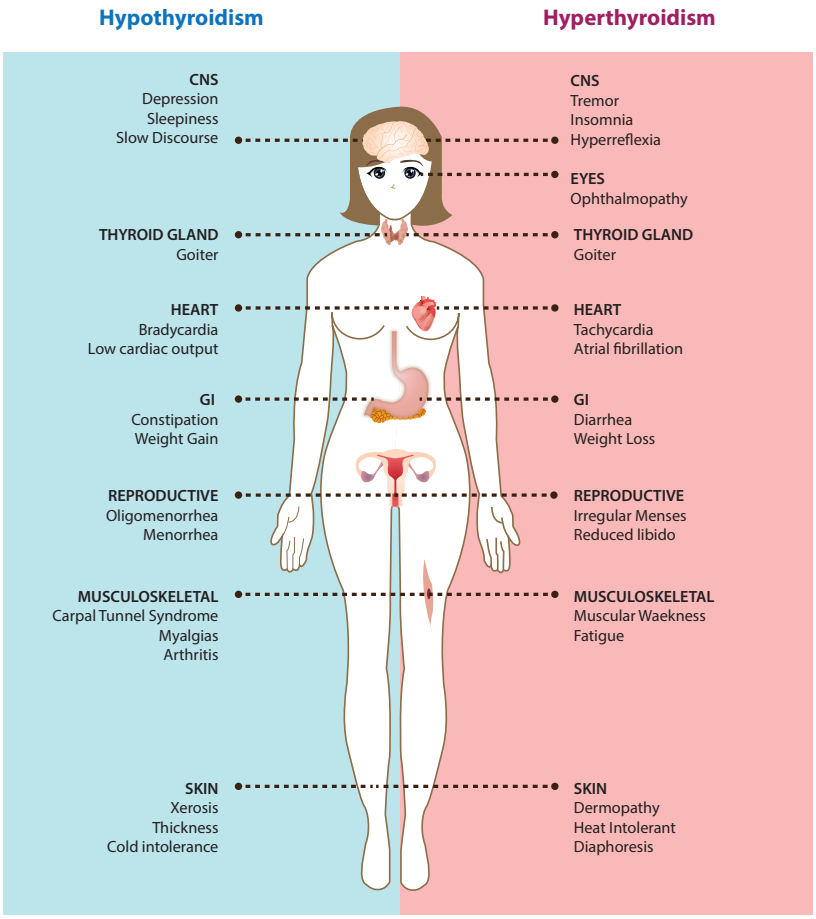

Figure 3. Clinical manifestations of hypothyroidism and hyperthyroidism. *GI*: gastrointestinal tract; *CNS*: central nervous system.

- Cardiovascular System: the most common finding is bradycardia followed by reduced cardiac output and low voltage on electrocardiogram (82,91).

- Musculoskeletal: fatigue, carpal tunnel syndrome, myopathy, and arthritis may be present (91). Myopathic symptoms consist of proximal muscular weakness accompanied by an increase in serum creatine kinase (91,92). The association between arthritis and hypothyroidism is well-known (93,94). Bland *et al*. (95) had previously described the arthritis that accompanied patients with hypothyroidism as characterized by affected knees, metacarpophalangeal joints, proximal interphalangeal joints, and metatarsophalangeal joints without the presence of sinovitis. It is thought to be a TSH-dependent increase in hyaluronic acid and proteoglycan synthesis in this subgroup of hypothyroid patients. This observation is supported by the symptomatic response to thyroid hormone replacement with concomitant TSH suppression (93,95).

- Neuropsychiatric System: sleepiness and slow discourse are frequent, along with depression, anxiety, and psychomotor retardation (82,91). Patients may also complain of memory impairment. Functional imaging studies have shown a decrease in cerebral blood flow and glucose metabolism (96).

- GI Tract: constipation is the main gastroenterological symptom secondary to dysfunctional motility (82,91). Other symptoms caused by decreased motility include dyspepsia and gastroesophageal reflux. Coexisting ADs may cause a decrease in gastric acid production and malabsorption (97).

- Endocrine System: oligomenorrhea and menorrhagia are the most common menstrual disturbances seen in these women (98). Men have lower concentrations of sex hormone – binding globulin and free testosterone (99).

- Other findings: patients with hypothyroidism may present with hyponatremia secondary to plasma dilution due to decreased free water clearance. This finding together with accumulation of mucopolysaccharides, reduced glomerular filtration rate, and low cardiac output results in edema (100). Hypothyroidism is characterized by abnormal lipid values; more than 90% of patients

present with increased low–densitiy lipoproteins (LDL) and apolipoprotein B because of reduced hepatic clearance secondary to a decrease in hepatic LDL receptors (101).

Some patients with HT, especially those with the goitrous form, might not present with the classic symptoms of overt hypothyroidism, but with symptoms secondary to the presence of a mass on the neck. The main compression symptoms are dyspnea, dysphagia, and dysphonia (82).

Laboratory Findings. Even though the clinical manifestations are very sensitive, their specificity is rather low (102). This is the reason the laboratory findings constitute the cornerstone for the diagnosis of thyroid dysfunction.

Secretion of the thyroid hormones, thyroxine (T4) and T3, is regulated by the TSH. In turn, TSH secretion is controlled by thyroid hormones. There is a negative relationship between free T4 (fT4) and TSH, where small changes in fT4 concentration induce very large reciprocal changes in TSH concentration (103). This means that serum levels of TSH are best for assessing the thyroid function. However, there is considerable controversy regarding the normal upper limit of serum TSH. Several authors have addressed this issue but, currently, there is no consensus (104–107). Even though most laboratories have used values of 5.0 IU/mL concurrent with the American Academy of Clinical Endocrinology (AACE) (106), an article published by the National Academy of Clinical Biochemistry argues that the upper limit should be reduced to 2.5 IU/mL based on their results where 95% of the euthyroid volunteers had serum values between 0.4 – 2.5 IU/mL (107). Jensen *et al*. and Hamilton *et al*. (104,105) found a normal upper TSH level of 4.1 IU/mL, which is more clinically acceptable for initiating therapy. Regarding T4 and T3, the free hormone hypothesis states that the unbound or free hormone is the one available for uptake into cells and interaction with nuclear receptors. The bound hormone, on the other hand, represents a circulating storage pool that is not immediately available for cell uptake. Free T3 (fT3) and fT4 are usually measured rather than serum total T3 and T4 concentrations.

The most common tests done in HT are TSH, fT4, TPOAb, and TgAb. The usual findings in patients with hypothyroidism include high TSH and low fT4 levels. Patients with high TSH but normal fT4 have a condition known as subclinical hypothyroidism (91). High serum TPOAb concentrations are present in 90% of the patients, and high serum TgAb are found in 50–90% of these patients. As discussed earlier, some patients with HT might have TRAb levels (i.e., up to 10%), but their role in the diagnosis is uncertain (91). The diagnosis of HT can be made in patients with high TSH levels, low fT4, and positive TPOAb.

Other tests that may be done include thyroid gland ultrasound (US) that will reveal an enlarged or shrunken gland depending on the clinical presentation of the HT (82). Radioiodine uptake (RaIU) over 24 hours does not provide useful information for diagnosis as it might be normal, reduced, or high depending on the functional phase of the disease (6,82). The fine needle aspiration (FNAB) should be done on every patient who presents with a dominant thyroid nodule and HT since, as discussed previously, lymphoma and thyroid carcinoma must be ruled out. A FNAB positive for HT will reveal the classical pathological findings that include a marked lymphocytic infiltration and the Hürthle cells (6,82).

Patients with hypothyroidism must be assessed on their cardiovascular risk as severe hypothyroidism leads to hypercholesterolemia and hypertriglyceridemia with a higher risk of atherosclerosis and acute coronary syndrome (101). Lipid profile tests should be done periodically on these patients. Patients with dyslipidemia, in turn, should always be screened for hypothyroidism (91).

Treatment. Thyroid hormone replacement therapy constitutes the main therapeutic strategy for patients with hypothyroidism (82,108). The treatment of choice is oral supplementation with synthetic thyroxine (levo–thyroxine, L-T4). L-T4 is a prohormone with very little intrinsic action. It is, in turn, peripherally deiodinated into T3, the active thyroid hormone. As the L-T4 has a prolonged half–life (i.e., approximately 7 days), once a day treatment ensures steady concentrations of T4 and T3 (109).

The goals of the treatment are improvement of the symptoms and normalization of TSH secretion. In addition, in patients with the goitrous form of presentation, a decrease in the size of the goiter is also considered a therapeutic objective (110). These goals are usually achieved with an average replacement dose of 1.6 mcg/kg/day (111). Elderly patients with coronary disease or multiple coronary risk factors should be treated conservatively since thyroid hormones increase myocardial oxygen demand which can induce angina, arrhythmias, or even myocardial infarction (82). The starting dose for these patients should be 50 mcg/day, and those with a prior history of coronary heart disease must be treated with 25 mcg/day (82).

Patients with replacement therapy must be monitored by assessing serum TSH levels. Although symptoms may begin to resolve a couple of weeks after initiating the treatment, steady–state TSH concentrations are not achieved for at least six weeks. Thus, these patients should be reevaluated with serum TSH in six weeks (112). If the TSH level persists at higher than the normal upper limit, the dose must be increased 12.5–25 mcg per day. Serum fT4 measurements are very insensitive for assessing the appropriateness of the dose (112). In the case of subclinical hypothyroidism, the decision to initiate replacement treatment depends on serum TSH levels. If the TSH level is >10 IU/mL, treatment with L–T4 is indicated to prevent progression to overt hypothyroidism. Patients with TSH levels that are between 4–10 IU/mL who also present with goiter, nonspecific symptoms of hypothyroidism, or high titers of TPOAb must be started on replacement therapy (113). Other therapeutic strategies such as

the use of immunosuppressive agents [e.g., glucocorticoids (GCs)] are not required since the lifelong administration of L–T4 is enough for these patients (82).

PAINLESS THYROIDITIS

PT, also called silent thyroiditis, is characterized by hyperthyroidism, followed by hypothyroidism, and finally, recovery to a euthyroid state (6,114). It is considered a variant form of HT, suggesting that it is part of the spectrum of AITD (114,115). There are many similarities with PPT and SAT (6,114,115).

The incidence of PT is not well-delineated and accounts for 1–5% of the cases of hyperthyroidism. It seems to be more prevalent in areas of higher iodine intake (117). Women are affected more commonly than men at a ratio of 4:1. The mean age of presentation is between 30–40 years (115). It is associated with a specific HLA, most often *HLA–DR3*, which suggests an inherited susceptibility. However, this association is weaker compared with *HLA–B35* and SAT (16).

Factors postulated to initiate PT include excess iodine intake and various cytokines (115,116). It has been reported in patients following cessation of GCs and in external radiation of the neck for Hodgkin lymphoma (117–120). The resulting thyroid inflammation damages thyroid follicles and activates proteolysis of the Tg stored in the colloid. This causes an unregulated release of T4 and T3 into the circulation and results in hyperthyroidism. Once the Tg is exhausted, hormone synthesis ceases and a state of hypothyroidism may develop. As the inflammation subsides, the repaired thyroid follicles resume normal synthesis and secretion of thyroid hormone (6,115).

Approximately 5% to 20% of the patients with PT present with the triphasic course of hyperthyroidism, hypothyroidism, and restoration to normal thyroid function (Figure 4). This clinical course is also described for PPT and SAT (115). The signs and symptoms that characterize hyperthyroidism and hypothyroidism are shown in Figure 3. The hypothyroid phase is recognized and diagnosed more often compared to the hyperthyroid phase (121). The thyroid gland is not painful, non-tender, but it is usually minimally enlarged sometimes firm in texture upon palpation (115).

As expected, thyroid function tests vary during the clinical course of the PT, and changes in serum TSH usually lag behind those in fT4 and fT3 (114,115) as explained in Figure 4. Thyroid function tests should be done every four to eight weeks to confirm resolution of hyperthyroidism and detect the development of hypothyroidism (115). Up to 50% of the patients with PT have increased titers of TPOAb but not to the same extent as those with HT (122). Other laboratory results as well as common imaging results are described in Table 2.

Most of the patients do not require any treatment at all since the thyroid dysfunction is not severe enough to make them symptomatic. However, patients who develop clinical manifestations do need treatment based on the phase of the clinical course they are in. During the hyperthyroid phase, the treatment of choice is beta-blockers (BB) (Table 3). In the hypothyroid phase, the replacement therapy is L-T4. Based on the results of the thyroid function tests, the course of the supplementation therapy must be defined (115). Although most patients recover full thyroid function, up to 20% will develop permanent hypothyroidism (123).

POSTPARTUM THYROIDITIS

Postpartum thyroiditis (PPT) is a destructive thyroiditis induced by an autoimmune mechanism within a year of parturition. It can also occur after spontaneous or induced abortion. Like PT, PPT is considered a variant form of HT (57,124–126).

The prevalence of PPT varies globally and ranges from 8 - 11% (71). Several risk factors have been identified that have a higher probability of developing PPT. For instance, women with positive TPOAb at the end of the first trimester have a 30-50% higher risk of progression to PPT. Patients with a prior history of thyroid dysfunction have a 40% risk, and for those with type 1 diabetes mellitus (T1DM) or family history of thyroid disease, the probability is about 20% (71,127). PPT has been related to *HLA-B* and *HLA-D*, suggesting an inherited pattern (128).

BB	MECHANISM OF ACTION	ADVERSE REACTIONS	DOSE	CONSIDERATIONS
Propranolol	Blocks adrenergic signaling, which is potentiated in hyperthyroidism; tissues with augmented action of cathecolamines include heart, skeletal muscle, bone and fat	Airway obstruction in patients with asthma or obstructive lung disease. Symptomatic Raynaud syndrome	10-40mg TID/QID	Nonselective BB Longest experience Preferred agent during breastfeeding
Atenolol			25-100mg QD/BID	Relative beta-1 selectivity Increased compliance
Metoprolol			25-50mg QID	Relative beta-1 selectivity

Table 3. Beta-blockers used for management of symptomatic hyperthyroidism. *BB*: beta-blockers; *BID*: two-times per day; *QD*: once per day; *QID*: four-times per day; *TID*: three-times per day. Adapted from Bahn *et al.* (149).

The presentation of PPT can be identical to painless thyroiditis but with a more variable course. Around 20–30% of the patients have the characteristic triphasic course with hyperthyroidism, hypothyroidism, and euthyroidism that lasts 12 months, 20–40% present with just hyperthyroidism, and 40–50% with overt hypothyroidism (125,129). Many of these symptoms are usually attributed to breastfeeding or the stress of having a newborn, making the recognition of PPT difficult at times. Patients with PPT may have a mildly enlarged, diffuse, non-tender thyroid gland that typically disappears with recovery of thyroid function (115).

The thyroid function test results are described in Figure 4. Positive TPOAb are found in 60–85% of the patients (78,124). Table 2 shows the common results of other laboratory tests and imaging studies.

The management of PPT is similar to the treatment of PT with some exceptions. When using BB for hyperthyroidism, propranolol is indicated given its low concentration in breast milk. Regarding hypothyroidism, the replacement should be halved after 6-12 months, unless the woman is pregnant, attempting pregnancy or breastfeeding (130,131). Up to 30% of the patients never recover from the hypothyroid phase and thus, require lifelong supplementation (127). Progression to permanent hypothyroidism may be related to higher initial TSH concentrations and TPOAb titer, maternal age, and female sex of the infant (71).

SUBACUTE THYROIDITIS

This type of thyroiditis, which is also known as De Quervain thyroiditis in honor of the Swiss surgeon who formally described the disease in 1904, is the main cause of thyroid pain (115,132). It is a self-limiting inflammatory disorder secondary to viral infections with a predictable clinical course of thyroid function evolution as seen in PT and PPT (133).

The estimated incidence is 3 cases per 100,000/year, and it affects four times more women than men. It occurs between 40 – 50 years of age (132–134) and decreases with age (132). The disease was thought to have a seasonal incidence, especially during summer, and clusters have been associated with a number of viruses (135). There is an association between SAT and *HLA–B35* in many ethnic groups (11).

The pathogenesis mechanism is the same as the one described for PT. However, the main trigger for the autoimmune disease is a viral infection as the majority of the patients have a prior history of upper respiratory tract infection days before the SAT (133). The main viral microorganisms identified are Echovirus and Coxsackevirus groups A and B, but other reports include measles, mumps, Epstein–Barr virus, and adenovirus.

The clinical course includes the triphasic phase described for PT and PPT. One of the main differences found in SAT is that thyroid pain represents the presenting symptom in up to 96% of the patients. The onset may be sudden or gradual and is usually radiated to the upper neck, jaw, throat, or upper chest. It is exacerbated by coughing or neck movements (136). On physical examination, the thyroid gland may be enlarged with tenderness upon palpation. Up to 50% of the patients have an initial thyrotoxicosis phase with the typical symptoms of hyperthyroidism (115). Another clinical variation found in patients with SAT is the appearance of a prodromic phase during the subclinical

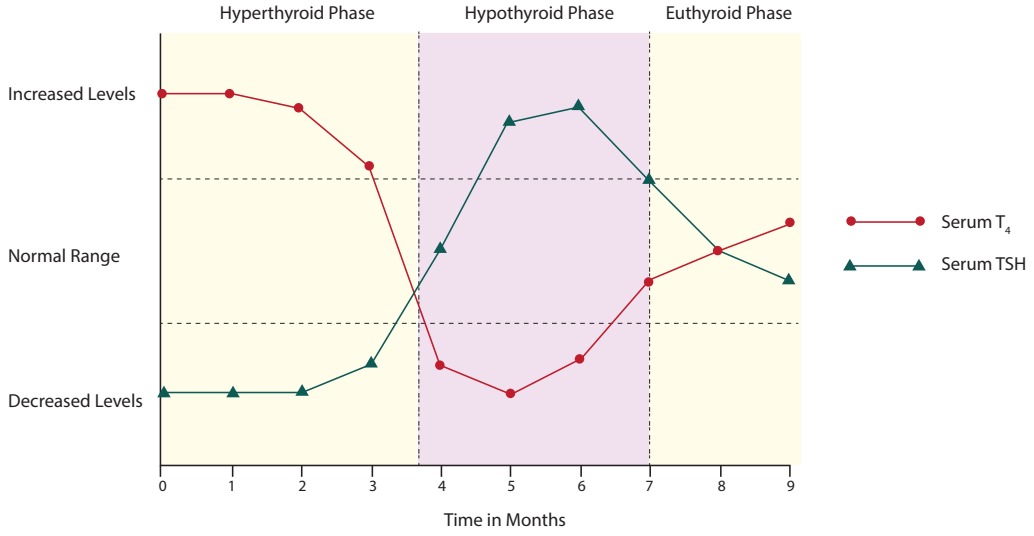

Figure 4. Naturla history of Painless, Postpartum and Subacute thyroiditis. These diseases share in common triphasic clinical course consisting in hyperthyroidism, hypothyroidism and return to normal thyroid function. During the hyperthyroidism phase, there is elevated fT4 and fT3, with low TSH; the serum fT3 concentrations are not disproportionately elevated as in GD, since the mechanism of elevation is due to a higher release from the gland rather than secondary to activity of the thyroid deiodinase. Likewise, in the patients who become hypothyroid, fT4 may decrease before the TSH levels increases. *fT4*: free-fraction thyroxine; *TSH*: thyroid-stimulating hormone. Adapted from Pearce *et al.* (6).

viral infection characterized by nonspecific symptoms such as myalgias, pharyngitis, low-grade fever, and fatigue (136).

The most relevant laboratory results and imagining studies are shown in Table 2 (137–140). The main goals in the treatment of SAT are pain relief and management of hyperthyroidism symptoms (115,132). Pain control strategies involve the use of both non-steroidal anti-inflammatories (NSAIDs) and/or systemic GCs (132). First line therapy is NSAIDs, so the patient is usually given ibuprofen or naproxen initially. If after two to three days there is no improvement in the symptoms, the NSAID should be discontinued and prednisone initiated at a dose of 40 mg/day. When the pain is controlled, a tapering dose of 5–10 mg per week should be encouraged to discontinue the medication within 4-8 weeks (132). About 4% of the patients have a recurrence of SAT (141).

GRAVES' DISEASE

Graves' disease (GD), named after the physician Robert Graves who first described the association in 1835, is clinically characterized by the presence of hyperthyroidism, diffuse goiter, ophthalmopathy (GO), and dermopathy (142,143).

Epidemiology. GD is the most common cause of hyperthyroidism, representing up to 80% of the cases (144). The annual incidence of GD is 20–25/100,000 in Caucasian populations. It is lower among people with African ancestry (142). It affects ten times more women than men. A peak frequency between the ages of 40–60 years has been reported (143,144). The concordance rate for GD between monozygotic twins is 38% (10). A family history of thyroid disease, especially among maternal relatives, is associated with increased risk of GD and a younger age of onset (145).

Pathogenesis. GD is the result of the presence of circulating TRAb that bind and activate the TSH receptor, thus stimulating follicle hypertrophy and hyperplasia as well as increasing the synthesis of thyroid hormones and the fraction of T3 relative to T4 (74,142). In GD, as discussed previously, there is an environmental factor that triggers an autoimmune response to the thyroid either by molecular mimicry or by bystander activation (142). Thyroid cells expressing HLA-R molecules may act as APC activating local T cells through the expression of co-stimulating molecules. As a result, there is a production of cytokines which in turn, activates thyroid–specific T cells and B cells to produce TRAb (142).

Clinical Manifestations. The clinical manifestations can be classified into those secondary to the excess of circulating thyroid hormones (hyperthyroidism) and those specific to GD (142). Typical signs and symptoms of hyperthyroidism are shown in Figure 3 (143,145). Some signs and symptoms are highly specific to GD including the following:

- Goiter: diffuse enlargement of the thyroid gland that can vary in size (142).

MAJOR CRITERION	MINOR CRITERIA
Hyperthyroidism (clinical or laboratory)	GO or dermopathy TRAb Diffuse radioiodine uptake

Table 4. Diagnostic criteria for Graves' disease. Diagnosis of GD requieres the major criterion plus one minor criteria. *GO*: Graves' ophthalmopaty; *TRAb*: TSHR antibodies.

- Ophthalmopathy: approximately 20–25% of patients with GD have clinically obvious GO (33). The estimated incidence of GO is 16/100,000 persons in women and 3/100,000 in men (146). It appears before the onset of hyperthyroidism in 20% of the patients, concurrently in 40%, and six months after diagnosis in about 20% (147). The characteristics signs are proptosis and periorbital edema. The major symptoms include sense of irritation in the eyes, excessive tearing, retroorbital discomfort or pain, blurring of vision, diplopia, and occasionally, loss of vision (148). Most of the patients have mild non–progressive ocular involvement, but in 5–10% of the cases, there is severe ophthalmopathy associated with significant visual impairment that can lead to visual loss (142). The pathogenesis of GO is a result of a cross-reaction between the TRAb and TSHR found on orbital fibroblasts, which induces the production of glycosaminoglycans, especially hyaluronic acid. (33).

- Dermopathy: present in 1-2% of the patients with GD, most commonly in those with severe ophthalmopathy (142). It is characterized by a non–pitting edema with occasional hyperpigmented papules, located on the pretibial area (142).

- Thyroid acropachy: clubbing caused by soft tissue swelling and periosteal bone changes in the fingers and toes. This sign represents a rare manifestation of GD (142).

Laboratory Findings. The laboratory findings in patients with GD are completely opposite to those found in hypothyroidism (Table 2). Monitoring of the patients with GD involves obtaining fT3 periodically to asses response to treatment when medical management is indicated (see below) (149). The main thyroid autoantibody present in patients with GD is the TRAb in >95% which, in contrast to HT, are stimulating antibodies (51,78,142,143). Other autoantibodies are also present. TPOAb is elevated in 40-70% of the patients and TgAb is found in 20–40% of GD (78,142). Thyroid gland US shows a diffusely enlarged thyroid (142). Color Doppler will show increased blood flow (143). RaIU in 24hrs reveals an increased uptake from the gland, which can be helpful to differentiate from the hyperthyroidism phase of thyroiditis (142). Diagnostic criteria are used based on clinical and laboratory findings (142) (Table 4).

Treatment. The treatment of GD is based upon one of three therapeutic strategies: medical management, using medi-

cation to suppress the hyperthyroid symptoms (i.e., BB), and blocking thyroid hormone synthesis with antithyroid medication (i.e., PTU, MMI); radioactive iodine therapy; or surgical approach with total thyroidectomy (149). The decision to choose one over the other depends on each individual and is based on the severity of the thyrotoxicosis, the presence of goiter and ophthalmopathy, and the patient's preference (149) (Table 5).

POLYAUTOIMMUNITY

Several clinical signs and symptoms that are shared among ADs –physiopathological mechanism and risk factors– indicate that they have a common origin, which has been called the autoimmune tautology (5,8). The clinical evidence of the autoimmune tautology highlights the co-occurrence of more than one AD in a single patient, namely polyautoimmunity, or MAS, a form of polyautoimmunity which corresponds to the coexistence of two or more well-defined ADs (5,9). The importance of these terms is due to the fact that patients with polyautoimmunity or MAS may have a modified disease course (i.e., with a worse prognosis or a better one) and a modified clinical presentation (9).

AITD has been described as the most prevalent AD as well as being associated with other organ-specific and non-organ specific ADs (150). In a study in which we analyzed 1,083 patients, polyautoimmunity was observed in 34.4% of the cases, and AITD was the most frequent polyautoimmunity found (9). This finding was supported by systemic literature review where three basic, large clusters were found. According to the analysis, the most hierarchical (i.e.,

TREATMENT	MECHANISM OF ACTION	ADVANTAGES	DISADVANTAGES	ADVERSE REACTIONS	DOSES
ATD PTU	Interfere with TPO - mediated iodination of tyrosine residues in Tg, by serving as an alternative substrate for the iodine intermediate and diverting oxidized iodide away from hormone synthesis	High likelihood of remission* High surgical risk Low-life expectancy Previously irradiated or operated neck Moderate to severe GO	Previous known major adverse reaction	Agranulocytosis ANCA-positive small vessel vasculitis Fulminant hepatic necrosis	Starting dose: 50-150mg TID Maintenance sose: 50mg BID or TID
MMI				Agranulocytosis Hepatotoxicity (cholestatic) Aplasia cutis Teratogenesis Lupus-like syndrome	Starting dose: 10 – 20mg QD Maintenance dose: 5 – 10mg QD
Radioiodine Therapy	Radiation - induced thyroid destruction	Planning pregnancy in more than 4 - 6mo High surgical risk Adverse reaction to ATD	Pregnancy or lactation Suspected or documented thyroid cancer Unable to comply with therapy sessions	Hypothyroidism Radiation thyroiditis Worsening of GO Transient reduction in testosterone levels.	Fixed dose: 10mCi Calculated dose depending on percentage of iodine uptake
Surgery	Directly removes most or all hyperfunctional thyroid tissue	Symptomatic compression or large goiter (>80g) Thyroid malignancy suspected or documented Hyperparathyroidism requiring surgery Moderate to severe GO	Comorbidities First and third trimester	Complications related to procedure	NA

Table 5. Treatment strategies for Graves' Disease. *ANCA*: anti-neutrophil cytoplasmic antibody; *ATD*: anti-thyroid drugs; *GO:* Graves' ophthalmopathy; *MMI*: methimazole; *NA* not applicable; *PTU*: propylthiouracil; *QD*: once per day; *Tg*: thyroglobulin; *TID*: three-times per day *TPO*: thyroperoxidase; *TRAb*: TSH receptor antibodies; *Mild disease, small goiter, low-titer TRAb.

chaperon) AD in the MAS cases is represented by AITD. It was associated with sclerodema (SSc) in 23% of the cases, rheumatoid arthritis (RA) in 21% , SLE in 17.9%, and multiple sclerosis (MS) in 9.1% . Female gender was a shared factor that was significantly associated with polyautoimmunity in the four ADs (9). Possible explanations for the relationship of these AD include the immunomodulatory effects of antithyroid antibodies, molecular mimicry between thyroid and disease-specific epitopes, and a genetic link between thyroid autoimmunity and the susceptibility to AD. Other studies have demonstrated the association between AITD and non–organ specific AD, particularly with RA, SLE, and Sjögren's syndrome (SS) (150-152) (Table 6).

Familial autoimmunity (FA) is defined as the presence of any AD in first-degree relatives (FDRs) of the proband, which are at increased risk of developing an AD (1,4–6). Recently, we found FA to be strongly associated with AITD (69). For instance, if the proband has AITD, the most common AD in the FDRs are: Addison's disease (AdD), celiac disease (CD), inflammatory bowel disease (IBD), myasthenia gravis, MS, pernicious anemia (PA), RA, SLE, T1DM, vitiligo (VIT), localized SSc, and discoid lupus erythematosus. On the other hand, if AITD is the AD in FDRs, the probands are predisposed to T1DM, SLE, RA, MS, SS, SSc, IBD, VIT, juvenile rheumatoid arthritis, juvenile lupus erythematous, inflammatory idiopathic myositis, CD, and alopecia areata. These finding shows that there is familial clustering of AITD in FDRs, particularly in female relatives (7).

Boelaert *et al*. (153) described FA among probands with HT or GD. Both ADs were significantly associated with the presence of T1DM, RA, PA, SLE, CD, VIT, and MS. Only GD was associated with AdD and IBD. Compared to the general population, FA in GD probands disclosed PA as the strongest association (RR:14.1) followed by RA (RR:13.5). Hemminki *et al*. (154) assessed FA only in probands with GD from Sweden. To calculate familial risk within a large community based cohort, they calculated standardized incidence ratios (SIR) as the ratio between the observed and the expected frequency for each disease. A value over one indicates a higher frequency of what is expected whereas a value below one indicates a decreased frequency. The analysis was stratified based on the FDR involved. For a single parent affected, HT, PA, and RA were the only diseases significantly associated as they had a SIR of 2.04, 1.82, and 1.48 respectively and thus, showed a frequency that was higher than what was expected. Significant associations for singleton siblings were found for T1DM, discoid lupus, and localized SSc if a parent and a sibling were affected with the same AD. The significant association was between HT with a SIR of 37.41 and SLE with a SIR of 14.33 (154). In another study, FA was significantly associated with polyautoimmunity in SLE and SSc patients (9), while Walker *et al*. (155) found an excess risk for AITD in RA multicase families compared to the general population.

AITD AND RHEUMATOID ARTHRITIS

For several decades, an increasing occurrence of thyroid disorders in patients suffering from RA has been documented, both autoimmune and non-autoimmune in nature (94,156–158). In addition, rheumatological and non-rheumatological manifestations of AITD have been described (55). Within these manifestations, it is noteworthy that the most common symptoms are polyarthralgia and unclassified arthritis, which are the main features of RA. Genetic background is an important aspect in autoimmunity. Genetic risk factors shared among diseases have been described in AITD and RA (55,159,160) .

POLYAUTOIMMUNITY	GENETICS	AITD (%)	HYPOTHYROIDISM (%)	HYPERTHYROIDISM (%)	TPOAb (%)	TgAb (%)	CONSIDERATIONS
RA	*PTPN22* and *CTLA-4*	9.8	NA	NA	37.8	20.8	AITD is a risk factor for CVD in RA Association between AITD and EAM seems to be link to CVD
SLE	Locus *5q14.3–q15*	7	4-14	1.7	27.7	15.4	Thyroid dysfunction risk increases in subgroup of patientes with higher disease activity
SS	*HLA-B8* and *HLA-DR3*	10-18	4	1.8-18	17.6	13.4	Histopathological similarities suggest common pathogenic mechanism 32% patients with AITD have sicca symptoms

Table 6. Polyautoimmunity and AITD. *AITD*: autoimmune thyroid disease; *CTLA-4*: cytotoxic T lymphocyte antigen-4; *CVD*: cardiovascular disease; *EAM*: extra-articular manifestations; *HLA*: human leukocyte antigen; *NA*: not available; *PTPN22*: protein tyrosine phosphatase, nonreceptor type 22; *SLE*: systemic lupus erythematosus; *SS*: Sjögren syndrome; *RA*: rheumatoid arthritis.

The prevalence of AITD in RA cases has ranged from 0.5% in Morocco (161) to 27% in Slovakia (78). It varies between regions and ethnicities [i.e., 1% in Germany (162), 2.1-9.8% in North America (163-166)]. For more details, see Figure 5. In Latin America, the prevalence of polyautoimmunity was reported to be 9.8% in a cross sectional analytical study of Colombian RA patients. This association, adjusted by gender and RA duration, was related to the presence of diabetes, thrombosis, and abnormal body mass index. Furthermore, a lower AITD frequency was demonstrated for the lowest educational level than for the highest one. This is also true when antimalarials are used (150).

It is widely accepted that, among the thyroid antibodies, the most frequent is TPOAb compared to TgAb (80). The prevalence for TgAb ranged from 5% in men from the UK (167) and 6% regardless of gender in Egypt (168), to 31% in RA patients from Japan (169). The prevalence for TPOAb was within the range of 5% in Egypt (170) to 37% in Italy (151). Cárdenas-Roldán *et al*. (150) in a recent cross-sectional analysis of 800 RA patients found that the presence of antibodies was 37.8% for TPOAb and 20.8% for TgAb. In contrast, Ruggeri *et al*. (171) show the assessment of thyroid antibodies at three points in time. There were more patients with TPOAb and TgAb than with a clinical diagnosis of AITD. Considering the idea that autoantibodies are predictors of disease (45,172), it is important to remain vigilant in following the clinical course of these patients. TPOAb and TgAb are known to predict AITD. This was demonstrated in the Wickham cohort (173). Patients within the accepted TSH reference range and who had the above mentioned autoantibodies had a greater risk of developing overt hypothyroidism. A careful assessment of those patients with a normal range of TSH but presenting specific antibodies should be done. For more details, see Figure 5.

The association between AITD and extra-articular manifestations (EAM) seems to be linked to CVD (174), and it is considered a major predictor of poor prognosis (175). In fact, Raterman *et al*. (156) agreed that the presence of hypothy-

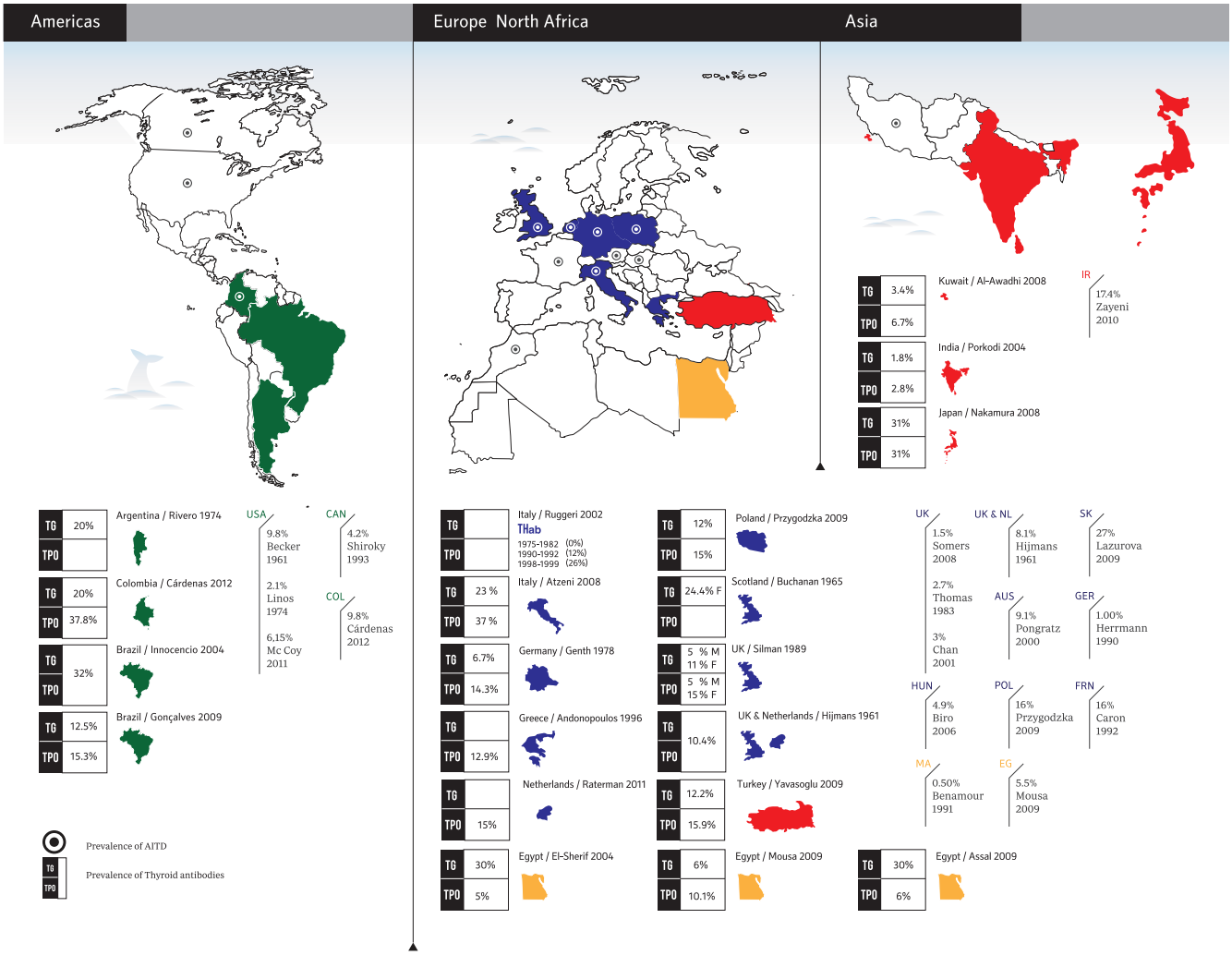

Figure 5. Prevalence of AITD and thyroid antibodies worldwide. *Tg*: thyroglobulin; *TPO*: thyroperoxidase.

roidism, including HT, is a risk factor for CVD in patients with RA. Moreover, McCoy *et al*. (165) found that L-T4 supplementation was significantly associated with CVD, which supports the fact that the administration of this medication does not decrease the occurrence of this outcome. Autoimmunity itself may be an independent risk factor for CVD. As both diseases increase inflammatory parameters and cytokines and cause endothelial dysfunction, a relationship between polyautoimmunity and the occurrence of CVD is not surprising.

With respect to RA severity, the literature is scarce. Charles *et al*. (176) did not find a relationship between the presence of thyroid antibodies and the occurrence of anti-cyclic citrullinated peptide antibody (anti-CCP), although they did the analysis with the *PTPN22 R620W* allele. Likewise, another cohort did not find a correlation between AITD and proxy variables for RA severity such as erosions, biological agent use, the presence of anti-CCP, and EAM (150). One cannot but hypothesize that many of these studies are cross-sectional in nature and because the importance of DAS28 and HAQ is along a timeline, it is not relevant to include these variables in the analysis.

AITD AND SYSTEMIC LUPUS ERYTHEMATOUS

White *et al*. (177) and Hijmans *et al*. (158) first described the association between SLE and AITD by showing an increased presence of thyroid dysfunction in patients with SLE compared to the general population. There are several studies that have suggested an increased prevalence of AITD in SLE (178–181). However, this is a subject of controversy to some authors (182).

Thyroid dysfunction in SLE may have various pathogenic mechanisms, but the underlying processes are unknown (183). For instance, genetic influence might play a role as suggested by Namjou *et al*. (184) in a study in which they found a gene of susceptibility identified in *5q14.3–q15*, a major locus of susceptibility for SLE also found in AITD. This result highlights a potential genetic link between these diseases.

The most common thyroid function abnormality found in patients with SLE is hypothyroidism with a prevalence of 4–14% (178–181,183), which is significantly higher than the general population prevalence of 1%. Additionally, subclinical hypothyroidism is present in up to 12% of the patients with SLE (178,179).

Whether hyperthyroidism is more prevalent in SLE than in the background population is still debatable (180,183). While the prevalence in different studies is up to 1.7%, the frequency in the general population is approximately 1.9%, which suggests that there is no significant difference (180).

When assessing the thyroid function of patients with SLE, some factors should be considered such as age, use of immunosuppressant, and disease activity (183). Kumar *et al*. (178) found a significantly higher prevalence of thyroid disease in the SLE cohort when compared to age and sex matched controls (36% vs. 8%). The studies assessing the relationship between disease activity and thyroid dysfunction are controversial with non–conclusive results (183). However, patients with greater clinical activity and severity of SLE have significant changes in the hypothalamus – pituitary – thyroid axis even with no evidence of thyroid disease (179). There is a subgroup of patients in which the disease activity affects the thyroid function, and are those with the "euthyroid sick syndrome." These patients had a SLEDAI that was statistically significant different from those without thyroid dysfunction, implying the effect of disease activity on this type of thyroid abnormality in SLE patients (178,185). Disease duration, in turn, has no statistically significant effect on thyroid function abnormalities (178).

Both TgAb and TPOAb are found with greater frequency in patients with SLE than in the general population even in those with no thyroid function abnormalities (178,183). In a recent study, 30% of the patients were positive for antithyroid antibodies, and 12% of them did not have thyroid function abnormality (178). Overall, there is a trend toward TPOAb being found more frequently than TgAb. Antonelli *et al*. (179) reported a 27.6% prevalence of TPOAb in patients with SLE as opposed to 15.4% of TgAb.

Thyroid involvement being non-life threatening in SLE compared to organ involvement, it can be undetected for a long time while contributing to the morbidity of the illness. Symptoms of thyroid disease can be confused with those of SLE. Therefore, it is necessary to identify the thyroid dysfunction in these patients and treat them accordingly (183).

AITD AND SJÖGREN'S SYNDROME

The most common association reported between an endocrine and rheumatological autoimmune disease is that found between SS and AITD (183). However, the controlled studies looking at this association have been few and the results have not been uniform thus, making them highly variable (186).

The lacrimal, salivary, and thyroid glands have a number of histological and functional similarities including the uptake and concentration of iodine by these glands (187). From the histopathological point of view, they share three characteristics (183,188). First, focal or diffuse infiltration by activated T lymphocytes similar to those from HT are found in patients with SS, which suggests that the same autoimmune response is directed towards the thyrocytes and salivary gland epithelium (183,187). Second, the participation of HLA of the haplotypes *HLA-B8* and DR3 in both SS and AITD is demonstrated by the higher frequency of those haplotypes in Caucasian patients with these diseases (189). Third is clonal B cell expansion (183,189). These observations may suggest a common pathogenic mechanism in both diseases. It is possible that antigens shared by the salivary and thyroid glands are responsible for the associated autoimmunity directed toward each organ (187). In addition, Hansen *et al*. (190) reported five cases of focal autoimmune sialadenitis in 19 patients with AITD. Thus, it is sometimes hard to establish whether the salivary and eye involvement represent an extra-thyroidal manifestation of AITD, or rather, an extra-exocrine manifestation of SS (183).

The prevalence in the association between AITD and SS varies between studies (186,187,189,191) and ranges from 10-18%. The major cause of thyroid disease is hypothyroidism. Lazarus *et al*. (192) reported that the most common AD developed by patients with SS in their cohort, approximately 16%, was hypothyroidism. Moreover, Foster *et al*. (193) showed a high prevalence of thyroid disease and anti-thyroid antibodies in female relatives of 42 patients with SS (13.7% vs. 3.3% in female controls). SS is ten times more frequent in patients with AITD, and HT is nine times more frequent in SS as compared to the general population (183). In a study of 479 patients with SS, the frequency of HT was greater than that in the general population, 6.25% and 1-2%, respectively (194). Ramos-Casals *et al*. (187), in turn, found that the most frequent hormonal profile observed was subclinical hypothyroidism, detected in 51% of their cohort.

Only a few studies have focused on the prevalence of hyperthyroidism in patients with SS, which is why it is thought to be infrequent (187,191,192). The prevalence in these studies is as low as 1.8% (192) and as high as 18% (187); thus, further studies are needed regarding this specific association.

The frequency of anti-thyroid antibodies in SS is approximately 11% (186). Punzi *et al*. (191) reported a 17.6% increase in the frequency of TPOAb and of 13.4% in TgAb in 119 female patients with SS, compared to 199 female controls.

Currently, there are no robust studies reporting the clinical manifestations of patients with AITD that will develop SS. Petri *et al*. (195), looking at the reverse association, found that there was no increase in the rheumatological symptoms in patients with AITD. However, it has been found that around 32% of patients with HT present with conjunctivitis sicca and xerostomia (194). According to this, it is justifiable to periodically screen patients with SS for AITD, especially HT, given the frequency of this association apart from the presence of symptomatology.

CONCLUSIONS

AITD is a term used to bring together a group of pathologies that include thyroid dysfunction and an autoimmune response to the thyroid gland. Even though the prevalence of this disease in the general population varies between countries, AITD can be regarded as the most common autoimmune endocrine disease. It includes a group of AD clustered together with a diverse clinical presentation that depends on whether it causes hypothyroidism, hyperthyroidism, or both. An international consensus to accurately diagnose AITD is warranted.

AITD is frequently associated with other organ specific and non-organ specific AD, most commonly RA, SLE, and SS. AITD is clinically important in the context of autoimmunity, and it is mandatory to screen patients with hypothyroidism or hyperthyroidism symptoms for the autoimmune etiology when there is suspicion of the coexistence of AITD (i.e. polyautoimmunity).

Routine screening for CVD among these patients should be considered. These results may help to further the study of the common mechanisms of AD, to improve patient outcome, and to define public health policies, especially for RA patients.

ABBREVIATION LIST

- **AACE:** American Academy of Endocrinology
- **AD:** autoimmune diseases
- **AdD:** Addison Disease
- **AITD:** autoimmune thyroid disease
- **ANA:** antinuclear antibodies
- **ANCA:** anti-neutrophil cytoplasmic antibodies
- **APC:** antigen presenting cell
- **ATD:** anti-thyroid drugs
- **BB:** beta-blockers
- **BID:** two-times per day
- **CBC:** complete blood count
- **CRP:** C-reactive protein
- **CTLA-4:** cytotoxic T lymphocyte antigen-4
- **CVD:** cardiovascular disease
- **EAM:** extra-articular manifestations
- **ELISA:** enzyme-linked immunosorbent assay
- **ESR:** erythrocyte sedimentation rate
- **FA:** familial autoimmunity
- **fT3:** free-fraction triiodothyronine
- **fT4:** free-fraction thyroxine
- **FNAB:** fine-needle aspiration biopsy
- **GCs:** glucocorticoids
- **GD:** Graves' disease
- **GO:** Graves' ophthalmopathy
- **HLA:** human leukocyte antigen

- **HT:** Hashimoto thyroiditis
- **IBD:** inflammatory bowel disease
- **IFN:** interferon
- **L-T4:** levothyroxine.
- **MAS:** multiple autoimmune syndrome
- **MMI:** methimazole
- **MS:** multiple sclerosis
- **NIS:** sodium/iodine symporter
- **NSAIDs:** non-steroidal anti-inflammatory drugs
- **PA:** pernicious anemia
- **PPT:** postpartum thyroiditis
- **PTPN22:** protein tyrosine phosphatase, nonreceptor type 22
- **PT:** painless thyroiditis
- **PTU:** propylthiouracyl
- **QD:** once per day
- **QID:** four-times per day
- **RA:** rheumatoid arthritis
- **RaIU:** radioactive iodine uptake
- **SAT:** subacute thyroiditis
- **SLE:** systemic lupus erythematosus
- **SLEDAI:** systemic lupus erythematosus disease activity index
- **SS:** Sjögren syndrome.
- **SSc:** scleroderma
- **T1DM:** type 1 diabetes mellitus
- **T3:** triiodothyrosine
- **T4:** thryoxine
- **Tg:** thyroglobulin
- **TgAb:** thyroglobulin antibodies
- **TID:** three-times per day
- **TPO:** thyroperoxidase
- **TPOAb:** thyroperoxidase antibodies
- **TRAb:** thyroid stimulating hormone receptor antibodies
- **TSH:** thyroid stimulating hormone
- **TSHR:** thyroid stimulating hormone receptor
- **US:** ultrasonography
- **VIT:** vitiligo

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31

AUTOIMMUNE HEPATITIS

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INTRODUCTION

Due to its location and function, the liver is continually being antigenically challenged by pathogens, toxins, tumor cells, and less harmful antigens such as those acquired with food intake. The bloodstream travels along the hepatic sinusoids which are covered by an endothelial layer devoid of basal membrane and presenting "pores." This sinusoid structure allows the transmigration of cells towards the hepatic parenchyma. Soon after differentiation and activation, circulating T cells are capable of entering non-lymphoid peripheral organs. Differentiation and activation of T cells within the peripheral lymphoid organs are known to take roughly 48 h. The liver is an exception to the general time course lymphoid trafficking due to its ability to retain mature naïve T cells, mainly CD8+ T cells which, after a 24 h period, are delivered into the peripheral circulation as activated cells (1).

Chronic inflammation of the liver occurs as a consequence of the recruitment and retention of lymphocytes in the tissue. The recruitment of effector and regulatory T cells (Tregs) from the circulation is dependent on interactions between lymphocytes and specific cell surface molecules expressed on endothelial cells (2). Once captured, the retention and positioning of leukocytes within tissue requires certain signals to locate and retain them at sites of target cell damage. These receptors and chemokine signals follow the accepted multistep paradigm of leukocyte adhesion to vascular endothelium, which is relevant to most organ systems although the specific signals involved differ among tissues. Chemokines are critical components of this adhesion cascade and are believed to play two crucial roles: triggering integrin-mediated stable adhesion and directing migration. Chemokines can bind to endothelial glycosaminoglycans and thus allow them to be presented to flowing leukocytes and also provide a mechanism for the paracrine presentation of chemokines secreted by other cells within the microenvironment. Similar mechanisms are believed to be involved in both normal immune surveillance and in inflammatory disease although the chemokines involved differ with constitutive chemokines playing the dominant role in physiological trafficking and inducible inflammatory cytokines involved in inflammation. Chemokines can be classified into four groups based on their amino acid sequence. The two largest groups are the CC chemokines, where conserved cysteine residues lie adjacent to each other, and CXC chemokines, where an amino acid separates the first two cysteine residues. Within the group of CXC chemokines, CXCL10 or interferon (IFN)-inducible protein (IP-10), CXCL9 or monokine induced by IFN-γ (MIG), CXCL11 or IFN-γ - inducible T-cell -chemoattractant (ITAC) display potent lymphocyte chemotactic activity and bind a common receptor and CXCR3, the expression of which is increased on tissue-infiltrating regulatory and Th1-polarized T cells. T cells infiltrating the inflamed liver express high levels of CXCR3. Moreover, CXCR3 ligands are up-regulated on hepatic endothelium at sites of T-cell infiltration in chronic hepatitis, and their presence has been correlated with outcome of inflammatory liver disease (3).

The liver is a residence organ for dendritic cells (DC), whose precursors derive from peripheral blood. The antigenic uptake that occurs within the liver by immature DC promotes their maturation and migration to peripheral lymph nodes, where they act as efficient antigen presenting cells. This is a key process in the initiation of the immune response. Tregs may be attracted to DC residing within the liver to modulate their local function and survival. In secondary lymphoid tissues, Tregs use multiple mechanisms to inhibit DC function and block initiation of autoimmunity. Tregs can suppress effector cell proliferation and function within the liver by secreting immunosuppressive cytokines (IL-10, TGFβ)

or through contact-dependent mechanisms to restore immune homeostasis and promote resolution of hepatitis. Effector T cells cause damage to hepatocytes resulting in interface and lobular hepatitis. Infiltrating T cells, macrophages, and stromal cells secrete IFNγ, TNFα, and other proinflammatory cytokines that result in a microenvironment that promotes the persistence of chronic liver inflammation.

Apart from DC, hepatocytes, kupffer cells, and sinusoidal endothelial cells can present antigens to CD8+ T cells. The local presentation of antigens within the liver leads to activation-triggered T cell apoptosis. The balance between the different activation processes that take place within the liver leads to immunological tolerance, which explains the long survival of hepatic grafts and the chronic persistence of hepatotropic viruses (4). The development of intrahepatic tolerance would be due to the low expression of co-stimulatory molecules by hepatocytes (5).

Another feature that makes the liver a specialized immune organ is the type of resident T cells in the parenchyma (6). Fifty percent of intrahepatic lymphocytes (IHL) are non-conventional cells expressing membrane proteins that are typical of T cells and proteins belonging to natural killer cells (CD56, CD161, and/or KIRs, which are receptors that inhibit the cytotoxic response). These cells are termed NKT cells or "natural T cells." IHLs represent 20% of the total NK cell population, 25% of the T cells, and 5% of the B cells. Classical NKT cells (type I) express only one α chain of the TCR called the invariant chain (iNKT) by which the recognition of antigenic epitopes presented by the non-classical histocompatibility molecule CD1 (7) is accomplished. These cells also express CD45RO, an isoform of the CD45 which features activated lymphocytes and CD161 (NKR-P1A), a co-stimulatory molecule that contributes to the antigenic presentation mediated by CD1.

The interaction between antigen presenting cells and NKT cells results in the rapid expression of regulatory/Th2 and proinflammatory (IFN-γ and TNF) cytokines by NKT cells. Activated NKT cells are a source of IFN-γ and IL-4, two cytokines with hepatocytotoxic properties (Figure 1).

AUTOIMMUNE HEPATITIS (AIH)

Autoimmune hepatitis (AIH) is a chronic and progressive hepatitis of moderate to severe activity. AIH does not cause granulomatous biliary lesions, siderosis, or copper depots (8). The classification of AIH is based on the specificity of the autoantibodies found in the patient's serum (Table 1). In this chapter, special reference will be made to type I AIH.

TYPE I AIH

Type I AIH (AIH-I) is a disease of unknown etiology featured by the presence of polyclonal hypergammaglobulinemia, autoantibodies, and a local progressive necroinflammatory response which frequently evolves to cirrhosis and hepatic failure. The actual prevalence of AIH is unknown. The first study to use the International Autoimmune Hepatitis Group scoring system on Alaskan natives reported a prevalence of definite AIH of 35.9 cases per 100,000 (11). A report from the United Kingdom showed that the annual incidence of AIH is 3.0 per 100,000 inhabitants (12). This disease is more frequent in females (70-90%) with a female:male ratio of 3.6:1 (13), and it responds favorably to treatment with immunosuppressant steroids and azathioprine (14,15).

LABORATORY FINDINGS

AIH-I is featured by the presence of antibodies to actin filaments of the smooth muscle (SMA) and/or antinuclear antibodies (ANA). These antibodies can also be accompanied by perinuclear anti-neutrophil cytoplasm antibodies (p-ANCA). Although these antibodies are useful in the diagnosis of the disease, an immunopathogenic role has not been attributed to them (16). Therefore, no direct correlation exists between the necroinflammatory activity and the presence of such antibodies though their titers are known to decrease upon corticosteroid treatment (15). The activity of aminotransferases and specific IgG titers do not correlate with histological damage and consequently provide limited help with respect to treatment initiation (17). Bilirrubin levels as well as alkaline phosphatase activity are slightly higher in 80% of patients and 75% of them present low serum albumin values (8).

HISTOLOGICAL ABNORMALITIES

Since aminotransferases and IgG levels do not reflect the extent of histological inflammatory activity, or the presence or absence of cirrhosis, liver biopsy is mandatory to not only confirm the diagnosis but also evaluate the severity of liver damage. The histological evaluation of chronic hepatitis, including AIH-I, consists of the assessment of the necroinflammatory activity (activity degree), the presence of fibrosis, and the disruption of the hepatic parenchyma architecture (stadification). Results of this assessment are expressed as the Knodell's histological activity index (18, 19). The most frequent histological findings are the presence of interface hepatitis with abundant inflammatory infiltrate of a lymphocytic nature with or without lobular (acinar) and portoportal or portocentral fibrotic bridges accompanied by rosette formation and nodular regeneration.

CLINICAL MANIFESTATIONS AND DIAGNOSIS

In individuals with unresolved hepatitis, it is common to find symptoms that are similar to those of acute viral hepatitis. In these patients, a combination of signs and symptoms can be found, e.g. jaundice, acolia, coluria, hepatomegaly, splenomegaly, spider veins, acne, malaise, fatigue, evidence of cirrhosis, or fulminant liver failure. In the first five years after the onset of the disease, 50% of untreated patients die while spontaneous remission is a less frequent phenomenon. Diagnosis of AIH-I is difficult, since clinical, biochemical,

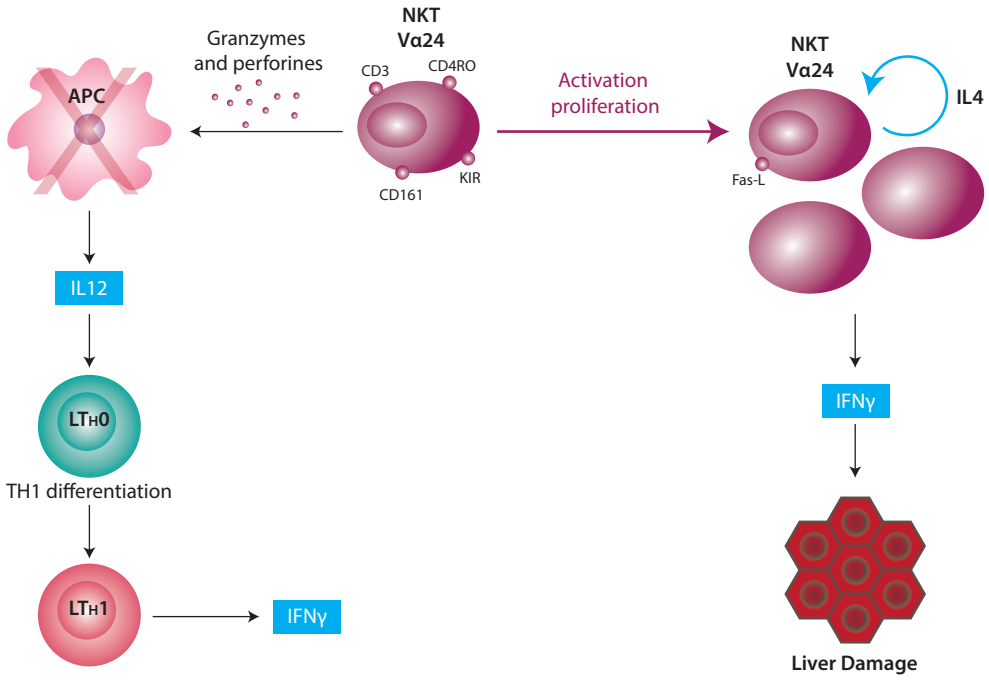

Figure 1. Myeloid dendritic cells (DC) present the antigen to NKT cells within the context of CD1 HLA complex through the TCR Vα24. During antigen presentation, DC release IL-12 which 1- promotes NKT cytotoxicity and regulates the number of DC (negative regulation) and 2- promotes the expression of Fas lingand (FasL) on the surface of NKT cells. The latter ones thus acquire a cytotoxic potential on hepatocytes via Fas/FasL and granzime/perforin pathways. IL-12 is, in turn, the main stimulating cytokine for the differentiation of type 1 helper T cells (Th1 cells).

CLASSIFICATION OF AUTOIMMUNE HEPATITIS BASED ON THE AUTOANTIBODY PROFILE	
Autoimmune hepatitis type	Autoantibodies
I	ANA, SMA, p-ANCA, anti-ASGP-R, anti-SLA/LP or anti-SEPSECS
II	Anti-LKM-1, anti-LKM-3, anti-LC1

Table 1. Abbreviations. *ANA*: Antinuclear antibodies, *SMA*: anti-smooth muscle antibodies, *p-ANCA*: perinuclear anti-neutrophil cytoplasm antibodies, *anti-ASGPR*: anti-a sialoglycoprotein receptor antibodies, *antiSLA/LP*: anti-soluble liver antigens/liver-pancreas antigen antibodies, *anti- SEPSECS*: anti-Sep (O-phosphoserine) *tRNA*:Sec antibodies (9), *anti-LKM*: anti-liver and kidney microsome antibodies, *anti-LC1*: anti-type 1 liver cytosolic protein. Adapted from (10).

or histological findings are not conclusive if taken in isolation. The criteria for the diagnosis of this disease have been established and revised by the International Autoimmune Hepatitis Group (20, 21). More recently, a simplified score intended to be used in clinical practice has been proposed by this group (22). The diagnostic criteria that make it possible to distinguish probable from definite AIH are in Table 2.

The current guidelines for the diagnosis of AIH are the following:

1. Diagnosis of AIH requires the assessment of aminotransferase activity, the levels of gammaglobulins, detection of ANA and/or SMA, or the presence of type 1 anti-liver and kidney microsome (anti-LKM-1) antibodies as well as the histological evaluation of a liver biopsy.

2. Diagnostic criteria of AIH must be applied to all patients.

3. In those cases where the diagnosis of AIH is not conclusive or when the clinical features of hepatitis are atypical, the scoring method must be used (Table 3).

4. Even though AIH-I presents histological and serological features that are common to pediatric and adult patients, previous work undertaken in our laboratory have shown that the clinical features and the genetic susceptibility for AIH-I differ between children and adults (Table 4).

TREATMENT OF AIH-I

AIH-I has been proven to respond favorably to the immunosuppressant treatment provided that there are no clinical signs of acute liver failure, a circumstance under which liver transplantation is mandatory.

REQUIREMENTS	DEFINITE DIAGNOSTIC CRITERIA	CRITERIA FOR PROBABLE DIAGNOSIS
Absence of liver disease of genetic origin	Normal phenotype for alpha 1 antitrypsin. Normal serum levels of ceruloplasmin, iron, and ferritin	Partial deficiency of alpha 1 antitrypsin. Non-specific abnormalities in the levels of ceruloplasmin, iron, and/or ferritin
Absence of active viral infection	Absence of markers for active A, B, or C virus hepatitis	Absence of markers for active A, B, or C virus hepatitis
Absence of toxic lesions or lesions caused by alcohol	Daily alcohol intake <25 g/d and no recent use of hepatotoxic drugs	Daily alcohol intake <50 g/d and no recent use of hepatotoxic drugs
Laboratory findings	Increase ≥1.5 fold of serum aminotransferases, levels of globulins, gammaglobulins or immunoglobulin G	Increase in serum aminotransferases. Any degree of hypergammaglobulinemia
Autoantibodies	ANA, SMA, or anti-LKM1 titers ≥ 80 for adults and ≥ 20 for children. Absence of AMA	ANA, SMA, or anti-LKM1 ≥ 40 in adults or the presence of other autoantibodies*
Histologic findings	Interface hepatitis. No biliar lesions, granulomas of changes suggesting other liver diseases	Interface hepatitis. No biliar lesions, granulomas of changes suggesting other liver diseases

Table 2. Diagnostic criteria for autoimmune hepatitis * Including perinuclear anti-neutrophil cytoplasm antibodies (p-ANCA) and antigens which are generally not exposed such as the soluble liver antigens/liver-pancreas antigen (anti-SLA/LP), actin and type 1 liver cytosolic protein antibody (anti-LC1), and anti-asialoglycoprotein receptor antibody. Abbreviations. *ANA*: antinuclear antibodies, *SMA*: anti-smooth muscle antibodies, *anti-LKM1:* anti-type 1 liver and kidney microsome antibodies, *AMA*: anti-mitochondrial antibodies.

The treatment of AIH-I comprises two phases. In newly diagnosed AIH, induction of remission is the main goal. The second phase of therapy is maintenance of remission with the lowest possible dose in order to maintain it while preventing significant side effects. If the diagnosis is correct, and the appropriate therapy is chosen, liver transplantation should be avoidable in patients with AIH.

The standard treatment consists of the administration of prednisolone with or without azathioprine. With this schedule, remission (normal liver tests) is achieved in more than 90% of the patients (26). In 20% of patients who have undergone remission, drugs can be withdrawn successfully after a few years of treatment, whereas in those patients suffering from AIH-II, the treatment must be maintained for the lifetime. A sustained remission of AIH has been reported in patients undergoing long-term treatment with azathioprine alone (27). Other effective drugs that are employed in the treatment of AIH-I are cyclosporine A and mophetil mycophenolate (28, 29)

RECURRENCE AND DE NOVO APPEARANCE OF AIH

Autoimmune hepatitis can recur or appear de novo after liver transplantation, resulting in hepatic fibrosis, graft loss, and the need for re-transplantation. Autoimmune hepatitis recurs in 8-12% of transplanted patients at 1 year and in 36-68% at 5 years. Recurrence may be asymptomatic and detected only by surveillance of liver test abnormalities or routine liver tissue examinations. Autoantibodies that characterized the original disease, hypergammaglobulinemia, raised serum immunoglobulin G levels, and characteristic histologic findings typify recurrence. Premature corticosteroid withdrawal and pre-transplant severity of the original disease are possible risk factors.

In patients with AIH, a decrease in the number of peripheral CD4+CD45RO cells has been observed together with the presence of an abundant intrahepatic infiltrate of mononuclear CD45RO cells. These findings have made it possible to suggest that a subset of memory cells could be preferentially migrating towards the liver. This cell subset remains unchanged even after the immunosuppressant treatment is administered and might be associated with occasional relapses of the disease.

A recent study demonstrated that AIH patients in remission have a significantly lower frequency of activated T cells in the peripheral blood, compared to both AIH patients with active disease and healthy subjects (30). De novo autoimmune hepatitis occurs in 1-7% of patients 0.1-9 years after transplantation, especially in children. The appearance of autoantibodies may herald its emergence, and antibodies to glutathione-S-transferase T1 seem to be predictive of the disease. While recurrent disease may reflect recruitment of residual memory T lymphocytes and host-specific genetic predispositions, de novo disease may reflect an allo-antigenic immune response and molecular mimicries that override self-tolerance. Treatment should be appropriate for autoimmune hepatitis and not based on anti-rejection drugs. Corticosteroid therapy alone or combined with azathioprine is the essential treatment (31).

IMMUNOPATHOGENESIS OF AIH-I

The establishment of this disease may be considered a consequence of a breakdown of tolerogenic mechanisms which govern the liver milieu. Although the autoantigen/s that trigger AIH-I have not been identified so far, the family of asialoglycoprotein receptors, xenobiotic substances, bacteria, and viruses have been postulated as candidates. Infection with hepatitis A, B, C virus (HAV, HBV, HCV) or measles virus, in particular, can precede the onset of AIH-I.

DIFFERENTIAL CHARACTERISTIC (23-25)	PEDIATRIC AIH	ADULT AIH
Sex	Female	+2
ALP/GOT (or GPT) ratio	>3 <1,5	-2 +2
Gammaglobulin or IgG (increase fold over the reference value)	>2,0 1,5-2,0 1,0-1,5 <1,0	+3 +2 +1 0
ANA, SMA, or anti-LKM1 titers	>80 80 40 <40	+3 +2 +1 0
AMA	Positive	-4
Markers of active viral infection	Positive Negative	-3 +3
Consuption of heptotoxic drugs	Yes -4 No +1	-4 +1
Alcohol ingestion	<25 g/d >60 g/d	+2 -2
Concomitant autoimmune disease	Any non-hepatic autoimmune disease	+2
Other autoantibodies	Anti-SLA/LP, actin, LC1, p-ANCA	+2
Histological findings	Interface hepatitis Plasma cells Rosettes None of the above mentioned Biliar changes Atypical characteristics	+3 +1 +1 -5 -3 -3
HLA	DR3 or DR4	+1
Response to treatment	Complete remission Remission with recurrence	+2 +3
PRE-TREATMENT SCORE		
Definite diagnosis Probable diagnosis		>15 10-15
POST-TREATMENT SCORE		
Definite diagnosis Probable diagnosis		>17 12 17

Table 3. Differential characteristics between pediatric and adult autoimmune hepatitis.

In Argentina there is a high prevalence of HAV infection. In children, the allele HLA II DRB1*1301 is linked to the development of both chronic HAV hepatitis and AIH-I. Due to this fact, it has been suggested that in children, the development of AIH-I requires the expression of yet unknown genes. The latter hypothesis is in line with the polygenic nature of autoimmune diseases (32). The treatment of chronic B and C hepatitis with IFN-α promotes the synthesis of serum autoantibodies and in some patients, these autoantibodies have been shown to be pathogenic. The neoantigen formation, the molecular mimicry or the induction of proinflammatory cytokines could be considered factors modifying the tolerogenic liver microenvironment that would lead to the development of AIH-I.

It is known that 10% of chronic HCV patients have autoantibodies typical of AIH-II (Table 1). The molecular mimicry between viral proteins and the host's antigens has been postulated to be the mechanism by which anti-LKM antibodies are generated (33).

Genetic expression studies done on liver biopsies obtained from pediatric patients with AIH-I have demonstrated the over-expression of primary transcripts of IFN-γ, IL-12p40 (a 40 kDa polypeptide), IL-12Rβ2 (the β2 subunit of the IL-12 receptor), IL-18, TGFβ1, IL-4, and Vα24 (34). The increase in the expression of these molecules, some of which were non-detectable in normal livers, has made it possible to infer their role in the mechanisms of liver injury. It is known that IL-2 is capable of inducing the synthesis of the IL-12 receptor chains in naïve T cells. Nevertheless, the rise in the expression of this receptor is dependent on the presence of IL-12, which is released by several antigen-presenting cells. Furthermore, the

CATEGORY	FACTOR	SCORE
Percentage of patients in which the onset resembles that of an acute viral hepatitis	70%	54%
Progression to fulminant hepatic failure	Occasional	Never
Percentage of patients presenting complete remission upon treatment with immunosuppressant drugs	68%	90%
Percentage of patients with extrahepatic autoimmune manifestations	13%	48% Arthritis, autoimmune thyroiditis, hemolytic anemia, celiac disease
Hypergammaglobulinemia	Marked	Less marked
Percentage of patients with antinuclear antibodies	55%	73%
Percentage of patients with anti-smooth muscle antibodies	95%	74%
Association with HLA in Argentine Caucasian population	HLA II DRB1*1301, present in a 66.6% of pediatric patients	Primary association with HLA I A11 present in 31% of adult patients. Secondary association with HLA II DR4 in 44% of patients

Table 4. **Scoring system for the diagnosis of atypical autoinmune hepatits in adults.**

IL-12-dependent production of IFN-γ is responsible for the maintenance of high levels of IL-12Rβ2 (35, 36), a molecule controlling the differentiation of the Th1 phenotype of T cells (37). One of the effects of the IL-12 is to enhance the expression of the IL-18R present in IFN-γ-producing cells thus attaining a synergistic response between IL-12 and IL-18 to produce IFN-γ (38). The high expression levels of IFN-γ, IL-12p40, and IL-12Rβ2 found in liver biopsies of AIH-I patients is evidence of the role of a Th1-skewed response in this disease.

Apart from the classical Th1 and Th2 subsets, an effector T cell subset named Th17 has also been described. Th17 cells are characterized by the production of interleukin-17A (IL- 17A), IL-17F, IL-22, IL-21, IL-6, and tumor necrosis factor α (TNF-α) (39). Although the differentiation and maturation of human Th17 cells is still a matter of controversy, transforming growth factor β (TGF-β), IL-6, IL-21, IL-1β, and IL-23 have been postulated to induce the human Th17 cell lineage (40). IL-23, a heterodimer of the IL-12 family, composed of the p19 and p40 subunits, appears to be essential to maintain and expand Th17 effectors. Meanwhile, IL-27, which is composed of the p28 and EBI3 subunits, suppresses the Th17 differentiation and IL-17 production and promotes Th1 differentiation (41). The presence of Th17 cells has also been reported in inflamed human tissues from patients suffering from a variety of inflammatory and autoimmune disorders (42-44). Only circumstantial signs of Th17 effector functions were found in liver biopsies of patients with AIH-I as deduced from the limited production of IL-17A and IL-17F transcripts (45).

REGULATORY T CELLS IN AIH-I

In addition to the Th1, Th2, and Th17 subsets, the functional quartet of CD4+ effectors also includes Tregs constitutively expressing the IL-2 receptor α-chain CD25 and the forkhead/winged helix (FoxP3) master regulatory transcription factor which is highly specific to the CD4+CD25high Treg lineage (46-48). Some studies carried out in mainly pediatric patients have addressed the possible impairment of Treg functions in AIH. These studies have shown that CD4+CD25+ Treg cells in peripheral blood seemed to feature both reduced frequencies and impaired suppressive functions (49, 50). Moreover, earlier studies have suggested that peripheral lymphocytes in autoimmune hepatitis exhibit increased immunosuppressive capability (51, 52). In these studies, however, Treg cells were defined only by staining for CD4 and CD25 since, at that time, it was not possible to distinguish human Tregs from activated effector T cells. In humans, a clear distinction of Tregs from activated conventional effector T cells is difficult since the Treg markers CD25 and Foxp3 can also be expressed transiently by activated effector T cells (53). More recently, a combination of more accurate methods has been developed. Those studies include the methylation status of the FOXP3 locus to search for demethylated FOXP3 TSDR (Treg-specific demethylated region) (54); the analysis of the expression of CD127, which is low in Treg cells and high in activated effector T cells (55); and the analysis of CD39, which seems to be one of the molecules that mediates the suppressive function of Treg cells by producing inhibitory adenosine (56). The results of these studies clearly established that Treg cells belonging to AIH patients are fully functional; Treg frequency is not decreased in AIH patients but rather seems to correlate with disease activity. Another important aspect of this study was the finding that the Treg frequency in the blood of AIH patients is associated with pharmacological immunosuppression, and therefore, not with the remission per se (30).

The complex cell and cytokine environment of the inflamed liver may have profound effects on Treg differentia-

tion, stability, and function as well as alter the susceptibility of effector T cells to suppression. In addition to the above mentioned interaction between Tregs and dendritic cells, local cytokines could reprogram Tregs to express either Tbet which would lead to a Th1 phenotype (57) or RORc and a Th-17 phenotype (58). We have described a higher Vα24, IFN-γ, FoxP3, p28, IL-12p40, and IL-21 expression at the moment of diagnosis as well as a positive correlation between IL-21 and aminotransferase levels. It is interesting that only IFN-γ and FoxP3 decreased during biochemical remission following immunosuppressive treatment (AIH-Ir). An "AIH-I phenotype" described as "high Vα24, IFN-γ, and FoxP3 expression" was observed in a low percentage of AIH-Ir children but not in healthy, age-matched controls. Overall, the presence of a local deregulation of the innate (i.e., NKT cells) and adaptive arms of the immune responses (i.e., Th1, Treg cells) was described (45). In addition, IL-21 was highlighted as a mediator of liver injury. Together with Th17 cells, iNKT cells are a source of IL-21. Since a seemingly limited presence of a Th17 subpopulation and an amplified expression of Vα24+ transcripts were detected, it has been suggested that iNKT cells may be the main source for IL-21 in the livers of patients with AIH-I.

IL-21, together with IL-2 and IL-15 are known to induce the proliferation of NKT cells (59). Apart from its role as a differentiation factor for Th17 cells from naïve cells, the inhibitory role of IL-21 on the differentiation of Treg FoxP3+ cells from the same precursor cells as those of Th17 cells is also noteworthy. The same is true of the ability of this cytokine to confer CD4+ cell resistance to the suppressing effect of Treg cells (60). The high levels of IL-21 found in the AIH-I inflammatory scenario but not in other inflammatory liver diseases (e.g. non-alcoholic fatty liver disease and HCV infection) make it possible to suggest that the immunoregulation of Treg and NKT cell subsets would constitute one of the most probable roles of IL-21 in the pathogenesis of AIH-I. The latter hypothesis implies that the high levels of FoxP3 found in the livers of pediatric patients reflects an increase in the number of Treg cells and that the prevailing function of IL-21 would be to confer resistance to the suppressant effector of Treg cells. Thus, and in spite of their local increase, FoxP3+ cells would not be able to suppress the autoimmune response. An increase in the recruitment of Treg cells from the peripheral compartment to the liver would constitute an attempt to suppress the local immune response. Treatment of AIH will fail if adoptively transferred Treg cells are inhibited or pushed into an effector differentiation pathway by local factors in the liver. Consequently, the need to shift the balance from damage towards resolution of inflammation emphasizes the importance of understanding the effects of the local inflammatory microenvironment (61). The fact that Treg cells are detected at high frequencies in the liver of patients with AIH suggests that suppression of function locally rather than a basic defect in Treg generation or function underlies the persistence of chronic autoimmune hepatitis.

Figure 2 depicts the interaction between genetic susceptibility, an antigenic peptide, and the production of different cytokines favoring the autoimmune attack (62).

OXIDATIVE STRESS

Reactive oxygen species (ROS) are highly reactive small molecules that are generated as by-products of oxygen metabolism during intracellular physiological processes. The production of ROS is finely regulated by enzymatic systems and reducing species that are able to clear these intermediates. Under physiological conditions, they play a crucial role in cell signaling pathways. However, when an imbalance between pro-oxidant and homeostatic anti-oxidant mechanisms occurs, a status known as oxidative stress arises. Under these circumstances, the ROS are cytotoxic due to their ability to react with most macromolecules. Thus, they induce the inactivation of enzymes, DNA damage, produce post-translational protein modifications, lipoperoxidation, and induce cell death (63).

Oxidative stress is not disease-specific, and its demonstration in animal models and humans with diverse liver diseases suggests that it has a complementary role in the pathogenesis of possibly all forms of acute and chronic hepatitis. It has been demonstrated that the livers of primary biliary chirrosis and AIH patients possess a higher expression of inducible nitric oxide synthase as well as nitrotyrosine (a result of an irreversible reaction between oxidant peroxynitrites and cell proteins) accumulation. It is likely that these findings suggest that oxidative damage to cell structures may play a role in the pathogenesis of AIH (64) and that complementary antioxidant therapy could be of value in selected patients (65).

ANIMAL MODELS

Due to the fact that AIH is often recognized during the late course of disease, it is difficult to know the immunological mechanisms responsible for initiation of the disease. Current AIH models have been helpful for understanding and modulating liver immune responses, but are not suitable for studying mechanisms in chronic AIH or for developing new therapies. The experimental AIH-I murine model induced by Concanavalin A has been useful for demonstrating that NKT cells are necessary for the development of the disease. Furthermore, the production of IL-4 by these cells plays an important role since this cytokine increases the autocrine cytotoxic activity by inducing the expression of granzime B and FasL (66).

While transgenic AIH models deal with short-term hepatitis, models with natural antigens are either self-limited or have unknown target antigens. Therefore, novel animal models with defined onset of AIH and a standard course of the disease are essential for a better understanding of the disease and its pathophysiology. To obtain a preclinical platform for new therapeutic approaches or to be able to prevent the onset of AIH, a positive impact of conventional, standard, therapeutic interventions in the model would be helpful. For decades, AIH re-

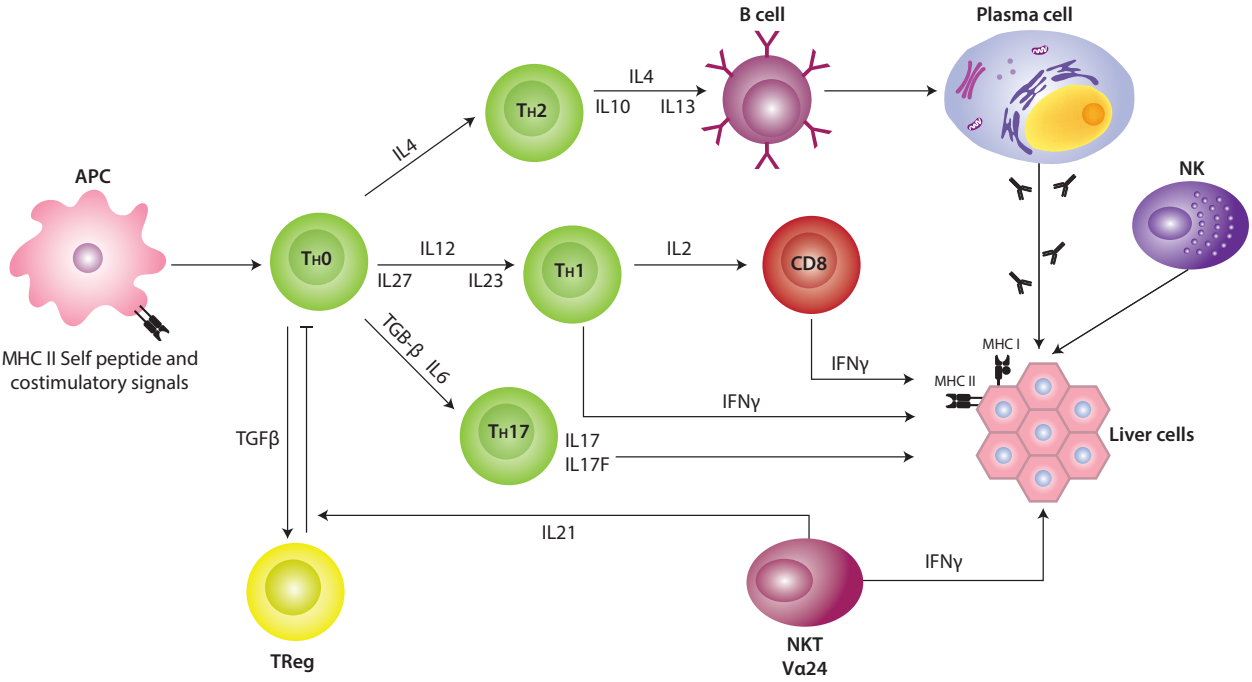

Figure 2 Autoimmune attack to hepatocytes. A specific autoantigenic peptide is presented to helper T cells (Th0 cells) within the context of class II HLA molecules together with co-stimulatory molecules present in the antigen-presenting cell (APC). Th0 cells become activated and, depending on the cytokine environment, give rise to different types of effector cells. The presence of IL-4 will allow the differentiation towards a Th2 phenotype featured by the secretion of IL-4, IL-13, and IL-10. These cytokines will favor the maturation of B cells into antibody-producing plasma cells. Antibodies bound to the surface of hepatocytes, in turn, will contribute to cell damage through antibody-dependent NK cell cytotoxicity together with complement activation. The presence of IL-27, IL-12, and IL-23 promote the differentiation of Th1 cells. The latter ones secrete IL-12 and IFN-γ which stimulate CD8 T cells, upregulate the expression of class I molecules, and induce the expression of class II molecules on hepatocytes, thus exacerbating the inflammatory process. TGFβ, IL-21, and IL-6 promote the differentiation of Th17 cells which secrete the pro-inflammatory cytokines IL-17A and IL-17F. Treg cells, which are specialized in suppressing responses to autoantigens, undergo differentiation under the influence of TGFβ and IL-10. In spite of the large number of Treg cells found in the livers of patients with AIH, their presence is not enough to hamper the damage to the liver. The latter finding suggests the participation of inflammatory mediators in the local milieu, such as IL-21, which hinders the normal functioning of this cell type.

search has lacked such a reliable preclinical model with chronic immune response against the liver. Initial results in breaking tolerance to hepatocytes have only led to mild and transient hepatitis. Transgenic models were helpful in understanding different aspects of hepatic immune regulation. At present, the fate of T cells, especially CD8+ T cells, is the focus of research. Ignorance, anergy, deletion, or TCR downregulation of T cells especially are the mechanisms of tolerance against hepatic antigens. Furthermore, the importance of professional antigen-presenting cells, and particularly liver sinusoidal cells, in liver tolerance has been demonstrated in many studies. Other models have shown the mechanism of interaction of adaptive and innate immune cells in the liver. Recently, approaches have been made to establish AIH models reflecting the situation in AIH patients. This will allow new studies in the field to be done and will provide an opportunity to study the onset and pathophysiology of AIH. Furthermore, new options for therapeutic approaches will be tried out in these models, and options to prevent onset of disease may be shown (67).

CONCLUSIONS

Due to the high incidence in many geographical areas as well as to the high percentage of liver transplantations due to autoimmune hepatitis, it is important to encourage more insightful studies to be done. Experimental evidence allows us to conclude that the liver damage in AIH-I would be a consequence of not only a Th-1-biased response but also the deregulation of hepatic Treg cell function. The molecular species responsible for the development of the different types of this disease in both children and adults remains to be determined. Knowing the autoantigen that triggers these processes is of paramount importance for it would broaden the frontiers for the development of more accurate methodologies of diagnosis, prognosis, and treatment of this pathology.

Follow-up studies are necessary to elucidate the mechanisms of the immune response that remain active despite the treatment and whether those pathways would be amenable to further therapeutic intervention.

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32

PRIMARY BILIARY CIRRHOSIS

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INTRODUCTION

Primary biliary cirrhosis (PBC) is an autoimmune liver disease with an incompletely understood etiology characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts leading to cholestasis and occasionally to fibrosis and secondary biliary cirrhosis (1).

The term "primary biliary cirrhosis" has been used for decades, but it is a source of confusion for patients and physicians since at the time of diagnosis many patients do not have structured fibrosis on biopsy.

EPIDEMIOLOGY

The PBC affects people worldwide although it has generally been considered more prevalent in Europe and North America (2). The disease predominantly affects women with a female:male ratio of 9:1. The symptomatology may differ based on sex. For example, males are at a higher risk of life-threatening complications such as gastrointestinal bleeding and hepatoma (3). The average age at onset is 50 years with a range of 20 to 90 years (4), an incidence of 0.7 to 49 cases per million a year, and a prevalence between 6.7 and 492 cases per million (2).

ETIOLOGY AND PATHOGENESIS

GENETICS

Various reports and case series have identified familial forms of PBC. In a study in the northeastern part of England, it was estimated that the relative risk for a first-degree relative of a patient with the disease to develop PBC was 10.5, a finding that is close to that observed in other autoimmune diseases (ADs) (5). The concordance rate for PBC in monozygotic twins is 63 percent (6). A higher report has been shown people with polymorphism of the gene for the vitamin D receptor (7).However, HLA polymorphisms are confirmed as the strongest association (e.g.HLA-DRB1*08) (8).

Non-HLA genes associated with PBC include interleukin 12A (IL12A) and IL12RB2 indicating that the IL-12 immunoregulatory-signaling axis is relevant to the pathophysiology of PBC (9). STAT4, IRF5, TNPO3, 17q12.21 locus, MMEL1, SPIB, and CTLA4 are other Non-HLA genes also influencing the risk of acquiring PBC (Table 1) (10-20).

MECHANISMS OF LIVER DAMAGE

The histological changes seen in the liver in PBC are mainly related to damage in biliary epithelial cells (BECs) in relation to T cell, B cell, macrophage, eosinophil, and natural killer (NK) cell infiltration in the portal area. A histological and immunological PBC paradox is the presence of primordial attack on the biliary epithelium but not on mitochondrial proteins despite the fact that antimitochondrial antibodies (AMAs) are marker of the disease (9). T cells, predominantly CD8 + type, are located in periductal areas and expression of Th1-type cytokines (IFN-γ) and Th2 (IL-4 and IL-5) is observed (21,22).

IL-1β and IL-6 promote the differentiation of Th17 cells, which may also be implicated in the pathogenesis of PBC. Th17 cells produce proinflammatory cytokines associated with autoimmunity, e.g., IL-17, IL-22, and TNF-α. The hepatocytes in PBC secrete IL-6 which further stimulates activation of Th17 (23,24). The serological hallmark of PBC is the AMAs which are mainly directed towards the E2 component of the pyruvate dehydrogenase complex (PDC-E2). There are other autoantigens for AMA targets: the branched chain 2-oxo-acid dehydrogenase complex, the ketoglutaric acid dehydrogenase complex, dihydrolipoamide dehydrogenase, and the binding related proteins (25). The expression

GENOTYPE	TYPE OF ASSOCIATION	REFERENCE
*HLA-DRB1*08*	Positive	9,11,12,13
*HLA-DRB1*0801*	Positive	11,19
*HLA-DRB1*08-C4B2*	Positive	17
*HLA-DRB1*08-C4Q0*	Positive	18
*HLA-DRB1*0501 P*	Positive	14
*HLA-DQB1*0402*	Negative	19
*HLA-DQA1*0102*	Negative	12
*HLA-DRB1*11*	Negative	15
*HLA-DRB1*11*	Positive	11
*HLA-DRB1*13*	Positive	11
HLA-DQB1	Positive	9
CTLA4	Conflicting results	8,9,10,11,20
IL1B	Positive	19
IL12A	Positive	9,11
IL12RB2	Positive	9,11
STAT4	Positive	9,11
IRF5	Positive	11
SPIB	Positive	11
TNPO3	Positive	11
MMEL1	Positive	11

Table1. Genes associated with susceptibility to developing PBC. *HLA*:Human leucocyte antigen, *CTLA4*: cytotoxic T-lymphocyte-associated protein 4, *IL1B*: interleukin 1β, *IL12A*: interleukin-12, , *STAT4*: signal transducer and activator of transcription 4, *IRF5*:interferon regulatory factor 5, *SPIB*: Spi-B transcription factor, *TNPOS*: protein-coding transportin 3, *MMEL1*: membrane metallo-endopeptidase-like 1.

of PDC-E2 in the membrane appears to be a trigger for the immune response during apoptosis of bile-duct cells (26-28). Specific xenobiotic modifications of the inner lysine–lipoyl domain of the PDC-E2 have been shown to be immune reactive when tested with patient serum (25-27). The signal induced apoptotic BECs are generated by cytotoxic effector cells (29) (Figure 1).

HUMORAL IMMUNITY - AUTOANTIBODIES

The presence of AMAs in PBC is pathognomonic. The pathogenetic role has been attributed to AMA IgA due to its transportation through BECs. AMA IgA has been shown to induce caspase activation. AMA specificity is 95% and sensitivity is nearly100% (31). AMAs are directed to a subunit of the 2-OXO acyl dehydrogenase complex (32-40). The enzyme catalyzes the transfer of a group from acetyl coenzyme A to the substrate for oxidation in Krebs's cycle.

Multidomain complexes which are directed towards the AMAs have subunits known as E1, E2, and E3. The E1 subunit corresponds to pyruvate dehydrogenase, E2 to dihydrolipoamide acetyltransferase, and E3 to dihydrolipoamide dehydrogenase (41). Multidomain complexes identified are:

Pyruvate dehydrogenase complex (PDC): E1α subunit, E1β, E2, and E3. E2 subunit protein is related to the «E3 binding protein» (E3BP) and the E3 subunit. It is attached to the latter through a «binding domain» rich in alanine and proline with catalytic activity.

Oxoglutarate dehydrogenase complex (OGDC) E1α subunit, E1β, E2, and E3. Not related to the E3BP.

Branching oxoacid dehydrogenase complex (ADC BCO): E1α subunit, E1β, E2, and E3. Not related to the E3BP.

The AMAs found most frequently in patients with PBC are directed against the subunits E2, E3, and E3BP of PDC (42-45). These antibodies are found in almost 100% of the patients.

In addition to the AMAs, the patients with PBC also show anti-nuclear antibodies (ANAs). About 30% of patients have positive ANAs which can also be predictive of the future development of other ADs, i.e., polyautoimmunity (46). ANAs in serum samples from patients with PBC often occur in a rim-like pattern in immunofluorescence. These findings suggest that targets of the ANAs are components of the nuclear envelope (NE). Two autoantibodies have been recognized that act against nuclear proteins. One is a nuclear membrane protein, the gp210. This is a type I membrane protein integral that anchors NPCs to the pore membrane. The other corresponds to the nuclear body protein sp100 (46). Anti-gp210 antibodies in PBC are immunoglobulin G isotype and primarily recognize a 15-amino acid linear stretch within the C-terminal domain of the protein. The prevalence of anti-gp210 in PBC ranges from 9.4% to 41.2% with a speci-

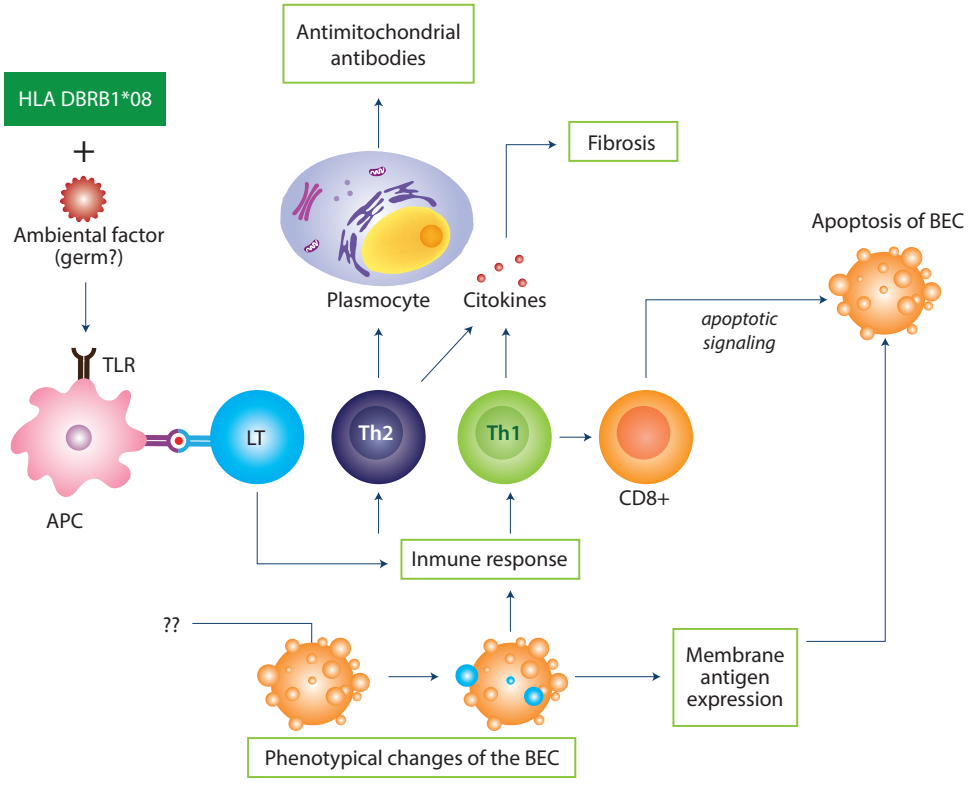

Figure 1. Mechanisms of damage of biliary epithelial cells (BEC) in PBC.

ficity> 96% (46-48). A thin nuclear dotted pattern is seen in indirect immunofluorescence (49) (Figure 2).

The prevalence of anti-sp100 antibody is about 25% in PBC and is highly specific, but they can be found in autoimmune hepatitis, systemic lupus erythematosus, and Sjögren's syndrome (SS) (31). Nuclear bodies are multiprotein complexes that have been observed in all mammalian cell lines. These antibodies generate a dotted pattern in immunofluorescence. Other known types of ANAs in PBC patients are directed against LBR (related protein inner nuclear membrane) (51), PLM (protein-nuclear bodies) (52), p62 (the protein complex the nuclear pore membrane) (53), and carbonic anhydrase II (54).

In addition to high titers of circulating AMAs, PBC patients have high levels of serum IgM that are not related to titers of AMAs. Antibodies in the portal tracts of PBC patients are found to be predominantly IgM while those cells predominantly express IgG in other forms of liver disease such as autoimmune hepatitis or chronic hepatitis associated with HCV infection (55). The presence of high levels of IgM in sera is directly related to the presence of interlobular bile duct lesions. Genetic analysis of the CD40L promoter on T cells from patients with PBC showed that there are significantly lower levels of DNA methylation of the CD40 ligand promoter. Furthermore, this decreased methylation was inversely correlated with levels of serum IgM in PBC patients (56).

CELL IMMUNITY

In patients with PBC, an enhanced ratio of Th1 to Th2 cells is one of the most important findings (25). A significant infiltration of T cells is found in liver lesions, mainly around the bile duct. These T cells invade the epithelium neighboring BECs with pathological changes. The infiltrate of T cells is mixed– CD4+ and CD8+. Both Th1 and Th2 type cytokines have been identified with a preponderance of Th1. This presumes a greater involvement of immune cell component (57). Antigen presenting cells, major histocompatibility complex (MHC) molecules, T cell receptors, co-stimulatory molecules, and adhesion molecules have been identified in liver lesions of PBC (59). The main characteristics of patients with PBC are summarized in Table 2.

POTENTIAL TRIGGERS

In patients with greater genetic susceptibility to PBC, immune responses to liver autoantigens could be triggered by molecular mimicry (24). Several candidates have been suggested as causative agents, including bacteria, viruses, chemicals, and xenobiotics. The association between bacterial infections and PBC has been investigated (31), particularly the role of Escherichia coli (60,61) (Chapter 19). Other mechanisms involved in the development of PBC are halogenated hydrocarbons which are capable of inducing

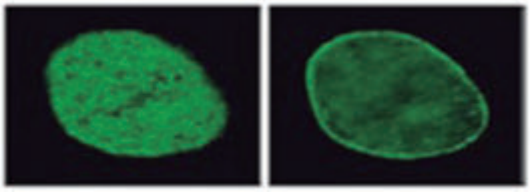

Figure 2. Indirect immunofluorescence in PBC. Using human HeLa cells and sera of patients with PBC, a fine dot pattern (left), and/or a perinuclear ring pattern (right) can be seen in the nucleus of cells. These later antinuclear antibodies are directed against the gp210 protein expressed in the nuclear membrane. Adapted from reference (49).

CHARACTERISTICS	COMMENT
Disease onset	40-60 years
AMA	95%
ANA	30%
Cytokines	Th1, Th17 increased
T regulatory cells	Diminished
Immunoglobulins	IgM Increased
Liver inflammation CD8+/CD4+Tcells	Increased
Fibrosis	Evident in late stage
Polyautoimmunity	AITD, Sjögren's syndrome

Table 2. Characteristics of patients with primary biliary cirrhosis. *AMA*: antimitochondrial antibodies, *ANA*: antinuclear antibodies, *AITD*: autoimmune thyroid diease. Adapted from reference (25).

antibodies that have an affinity for the human pyruvate dehydrogenase complex (62). Epidemiological studies have strongly implicated smoking as a risk factor for PBC, and it may also increase the risk of fibrosis (63). Immunosenescence may also contribute to the cell pathology of PBC (64) (Chapter 11) (Table 3)(60-73).

EPIGENETICS

Environmental influences could also be demonstrated by epigenetic studies. A recent study attempts to explain PBC female prevalence based on the hypothesis of an epigenetic component in the X chromosome. The results suggest that methylation at X-linked promoters is implicated in the pathogenesis of PBC (74). Another study showed that CD40 ligand promotor methylation is inversely correlated with IgM levels in patients with PBC (56).

CLINICAL FEATURES

ASYMPTOMATIC PHASE

Between 50 to 60 percent of patients are asymptomatic. However, CBP is now diagnosed earlier due to the wide spread use of screening laboratory tests (1). Clinical suspicion is based on the finding of elevated serum AMAs or alkaline phosphatase (ALP) in the tests requested as part of the study of other non-hepatic autoimmune diseases. Many individuals without symptoms and positive AMAs show features consistent with PBC on liver biopsy. Many of these patients develop clinical symptoms of cholestasis later (1,24).

SYMPTOMATIC PHASE

The most common symptoms are fatigue and pruritus that are found in up to two-thirds of the patients at diagnosis. The severity of the degree of fatigue is not connected to the degree of severity of the liver disease (1). Pruritus can occur at any time over the course of the disease and is characterized by intermittent episodes during the day and episodes that are more annoying at night (75). Jaundice occurs frequently in the later stages of the disease, and if it occurs persistently, it becomes a symptom of poor prognosis. Cutaneous hyperpigmentation, hepatosplenomegaly, and xanthelasma (i.e., cholesterol skin deposits) are frequently found in the physical examination of individuals with PBC in intermediate stages. Other common findings include hyperlipidemia, hypothyroidism, and osteopenia as well as symptoms associated with the development of polyautoimmunity. In advanced stages of the disease, the characteristic signs of extensive liver involvement, e.g., ascites, encephalopathy, and signs of portal hypertensionappear (76) (Table 4).

ASSOCIATED DISEASES

Polyautoimmunity is not uncommon, SS being one of the most frequent observed (77). Many of the diseases associated with the PBC are related to autoimmune phenomena

TRIGGER	COMMENT	REFERENCE
Gram positive	High frequency of urinary tract infections Phagocytic cells containing lipoteichoic acid MCP-2 and MCP-3 in mononuclear cell infiltrates portal tract	(65-67)
E. coli	Cross-reaction with peptide clones Bacterial LT and autoantigens	(60, 61, 68)
Chlamydia pneumoniae	Specific antigen of Chlamydia pneumoniae in liver tissue samples.	(69, 70)
Retrovirus	Reactivity of antibodies against retroviral antigens Viral particles in the biliary epithelium by electron microscopy Retroviral specific nucleotide sequences	(71-73)
Halogenated hydrocarbons	Induction of antibodies with affinity for the PDC.	(62)
Smoking	Alterations in cytokine production IL-13, IL-10, TH1 cytokine response, IFN-g	(63)
Immunosenescence	Decreased Treg response Increased CD127 and decreased CD39 on CD8+ T cells DNA damage gammaH2AX-DNA-damage-foci detected in BEC Increased apoptosis Apoptotic marker Bcl-2 in BEC (may increase PDC-E2 exposure) Increased apoptotic marker CD95 (Fas) on BEC Unmodified PDC-E2 found in apoptotic blebs (apotopes) in BEC Increased expression of senescence markers p16 and p21 in BEC Increased autophagy, increased LC3 expression in BEC	(64)

Table 3. PBC triggers.

such as those indicated in Table 5 (78). An overlap between autoimmune hepatitis and PBC might be observed (79). There also seems to be an increased incidence of malignancies and hepatocellular carcinoma (80).

Seventy percent of the patients with PBC have osteopenia and 11% have osteoporosis. The origin of the disease appears to be mainly due to a decrease in bone formation secondary to disruptions in the regulation of bone turnover through alterations in osteoclasts, osteoprotegerin, and the ligand (RANKL) (81). Deficiency of fat-soluble vitamins is uncommon in patients with PBC. A, D, E, and K are 33.5%, 13.2%, 1.9%, and 7.8% deficient respectively (82).

DIAGNOSIS

BIOCHEMICAL CHARACTERISTICS

The most common scenario is to find anicteric cholestasis with an increase in the serum ALP and gamma-glutamyl transpeptidase (GGT). While the albumin and prothrombin time (PT) are affected when disease progresses, hyperbilirubinemia may be present earlier than any other signs of liver failure (83). Hypercholesterolemia may occur in 85% of the patients at diagnosis. Approximately 70% of patients show elevated serum IgM (84).

SEROLOGICAL CHARACTERISTICS

Between 90-95% of the patients with PBC are AMA positive at diagnosis with titers above 1/40 (83,85). ANAs are positive in approximately 48% of the cases, anti-Sp100 antibodies may be found in 18% - 44%, and anti-gp210 is associated with aggressive disease and progression to liver failure (83). Patients who present with CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) also have anticentromere antibodies (7,86). Other autoantibodies observed are: rheumatoid factor (70%), antithyroid antibodies (40%), and smooth muscle antibodies (35%). The AMAs are negative in approximately 5-10% of patients when immunofluorescent techniques are used. Antibodies that act against the major M2 components may still be identified using enzyme-linked immunosorbent assay or western blotting techniques (83). However, negative AMAs with clinical features suggestive of the disease have been called «autoimmune cholangitis» (87) and natural history in these cases is similar to positive AMA PBC patients (88).

RADIOLOGICAL CHARACTERISTICS

Imaging studies are designed to rule out an extrahepatic cholestasis source and evaluate the presence of hepato-biliary disease; portal hypertension with cirrhosis stigmata, infiltrative disease, and malignancies. Ultrasonography gives good sensitivity and specificity to clarify this point. Other studies, e.g., computed tomography and magnetic resonance imaging provide additional information, especially in situations where there is doubt about the presence of associated neoplasia. Also, hepatic lymphadenopathy may be observed (83).

HISTOLOGICAL CHARACTERISTICS

Routine liver biopsy is rarely used in CBP, mainly because the treatment is limited to ursodeoxycholic acid (UDCA) at any stage of the disease and staging can be evaluated reasonably efficiently by serum markers of fibrosis, imag-

FINDINGS	FREQUENCY (%)
Fatigue	65-70
Pruritus	55-65
Jaundice	10
Hyperpigmentation	25
Hepatomegaly	25
Splenomegaly	15
Xanthelasma	10
Asymptomatic	25

Table 4. Clinical findings of PBC at the time of diagnosis.

DISEASE	FREQUENCY (%)
Keratoconjunctivitis sicca*	72-100
Renal tubular acidosis	50-60
Arthritis/arthropathy	40-42
Cholelithiasis	33
Autoimmune thyroiditis	15-20
Skin disorders (lichen planus, discoid lupus, pemphigoid)	11
Raynaud's phenomenon	8
Systemic sclerosis	3-7
Hepatocellular Carcinoma	1-2

Table 5. Diseases associated with PBC. * Sjögren's syndrome should be ruled-out (see Chapter 28).

ing, and fibroscan (83). However a liver biopsy is considered essential in the diagnosis of PBC with negative AMAs. The liver biopsy should be interpreted cautiously since the liver is not affected uniformly, and a single biopsy may demonstrate the presence of all four stages at the same time (83). The Ludwig histological classification is the most widely used (89). In stage I there is inflammatory destruction of intrahepatic septa and interlobar bile ducts that goes as far as a diameter of 100 μm. Portal tracts are also invaded by lymphocytes, neutrophils, and eosinophils. In stage II, the inflammation extends from the portal tracts into the parenchyma in a condition called interface hepatitis (formerly called «piecemeal necrosis»). Cholangitis and granulomas are also seen. Stage III is characterized by septal cirrhosis or bridging with cholestasis and ductopenia which results in increased copper deposits around the portal space and paraseptal hepatocytes. Stage IV represents the end stage of the lesion with cirrhosis and regenerative nodules (83,89).

NATURAL HISTORY AND PROGNOSIS

PBC is characterized by the slow progression of cholestasis with progressive liver inflammation towards the development of fibrosis and its associated complications. In the present era, patients are more likely than in the past to be asymptomatic at diagnosisand to receive medical treatment earlier. In at least 25 to 30 percent of the patients with PBC who are treated with UDCA, a complete response may occur (1). Asymptomatic patients have a higher rate of survival than symptomatic patients. However, their life expectancy is lower than the life expectancy for healthy subjects. The symptoms of the disease appear in approximately 40% of the patients over the course of 5 to 7 years of follow-up and once developed, the life expectancy is significantly reduced with a median survival of about 10 years (90). Among the factors that are associated with decreased survival are jaundice, irreversible loss of bile ducts, cirrhosis, enlarged liver, ascites, variceal bleeding, stage III or IV on liver biopsy, and the presence of other autoimmune diseases. One of the best predictors of survival is the bilirubin level (91).

TREATMENT

PHARMACOTHERAPY

There are a variety of medications that have been used in PBC. UDCA is the drug for treating PBC that has been evaluated the most. There are a variety of medications used for PBC that have had conflicting results, were poorly defined, andhad little or no effect on symptomatic improvement or delayed the decision to do a liver transplantation. The drugs used the most are: azathioprine, colchicine, methotrexate, glucocorticoids, and cyclosporine (CsA) (92-98).

UDCA mechanisms for action include inhibition of intestinal absorption of toxic endogenous bile salts, stabilizing the hepatocyte membrane, replacement of the endogenous bile acids, and reduction of expression of HLA class I and class II in the biliary epithelium. UDCA (13 to 15 mg/kg per day) delays the progression to end-stage liver disease and is well-tolerated. However, a third of the patients still develop progressive disease. Close to 10% of the patients require liver transplantation (99). A recent systematic review (100) did not demonstrate any significant benefits from UDCA for all causes of mortality or liver transplantation, pruritus, or fatigue. UDCA seemed to have a beneficial effect on liver biochemistry measurements and on histological progression compared to the control group. Farnesoid X receptor agonists are under investigation for potential therapeutic use in PBC in animal models (101). In addition, there may be some benefit in the use of combination therapy in patients who do not respond to UDCA, e.g., triple therapy with UDCA, budesonide, and mycophenolate mofetil(102).

SURGICAL TREATMENT

The treatment of choice for patients with liver failure secondary to PBC is liver transplantation, and this is one of the main indications throughout the world for this treatment. The disease may reoccur; however, the outcomes are usually favorable. The frequency of recurrent PBC usually increases progressively over time, and histological disease

is reported in about 30% of the patients 10 years after the liver transplant (103,104). Another consistent observation from many transplant programs is that the more potent immunosuppressive regimens using tacrolimus accelerate the onset and severity of recurrent disease. In contrast, CsA based regimens have been linked to a diminished incidence of recurrent disease (105).

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33

CELIAC DISEASE

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INTRODUCTION

Celiac disease (CD) is an autoimmune intestinal disorder affecting approximately 1% of the general population. The disease is produced by an immune-mediated enteropathy triggered by ingested prolamins present in wheat, barley, and rye (generically called gluten) occurring in predisposal individuals carrying the characteristic HLA haplotype DQ2 and/or DQ8. The disorder is characterized by almost invariable mucosal damage as a consequence of both the innate and adaptive immunologic response to the offensive proteins in the small intestine mucosa (1).

Celiac disease has been classified into a group of gluten-related disorders such as other autoimmune diseases (ADs) (gluten ataxia and dermatitis herpetiformis), wheat allergy (respiratory allergy, food allergy, wheat dependent exercise-induced anaphylaxis and contact urticaria) and non-autoimmune and non-allergic disease (gluten sensitivity) (2). Gluten comprises a broad group of prolamins (gliadins and glutenins) found in wheat. Other prolamins are also found in rye (secalins) and barley (hordeins) (3).

Due to lack of common definitions for the spectrum of terms and disorders related to CD, recently the different terms have been redefined. Therefore terms as "asymptomatic CD" or "silent CD" should be reclassified as "subclinical CD". The terms typical, atypical and latent CD should no longer be used. Table 1 summarizes some related terms and some recommended terms (3).

EPIDEMIOLOGY

Celiac disease is a common disorder that is very frequent in highly populated countries, where inhabitants have a white ancestry, mainly located in Europe and North America. However, CD has also been reported among people with Amerindian or African American origins (4,5). CD affects 0.6 to 1.0% of the population worldwide, the female-to-male ratio is 2.8:1 and the age distribution of onset shows a first peak between nine months and two years and a second peak during the fourth decade (6). Many epidemiological studies have shown that the prevalence of this disease varies according to the population (Table 2) (7–32).

The distribution of the CD has been associated with migratory patterns and changes in feeding habits over time. In the early days, man was not exposed to gluten-containing cereals. It was only 10 000 years ago in a small region called the "Fertile Crescent" of the Middle-East including Anatolia (Southern Turkey), Lebanon, Syria, Palestine and Iraq) where wild wheat and barley grains were successfully cultivated due to favorable environmental conditions. In this region some tribes settled, because the cultivation of the land allowed for storage of food. Then, people migrated to the Mediterranean area (Northern Africa and Southern Europe) and Central Europe searching for new lands for cultivation. The expansion continued from 9000 to 4000 BC to reach Northern Europe (Ireland, Denmark and the Scandinavian countries) (6).

The frequency of CD is likely to increase in many developing countries due to the progressing "westernization" of the diet, or by changes in wheat production or food elaboration. For instance, over the past 30 years, the prevalence of CD in the United States has increased five-fold, doubling approximately every 15 years (35).

Moreover, the prevalence of CD has increased among first-degree relatives of people affected with CD (10 to 15%), type 1 diabetes (3 to 16%), Hashimoto's thyroiditis (5%) or other ADs (Sjögren's syndrome, liver diseases, psoriasis and IgA nephropathy), IgA deficiency (9%), Down's syndrome (5 to 19%), and Turner's syndrome (3%) (35,36).

On the other hand, genetic susceptibility given mainly by HLA and other non-HLA genes are involved in the develop-

TERMS	CHARACTERISTICS
Classical CD, a term related to CD	Presents diarrhoea, steatorrhoea, and weight loss or growth failure. Moreover signs and symptoms of malabsorption (anemia, oedema secondary to hypoalbuminemia, alterations in calcium metabolisms, among others). Children with classical CD are often characterized by thrive failure, diarrhoea, muscle wasting, poor appetite, abdominal distension, emotional distress ('change of mood') and lethargy. Examples of classical CD are patients with diarrhoea and weight loss but also patients with weight loss and anaemia
Non-classical CD, a term related to CD	Patients do not suffer from malabpsorption (i.e., a patient with constipation and abdominal pain but no malabsorption). Patients with monosymptomatic disease (other than diarrhoea or steatorrhoea) usually have non-classical CD
Symptomatic CD, a recommended term	Characterized by clinically evident gastrointestinal and/or extraintestinal symptoms attributable to gluten intake. The clinical manifestations of CD vary from none (asymptomatic CD) to a broad spectrum of symptoms
Subclinical CD, a recommended term	It is below the threshold of clinical detection without signs or symptoms sufficient to trigger CD testing in routine practice
Potential CD, a recommended term	People with normal small intestinal mucosa who are at increased risk of developing CD as indicated by positive CD serology

Table 1. The Oslo definitions for celiac disease. Terms related to CD and recommended terms. Adapted from Ludvigsson *et al.* (3).

ment of the disease. The HLA-DQ2 haplotype (DQA1*0501/DQB1*0201) is present in 90% of the patients with CD and this haplotype is also present in one third of the general population. The HLA-DQ8 haplotype (DQA1*0301/DQB1*0302) is present in 5% of patients and the remaining 5% of patients have at least one of two genes encoding DQ2 (DQA1-05-01 or DQB1*0201) (Chapter 17) (35).

PATHOGENESIS

Celiac Disease is determined by both genetic and environmental factors. The presence of gluten is necessary to develop an autoimmune response against the epithelium of the small intestine, HLA-DQ 2 or HLA-DQ 8 haplotypes, other non-HLA genes, and failures in both innate and adaptive immunity (Figure 1).

Alterations in the tight junction (TJ) system cause changes in the permeability and integrity of the intestinal epithelium. TJ are regulated by zonulin, this protein apparently alters epithelial permeability increasing the passage of gliadin and favoring the trigger of an autoimmune response (37). The passage of gliadin through the epithelium may also be accomplished by transcytosis (38) or by retrotrancytosis of secretory IgA (sIgA) by the transferrin receptor, denominated CD71. In active CD, CD71 expression is strongly upregulated at the apical surface of small intestinal enterocytes allowing the binding of intraluminal anti-gliadin IgA complexed to gliadin peptides and their subsequent retrotransport into lamina propria via the recycling endocytic pathway (39,40).

The gluten proteins have high content in glutamine (Q) and proline (P) residues assembled in related repetitive sequences. Proline-rich peptides are resistant to proteolysis by digestive enzymes (41). Peptides with Q and P rich motifs (notably Q-X-P sequence) are excellent substrates for the interaction with the tissular transglutaminase 2 (tTG2). This enzyme (78-kDa) is present in liver, heart, retina, intestine, placenta as well as erythrocytes, endothelial cells, immune cells and fibroblasts. tTG2 is involved in the stabilization and remodeling of the extracellular matrix, regulation of cell death, differentiation and apoptosis (42).

The tTG2 fulfills two key roles in the pathogenesis of the disease: as a deamidating enzyme, enhancing the immunostimulatory capacity of gluten, and as a target autoantigen in the immune response. The deamidation is a pH and calcium - dependent process (43,44). At low pH and in the absence of donor polyamines, tTG2 converts glutamine (neutral charge) to glutamate (negative charge), and thus, a more tightly binding of the negatively charged aminoacids to HLA-DQ2 and DQ8. This mechanism is similar to what occurs in rheumatoid arthritis by deamidation of arginine to citrulline mediated by peptidyl arginine deiminase (PAD) for recognition of HLA (45,46).

The positively charged pockets P4, P6, and P7 in HLA-DQ2 and P1 and P9 in HLA-DQ8 have a preference for negatively charged anchor residues. HLA-DQ2 and –DQ8 preferentially bind a glutamate residue of gluten peptide at positions 6 and 1/9 respectively (46).

In addition to being involved in the deamidation process, it is proposed that the binding of tTG2 to gliadin peptides can also induce gliadin-tTG crosslinking, resulting in the formation of a gliadin-tTG inmmunogenic complex and in increased production of anti-tTG IgA antibodies. An antibody-mediated inhibition of tTG functions by still unclear mechanisms, is involved in a decreased cell adhesion that decreases stabilizing matrix proteins and alters the wound repair (45). However, the presence of anti-tTG2 IgA is not sufficient to induce tissue damage in patients with subclinical CD (47).

Gluten-reactive CD4+T cells are activated by the presence of different epitopes with the 33-mer gliadin fragment LQLQPFPQPQLPYPQPLPYQPQLPYPQPQPF being this one the most immunogenic peptide (41). Activated T cells produce high levels of pro-inflammatory mediators with IFN- γ as the predominant cytokine, and display a characteristic T helper 1 (Th1) pattern of immune response (48).Interleukine (IL)-1β together with IL-1α, IL-18, and IL-33, has been

COUNTRY	PREVALENCE	CHARACTERISTICS OF POPULATION STUDIED			ANTIBODIES TESTS			BIOPSY
		n	Age (yr) Mean/median (range)	Females %	AGA	tTGA	EMA	
Finland (8, 9)	1:42	4846	NA (30-64)	53		+	+	+
	1:47	2815	NA (52-74)	52		+		+
Argentina (10, 11)	1:79	2219	NA (3-16)	38		+	+	+
	1:167	2000	29 (16-79)	50	+		+	+
England (12)	1:83	7550	59 (45-76)	59		+	+	
Turkey (13, 14)	1:100	906	38,6 (20-59)	50		+		+
	1:212	20190	NA (6-17)	NA		+	+	+
Italy (8-15)	1:100	1002	33 (13-90)	56.6		+	+	
	1:145	2759	NA (30-64)	58		+	+	+
Iran (16,17)	1:104	2799	33.7 (18-66)	50		+	+	+
	1:400	2000	35.5 (18-65)	21	+		+	+
United States (18-19)	1:105	2845	NA	57	+	+	+	+
	1:141	7798	38 (6-80)	44		+	+	
Switzerland (20)	1:132	1450	NA (12-18)	60,1		+	+	+
Libya (21)	1:146	2920	NA (5-17)	49.7				
Iceland (22)	1:136	813	36 (17-64)	23,7		+		+
Brazil (24-26)	1:214	2045	32.8 (18-61)	12,4	+		+	+
	1:286	4000	31 (18-65)	NA		+	+	+
	1:681	2045	32.8 (18-61)	12.5	+		+	+
Australia (27)	1:251	3011	NA (30-50)	NA			+	+
Germany (8-28)	1:270	2157	42.6 (18-65)	51.9	+	+	+	+
	1:344	3098	NA (30-64)	51		+	+	+
Netherlands (29, 30)	1:286	1440	40.6 (20-59)	54		+	+	
	1:333	1000	NA	NA			+	+
Spain (31)	1:390	1170	44.9 (2-89)	55,3	+		+	+
Greece (32)	1:558	2230	46 (18-80)	55		+	+	+
Tunisia (33)	1:709	1418	27.5 (17-57)	27		+	+	+

Table 2. Prevalence of celiac disease in different populations. Abbreviations: *AGA*: Anti-gliadin antibody; *tTG*: Anti-tissue transglutaminase; *EMA*: anti-endomysium antibodies; *NA*: Not available; +: Positive in the study. Adapted from Kratzer *et al.* (28).

associated with the inflammatory condition in CD patients. This cytokine has also been demonstrated to control the secretion of IL-23 that lead to Th1/Th17 immune pathways. Although Th17 cells participate in the pathogenesis of CD, the relative importance of each T cell response and their role in the initial events of the disease need further investigation (49). The dominant Th1 cytokines promotes inflammatory effects, including fibroblast or lamina propria mononuclear cell secretion of matrix metalloproteinases, which are responsible for tissue remodeling, resulting in villous atrophy and crypt hyperplasia (50). Other cytokines involved in this process of remodeling are TNF-α and IL-21,whose production is regulated by IL-15 (51).

Gluten-reactive CD4+Tcells may participate in the activation of intraepithelial lymphocytes (IELs) via cross-priming or via the production of IL-21 that synergizes with IL-15 produced by enterocytes, DCs and macrophages present in the lamina propria (52).

Additionally, gluten-reactive $CD4^+$ Tcells may differentiate into Th2 cells that are capable of triggering activation and clonal expansion of B cells that lead to the production of anti-gliadin and anti tTG- autoantibodies. By interacting with the extracellular membrane-bound tTG, tTG-autoantibody deposits in the basement membrane region might induce changes in the enterocyte cytoskeleton with actin redistribution and epithelial damage (53).

ROLE OF INTRAEPITHELIAL LYMPHOCYTES IN CD

Intraepithelial lymphocytes (IELs) are a population of T cells interspersed with enterocytes of the intestinal epithelium. IELs can interact with enterocytes to maintain epithelium

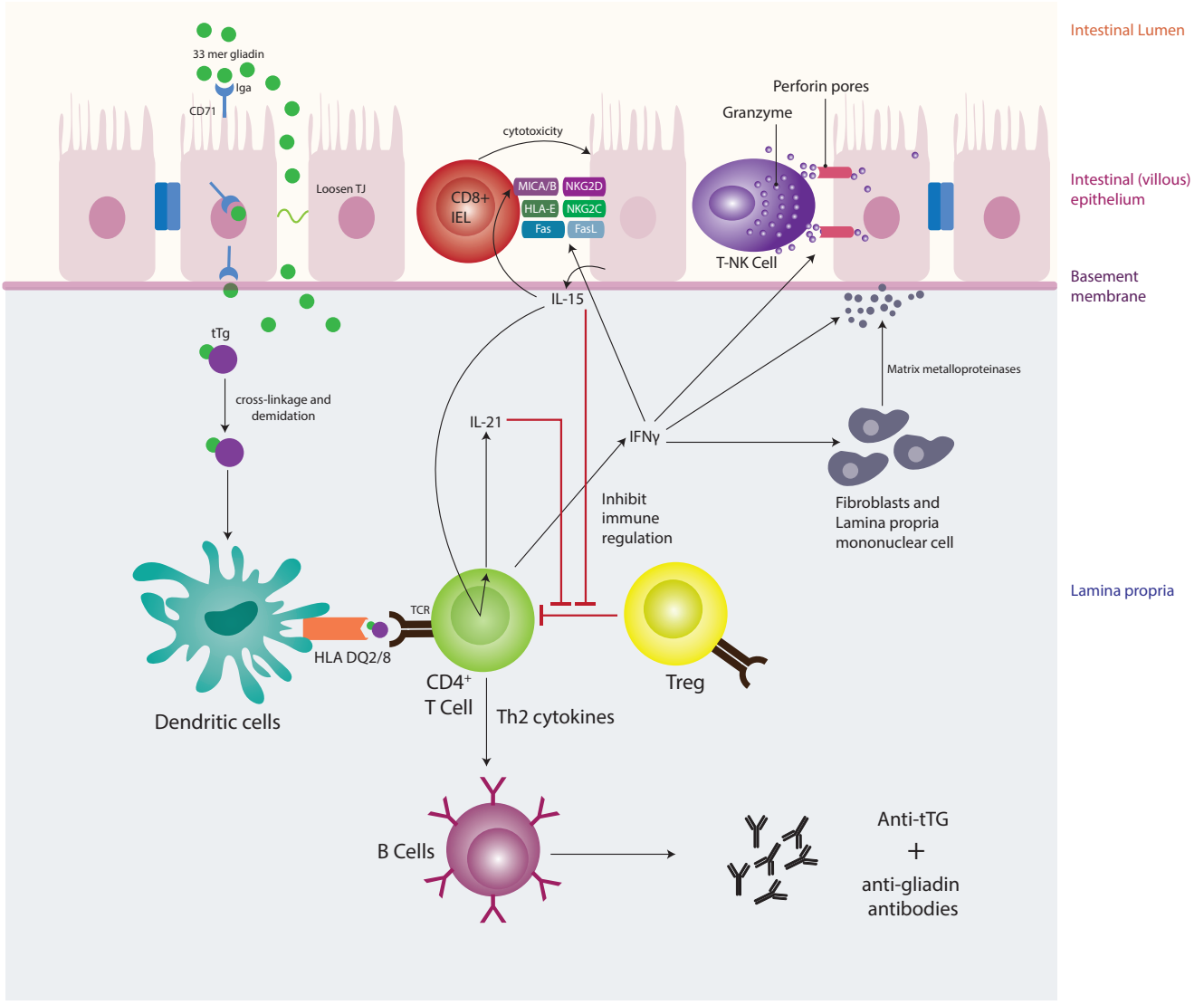

Figure 1. Steps involved in the development of celiac disease.

integrity and to prevent pathogenic damage. Small intestinal IELs are comprised mostly of TCRαβ+CD8αβ+ cells (75%), TCRαβ+ CD4+ (10%),TCRγδ+ cells (15%) and double negative (DN) or CD8+ T cells (<10%). Colonic IELs are mainly CD4+ T cells expressing TCR α and β chains, present in smaller numbers than duodenal IELs, and curiously displaying the ability to become activated in absence of costimulatory signals (54).

The main function of IELs is the immune protection by preventing the entry and spread of pathogens. Under steady-state conditions, these lymphocytes respond to stress signals produced by intestinal epithelial cells (IECs) and produce anti-inflammatory cytokines (IL-10, TGF-β) and keratinocye growth factor (KGF) to participate in IECs homeostasis, growth and wound repair. In non-pathological conditions, the IELs have a low cytolytic capacity, they express the inhibitory natural killer receptor (NKR) CD94/NKG2A, low levels of the activating NKR NKG2D, and low levels of perforin, while in inflammatory conditions the IELs and NKT cells exert their cytolytic functions to eliminate infected and damaged cells through several mechanisms mediated by granzyme B and perforins and by the production of INF-γ and TNF-α (54).

In the context of CD, TCRαβ+CD8αβ+ and TCRγδ+ IELs are involved in the intestinal tissue damage. In active CD, CD8+ IELs undergo marked expansion that is associated with enhanced intraepithelial expression of INF-γ, Fas/Fas ligand system, granzyme B, perforins and therefore epithelial apoptosis. There are two non-mutually exclusive hypotheses to explain the function of CD8+ T IELs in intestinal tissue damage. The first hypothesis is that of CD8+ IELs are activated through cross-presentation of gluten peptides. The second hypothesis suggests that the activation of CD8+ IELs is mediated by IL-15 and by NKR interacting with their ep-

ithelial ligands (52). The expression of NKR (CD 94/NKG2C and NKG2D) by CD8+ IELs is regulated by IL-15. Both CD 94/NKG2C and NKG2D recognize their respective ligands [HLA-E for CD 94/NKG2C and MHC class I polypeptide-related molecules (MICA and MICB) for NKG2D] expressed by IECs. These interactions between NKR and their ligands promote cell proliferation and secretion of inflammatory cytokines which leads to destruction of IECs and villous atrophy. In addition, IL-15 also acts as a costimulatory molecule for NKG2D cytolytic pathway in CD8+ IELs, which in turn, leads to the production and secretion of arachidonic acid, therefore promoting activation and recruitment of granulocytes and the generation of more intestinal inflammation (54). Surprisingly, IL-15 inhibits immune regulation produced by regulatory T cells (Treg) and high levels of this cytokine can promote the onset of T cells lymphoma (the most severe complication of CD) (52).

The role of TCRγδ+ IELs remains unknown. These cells might be involved in recognition of tissue-specific stress signals and might also help to maintain epithelium integrity by exerting cytotoxicity or by releasing soluble factors. Apparently, the accumulation of these cells may be a IL-15-driven process (52).

GENETIC FACTORS

The HLA DQ2 and HLA DQ8 are the most important genes associated with CD; however, HLADQ2/8 is necessary but no sufficient to explain CD development. Some polymorphisms in non-HLA genes are also necessary for disease development. Celiac disease has been associated with more than thirty non-HLA genes (55).The majority of these genes have also been reported to be related to other autoimmune diseases. These genes are involved in T cell and B cell functions such as antigen presentation and production of cytokines (46).These genes are involved in the differentiation (RUNX3, THEMIS, ETS1, SH2B3, IL12A, IL18R1, IL18RAP, IL1RL1, IL-1RL2), survival (FASLG, TNFSF18), migration (RGS1) and activation (CTLA4, ICOS, CD28, CD80, PTPN 2, IL2, FASLG, CD247, SH2B3, UBASH3A, PRKCQ, TAGAP, ARHGAP31) of T cells or in antigen presentation (CD80, TNFSF4, CIITA, ELM01, NFIA) (46,52). Genes associated with activation and migration of T cells such as MAP3K7, IL-21, CCR9 and RGS1 and genes associated with the activation and maturation of B cells such as ICOSLG, RGS1, BACH2, POU2AF1, TNFAIP3 and ZFP36L1 are also included (46,52).

Accordingly with the involvement of cytokines as IFN-γ and IL-21 in tissue damage, polymorphisms in genes involved in the synthesis of these cytokines such as STAT4, TNFRSF9, RUNX3, SOCS1, PTPN2 for IFN-γ synthesis, or IRF4 and ICOS for IL-21 synthesis have also been described to be associated with CD (52). (Chapter 18)

ENVIRONMENTAL FACTORS

Gluten coming from the diet is the most critical environmental antigenic factor recognized so far. Celiac disease is considered as an outstanding example of the loss of tolerance to a food protein in humans. To induce uncontrolled activation of the adaptive immune system this should be recognized in the context of "danger signals". Indeed several infectious agents have been associated with the development of CD such as *Campylobacter jejuni* (56), *Giardia lamblia* (57), rotavirus (58), hepatitis C virus (59,60), adenovirus type 12 (61,62) and enterovirus (63). It has been observed that that the treatment with IFN α of patients having hepatitis C virus might contribute to induction of the disease (64).

The composition of the intestinal microbiota has also been related to CD. Changes of its composition due to the treatment of patients with a gluten-free diet suggest that intestinal microbiota may play a role in the manifestation of the disease (65). Alterations in the microbiota have also been associated with other ADs where a loss of tolerance and uncontrolled induction of the immune system does occur (Chapter 19).

Other environmental factors that have been attributed to the development of CD are the timing of gluten ingestion, breast feeding cessation (23) and cigarette smoking (66).

NATURAL HISTORY

Potential CD (PCD) is applied to people with normal small intestinal mucosa who are at increased risk of developing CD as indicated by positive CD serology (Table 1). It can evolve into flat, active CD but this evolution is, however, uncertain. A recent retrospective study has compared PCD and active CD during a 12 year period in order to understand the natural history of adult patients with potential CD. Interestingly, age at diagnosis, laboratory data, and prevalence of symptoms, associated diseases, and familiarity for CD did not differ between patients with PCD and those with active CD. Furthermore, some patients with PCD maintained a normal duodenal mucosa for many years and their symptoms spontaneously improved despite maintaining a gluten containing diet. These observations suggest that PCD is not a prodrome of CD but is a separate entity that can only subsequently evolve into active CD (67). In other study, children with PCD were advised to remain on a gluten-containing diet and prospectively investigated form birth for a 2-years period. In that study, the prevalence of PCD and the percentage of negativisation of CD-related serology in the short-term were high. These results led to the conclusion that children without symptoms and with positive celiac serology, the decision of performing an intestinal biopsy should be preceded by positive serology results in more than one occasion (68).

CLINICAL MANIFESTATIONS

Celiac disease is recognized as a systemic disease that may affect various systems; mainly gastrointestinal but only 40% to 50% of patients have symptoms and signs as diarrhea, weight loss, recurrent abdominal pain and abdominal distention. CD affects people of any age and ethnic groups. Associated clinical manifestations include iron deficiency with

CLINICAL MANIFESTATIONS IN PATIENTS WITH CLASSICAL CD	CLINICAL MANIFESTATIONS IN PATIENTS WITH NON-CLASSICAL CD	ASSOCIATED AUTOIMMUNE DISEASES	ASSOCIATED GENETIC DISEASES
• Diarrhoea • Abdominal distension • Nausea and Vomiting • Steatorrhoea • Weight loss • Growth failure • Muscle wasting • Poor appetite • Emotional distress • Signs and symptoms of malabsorption (Iron deficiency with or without anemia, oedema secondary to hypoalbuminemia, and other metabolic and electrolyte changes)	• Recurrent aphtous -Stomatitis • Dental enamel hypoplasia • Artritis • Headache • Polyneuropathy • White matter lesions • Cerebellar ataxia • Epilepsy • Pubertal delay • Recurrent abortions • Infertility • Hypertransaminasemia • Coagulopathy • Chronic fatigue	• Autoimmune thyroditis • Type 1 diabetes • Autoimmune hepatitis • Primary biliary cirrhosis • Sjogren syndrome • Primary sclerosing cholangitis • Psoriasis • Vitiligo • IgA nephropathy • Myasthenia gravis • Addison disease	• Down's syndrome • Turner's syndrome • Williams' syndrome • IgA deficiency

Table 3. **Clinical manifestations of celiac disease.**

CD SHOULD BE CONSIDERED IN THE FOLLOWING CASES
• First-degree and second-degree relatives of celiac patients (10% and 5%, respectively) • Unexplained iron-deficiency anemia (3% to 15%) • Unexplained folic acid, iron, or vitamin B12 deficiency • Reduced serum albumin • Unexplained hypertransaminasemia (2% to 9%) • Osteoporosis and osteomalacia of premature onset (2% to 4%) • Recurrent abdominal pain or bloating • Other autoimmune disorders: type 1 diabetes mellitus (2% to 15%), thyroid dysfunction (2% to 7%), Addison's disease, and autoimmune hepatitis (3% to 6%) • Ataxia and idiopathic neuropathy • Down's syndrome and Turner's syndrome (5 to 19% and up to 6% respectively) • Irritable bowel syndrome (3%)

Table 4. **Populations at-risk of developing celiac disease.**

or without anemia, aphthous stomatitis, short stature, neurological symptoms, chronic fatigue, hypertransaminasemia, hypoproteinemia, hypocalcemia and reduced bone mineral density, among others (Table 3) (69).

Most patients are asymptomatic or have mild clinical manifestations, which makes diagnosis much more complicated. In some cases the disease is diagnosed in at-risk individuals (familiarity or association with other diseases such as genetic or autoimmune diseases) (Table 3-4) (70).

Celiac crisis is a life-threatening syndrome present in patients with untreated CD and is frequently observed in children. It is characterized by severe diarrhea, hypoproteinemia, and metabolic and electrolyte alterations (71).

DIAGNOSIS

Diagnosis of CD is based on small bowel histopathology in biopsies of the duodenum and accurate serological tests. Due to the wide variety of clinical manifestations of the disease, diagnostic criteria have been defined (Table 5) (72) and an algorithm has been proposed for the diagnosis and to identify asymptomatic patients but with positive serology (PCD or subclinical CD) (Figure 2).

Serological tests

Autoantibodies are fundamental for screening. Antibodies can be used with two purposes: initial testing in persons with clinical suspicion or to confirm the diagnosis in cases in which an enteropathy has been detected (Table 6) (35,73).

In the past, serological tests consisted of the detection of autoantibodies against gliadin and reticulin but nowdays they are considered obsolete for the diagnosis because of the low sensitivity and specificity (74). In the recent years these antibodies have been replaced by IgG or IgA anti-tissue transglutaminase (anti-tTG), IgA and IgG anti-endomysium (IgA and IgG EMA), and IgA or IgG anti- deaminated gliadin peptides (IgG DGP).

The initial screening is done with IgA anti-tTG in persons who do not have concomitant IgA deficiency because this test has high sensitivity (94%) and high specificity (97%) (75). In patients with IgA deficiency, the detection of IgG anti-TG is particularly useful. Measurement of IgA EMA has nearly

DIAGNOSTIC CRITERIA FOR CELIAC DISEASE (AT LEAST 4 OF 5 OR 3 OF 4 IF THE HLA GENOTYPE IS NOT PERFORMED)
1. Typical symptoms of celiac disease*
2. Positive serology with high titers of autoantibodies ¶
3. HLA-DQ2 or DQ8 genotypes §
4. Celiac enteropathy at the small intestinal biopsy‡
5. Positive response to GFD†

Table 5. Diagnostic Criteria for celiac disease. Notes: A family history of CD adds evidence to the diagnosis; in symptom-free patients, particularly young children, it is advisable to confirm antibody positivity on 2 or more samples taken at least 3 months apart; in selected cases, a gluten challenge after at least 2 years of GFD might be required for diagnosis confirmation.

*Examples of typical symptoms are chronic diarrhoea, growth faltering (children) or weight loss (adults), and iron deficiency-associated anemia.

¶Both IgA tTG and EMA in IgA-sufficient or IgG tTG and EMA in IgA-deficient subjects. The finding of IgG DGP adds evidence to the diagnosis.

§HLA-DQ2 positivity includes subjects with only half the heterodimer (HLA-DQB1*02 positive).

‡Including Marsh-Oberhuber 3 lesions, Marsh-Oberhuber 1-2 lesions associated with positive celiac antibodies positive at low/high titer, or Marsh-Oberhuber 1-3 lesion associated with IgA subepithelial depots.

†Histological in patients with seronegative celiac disease or associated IgA deficiency.

Abbreviations: IgA: immunoglobulin A; GFD: gluten-free diet; EMA: anti-endomysium antibodies; tTG: anti-tissue transglutaminase; DGP: anti-deaminated gliadin peptide

Adapted from Catassi *et al.* (72).

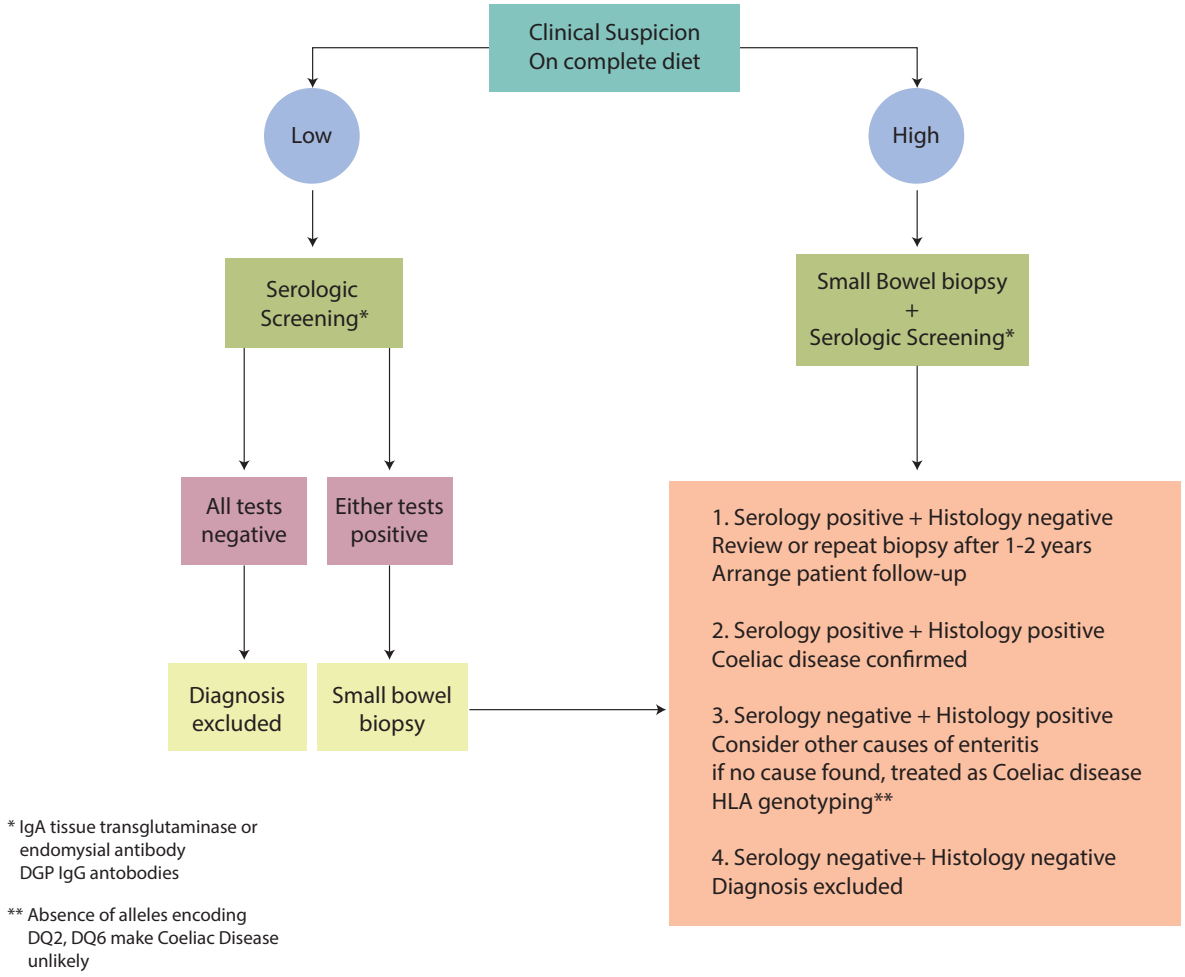

Figure 2. Diagnosis of celiac disease. Adapted from from Bai *et al.* (70).

TEST	SENSITIVITY (%)	SPECIFICITY (%)	PREDICTIVE VALUE (%)	
			POSITIVE	NEGATIVE
IgA AGA	55-100	65-100	30-100	70-100
IgG AGA	57-78	71-87	20-90	40-90
IgA tTg	77-100	91-100	>90	>95
IgGtTg	12.6-99.3	86.3-100	Unknown	Unknown
IgA EMA	86-100	98-100	98-100	80-95
IgA tTg and IgA EMA	98-100	98-100	>90	>95
IgA DGP	98	94	92	98
IgG DGP	97	100	100	97
IgA DGP and IgA tTg	100	93	91	100
IgG DGP and IgA tTg	100	97	97	100
HLA-DQ 2 or HLA-DQ 8	82.6-97	12-68	Unknown	Unknown

Table 6. Serological tests for the diagnosis of celiac disease. Abbreviations: AGA: anti-gliadin antibodies; tTG: anti-tissue transglutaminase; EMA: anti-endomysium antibodies; DGP: anti-deaminated gliadin peptide. Table adapted from Admou *et al.* (73).

100% of specificity for active CD, but it should be used only as a confirmatory test in the cases of borderline or possibly false positive results with anti-tTG, as occurs in other autoimmune diseases (type 1 diabetes, primary biliary cirrhosis, Sjogren´s syndrome, psoriasis and systemic lupus erythematosus). Low levels of anti-tTG have also been associated with infections, tumors, liver disorders and myocardial damage (76–79). Performing IgA anti-tTG and IgA EMA has a sensitivity and specificity of 100% (76). IgG DGP is other antibody used for the diagnosis of CD, but this test alone has not a better performance than the other two (73), except in the cases of IgA deficiency, in which it has proven to be more suitable (80). The DGP/tTG Screen assay could be considered as the best initial test for CD. Combinations of two tests, including a DGP/ tTG Screen, might be able to diagnose CD accurately in different clinical scenarios making biopsy avoidable in a high proportion of subjects (81)

It is known that not all of the patients with CD have positive serological tests; these patients are characterized by clinical, genetic and histological changes. Seronegativity in patients with CD is associated with IgA deficiency (72). The sensitivity of the serological tests is dependent on the gluten-containing diet; patients with gluten-free diet decrease the tests performance.

Intestinal Biopsy

An intestinal biopsy together with positive serology represents the gold standard in the diagnosis. The histological diagnosis of CD consists of an integrated assessment of IEL counts, mucosal architecture and intracytoplasmic alterations. Villous atrophy is defined by a decrease in villous height, alteration of normal crypt/villous ratio (3:1) to total disappearance of villi. Crypt hyperplasia reflects the regeneration of epithelial crypts associated with changes and the presence of more than 1 mitotic cell per crypt (82).

Histological classification of CD of Marsh, Marsh-Oberhuber and Corazza-Villanacci are commonly used (Table 7). Intestinal biopsy should be taken from the first and second portion of the duodenum. As in the case of serological testing, for the histological diagnosis, it is required that the patient has a normal gluten-containing diet at the time of the biopsy (82). It should be borne in mind that other pathologies may have similar histological characteristics to those found in CD (Table 8) (72). For these cases or for patients with negative serology the detection of subepithelial anti-tTG IgA depots by immunofluorescence is recommended (83).

The European Society for Pediatric Gastroenterology and Hepatology and Nutrition (ESPGHAN) recommends that the diagnosis in patients with signs or symptoms suggestive of CD and high anti-tTG levels (higher than 10 times the upper limit of the normal range) might be made without biopsy (76).

HLA testing for HLA-DQ2 and HLA-DQ8

Testing HLA is a useful tool to rule out or to consider CD unlikely in the case of a negative test for antibodies. HLA testing should also be performed in patients with uncertain diagnosis, for example, in patients with negative serology and alterations in the intestinal biopsy or patients in treatment with gluten-free diet without proper diagnosis (76).

Gluten Challenge

This test does not necessarily leads to diagnosis, but it may be performed under special circumstances. It is recommended for children with villous atrophy but without positive serology or for patients with gluten-free diet without proper diagnosis. The challenge should not be performed before the age of 5 years or during puberty (76).

COMPLICATIONS

Complications associated with untreated CD include osteoporosis, impaired splenic function, neurologic disorders, infertility or recurrent abortion, ulcerative jejunoileitis, and cancer (84). In addition, the enteropathy-associated T-cell lymphoma and adenocarcinoma of the jejunum are rare complications of CD (85).

Non responder to gluten free diet represents approximately 5% of CD patients whereas refractory CD (RCD) is present in 1% of CD patients. It is defined as persistence or recurrence of malabsorptive signs and symptoms (i.e., diarrhoea, abdominal pain, involuntary loss of weight, low haemoglobin and hypoalbuminemia) associated with persistent or recurrent villous atrophy despite a strict gluten- free diet for more than 12 months (or severe persistent symptoms

MORPHOLOGY OF DUODENAL MUCOSAL BIOPSY	CLASSIFICATION		
	Marsh	**Marsh-Oberhuber**	**Corazza-Villanacci**
Normal	Type 0	Type 0	Normal
Normal architecture and increased IEL (>25-30/100 enterocytes)	Type 1	Type 1	Grade A
Normal architecture and increased IEL (>25-30/100 enterocytes) with crypt hyperplasia	Type 2	Type 2	Grade A
Partial villous atrophy and increased IEL (>25-30/100 enterocytes)	Type 2 hyperplastic lesion: crypt hyperplasia, increased crypt height and influx of inflammatory cells	Type 3 3a. partial villous atrophy; blunt and shortened villi with a villous: crypt ratio, 1:1 3b. subtotal villous atrophy; atrophic villi but still separate and recognizable	Grade B1 atrophic, villous to crypt ratio is <3:1
Total villous atrophy (IEL >25-30/100 enterocytes)	Type 3	Type 3c total villous atrophy; rudimentary or absent villi; mucosa resembles colonic mucosa	Grade B2 atrophic, villi are no longer detectable
Atrophic hypoplastic lesion: flat mucosa, normal crypt height, no inflammation with normal IEL counts	No equivalent	Type 4	No equivalent

Table 7. Histopathological classification. Adapted from Ludvigsson *et al.* (3).

independently of the duration of gluten- free diet) in the absence of other causes of villous atrophy or malignant complications and after the confirmation of the initial diagnosis of CD. Most RCD patients are negative for EMA and tTG serological tests at the time of diagnosis, but the presence of persisting elevated titers of circulating EMA and/or tTG does not necessarily rule out RCD. In all cases, a careful dietary interview should be performed to exclude gluten exposure before diagnosing RCD. Not all diet non-responsive CD are cases of RCD (3). Over the past 15 years, multidisciplinary approaches have been developed to assess the mechanism of resistance to diet, and two distinct entities have been delineated. Type II RCD (RCDII) can be defined as a low-grade intraepithelial lymphoma. RCD II is characterized by accumulation of abnormal IEL that display an aberrant hybrid NK/T cell phenotype, the abnormal phenotype is supported by loss of normal surface markers CD3, CD4 and CD8 with preserved expression of intracytoplasmic CD3 (CD3 ε) in >50% of IELs as evaluated by immunohistochemistry or >20% as determined by flow cytometry, and by detection of clonal rearrangement of T-cell receptor chains (γ or δ) by PCR (3). This condition has a severe prognosis, largely due to the frequent transformation of IELs into overt aggressive enteropathy-type-associated T cell lymphoma (86). In contrast, in type I RCD, intestinal lymphocytes have a normal phenotype, and this generally milder condition remains often difficult to differentiate from uncomplicated CD except for the resistance to gluten-free diet. The mechanisms that underlie resistance to gluten deprivation are not completely understood (3).

TREATMENT

The main treatment for CD is a strict gluten-free diet for the lifetime. Foods containing gluten from wheat, rye or barley must be completely eliminated from the diet (Table 9). Oat is not toxic in more than 95% of patients with CD, but there is in <5% in whom this cereal grain is not safe. In addition, in some countries there is reluctance to advise liberal use of oat, because of difficulties in guaranteeing that commercially available oat are free of contamination with other grains (87, 88). This contamination is difficult to avoid, therefore the new Codex Alimentarius regulation endorses a maximum gluten

Intolerance to foods other than gluten (milk, soy, chicken, tuna)
Collagenous sprue
Tropical sprue
Whipple's disease
Radiation enteritis
Eosinophilic gastroenteritis
Giardiasis
Tuberculosis
Human immunodeficiency virus enteropathy
Intestinal lymphoma
Zollinger-Ellison syndrome
Autoimmune enteropathy
Crohn´s disease

Table 8. Causes of villous atrophy other than celiac disease. Adapted from Green *et al.* (69).

GRAINS THAT SHOULD BE AVOID	SAFE GRAINS (GLUTEN-FREE)	SOURCE OF GLUTEN-FREE STARCHES THAT CAN BE USED AS FLOUR ALTERNATIVES
• Wheat (includes splet, kamut, semolina, triticale) • Rye • Barley (including malt)	• Rice • Amaranth • Buckwheat • Corn • Millet • Quinoa • Sorghum • Teff (an Ethiopian cereal grain) • Oat	• Cereal grains: amaranth, buckwheat, corn (polenta), millet quinoa, sorghum, teff, rice (white, brown, wild, basmati, jasmine), montina (Indian rice grass). • Tubers: arrowroot, jicama, taro, potato, tapioca (cassava, manioc, yucca) • Legumes: chickpeas, lentils, kidney beans, navy beans, peanuts, soybeans • Nuts: almonds, walnuts, chestnuts, hazelnuts, cashews • Seeds: sunflower, flax, pumpkin

Table 9. Fundamentals of the gluten-free diet. Adapted from Green *et al.* (69).

contamination of 20 ppm in gluten-free products (89). The lowest amount of daily gluten that causes damage to the intestinal mucosa over time is 10 to 50 mg per day (a 25-g slice of bread contains approximately 1.6 g of gluten) (90).

Complete removal of gluten from the diet will result in symptomatic, serological, and histologic remission in most patients (91). The healing of the intestinal damage occurs within 6 to 24 months after initiation of diet. However, complete normalization of mucosal damage is rare in adult patients, despite negativisation of serological tests and disappearance of symptoms(92).

In addition, growth and development in children returns to normal with adherence to the gluten-free diet and many disease complications in adults are avoided, with an improvement in the life quality (93–95) .

Approximately 70% of patients present an amelioration of symptoms within 2 weeks after starting the gluten-free diet (91).

However, patients with poor adherence to the gluten-free diet or with RCD should have alternative treatments. These treatments may alternatively involve hydrolysis of toxic gliadin, prevention of gliadin absorption, blockage of selective deamination of specific glutamine residues by a tTG2 inhibitor, peptide vaccination, modulation of immune response to dietary gliadin (HLA-DQ blocker, interleukin blocker and NKG2D antagonists) and restoration of intestinal architecture (6).

FOLLOW-UP

Patients should be followed (usually on an annual basis) for their lifetimes in order to monitor adherence to the diet with serological tests (persistence or recurrence of abnormal levels of IgA tTG usually indicates poor dietary compliance) and monitoring for associated conditions (e,g, osteoporosis, other autoimmune diseases, anemia, among others) (35).

For the follow-up should also be carried out by a nutritionist, to assess the patient current nutritional status; to identify macronutrient and/or micronutrient intake and to detect deficiencies and/or excesses; to analyse eating habits and potential factors affecting access to the diet; to provide information and initiate the gluten-free diet; to provide dietary education; to monitor and evaluate dietary compliance and reinforce alimentary counseling. If patients are unable to adhere to the diet, they may require psychological counseling as well (70).

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34 DERMATOLOGICAL AUTOIMMUNE DISEASES

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INTRODUCTION

The skin is the largest organ in the human body, representing 16% of the total body weight. The skin is also one of the largest organs in humans and is formed by a layer (termed the epidermis) that enables the body to interact with the environment through physico-chemical mechanisms and sophisticated sensorial stimuli. Moreover, the epidermis provides protection for the human body through specialized cells involved in immunity, which are distributed throughout the organ. The epidermis is comprised of the following five layers (listed in order from the innermost layer to the outermost layer): the basal or germinate layer that consists essentially of keratinocytes that are attached to the basement membrane by a structures called the hemidesmosome and the focal contact. The hemidesmosome plays an important role in autoimmune bullous skin. Next to the basal layer are the basal cells, which are cuboidal and migrate to the surface in a process termed cell differentiation. These cells allow the expression of certain types of keratins in the keratinocytes. The next layer is the stratum spinosum, which consists of five rows of polygonal flattened cells. The cytoplasm of these cells exhibit discrete basophilic staining, and in this stratum, the presence of tonofibrils is evident, and the cells are joined by structures called desmosomes. The desmosome is a molecular complex formed by desmogleins proteins (Dsg) that are involved in triggering a pathogenic immune response in blistering autoimmune diseases, such as pemphigus. The next layer is the granular stratum, the surface of which is formed by three rows of cells containing round nuclei. The keratinocyte layer is characterized by the presence of electron-dense granules composed of sulfur-rich amino acids present in the precursor molecule of filaggrin. The next layer is the stratum lucidum, which is comprised of two rows of flattened cells that do not contain nuclei and have poorly defined shapes. The cells produce a thin eosinophilic zone containing large amounts of keratins and are found mainly in the palms and soles, which is of clinical relevance in autoinflammatory diseases, such as keratoderma palmoplantar. Finally, the stratum corneum corresponds to the outermost layer of the epidermis, which consists of between 15 and 20 layers of flattened cells with a dense keratin content, termed corneocytes. Corneocytes are insoluble, and the process of the cornified cell envelopes is determined by the molecule involucrin. In the cornification process, the multiple bridges that crosslink the structure are induced by epidermal transglutaminase. The corneocytes are replaced by cells from the basal layer, and the epidermic renewal process takes approximately 21 to 28 days. The last step in this process is termed desquamation, which involves the degradation of lamellar lipids in the intercellular spaces, and this process is accelerated in autoinflammatory diseases, such as psoriasis.

The dermis is another component of skin tissue and is located below the epidermis. The dermal tissue is distributed in two regions; the innermost region is termed the reticular area, which is extensively vascularized and hosts appendices, such as the hair follicles and the sweat and sebaceous glands. The reticular zone has clinical significance in a number of autoimmune diseases of the skin that affect the ability to sweat, such as scleroderma. The upper area of the dermis is termed the papillary dermis, and in this area, the blood vessels are involved in superficial vasculitis processes in diseases such as lupus.

THE ORIGIN OF THE SKIN

Skin emerges during the very early stages of embryonic development. In the early gastrula, the mesoderm migrates and generates the dermis. The mesoderm is also essential

for the differentiation of epidermal structures including the hair follicle, and in turn, the dermis is essential for maintaining the adult epidermis. The development of the epidermis involves a fine balance, which plays a key role, between the opposing signals of Notch and Wnt (wingless related) and involves beta-catenin, Lef1, and the Notch peptide. The sonic hedgehog pathway signaling promotes neural tube development, and morphogenic proteins (BMPs) that promote signaling during the development of the ectoderm are also involved; therefore, these elements establish complex interactions and justify the presence of nerve terminals and Merkel cells in the skin. The fibroblast growth factor (FGF) induces an additional control on the Wnt pathway that influences the epidermal development. Hemidesmosomes and desmosomes appear at approximately the 10th week of development and are followed by the emergence of keratins after 14 weeks, and the filaggrin protein is present in the granular layer at 15 weeks (1). The rudiments of hair follicles emerge at approximately 9 weeks, and after the apocrine glands appear between the 13th and 15th week of development. Eccrine or sweat glands develop from the germinate layer at 14-15 weeks, after which they penetrate deep into the dermis, and the intra-epidermal duct is formed by the coalescence of intra-cytoplasmic cavity groups formed by two adjacent layers of cells. Nail development begins between 16 and 18 weeks, after which the keratinized cells of the dorsal and ventral matrix are differentiated (1).

Approximately 90% of the cellular components of the epidermis correspond to squamous epithelium cells; keratinocyte growth in culture depends initially on fibroblast support; however, epidermal growth factor (EGF) delays senescence in epidermal cells (2,3). Another component of development is the melanocyte cell lines that are generated in the neural crest; these cells appear transiently and re-emerge after 4-6 months of gestation. Melanocytes are responsible for the production of melanin, which produces skin color and are attacked in vitiligo disease (4). Langerhans cells belong to the histiocytic lineage and are antigen-presenting cells derived from the monocyte-macrophage line that migrate from the blood to the skin after the 12th week of gestation (5). Finally, Merkel cells are sensory cells that appear in the nails, fingertips, lips, and other parts of the skin at 16 weeks of intrauterine life (6). The cells of the dermis, such as the mesenchymal stem cells, are widely expressed in a variety of cells, including blood-derived cells, cells that form the connective tissue including fibroblasts, mast cells, and skin-derived precursor cells (SKF). Other skin cells include adipocytes, smooth muscle cells, neurons, etc (1).

CELL JUNCTIONS RELEVANT FOR AUTOIMMUNITY

Different cell junctions bind keratinocytes to maintain the mechanical integrity of the skin and are responsible for biochemical and mechanical interactions and cellular communication; these junctions include hemidesmosomes, desmosomes, adherent junctions (AJ), gap junctions, and tight junctions. *The basement membrane zone* (BMZ) is an ultra-structurally adhesive zone located between the internal part of the epidermis and the external part of the dermis. The BMZ is subdivided into four distinct ultrastructural areas: 1) the hemidesmosome and top of the lucid membrane, 2) the lower lucid lamina, 3) the lamina densa, and 4) the sub-lamina densa. The components of the BMZ that are best characterized are the hemidesmosome and the upper lucid lamina. The hemidesmosomes are composed of the pemphigoid antigens BP230 and BP180, integrins 6β4 and 7, and plectin. The lower part of the lucid lamina includes the laminins 1, 5, and 6, p105, and enactin/nidogen. The lamina dense is formed by IV collagen and perlecan. Finally, the sub-lamina densa is formed by collagen VII (COL7), which is the antigen of the epidermolysis bullosa acquisita (7). In 1956, Keith Porter defined the ultrastructure of the desmosome as an adherent complex that anchors the intermediate keratin filaments and the cell membrane of two adjacent cells, which interact to form symmetrical joints with spaces of 30 nm. Electron microscopy can distinguish the plaque formed by electro-dense material near the BMZ, a less dense band, a fibrillar area, and the intermediate filament that crosses the plaque. Desmosomal molecules are products of the cadherin gene superfamily and include desmosome cadherins, armadillo family proteins, and plakins (desmoplakin and plakofilin stabilize keratinocyte adhesion). Transmembrane cadherins are involved in the heterophilic associations between desmogleins (Dsg) and desmocollins (Dsc). There are four types of Dsg (Dsg 1-4): Dsg1 is expressed primarily in the surface layers of the epidermis, whereas Dsg3 is expressed mainly in the basal layer, and this difference is important for the classification of the disease pemphigus (1,8,9). Dsg4 is expressed in the hair, and Dsg2 is expressed at low levels in the stratified epithelia, such as the human epidermis, and is restricted to the proliferative basal cell layer. *AJ* correspond to electrolucid transmembrane structures related to cellular shape. These structures participate in cellular interactions, and the major component of AJ is E-cadherin, which links the cytoskeleton via α-catenin and other components (p120ctn, β-catenin, plakoglobin, α-actinin, and vinculin). The AJ joints are important for actin polymerization. *Gap junctions* are groups of intracellular channels (connexons) formed by six connexin subunits that connect the cytoplasm of two adjacent keratinocytes. *Tight junctions* regulate the epithelial permeability via proteins called claudins, and occludin, a member of this family of proteins, is altered in autoinflammatory diseases such as psoriasis (1).

AUTOIMMUNITY

Over a hundred years ago, Paul Ehrlich wrote, *"We pointed out that the organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements and so giving rise to autotoxins . . . so that one might be justified in speaking of a 'horror autotoxicus'"* (10). His

proposal was mistakenly interpreted as "autoimmunity cannot occur," which remained a dogma for 50 years until Noel Rose described his model of experimental thyroiditis in 1956 (11). In reality, Ehrlich was proposing the existence of "mechanisms to prevent the immune reaction against their own elements," which is nothing less than what we now know as immune tolerance. Clearly, these mechanisms prevent or stop the production of autotoxins (autoantibodies) to avoid autointoxication (self-damage). This seminal but imperfect concept of *horror autotoxicus* by Ehrlich was indeed very wise because he realized the need for some type of immunological control to avoid autoimmunity, and at the time when his hypothesis was proposed, there were no experimental data at the cellular and/or molecular levels to begin to understand tolerance and autoimmunity (12,13).

Historically, Donath and Landsteiner described paroxysmal cold hemoglobinuria as the first autoimmune disease in 1904 (14). It is a rare form of hemolytic anemia caused by complement-dependent cold-acting autoantibodies that produce hemolysis in vivo in a temperature-dependent reaction that occurs between 18 and 20ºC. Another advance in the understanding of the spectrum of autoimmune disease was made in 1962, when Milgrom and Witebsky proposed a number of postulates for the classification of autoimmune diseases (15) as follows: 1) direct evidence of the transfer of pathogenic antibody, 2) indirect evidence based on the reproduction of autoimmune diseases in experimental animals, and 3) circumstantial evidence from clinical clues. Later, Rose and Bona revised the criteria, assembling the original postulates with autoantibody markers of certain autoimmune diseases (16). Next, autoimmune diseases were classified as localized or organ-specific autoimmune diseases in which pathogenic autoantibodies were directly related with the affected tissue, as in the case of pemphigus. The other category was systemic autoimmune diseases; however, their pathophysiology was complex, and organ non-specific autoantibodies were considered markers rather than pathogenic autoantibodies because in most cases, they were not directly involved in tissue damage; the best example in this category is likely systemic lupus.

Nevertheless, the classification of autoimmunity has become more complex since the rapid evolution of the understanding of genetics and the molecular mechanisms involved in the pathophysiology, which has challenged the old autoimmunity paradigm of classification. In addition, recent efforts to reappraise autoimmunity takes into account the genes and cells involved in certain types of autoimmunity and other diseases that are not strictly autoimmune but are autoinflammatory. These efforts have fostered the development of a classification system that provides for five types of diseases as follows: 1) monogenic autoimmune diseases, 2) polygenic diseases exhibiting a prominent autoimmune component, 3) monogenic autoinflammatory diseases, 4) polygenic disease exhibiting a prominent autoinflammatory component, and 5) mixed pattern diseases (17). Most autoimmune skin diseases belong to the second category, as is the case for autoimmune bullous disease (pemphigus and pemphigoid), and one example of autoinflammatory skin disease is psoriasis. Differences between autoimmunity and autoinflammation are as follows: autoimmunity is a self-directed inflammation caused by aberrant dendritic cells and T and B cell behaviors that disrupt tolerance, resulting in an adaptive immune response that plays a central role in the phenotypical clinical expression of autoimmune diseases. In sharp contrast, autoinflammation leads to the activation of the innate immunity and may result in tissue damage through the alteration of cytokine cascades, which induce site-specific inflammation and is independent of the adaptive immune response (17).

In addition to understanding autoimmunity classification, it is important to understand tolerance mechanisms and the reasons why physiological control is disrupted. In this chapter, we will discuss a number of autoimmune diseases that affect mainly the skin and mucous membranes (pemphigus, pemphigoid, dermatitis hepetiformis, and vitiligo), and we will discuss one common autoinflammatory disease (psoriasis).

PEMPHIGUS

Pemphigus (in Greek, pemphix means blister) is a group of blistering diseases with an organ-specific autoimmune pathogenesis that affects the skin and mucous membranes. The disease is characterized by blisters and erosions caused by intraepidermal cell detachment in a process termed acantholysis. The lesions are induced by the presence of autoantibodies against proteins in the desmosomes, which are the attachment structures of keratinocytes (18). The clinical hallmark of this group of diseases is the presence of intraepidermal bullae; the lesion can be produced artificially by pressing the skin between two fingers and the positive Nikolsky sign consisting of the detachment of the surface layers of the skin (19). Another simple assay for this disease is the Tzanck test in which the microscopic analysis of a sample of cells from the blister basement demonstrates the presence of acantholytic cells (20). Pemphigus has a worldwide distribution, with an incidence of 0.1 to 0.5/100,000 and occurs more often in women (1.5:1) between the fourth and fifth decade of life (21). In addition to the disease in humans, this disease is also observed in horses, dogs, and cats. The histopathology findings reported by Civatte in 1943 described the loss of cell-cell adhesion, and the process was designated "acantholysis." This term is important because it reflects its mechanism, which is the disease hallmark (22). After keratinocyte detachment, the cells become deformed and acquire a spherical shape because of the loosening of the intercellular connections; therefore, the keratinocytes are isolated within the blisters as acantholytic cells (23). The epidermal location of the blisters allows the histological classification as suprabasal pemphigus, sub corneal, or intragranular pemphigus.

A milestone in the study of pemphigus was made in 1964 when Beutner and Jordon (24) first described the pemphigus antibodies that reacted to the keratinocyte surface. This

description allowed the recognition of the autoimmune nature of the disease. Beutner and Jordon used an immunofluorescence technique, describing the "honeycomb" pattern of the pemphigus antibodies induced by the antibody recognition of the structures attached to the epidermal cell surface. They also proposed the quantification of circulating antibodies in the serum of patients by reciprocal dilution of the patients' serum, and the titers correlated with the extent of the blistering. This anti-epithelial antibody determination has been used as a predictor for disease activity and therapy response (25). Pemphigus autoantibodies are IgGs (IgG1 and IgG4 isotype) and target different proteins in the desmosome complex. The pathogenic properties of these antibodies were demonstrated elegantly by Anhalt and Diaz (26) in 1982 when they reproduced the disease in Balb/c newborn mice by the passive transfer of human pemphigus IgG. This experimental model has been established as the principal tool for studying the effect of pemphigus autoantibodies *in vivo* by inducing acantholysis. Additionally, the pathogenesis of pemphigus autoantibodies has been demonstrated during pregnancy in pemphigus patients as a "phenomenon of nature." Pemphigus depends on the maternal autoantibody titer and has been found in newborns as neonatal pemphigus. Therefore, the transfer of the pemphigus IgG4 isotype maternal autoantibodies via the trans-placental pathway induces blisters in newborns; however, this transient disease disappears after weeks or months when the maternal pemphigus autoantibodies become degraded (27,28) Pemphigus antibodies recognize Dsg1 and Dsg3; however, according to the type of pemphigus, the antibodies may recognize other desmosome proteins, such as Dsc, envoplakin, periplakin, etc. (20,25).

Keratinocytes are firmly bound together by desmosomes, which consist of two types of proteins: a) the transmembrane glycoproteins, Dsg, and Dsc and b) the cytoplasmic plate proteins desmoplakins and plakoglobin. Dsg are transmembrane glycoproteins that require Ca^{+2} for their adhesive function. Dsg and Dsc belong to the type 1 desmosomal cadherin family. Both Dsg and Dsc contain an extracellular domain with four cadherin repeats and an intracytoplasmic domain at the carboxyl terminal (23). The expression of these proteins in the epithelia varies; Dsg1 is expressed in the stratum granulosum of the epidermis, and Dsg3 is restricted to the lower layers of the epidermis, practically distributed above the basal layer. Based on this finding, John Stanley (29) explained the involvement of these cells in the pemphigus disease subsets; those affecting the skin exhibit anti-Dsg1 activity, and pemphigus in the mucosa exhibits anti-Dsg3 activity, while patients with anti-Dsg1 and anti-Dsg3 specificities exhibit skin and mucous membrane involvement (Figure 1). In addition to Dsg, other autoantibodies have been described in pemphigus, including autoantibodies against adhesion molecules, cell membrane receptors, anti-muscarinic receptors, annexins, hematological antigens, carcinoembryonic cells and microsomal antigens, the clinical and pathogenic significance of which is unknown (30). The pathophysiological mechanism that induces acantholysis affects the amino terminus of the extracellular domain 1 of Dsg; therefore, the interaction between anti-Dsg antibodies and epitopes of this immunodominant region triggers a cascade of events including the following: 1) steric hindrance that affects desmosome adhesion directly; 2) induction of a downstream signaling transduction cascade mediated by protein kinases and calcium that induces Dsg phosphorylation and is followed by plakoglobin dissoci-

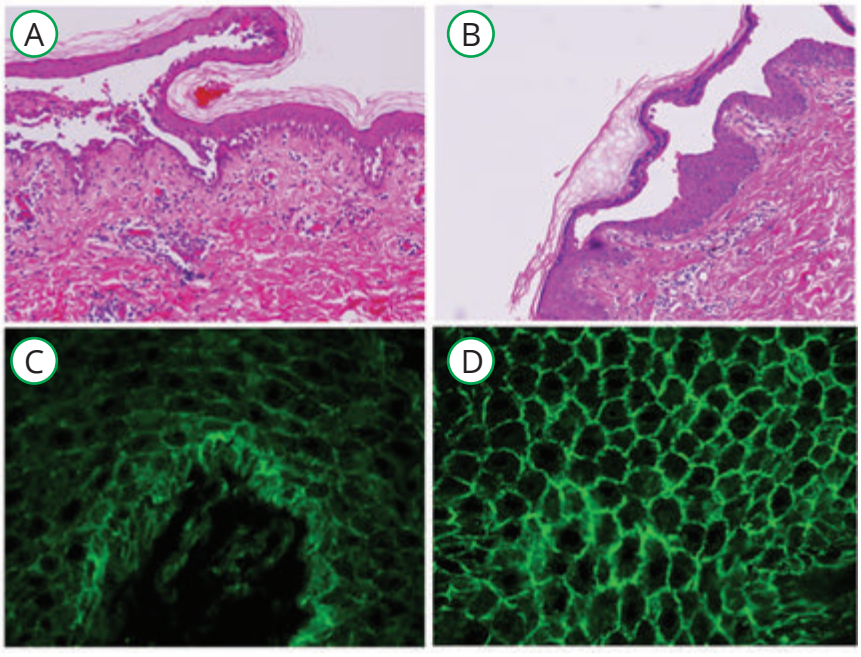

Figure 1. Skin biopsies of Pemphigus. A. Pemphigus vulgaris (H&E) showing a suprabasal blister. B. Indirect immunofluorescence showing IgG deposition on intercellular spaces at basal keratinocytes (Dsg3). C. Pemphigus foliaceous (H&E) showing blister at the granular layer. D. Indirect immunofluorescence with IgG deposition along intercellular spaces (Dsg1).

ation and desmosome break–down; 3) plasminogen activation (urokinase) with the conversion of plasminogen in plasmin, followed by the digestion of the extracellular domains of the desmosome resulting in acantholysis; 4) clustering of Dsg3 induced by pemphigus IgG binding, with the clustering followed by endocytosis and desmosome degradation in a p38MAPK-dependent manner; 5) digestion with metalloproteinases; and 6) apoptosis resulting in cell detachment, which creates or feeds a pathogenic cycle through the Fas pathway; this mechanism involves other mediators of inflammation including TNF (18,19,25,31-33). The genetic factors associated with pemphigus susceptibility are the HLA class II genes. Population studies have shown an association between certain class II alleles and pemphigus in different ethnic groups; for example, HLA-DRB1*0402 is associated with over 90% of Ashkenazi Jews with pemphigus vulgaris (PV), and HLA-DQB1*0503 is associated with non-Jewish populations. Likewise, DRB1*1404 is the most important risk factor in an Indo-Asian population, and in Brazilian pemphigus, HLA-DRB1*0102 is a risk factor (34). In the year 2000, studies of SNPs (single nucleotide polymorphisms) corresponding to a variation in DNA sequences by a single nucleotide change demonstrated that the genome consists of physically linked SNP variants. The DSG3 gene contains 46 coding SNPs, and one study demonstrated an association between 2 SNPs and PV; notably, these 2 SNPs were in the context of the PV alleles. Below, we describe the main pemphigus subsets (31,34).

Pemphigus Vulgaris (PV), the most common form of pemphigus, affects men and women equally. The disease is chronic and progressive and is characterized by flabby blisters and erosions on the skin and/or mucous membranes. The oral mucosa is affected primarily in approximately 70% of cases, causing dysphagia for months after the blisters appear on the skin surface (trunk, buttocks, and feet). The genitourinary tract may be particularly affected in women. Blisters can erupt and leave erosions and crusts, and if the lesions are extensive, then electrolyte abnormalities, fluid loss, and hypoproteinemia may be observed. The most common complication of this disease is secondary infection. Histopathology demonstrates blisters above the basal cells, which form a layer resembling headstones. Eosinophilic infiltration can be detected. The antibodies are directed against Dsg3 in the mucosa involved, against Dsg1 in the skin involved, or against both proteins in the mucocutaneous features (18,20,21). Figure 2.

Pemphigus Foliaceous. In 1844, Cazenave described the pemphigus foliaceus, then two clinical features are recognized: the non-endemic pemphigus foliaceus and the endemic or fogo selvagem. The first occurs in middle age or late adulthood and affects the face, scalp, trunk, and back. The blisters are superficial, fragile, and easily broken, with the erosion of the erythema and crusting being the common lesions; the mucous membranes are not affected, and the patients test positive for the Nikolsky sign (18,20). Endemic pemphigus foliaceus occurs in children, young adults, and genetically related family members in Brazil, Colombia, and Tunisia (35). The lesions do not differ from non-endemic pemphigus. Diaz et al. (36) studied this type of pemphigus at the molecular, cellular and epidemiological levels and reported the presence of endemic foci, suggesting that a vector triggers the disease (37). Skin biopsies revealed superficial intraepidermal blisters and antibodies directed against Dsg1 (38,39).

Pemphigus erythematosus (PE) is also known as Senear-Usher syndrome. In PE, the blistering coincides with

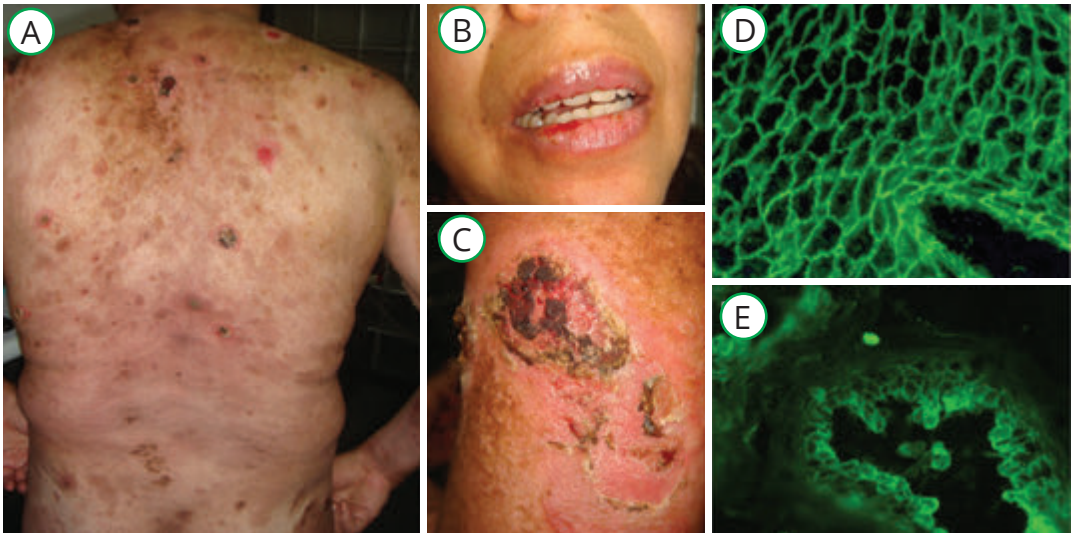

Figure 2. Pemphigus vulgaris show blisters, erosions and crusts (A, B, C), positive anti-epithelial antibodies detected in cow nose (D), and direct immunofluorescence that show intraepidermal blister with acantholytic cells (E).

a seborrheic erythematous rash resembling the rash associated with lupus. Serologically, PE patients have autoantibodies similar to individuals with pemphigus foliaceus and cutaneous lupus erythematosus (18-20). Likewise, the skin immunopathology of PE is characterized by acantholysis with immunoglobulin deposition in desmosomes and at the dermal-epidermal junction (lupus band test). The histology and anti-Dsg1 serological marker of pemphigus foliaceus and PE are the same. The clinical hallmarks of PE are seborrheic lesions in the nose, nasolabial folds, and malar areas that resemble the "butterfly" distribution of lupus. Lesions may also affect the preauricular region. Hyperkeratotic scars with erythema and superficial blisters can be present on the chest. The immunopathology of PE was described by Chorzelski et al. (40), demonstrating the presence of immunoglobulin and complement at the dermo-epidermal junction (DEJ) resembling the lupus band test. PE patients also exhibited antinuclear antibodies, and the report proposed the coexistence of pemphigus and lupus erythematosus. We recently described Dsg1 and Dsg3 and antinuclear antibodies specific for Ro, La, Sm, and double-stranded DNA antigens in patients with this form of the disease. After eluting specific anti-epithelial or anti-nuclear antibodies, a lack of cross-reactivity was demonstrated between desmosomes and nuclear and cytoplasmic lupus antigens (41). This result suggests that the autoantibodies in PE are directed against different antigens and that independent clones produce these autoantibodies. The DQB1*0301 allele may handle desmosomal and/or hemidesmosomal epitopes, and under different stereochemical conditions, the same allele interacts with ribonucleoproteins, raising two independent clones that produce different and unrelated autoantibodies. Taking into account these clinical and serological data, we suggest that PE behaves like a multiple autoimmune disease (18,19,23,34,42).

Drug-induced pemphigus is induced by thiol or sulfide groups such as penicillamine, penicillin, captopril and propranolol, indomethacin, beta-blockers phenylbutazone, piroxicam, and tuberculostatic agents. The clinical picture of drug-induced pemphigus resembles pemphigus foliaceous but can be similar to that of PV or PE; the autoantibodies recognize Dsg3 and Dsg1. Interestingly, this syndrome may also occur after treatment with interferon-γ and interleukin 2 or may precede a lymphoma or lung cancer (18,42).

IgA pemphigus is characterized by the presence of confluent pustules prone to forming circinated blister patterns. The condition is rare, and immune deposits of IgA antibodies that recognize the epidermis and the Dsc I and II can be found in the skin lesions (42).

Pemphigus vegetans was described by Neumann in 1886 and is considered a subtype of PV. The lesions present as vesicles and erosions in the intertriginous areas, such as the axillae and groin. In addition to blisters and pustules, vegetations or papillomatous verruciform may be found and oral lesions may occur. Pemphigus vegetans of the Neumann type can resemble PV, and the Hallopeau type presents with pustules and has a more benign prognosis (18,42).

Paraneoplastic pemphigus. This rare subset was described in 1990 by Anhalt (43) and usually accompanies a lymphoproliferative or hematologic malignancy or is associated with benign tumors, such as thymoma and Castleman's tumor. The lesions are distributed in the oral mucosa, lips, and pharynx. Erosions, ulcerations, and crusting blood can be found, which can leave synechiae pseudomembranous conjunctivitis, and erythema multiform-like lesions on the palms and soles can be observed. This pemphigus subset may involve the lung epithelium, causing respiratory failure, which is a terminal complication in 30% of patients. Biopsies reveal antibodies against the epithelial intercellular space; however, immune deposits in the dermal-epidermal BMZ are found in virtually all cases. The autoantibodies are directed against Dsg1, Dsg3, and other desmosome proteins, such as desmoplakins I and II, bullous pemphigoid (BP) antigen 1 (BP230), envoplakin, and periplakin, among others. It is also important to identify the anti-epithelial antibodies attached to the skin overlying the tumor (18,42).

The diagnosis of autoimmune blistering diseases is based on the evaluation of clinical outcomes, histopathology, direct immunofluorescence (DIF), and indirect immunofluorescence (IIF). Histological examination should be performed in a recent intact blister, including adjacent skin. The biopsy for DIF should be performed on perilesional normal-appearing skin. The determination of antibodies by IIF for anti-epithelial antibodies remains the gold standard for diagnosis. However, anti-Dsg1 and anti-Dsg3 antibody determination using ELISA is important because antibody follow-up allows the assessment of the treatment response or the prediction of a relapse. Accurate diagnosis is a prerequisite for accurate forecasting and effective treatment (25,39,42).

Treatment. Conventional treatment involves the administration of corticosteroids and immunosuppressive agents. In the case of resistant pemphigus, using IVIG and Rituximab is effective (44,47). Other experimental therapies have been proposed to neutralize the pemphigus IgG by means of anti-idiotype antibodies or apoptosis inhibitors, and these have demonstrated experimental success in controlling blistering (45,46). Additionally, support therapy including the treatment of infections and fluid control is important for a positive outcome (47).

BULLOUS PENPHIGOID (BP) DISEASES

BP is a blistering autoimmune disease described in 1953 by Walter Lever (48). BP is characterized by the separation of the dermal-epidermal junction (DEJ) accompanied by inflammatory cell infiltration in the upper dermis. Another important

contribution was made by Jordon et al (1967), who demonstrated that the BP autoantibodies were reactive to the basal membrane, which is the hallmark of the disease (49). The autoantibodies recognize the BP180 antigen (BPAg2), a type of XVII collagen and the BP230 antigen (BPAg1), a cytoplasmic plakin protein family member that links the hemidesmosome to the keratin of intermediate filaments; both antigens are components of the hemidesmosome. The disease affects mainly the elderly; however, a small number of cases have been reported in children (50,51). The incidence of BP is 1.2 to 2.1 cases per 100,000 people (52,53). The disease is clinically characterized by subepidermal blisters that form tense bullae, which do not disrupt easily. Other characteristic lesions include erythematous urticarial plaques that cause pruritus and blistering that appears along flexural areas and that is distributed on the chest and abdomen (over time, these lesions often become excoriated). Oral involvement is rare even though a special pemphigoid subset affects the mucosa.

In antigenic triggering, two major antigens have been described: the BP180 antigen described by Luis Diaz group (54-56), and the BP230 antigen described by John Stanley et al. (57). BP180 is a transmembrane glycoprotein that extends from the lamina lucida to the lamina densa in a stick-like form that contains an extracellular collagen domain. The immunodominant region of the molecule is a non-extracellular collagen domain (NC16A), and most patients generate pemphigoid IgG class antibodies (58). Likewise, reactivity with epitopes in the C-terminal region appears to be associated with the participation of the mucosa and a more severe disease of the skin. The pathogenicity of the BP180 antibodies is well established in animal models. BP230 is a 230-kDa protein with an intracellular component associated with the hemidesmosome plate belonging to the family of plakin proteins. The immunodominant epitopes are located in the C-terminal globular region. The mechanism of tissue damage begins with the antibody binding to the BP180 and/or BP230 antigens, which induces complement activation leading to the accumulation of the eosinophils and neutrophils that release proteolytic enzymes. Additionally, the autoantibodies interfere directly with the function of BP180 and BP230 and induce pro-inflammatory cytokine release. Furthermore, there is evidence of clinical association with disease activity from the high concentrations of IgG autoantibodies reactive to the NC16A ectodomain epitope from BP180 (59). Anti-BP180 antibodies of the IgE class might contribute to tissue damage by stimulating basophil and mast cell degranulation. The BP230 antigen is a disease marker, and 60% of patients develop antibodies against the BP230 epitopes. Autoantibodies of the IgG class recognize the COOH-terminal domain of BP230; however, the pathogenic role of these autoantibodies remains to be defined. The understanding of the pathophysiology of BP has been advanced in animal models. Liu et al. (60) raised antibodies in rabbits directed against the murine NC16A region of BP180, and the antibodies induced a disease similar to BP in neonatal mice. In contrast, other animal models for assessing the role of BP230 in blister induction were inconclusive and will require further investigation. Additionally, numerous non-immune factors have been implicated as BP triggers, such as trauma, burns, radiation, ultraviolet radiation (UV), and a variety of drugs (aldosterone antagonists and phenothiazines) (58).

Clinically, BP blisters are large and tight and are surrounded by erythema and urticarial plates. After several days, the blisters can erupt and cause erosion and scabs in flexural areas of the abdomen; most patients have accompanying itching that is severe. Diagnosis is based on clinical criteria, with DIF data from a perilesional biopsy, demonstrating linear immune deposits of the IgG class at the dermal-epidermal junction, although deposits of IgA and IgE can also be observed (Figure 3). The source of the antigen for the IIF is the splitting of the normal skin using 1 mol NaCl; the separation allows the differentiation of the antibody (BP180/BP230) binding to the roof blister (61). Likewise ELISA tests are useful for the detection and quantitation of both antibodies. BP can also occur in dogs, small pigs, horses, and cats, exhibiting the same clinical and immunopathological features as in humans. Immunogenic studies have demonstrated that HLA-DQB1*0301 is a susceptibility gene involved in BP development.

The BP subsets that have been described are as follows: *Mucous membrane pemphigoid* is a BP subset that chronically affects the DEJ, predominantly at the mucosal level. This subset of BP is also known as cicatricial pemphigoid; however, this term is now used to define a clinically severe form of the disease affecting the skin that leaves scars and involves the

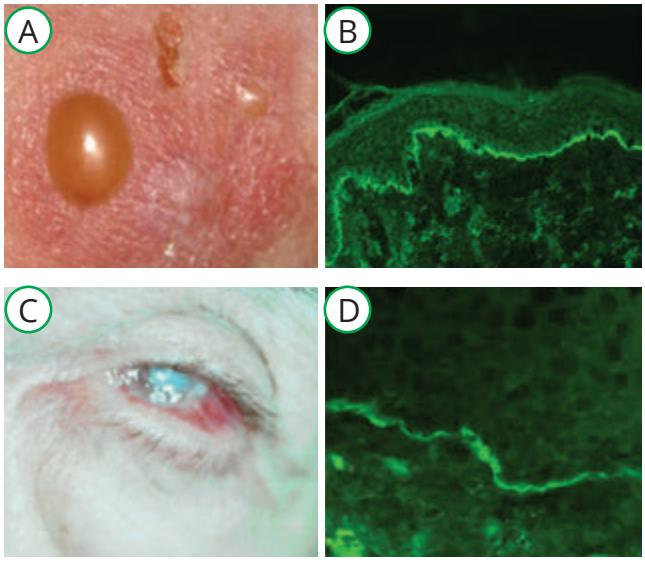

Figure 3. A. BP blisters are large and tight and are surrounded by erythema and urticarial plates. B. IgG deposits along BMZ by direct immunofluorescence. C. Mucous membrane pemphigoid. D. BP IgG autoantibodies detected by indirect immunofluorescence.

mucosa. The incidence of this BP subset is 1.3 - 2 per 1 million/year, the disease onset occurs after 60 years of age, and the susceptibility gene is HLA-DQB1*0301, similar to BP. Clinically, this disease affects the mucous membranes, with a predilection for the oral and conjunctivae mucosas, which causes a foreign body sensation in the dry mucous membranes, and conjunctive synechiae can progress to blindness. The diagnosis is similar to BP, and the antibody profile recognizes several of the hemidesmosome proteins, such as BP180, BP230, laminin 332, α6β4 integrin, and collagen VII (58,59). Treatment is difficult because it is a progressive condition, and therapy includes systemic steroids, dapsone, immunosuppressants such as cyclophosphamide (daily or bolus), intravenous immunoglobulin, and Rituximab. The treatment should be multidisciplinary, with an emphasis on ophthalmology (62). *Pemphigoid gestationis disease* was previously known as herpes gestationis and is a bullous autoimmune condition associated with pregnancy that is mediated by autoantibodies against BP180 NC16A. The frequency is 1 case per 10,000 to 40,000 pregnancies, and the disease is expressed in the 2nd or 3rd trimester of pregnancy and in 10% of patients, within 4 weeks after delivery. Clinically, the disease onset exhibits pruritic papules and urticaria in the periumbilical area that can later become generalized blisters. The risk of preterm delivery occurs in 20% of the cases. Relapses are frequently observed in subsequent pregnancies, during menstruation, or during contraceptive oral therapy. Few cases (5%) evolve to BP. *Childhood BP* is a rare pathological condition, similar to the adult disease, which occurs most frequently before 8 years of age, and 60% of patients exhibit generalized blistering. Criteria have been proposed for the early diagnosis of the disease, which include the following: 1) patients under 18 years with tense bullae on erythematous or normal skin, with or without the involvement of mucosal epithelia, and histological subepidermal blister with eosinophils, and 2) perilesional skin biopsy exhibiting linear deposits of IgG and C3 on the BMZ or circulating IgG antibodies against the BMZ, observed using IIF. *Lichen planus pemphigoid* is an infrequent variant that combines two entities: pemphigoid and lichen planus. The disease is characterized by the development of blisters in patients with active lichen planus, antibodies against BP180 and BP230 and anti-p200/anti-laminin-γ1. Lichen planus pemphigoid occurs in the fifth decade of life, and the course of the disease is relatively benign.

DERMATITIS HERPETIFORMIS (DH)

DH is a pruritic papulo-vesicular skin disease that affects extensor surfaces and is considered a cutaneous component among the celiac disease spectrum. DH is characterized by rash and blisters associated with cutaneous IgA deposition. Duhring identified DH disease in 1884, and this pathology affects middle-aged patients, causing intense itching and erythema or urticarial-like wheals. In some cases, the lesions disseminate, particularly to the elbows, buttocks, and knees. Usually, mucous membranes are unaffected. The skin histopathology demonstrates a neutrophilic infiltration and micro abscesses, and the immunofluorescence studies detect granular deposition of IgA in the dermic papillae. Because DH belongs to the celiac disease spectrum and this pathology is caused by a wheat allergy, wheat proteins could trigger the skin inflammation in some DH patients. The disease is more prevalent in Caucasians (1 case per 10,000 individuals), affecting them at any age but is observed more frequently in the fourth decade of life and is more frequent in men than in women. These patients exhibit a high prevalence of the HLA-DQ2 haplotype in approximately 90% of cases, and the HLA-DQ8 allele is associated with a low prevalence (approximately 5%) (63). The pathogenesis is not fully understood because the factors that determine the clinical expression in the bowel or the skin are unknown. The immunopathology is characterized by the granular deposition of IgA in the dermic papillae of the skin (64), which is the hallmark of DH. The IgA autoantibody deposition induces clusters of neutrophil infiltration, and these cells infiltrate the vesicles in the lamina lucid. Experimental data have shown that transglutaminase-3 (TG3) triggers the IgA deposition and induces the *in situ* immune complex formation, which subsequently triggers the neutrophil infiltration and vesicle formation (65,66). In 1996, Dietrich demonstrated the importance of tissue transglutaminase (tTG), and this enzyme was defined as the autoantigen of celiac disease (67). The enzyme belongs to a protein family containing nine Ca^{2+} isoform members that catalyze the cross-linking reaction that results in the N-isopeptide bond formation between the substrates (68). TG3 is involved in epidermal differentiation, and Sárdy demonstrated that TG3 was the major antigen in DH (65). Interestingly, there are two TG3 isoforms produced by alternate splicing, which is not regulated during differentiation, and whether the isoforms are involved in pathogenesis is not known (69). Another interesting observation is that in celiac disease, the antigenic peptide presentation by antigen-presenting cells to $CD4^+$ T cells is restricted to DQ2 or DQ8 alleles, and the pockets of these class II molecules better accommodate glutamic acid residues of epitopes, which are deaminated by the tTG enzyme (70,71). Therefore, it would be reasonable to assume that the initial antigen of DH is the gluten-derived epitope, which after deamination becomes a posttranslationally modified epitope that in complex with the TG3 enzyme might induce epitope spreading and ultimately become the major target of DH $CD4^+$ T cells. The therapy is integrative, based on the new pathophysiology notions, and includes a gluten-free diet combined with dapsone or steroids at low doses. Alternatively, sulphasalazin is used, and other therapies are directed to immunomodulatory targets, such as IL-10, to promote tolerance. Additionally, promising biologics, such as anti-IL-15, are under evaluation (72).

EPIDERMOLYSIS BULLOSA ACQUISITA (EBA)

EBA is a rare acquired sub-epidermal bullous disease that exhibits certain clinical similarities to the genetic forms of dystrophic epidermolysis bullosa. Autoimmune EBA affects

the structures that anchor the BMZ to the dermis. These structures are the anchoring fibrils (AF), and the principal component of these fibrils is collagen VII (COL7). In this disease, patients develop anti-COL7 autoantibodies, causing skin fragility, erosions, and blisters (73,74). EBA prevalence is as low as 0.2 per million but is variable according to race and has been reported at a higher prevalence in Koreans. This pathology can present in a wide range of ages from childhood to middle age; however, most cases begin at 40 and 50 years of age. The genetic factor is HLA-related, and the DR2 phenotype is associated with EBA. Evidence that EBA is an autoimmune disease includes the deposition of class IgG autoantibodies reactive to COL7; these autoantibodies deposited along the DEJ can be detected by immunofluorescence studies (75). Different clinical subsets have been described and include the following: 1) *classical feature*, consisting of skin fragility and mechano-bullous disease with acral involvement. The healing process produces scarring and milia (cysts), the oral mucosa is frequently implicated, and the pathology demonstrates dermal-epidermic splitting at the BMZ with discrete inflammation. 2) *BP-like feature*, which is a form of EBA in which vesicles and bullous pruritic lesions are disseminated along the trunk, abdomen, and extremities, and inflammatory infiltrates are prominent and are composed of neutrophils, mononuclear cells, and a small number of eosinophils. 3) *cicatricial pemphigoid-like feature* involves different mucous epithelia including the mouth, esophagus, conjunctiva, anus, and vagina, with a histopathology demonstrating the splitting of the DEJ with little infiltrates. 4*) Brunsting-Perry pemphigoid-like feature* is located in the neck, the bullous lesions are recurrent, and the immunopathology demonstrates IgG deposition along the DEJ. 5) *linear IgA bullous dermatosis-like disease* is accompanied with linear IgA deposition along the BMZ. Clinically, the disease is characterized by the presence of tense vesicles arranged in an annular pattern, and the autoantibodies against COL7 are of the IgA and IgG classes (75). Therefore, anti-COL7 autoantibodies target different epitopes. This differentiated molecular reactivity to epitopes is associated with the different clinical features (76,77). EBA is linked to other systemic diseases including inflammatory bowel disease (78). The diagnosis can be made using the following guide: A) a bullous disorder within the defined clinical spectrum, B) absence of a family history of a bullous disorder; C) skin biopsy with subepidermal blistering, D) DIF of perilesional skin showing IgG deposition within the DEJ, E) immunoelectron microscopy of perilesional skin showing IgG deposition within the lower lamina densa; and F) alternative laboratory tests to demonstrate autoantibodies, including immunofluorescence, ELISA, western blot, and other tests (79,80). The treatment depends on the clinical features of the EBA and includes general measures to avoid skin trauma. The classical therapy includes colchicine, dapsone, steroids, and immunosuppressive therapy, and recently, the use of anti-CD20 that targets mature and immature B cells has been used with success (78,81).

VITILIGO

Vitiligo is a chronic depigmentation disease that affects the melanocytes, and the destruction of the melanocytes is the central pathological event that causes the depigmentation. This pathology can be presented clinically as a primary disease or can be a component of multiple autoimmune processes such as thyroid disease, pernicious anemia, rheumatoid arthritis, lupus, adult onset autoimmune diabetes, and Addison's disease (Figure 4). Vitiligo is present in 0.5% of the population (82), is distributed equally in males and females, and the disease starts during the second decade of life. Autoimmune depigmentation is expressed clinically as generalized vitiligo (GV), which is a result of diverse mechanisms involving multiple genes and environmental factors that are not well determined. However, clinical and experimental data have demonstrated enough evidence to support that GV is an autoimmune disease (83,34). Several chromosomal loci have been implicated in autoimmunity or in autoinflammatory disease. In vitiligo, one locus of particular interest is on chromosome 17p13 and is linked in lupus patients who simultaneously develop vitiligo (85). This locus is located in the *NALP1* gene, which encodes NACHT leucine-rich-repeat protein 1, a regulator of the innate immune response, and contributes to

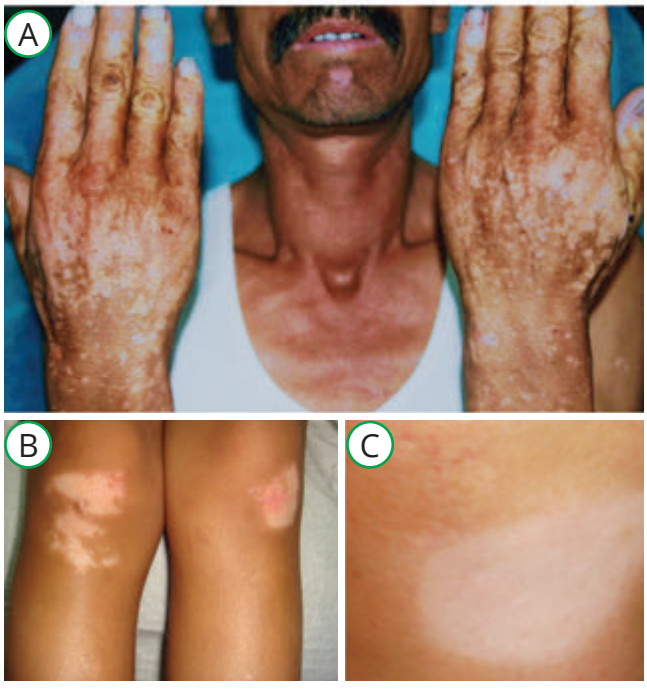

Figure 4. Vitiligo. A. Depigmentation as component of multiple autoimmune processes such as thyroid disease, note the thyroid enlargement. B and C. Lesions clinically presented as a primary disease.

the risk of GV susceptibility (86,87). Another gene involved in vitiligo is *TYR* (encodes tyrosinase), which is involved in melanin biosynthesis and is the major GV autoantigen (88). The major histocompatibility complex (MHC) demonstrates the association of HLA-A02 with GV (89). The pathophysiology of GV is the result of infiltrating $CD8^+$ T cells, which are activated by melanocyte-specific peptides (tyrosinase), melanoma antigen recognized by T-cells-1 (MART1), melanin-concentrating hormone receptor-1 (MCHR1), gp100, and tyrosine hydroxylase (TH). In response, the $CD8^+$ T cells express IL-17, TNF-α, and IFN-γ and cytotoxic molecules, e.g., as granzyme B, that induce melanocyte apoptosis. The expression and extension of the disease depends strongly on IFN-γ and CXCR3; therefore, antagonists of these molecules have been proposed as possible therapies to preserve skin pigmentation (90). The humoral immune response is frequently found in GV, and autoantibodies against tyrosinase, MART1, MCHR1, gp100, TH, and PMEL17 can be detected in approximately 42% of the patients. However, the autoantibodies do not correlate with disease activity (89). Different therapeutic approaches have been used with variable results, including the traditional therapy using corticosteroids, calcineurin inhibitors, and narrow-band UVB or UBA radiation combined with the administration of oral photosensitizing molecules such as psoralen. More recently, immunomodulatory therapies have been studied using an engineered mouse model of this condition (91,92).

PSORIASIS

Psoriasis is a papulo-squamous and desquamative disorder characterized by sharply demarcated erythematous plaques covered with silvery whitish scales. Psoriatic lesions are distributed mainly on the scalp, elbows, knees, trunk, and gluteus creases. The fingernails are involved in 50–80% of patients, displaying pitting, leukonychia, nail plate crumbling, red spots in the lunula, and nail-bed psoriasis (onycholysis, oil-spots, hyperkeratosis, erythronychia, and/or splinter hemorrhage). The clinical subsets of psoriasis are guttate, plaque, pustular, erythrodermic, and inverse (Figure 5). Psoriatic arthritis is an extra-cutaneous manifestation observed in 5-20% of patients. The clinical evolution of psoriasis exhibits a cyclical pattern, improving during the summer and worsening in the winter (93-95). Psoriasis is associated with a group of susceptibility genes: *PSOR 1–7, the LCE3B/3C* gene (related to epidermal differentiation), the *IL-23* gene, and the gene encoding transcription factor NF-kB (96). The HLA-Cw6 allele constitutes a risk factor for the development of psoriasis (96,97). In summary, psoriasis is associated with a genetic background accompanied by autoinflammation. The inflammasome plays a role in the pathogenesis of psoriasis, and for this reason, it is considered an inflammasome-mediated pathology (98,99). There are biochemical differences according to the clinical subsets; for example, chronic plaque and guttate psoriasis differentially express the skin proteins SCCA2, cytokeratin 14, cytokeratin 17, enolase, superoxide dismutase, and galectin (100-103). The psoriatic abnormality is related to epidermal proliferation, hyperkeratosis, skin regeneration, skin metabolism, and inflammation. The transcription of genes involved in the disease induces pathologic changes in the skin and other tissues such as entheses and joints. The pathophysiology depends on a complex network of cellular interactions between T cells, monocytes, and activated macrophages, which results in the over-regulation of cytokines, e.g., TNF, IL-6, IL-12, IL-2, and IFN-γ and produces psoriatic inflammation of the skin and joints (104,105). An initial trigger, such as an infection

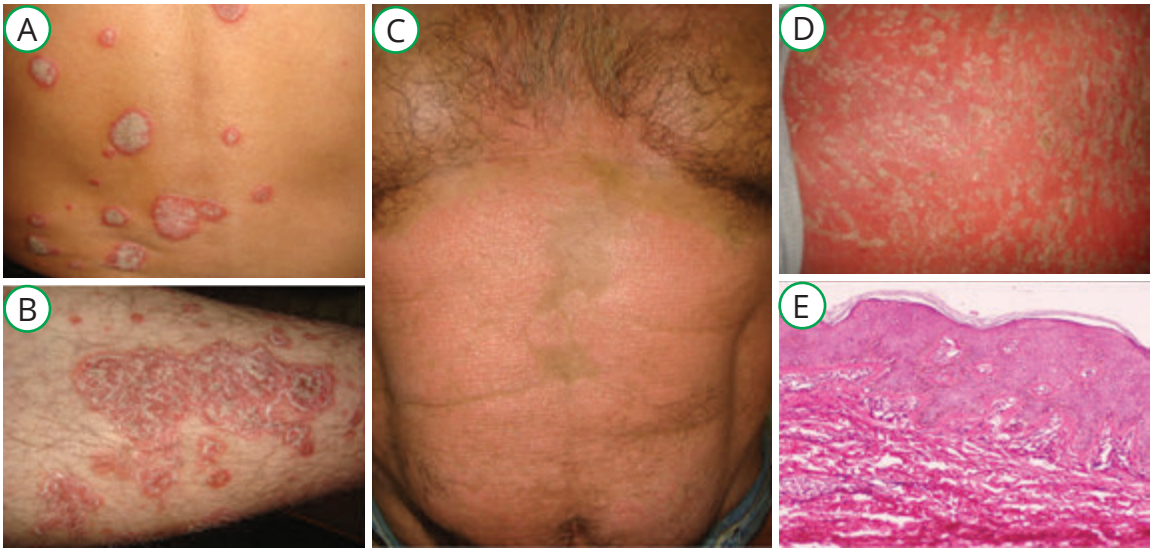

Figure 5. Clinical subsets of psoriasis are: A. guttate, B. plaque, C. Generalized, D. erythrodermic, E. Histology.

(*Streptococcus, Klebsiella*, or a virus), trauma (Koebner phenomenon), stress, or drugs could set off the disease; next, a number of different transcription factors, receptors, and cytokines may induce and support keratinocyte hyperproliferation and inflammatory infiltrates along with plaques. These infiltrates become recurrent through IL-8 participation, which enhances neutrophil accumulation in psoriatic plaques. Because psoriasis is an autoinflammatory disease, beyond the traditional treatments, current therapeutic approaches focus on biological agents to ameliorate inflammation, including the use of chimeric or human anti-TNF monoclonal antibodies and recombinant anti-cytokine receptors. Lately, IL-12 and IL-23 blockage has been suggested as a new strategy to prevent the production of TNF, IFN-γ, and IL-2 cytokines and may represent an effective way to arrest the differentiation of the Th17 phenotype via IL-1 signaling, which drives the inflammation pathway in psoriasis (106,107).

CONCLUDING REMARKS

The various autoimmune blistering diseases reviewed in this chapter have in common the blistering triggered by autoimmune reactions that lead to the loss of cell adhesion in skin components at different levels, depending on the disease. Early diagnosis and rational therapies are required to terminate the fatal or disabling course of skin autoimmune diseases. New therapeutic approaches based on a pathogenic approach are the current challenges. However, future therapeutics point toward restorative tolerance approaches that are not yet available for clinical use. Therefore, these diseases should be taken as research challenges with the long-term task of eradicating autoimmunity issues.

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35

SYSTEMIC SCLEROSIS

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INTRODUCTION

Systemic Sclerosis (SSc) is a chronic disease of the connective tissue (CT), of unknown etiology, and variable severity/course. The CT supports, connects, or separates different types of tissues and organs of the body. It is one of the four general classes of biological tissues, the others of which are epithelial, muscular, and nervous tissues. The CT has three main components: cells, fibers, and extracellular matrices.

The hallmarks of SSc are extracellular matrix alterations and fibrosis, secondary to the deposit of large amounts of CT. Clinically, this results in hardening and thickening of the skin, micro vascular and blood vessel problems subsequent to changes in endothelial cells that result in Raynaud's Phenomenon (RP), autoimmunity disruptions, and degenerative fibrotic changes at musculoskeletal and visceral levels (1).

In 1980, the American College of Rheumatology (ACR) classified SSc into 2 subtypes: Diffuse Systemic Sclerosis (DSSc) and Limited Systemic Sclerosis (LSSc) (2) based on the extent of skin involvement, specific antibodies, and internal organ damage. In 2001, abnormal capillaroscopy results were added to these criteria (3), thus improving the disease inclusion criteria as well as its early detection.

LSSc has an insidious course and the main features include cutaneous involvement limited to distal elbow and knee areas, and occasionally, the face. Rheumatoid factor (RF) may precede cutaneous manifestations by many years and the vascular component is mostly related to clinical signs such as pulmonary artery hypertension (PAH), digital ulcers (DU), telangiectasias, and renal crisis.

DSSc is characterized by a more progressive and widespread skin involvement including all extremities—face, neck, and upper body. Visceral involvement is earlier, and both interstitial pulmonary disease and gastroesophageal involvement are more frequent. RF usually initiates simultaneously along with the skin activity (3).

Incidence and prevalence rates of SSc are similar in Europe, United States, Australia, and Argentina, suggesting a prevalence of 150-300 cases/million with a low prevalence in Scandinavia, Japan, United Kingdom, Taiwan, and India (4). The estimated incidence during the 70's and 80's was 0.6/million per year, and during this last decade, it was up to 122/million (5). As in most of the autoimmune diseases (ADs), SSc is more frequent in females than in males with a 3-4:1 ratio. Incidence increases with age, and the peak is observed between 45 and 64 years old in both sexes. African-Americans tend to have a higher risk than Caucasians (6), and along with those patients with delayed presentation of the disease, they tend to show a more aggressive course (4).

There is evidence suggesting an association between exposure to environmental factors and SSc. Nevertheless, these environmental factors make only a limited contribution to these cases. Smoking has not been found to increase the risk of developing SSc, but it might contribute to the severity of the disease (4).

Physiopathology is based on 3 pillars: vasculopathy, fibrosis, and autoimmunity. In the natural course of SSc, the dysfunction of regulatory mechanisms in response to an injury or to inappropriate stimuli could result in a vascular disease with inadequate vascular proliferation and remodeling and/or altered response of the blood vessel wall. At the beginning, there is an increase in the vasoconstrictor or vasospactic activity (i.e., 95% of the patients present RP) that evolves to a structural alteration (i.e., microcirculation vasculopathy) (7). The tiny arteries and arterioles develop a concentric fibrotic injury of the intimae. This injury may be accompanied by intravascular thrombosis occluding the light. Platelet activity seems to be higher, and the fibrinolytic system altered. The endothelial injury may develop

early and present damage to the endothelial cells as well as apoptosis, excessive platelet adhesion and decreased plasma concentration of von Willebrand factor.

Atrophy and capillary growth produce architectural distortion. Pathological findings suggest a significant loss of the vascular net that is not proportional to the appearance of new vessels, which could lead to tissue ischemia (8).

Fibrosis pathogenesis in SSc includes a complex set of interactions between the fibroblast and its environment. Two important mechanisms have been identified: production stimulation of the extracellular matrix by cytokines and fibroblast growth that produces a higher amount of extracellular matrix. Multiple types of fibrosis are activated. However, the mechanisms leading to this activation are unclear. Disproportionate fibroblastic activity might be due to a combination of defects including fibrillin alterations, antibody formation, excessive endothelial reaction in the injury, and excessive response to the transforming growth beta (TGF β) factor together with an increase in IL1 and IL4 that stimulates extracellular matrix production (9-11).

Survival rates have improved during the last few years. Nevertheless, pulmonary involvement, both vascular and interstitial, is the main cause of death related to SSc (4). Early diagnosis is essential for the initiation of effective therapeutic measures aimed at modifying the natural course of the disease. Otherwise, when the diagnosis is delayed and complications are established, the disease is irreversible and fatal. Next, we will use clinical examples to illustrate the course of the disease.

CASE REPORT 1

This was a 22-year old woman, with a four-year evolution of change in hand coloration related to low temperature exposure, paleness and cyanosis in fingers associated with pain and subsequent erythema, and no other associated symptoms. As relevant background, she refers to a mother with Sjögren's Syndrome (SS) and a maternal cousin with systemic lupus erythematosus (SLE)—no personal or expositional background. During physical examination, finger edema was evident, as was triphase RP and skin activity with a Rodnan Score of 6 (hands, thighs, feet). In order to make the differential diagnosis between primary and secondary RP, a videocapillaroscopy was done and megacapillary hemorrhage, capillary loss, capillary branches, and disorganized capillary architecture corresponding to an SSc active pattern was found. Within the autoimmune profile requested, positive antinuclear antibodies (ANA), 1280 nucleolar pattern, and positive Anti SL 70, and elevated acute phase reactants. Anti-aggregate therapy with acetyl salycilic acid (ASA) (100 mg/day), preventive measures regarding temperature changes and repetitive trauma, lubricating and moisturizing skin lotions, Methotrexate (15mg/week by mouth), Folic Acid (1 mg/day by mouth), extended-release Nifedipine (30 mg/day by mouth) resulted in skin improvement and spacing with a decrease in the severity of RP episodes.

In 1862, Maurice Raynaud described the phenomenon given his name, which consist of an episodic vasospasm of the digital arteries that causes chromatic sequential changes in the skin on the hands, toes, and fingers as well as the other acral parts of the body (Figure 1). Within the RF Physiopathology, several physiological processes that contribute to the balance alteration that normally must exist between substances or vasodilator and vasoconstrictor processes have been involved. It is estimated that 3 to 22% of the general population suffers from coloration changes in the fingers with a cold stimuli (12-15). Since 1886, it has been postulated that the physiopathological difference between the primary RF that is the one not related to any pathology and the other, secondary RF, was related to other pathologies that were predominantly autoimmune in nature, e.g., SSc (90%), SLE (10-45%), SS (30%), inflammatory myositis (20%), and rheumatoid arthritis (RA) (10-20%).

In a meta-analysis published by Spencer-Green where 639 patients from ten studies were included for analysis, it was shown that approximately 12% of the patients with RP will present a pathology that explains this phenomenon, and the average time for the symptoms of an associated pathology to show up was 10.4 years from the initiation of the phenomenon. Transition predictors from primary RP to secondary RP are: abnormal capillaroscopy [Odds ratio (OR): 18.8, 95% Confidence Interval (CI): 8.4-42, positive predictive value (PPV): 47%, negative predictive value (NPV): 93%], and the presence of positive ANA results (OR: 9.3, 95%CI: 5.3-16.6, PPV: 30%, PNV: 93%) (16). Current classification criteria for primary RP were described by Le Roy and are summarized in Table 1 (17).

When facing RP, the most important action to take is to classify it as either primary or secondary (Table 2) (18).

The "Gold Standard" to make this differentiation is videocapillaroscopy, a simple, non-invasive, economical technique that evaluates the microvasculature and detects morphological abnormalities. Videocapillaroscopy is a predictor of secondary RP (OR: 18.8, 95%CI: 8.4-42, VPP 47% VPN 93%) (Figure2). Autoimmune systemic pathologies related to RP in its physiopathology have an important vascular component, specifically at the microvascular level, and some of them occur during an early phase. The main anatomical abnormalities predicting the microvascular injury are summarized in Table 3.

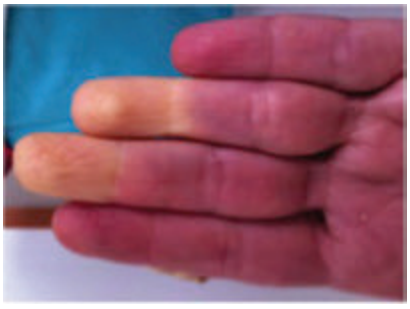

Figure 1. Triphase Raynaud's phenomenon.

Vasospastic attack caused by cold or emotional stress
Symmetric Attack including both hands
No digital necrosis or gangrene
No history or suggestive findings of secondary causes for the phenomenon
Normal capillary Nail Bed (i.e., normal capillaroscopy)
Normal erythrocyte sedimentation rate
Negative serological findings, specifically ANA tests.

Table 1. Criteria for the diagnosis of primary Raynaud's phenomenon.

PRIMARY RAYNAUD'S PHENOMENON	SECONDARY RAYNAUD'S PHENOMENON
• Absence of underlying disease • Absence of tissular necrosis or gangrene • Normal capillaroscopic pattern • Normal acute phase reactants • Negative to antibodies	• Secondary disease suspected • Presence of tissular necrosis • Abnormal capillaroscopic pattern • Elevated ESR • Positive to antibodies

Table 2. Differentiation between primary and secondary RP.

Although a modest number of tortuosities can be observed in healthy individuals, especially in the elderly, the presence of significant capillary tortuosity (higher than 20%) has been reported in several rheumatic diseases such as SLE, Behçet Disease, SSc, and the mixed connective tissue disease (MCTD). Ectasia is defined as the increase in the diameter of the capillaries to over 20 microns. This alteration is present in a wide range of pathologies including SSc, dermatomyositis (DM), MCTD, acrocyanosis, and benign hemorrhagic teleangiectasias. Megacapillary terminology is reserved for homogeneously dilated loops having a diameter greater than 50 microns. Ectasias and megacapillaries are considered structural abnormalities and are characteristic of the early pattern in SSc. Angiogenesis is a secondary process countervailing the progressive reduction of capillary density. It is characterized by the presence of "tree-shaped" capillaries, or the presence of four or more capillaries within the dermal papilla. It is particularly visible in patients with dermatomyositis (DM), diabetes mellitus, and psoriasis. Hemorrhage and thrombosis may also be observed. A hematic extravasation secondary to capillary wall injury is not uncommon, and in the case of a long duration of disease, hemosiderin deposits can be found. The presence of hemorrhagic disruptions can be observed also in healthy subjects due to microtraumas, onychophagia, or manicure. They may also be present in active phases of SSc and MCTD. Reduced capillary density is characterized by the appearance of avascular areas, defined as the absence of capillaries in a field greater than 500 microns. This finding is one of the most characteristic expressions of the SSc. This abnormality has a prognostic significance and is characteristic of the most aggressive and major progression types of SSc (19, 20).

Among the vascular abnormalities that can be found in the videocapillaroscopy and that predict the presence of SSc are the following: Increase in capillary size (OR 7.2, 95%CI: 4.2-12.4, p=0.001) and loss in the number of capillaries (OR: 2.5 95%IC: 1.2-4.9, p=0.001) (21).

Figure 2. Capillaroscopy technique.

• Tortuosities • Ectasia • Angiogenesis • Hemorrhage and thrombosis • Reduced capillary density

Table 3. Microvascular injury. Predictive findings of nailfold capillaroscopy.

Videocapillaroscopy patterns. These have been described for SSc based on the alterations found.

- EARLY SSc Pattern: This is characterized by occasional hemorrhage and giant capillaries preserving the capillary architecture and its distribution (Figure 3).
- ACTIVE SSc Pattern: giant capillaries, hemorrhages, moderate capillary loss, and disorganization of capillary architecture (Figure 4).
- LATE SSc Pattern: decrease in the number of capillaries, loss of architecture, branching, and capillary disorganization showing avascular zones (22) (Figure 5).

Cutaneous involvement is a pathognomonic feature of SSc and in order to evaluate its degree of activity, the Rodnan Score, which is a semi quantitative score that evaluates

the skin in 17 body areas, is used. The score goes from 0 to 3 with 0 being normal and 3, skin thickening without fibrosis (Figure 6). The maximum possible score to measure cutaneous activity is 51. Since the extension of the skin involvement is responsible for classifying different subtypes of SSc, a Rodnan Score higher than 21 is a diagnosis of DSSc. In addition to being a useful tool for measuring the cutaneous activity in SSc, Rodnan Score is predictive, prognostic, and measures the SSc therapeutic response. (23).

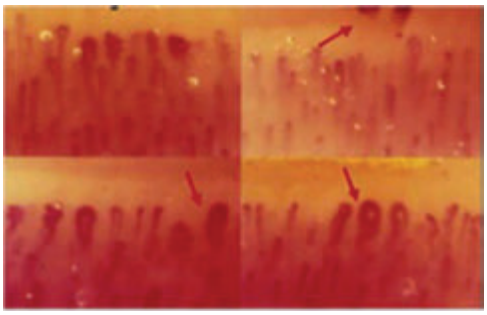

Figure 3. EARLY SSc pattern. This is characterized by occasional hemorrhage, giant capillaries preserving the capillary architecture and its distribution.

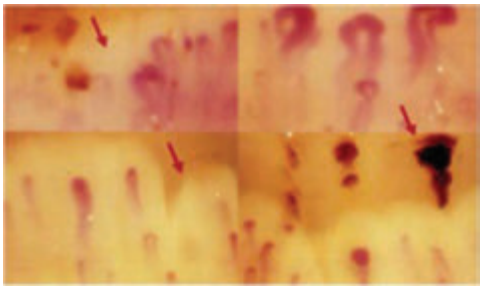

Figure 4. ACTIVE SSc pattern. Giant capillaries, hemorrhage, moderate capillary loss, and disorganization of capillary architecture.

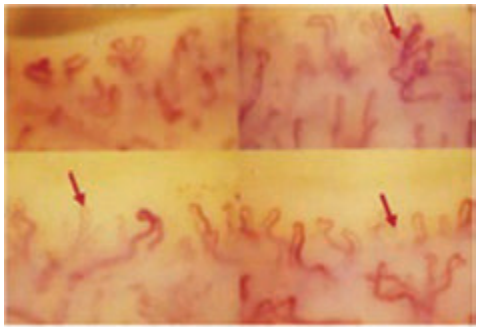

Figure 5. LATE SSc pattern. Decrease in the number of capillaries, loss of architecture, branching, and capillary disorganization showing avascular zones.

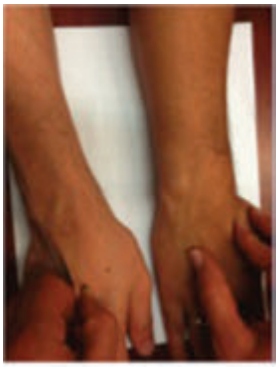

Figure 6. Technique for Rodnan score.

In patients with RP, ANA positive has an OR between 3-9 for collagen diseases, and among them, the strongest association is with SSc (24). Furthermore, anti Scl70 positive or topoisomerase I increases the SSc diagnostic probability even more, specifically with the diffuse disease subtype (24, 25).

For this patient, a young woman, with RF of several years of evolution, a background of family autoimmunity, ANA and Anti Sl70 positivity, videocapillaroscopy pattern secondary to SSc, and cutaneous activity based on the Rodnan Score, the most probable diagnosis is secondary RP with active early diffuse SSc.

Within non-pharmacological measures for the treatment of RP, the following are included: avoid or stop smoking, avoid exposure to cold and traumatic work activities (vibrational), do more exercise, and get relaxation therapy. Simultaneously, the following pharmacological measures must be initiated: use of calcium antagonists (Nifedipine or diltiazem) for their vasodilating action that decreases the number of episodes and their severity and angiotensin converting enzyme (ACE) inhibitors (enalapril or losartan) for their vasodilator effect. In refractory cases, use inhibitors of phosphodiesterase 5 (Sildenafil, or tadalafil) or endothelin receptor antagonist I (Bosentan), alpha-adrenergic blockers, e.g., Prazocin, which have very limited use due to their greater adverse effects. In severe, acute, or refractory cases, Prostanoids such as (Prostacyclin IV or iloprost) are indicated due to their vasodilating action and their antiplatelet action.

Recently, the usefulness of Serotonin reuptake inhibitors such as fluoxetine to treat RP has been reported. Other therapies include botulinum toxin, spinal stimulation and laser therapy, palmar digital artery sympathectomy, and surgery in cases of severe digital ischemia (26). Two

Strict monitoring of patients with RP impacts morbi-mortality. Capillaroscopic evaluation is mandatory in patients with RP in order to differentiate primary and secondary RP. Secondary RP should be treated to modify the course of vascular damage. If capillaroscopic abnormalities are detected, further study should be done to search for early SSc.

randomized, controlled studies showed improvement in the Rodnan Score after using Methotrexate during the SSc early phase (27, 28)

CASE REPORT 2

This is a 24-year old woman, with a 3-month evolution of a painful injury on her right hand, third finger pad. As background, she was diagnosed with hypothyroidism 10 years ago and is an occasional smoker. When asked, a 5-year evolution of RP and finger edema for six months, were detected, but she did not mention dysnea or cardiovascular symptoms. On physical examination, a triphase RP was evident as well as finger edema, oral mucosa telangiectasias, (photo), cutaneous activity with a Rodnan Score of 16 (hands, forearms, trunk, face, thighs, feet), and a deep DU showing irregular edges, edema, and perilesional skin inflammation with necrosis. Diagnostic aids requested: Positive ANA 640 homogeneous, positive Anti SL 70, positive anti-thyroid, carbon monoxide diffusion (DLCO) 68% of predicted (decreased), unaltered trans-thoracic echocardiogram (TTE) with pulmonary artery systolic pressure (PASP): 25 mm Hg, abnormal videocapillaroscopy with a pattern of active SSc. Treatment with non-pharmacological measures was initiated including: avoid cold, nicotine, and repetitive trauma exposure as well as strict lipid control. For skin activity, treatment with Methotrexate subcutaneous (25 mg/week) and folic acid (5 mg/week by mouth) was initiated. To control vasculopathy, management with vasodilators was initiated: Sildenafil (50mg /12hr increasing dose to 200mg/day), Atorvastatin (20mg/day), Dicloxacillin (500 mg by mouth every 6 hr for 10 days), SA (100 mg/day), topical Vitamin E, and pain management with opioids. Three months later, patient mentions decrease in skin activity, Rodnan 12, digital ulcer healing but reported strong adverse events with Sildenafil. Therefore, vasodilator treatment scheme was changed to Bosentan (62.5 mg every 12 hr for 4 weeks by mouth, with maintenance dose of 125 mg every 12 hr for 16 weeks). As a result, patient maintained the DU healing process, and for 12 months she did not present new injuries (Figure 8).

RP in a young woman must always be considered a predictor of an autoimmune disease with vascular involvement. Therefore, the differentiation between primary and secondary RP is mandatory. In the event that secondary RP is present at an early age, it is considered a risk factor for the subsequent development of DU in SSc (OR: 3.62, 95%CI: 1.66-7.91, p=0.001) (29).

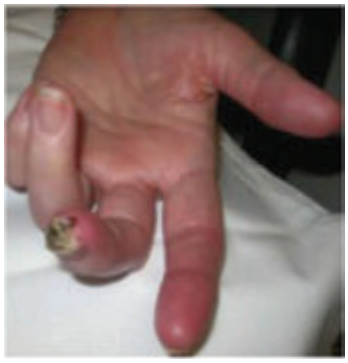

Figure 8. Active digital ulcer.

DU's are the most common clinical manifestation of vasculopathy in SSc. They are defined as a denuded area with loss of dermis and epidermis that is located on the volar surface of the fingers and distally of the proximal interphalangeal joints. They are characterized as being immensely painful with distal tissue loss, delayed healing, and at high risk of infection, progression to gangrene, and amputation thereby becoming a major cause of morbidity (30). Depending on their location, DU's are classified as: DU in distal area of the fingers and DU located over bone prominences. In the first case, the pathophysiological mechanisms involved are: progressive tissular ischemia due to injury induced by free radical oxygen; persistent vasospasm; secondary RP, leading to deterioration in oxygenation; vasculopathy for intimal proliferation with decreased vessel lumen; endothelial injury by antibody action; and increased vasoconstrictor and intraluminal thrombosis. In DU's located on the bone surfaces, the repeated trauma on chronic contracture sites, and chronic atrophic surrounding tissues make these areas vulnerable to injury and deterioration of the healing process are responsible for the appearance of these lesions and, unlike DU located in distal areas, these do not respond to vasodilator treatment (30).

According to the Pittsburgh database, of 2,080 patients with SSc, 58% developed DU. Of those, 68% had a limited form of the disease, 32% had recurrence, and 30% had severe complications such as gangrene and amputation. Videocapillaroscopy can predict the development of DUs using the diameter, number of capillaries, and presence of megacapillaries: Diameter x No. Megacapillaries/ (No. Capillaries)^2. with a sensitivity of 94%, and a specificity of 85% (31). Support treatment for DUs includes: the use of vasoconstrictors and avoidance of low temperatures, direct traumas, and cigarette smoking (32).

Intense pain generated by active DU produces anxiety and worsens RP episodes, which contributes to a delay in healing—avoid NSAIDs, stagger opioids, and improve oxygenation of the affected area (32). DU infection should be suspected if there is increasing pain and presence of pus. In these cases, a culture of the lesion should be taken and antibiotics prescribed based on the antibiogram. In the case of osteomyelitis, interdisciplinary management should be started.

Pharmacological treatment objectives are based on the improvement of digital circulation, healing promotion, and appearance of new DU prevention. In a 16-week, randomized, controlled study of oral Nifedipine vs IV Iloprost, a decrease in the number of ulcers was evident with no changes in either the microvascular blood flow nor in the temperature of the hands (33). The use of phosphodiesterase 5 (Sildenafil, Tadalafil) induces vasodilatation by increasing nitric oxide levels. This medication has resulted

in a decrease in the number of ulcers (34) and an improvement in healing by preventing the appearance of DU. In a randomized, placebo controlled study of 122 patients with pre-existing DU and under DU treatment, Bosentan, one endothelin antagonist receptor showed an improvement in the functionality and reduction of new DU (35). The use of IV prostacyclin analogues (i.e., Iloprost) diminished the severity of RP and reduced the number of DU by 50% (36). The use of statins decreased the severity of RP and prevented the appearance of new DU while it also decreased the number of new DU and improved the functional scales (37,38). Sympathectomy and revascularization are used in cases refractory to pharmacological measures, and they have shown improvement in healing UD (39,40).

> Early secondary RP diagnosis impacts morbidity, and the initiation of an early treatment will decrease the risk of DU appearance. Early DU therapeutic intervention decreases infection, gangrene, and amputation as well as deterioration in functionality and quality of life.

CASE REPORT 3

This is a 42-year old woman diagnosed with SSc 8 years ago, who consulted about a progressive dysnea after moderate exertion during the last 6 months with a background of RP for the last 10 years, gastrointestinal involvement, calcinosis, and DU secondary to the base disease. Pulmonary thromboembolism and coronary acute disease were discarded. Diagnostic test report: Brain natriuretic peptide (BNP) 658 pg/ ml (N <125); pulmonary function testing (PFT) with forced vital capacity (FVC) < 80% (N> 80%); DLCO adjusted to alveolar volume (AV) 50% predicted value (N>80% predicted value); a thorax computed tomography (CT) with dilatation of the proximal segment of the pulmonary artery, transverse diameter of 29.6 mm (N<25 mm) with no evidence of interstitial lung disease (ILD); ETT with an ejection fraction of 65%, tricuspid regurgitation velocity of 3.4 m/seg (N<3m/seg), PSAP of 55 mm Hg (N<35 mm Hg) without valvulopathy. Hemodynamic results related to cardiac catheterization showed a pre capillary PAH: PASP of 50 mmHg (N = 30), average PASP 36 mm Hg, right atrial pressure 15 mmHg (N = 5), right ventricular pressure of 52 mmHg (N = 30). A treatment with diuretics, oral anticoagulants, oxygen, immunosuppressants, and a combination of vasodilators with Sildenafil plus Bosentan showed functional improvement 12 weeks after initiation of therapeutic plan.

Pulmonary involvement, especially vascular, can be found in 5-12% of SSc patients, and it is considered the first cause of mortality within this population. PAH early diagnosis is vital for the initiation of treatment before the functional and hemodynamic deterioration is irreversible (41).

Previous studies in patients with SSc have determined that LSSc type of the disease (42), RP duration (43), DUs (44), gastroesophageal reflux (45), etc. are risk factors for PAH.

Within the screening for PAH early detection in SSc, the following is included: BNP measurement, which elevates in early stages of the pulmonary vascular involvement in patients with SSc, has been correlated with hemodynamic measures and survival (46-48). PFT are a very relevant tool for the evaluation of pulmonary complications. In SSc, the severity of the pulmonary disease through the PFT is defined as: mild (70-79% of predicted), moderate (50-69% of predicted) or severe (<50% of predicted) (49). Values lower than 80% of the predicted in FVC in the presence of RP are independent risk factors associated with PAH in patients with SSc (50). An alteration in DLCO is considered an early marker of ILD and/or PAH in SSc, and it is correlated with their severity (51-53). The decrease in DLCO is associated with an increase in mortality and a greater risk of developing PAH (54, 55). It is also considered a predictive tool for the detection of PHT in patients with SSc (56).

The thorax CT is very useful in the study of ILD associated with PAH. It is not useful for a direct diagnosis of PAH, and only the dilatation of lung vessels can, in later stages, help with diagnosis (57).

TTE is the easiest non-invasive, screening test available for the PAH diagnosis, and it is very important for excluding other possible PAH causes such as valvulopathies or cardiomyopathies (58). Tricuspid regurgitation strongly reflects right ventricular function and predicts survival in patients with PAH and SSc (59). However, TTE has limitations: it is an operator-dependent test, and that is why there are many false-positive results (60). TTE can be useful in the PSAP estimate, in visualizing structural and hemodynamic changes, but the PAH diagnosis should always be confirmed by cardiac catheterization (61, 62).

Cardiac catheterization is considered the gold standard for confirming the suspected PAH diagnosis through TTE, and it is recommended for all PAH patients prior to the initiation of specific treatments (63, 64). It is also used to directly measure the severity of hemodynamic changes and to assess the vasoreactivity of pulmonary circulation, using short-acting pulmonary vasodilators. However, the test in patients with SSc vasoreactivity is not considered cost-effective since only 6 to 10% of patients are reactive to calcium antagonists, and this low reactivity is depleted over time, making this type of therapies not cost-effective.

In patients with PAH and SSc, follow the same treatment algorithm as used in patients with idiopathic PAH. Nonetheless, treatment seems to be more complex compared to other forms of PAH as it should be addressed to correct both the symptoms and the causes. The use of diuretics, digoxin, and anticoagulants should be considered on the basis of individual cases (65). Steroid use in SSc should be limited considering the risk of triggering a renal crisis (66). Control

of ILD with cyclophosphamide or mycophenolate mofetil can reduce the severity of secondary PHA to interstitial involvement in the early stages, not in fibrosis (67). Phosphodiesterase type 5 (PDE5) is an enzyme that rapidly degrades cGMP and limits pulmonary vasodilatation mediated by nitric oxide. The use of PDE5 inhibitors (e.g., sildenafil, tadalafil) improves exercise capability, functional class, and some hemodynamic measurements. The difference between the two is the time of action with tadalafil being the one with the longest action—36 hours compared to 4-5 hours for Sildenafil (68, 69). SSc patients with PAH show an increase in endothelin-1 production (ET-1). The use of antagonists of this vasoconstrictor lowered morbidity. There are three ET receptor antagonists. 1: bosentan, a dual antagonist of receptor ET-1, improves a six-minute walk and survival; 2: sistaxentan and ambrisentan, both antagonists of receptor ET-1 improve exercise capability. However, sistaxentan was withdrawn from the market due to its marked liver toxicity (70). The analogues of prostacyclin (epoprostenol, iloprost, and trepostinil), potent vasodilators, have improved functional and hemodynamic parameters; 3: epoprostenol, has a short average life, and its route of administration may induce multiple adverse effects (70). Combination therapy is a therapeutic strategy that maintains stability in functional improvement (70).

PAH in patients with SSc is a major cause of morbidity and mortality. Its early diagnosis and intervention impact survival. A rigorous and regular PAH screening of patients with SSc allows early detection of this serious complication and should be done systematically on risk populations.

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36 SYSTEMIC VASCULITIS

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INTRODUCTION

Vasculitis is an inflammatory process that affects the vessel wall as the primary site of inflammation. This process causes damage to the vessel wall and fibrinoid necrosis, it leads to the narrowing of the lumen due to thickening or to total occlusion due to thrombosis resulting in tissue ischemia and necrosis. Moreover, the focal lesions within the vessel wall may also cause weakening with the subsequent development of aneurysms and/or rupture with bleeding into surrounding tissues (1,2).

Vasculitic syndromes are heterogeneous disorders whose disease manifestations may arise from the involvement of different types and sizes of blood vessels (i.e., small, medium, and large vessels) in different organs and systems (3). When only one organ is affected by the vasculitic process, it is regarded as a single-organ vasculitis (SOV) while the involvement of several organs and systems characterizes a systemic vasculitis (4). Systemic vasculitis can be primary when no etiological factor is identified or secondary to infections (e.g., secondary to hepatitis C or HIV infection), drug-induced (e.g., propylthiouracil, hydralazine), drug abuse (e.g., levamisole-induced vasculitis), systemic autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis, and Sjögren's syndrome), or to cancer (5).

CLASSIFICATION OF VASCULITIS

Several classification systems have been developed to categorize vasculitis syndromes since the first report of a patient with necrotizing arteritis referred to as periarteritis nodosa in 1866 by Kussmaul and Maier (1,2). The first attempt to classify vasculitis was published by Zeek and included five categories: hypersensitivity angiitis, allergic granulomatous angiitis, rheumatic arteritis, periarteritis nodosa, and temporal arteritis. This first classification of vasculitis did not include granulomatosis with polyangiitis (GPA – formerly Wegener's granulomatosis) or Takayasu arteritis. A microscopic form of periarteritis nodosa was recognized by Davies in 1948, and later on, it was considered to be closely related to GPA and to eosinophilic granulomatosis with polyangiitis (EGPA – formerly Churg-Strauss syndrome). The most widely accepted way of classifying systemic vasculitis included the predominant size of affected vessels, the association with an etiological agent (i.e., primary or secondary vasculitis) and the presence of antineutrophil cytoplasmic antibodies (ANCA) (1-3).

In 1990, the American College of Rheumatology (ACR) developed classification criteria for systemic vasculitides including: Takayasu arteritis (TA), giant cell arteritis (GCA), polyarteritis nodosa (PAN), GPA, EGPA, IgA vasculitis (formerly Henoch-Schölein purpura), and hypersensitivity vasculitis. Those criteria were developed to be used in clinical and epidemiological research in vasculitis. The range of sensitivity was 71.0%-95.3%, and for specificity, it was 78.7%-99.7%. TA, GCA, and EGPA had the best sensitivity and specificity criteria. Although the ACR criteria help to distinguish one vasculitic syndrome from another in patients with an established diagnosis of vasculitis, they are not meant to be used in patients prior to the diagnosis of vasculitis, and they do not distinguish vasculitic syndromes from other diagnosis (3). Microscopic polyangiitis (MPA) was not included in the ACR criteria, and it was not recognized as a separate entity until the first International Chapel Hill Consensus Conference (CHCC) published in 1994 (2). Moreover, the use of ANCA tests for ANCA-associated vasculitis (AAV) only became widespread after the publication of the ACR criteria for vasculitides and thus were not included either (1-3).

The first CHCC was a consensus of the names and definitions of the most common forms of vasculitides. How-

ever, the CHCC was not meant to be a classification or a diagnostic system. Ten vasculitic syndromes were defined based on clinical and histological features while those entities were grouped based on the predominant size of the affected vessel (Table 1). CHCC introduced MPA as a separate entity and replaced the term hypersensitivity vasculitis with cutaneous leukocytoclastic angiitis (6). Later on Sorensen et al evaluated the use of CHCC definitions together with surrogate markers for vasculitis. They found that this approach failed to act as diagnostic criteria for GPA and MPA and proposed a new set of criteria for both entities (7). However, when evaluated prospectively in a cohort of AAV patients, Sorensen criteria were not helpful for classifying patients with MPA while they were limited for GPA due to the use of eosinophilia as an exclusion criterion (8). Then the European Medicines Agency (EMEA) developed an algorithm to improve the classification of patients with AAV and PAN for epidemiological studies using the ACR classification criteria, 1994 CHCC, and Lanham criteria in a four-step algorithm (Figure 1) (9).

A second International CHCC was held in 2012 in order to improve the original 1994 nomenclature based on advances in the understanding of vasculitis (Table 2). In the updated CHCC, an effort was undertaken to replace eponyms with descriptive terms that had a pathophysiological representation for that specific vasculitic syndrome (e.g., IgA vasculitis instead of Henoch-Shönlein purpura). In the case of TA and GCA, the two large vessel vasculitis variants with indistinguishable histopathological features, the age of 50 years was used as a reference point for the onset of symptoms since TA is a disease affecting younger and GCA a disease affecting older individuals. In medium vessel vasculitis, the absence of ANCA was included in the definition of PAN, especially to discriminate it from MPA. Small vessel vasculitis was subdivided into two categories based on the presence or absence of immune complex deposits on vessel walls namely ANCA-associated vasculitis (i.e., necrotizing vasculitis with few or no immune deposits) and immune complex vasculitis that included anti-glomerular basement membrane (anti-GBM) disease, IgA vasculitis, and hypocomplementemic urticarial vasculitis (HUV). A limited expression of GPA and EGPA affecting the upper and/or lower respiratory tract or the eyes, in the case of GPA, was recognized. In MPA, the updated CHCC highlighted the absence of granulomatous inflammation. Other categories were included in the 2012 CHCC such as variable vessel vasculitis, single-organ vasculitis, and secondary vasculitis including vasculitis associated with systemic diseases and vasculitis associated with probable etiology (Table 2) (10).

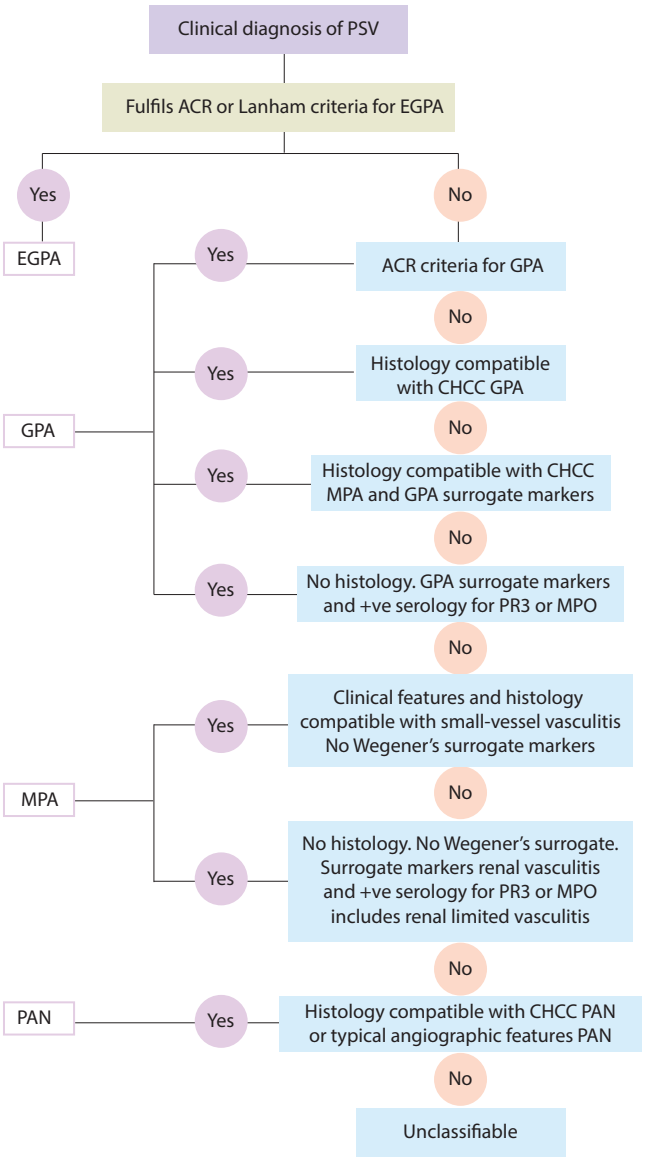

Figure 1. EMEA Classification algorithm for ANCA-associated vasculitides and polyarteritis nodosa. Adapted from reference (9).

Large vessel vasculitis
Takayasu arteritis
Giant cell arteritis
Medium sized vessel vasculitis
Polyarteritis nodosa
Kawasaki disease
Small vessel vasculitis
Wegener's granulomatosis
Churg Strauss syndrome
Henoch-Schönlein purpura
Essential cryoglobulinemic vasculitis
Cutaneous leukocytoclastic angiitis

Table 1. 1994 Chapel Hill Consensus Conference classified vasculitic syndromes based on the predominantly affected vessel size. From Jennette *et al.* (6).

Large vessel vasculitis
Takayasu arteritis
Giant cell arteritis
Medium sized vessel vasculitis
Polyarteritis nodosa
Kawasaki disease
Small vessel vasculitis
Small vessel vasculitis
ANCA – associated vasculitis Microscopic polyangiitis Granulomatosis with polyangiitis (Wegener's) Eosinophilic granulomatosis with polyangiitis (Churg-Strauss)
Immune complex small vessel vasculitis Anti-glomerular basement membrane antibody disease Cryoglobulinemic vasculitis IgA vasculitis (Henoch Schönlein) Hypocomplementemic urticarial vasculitis (anti-C1q vasculitis)
Variable vessel vasculitis
Behçet's disease
Cogan's syndrome
Singel-organ vasculitis
Cutaneous leukocytoclastic angiitis
Cutaneous arteritis
Primary central nervous system vasculitis
Isolated aortitis
Others
Vasculitis associated with systemic disease
Lupus vasculitis
Rheumatoid vasculitis
Sarcoid vasculitis
Others
Vasculitis associated with probable etiology
Hepatitis C virus – associated cryoglobulinemic vasculitis
Hepatitis B virus – associated vasculitis
Syphilis-associated aortitis
Drug-associated immune complex vasculitis
Drug-associated ANCA-associated vasculitis
Cancer-associated vasculitis
Others

Table 2. Updated vasculitis nomenclature adopted by the 2012 Chapel Hill Consensus Conference (10).

PATHOGENESIS

The etiology of primary systemic vasculitides is unknown. However, several immunological mechanisms are thought to play a role in the pathogenesis of vasculitis, and each entity has its own features. General proposed mechanisms include immune complex deposition on vessel walls, autoantibodies (e.g., anti-endothelial antibodies and ANCA), cell and molecular immune responses, granuloma formation, and injury to endothelial cells (11). Aberrant $CD4^+$ T cell polarization may be involved in different pathogenic vasculitic mechanisms. These include a Th1 response with the participation of T cells and macrophages secreting large amounts of interferon (IFN) γ, a Th2 pattern with the participation of B cells and the secretion of Th2 cytokines [e.g., interleukin (IL)-4, IL-5, and IL-10], and the production of autoantibodies. Furthermore, the newly described Th17 polarization has been involved in the pathogenesis of vasculitis as another factor involved in vascular inflammation (12-15). All pathogenic processes lead to inflammation, damage, and necrosis in the vessel wall resulting in manifestations of vasculitic syndromes (11).

Large-vessel vasculitides are mainly associated with cell mediated reactions by lymphocytes, macrophages, and usually multinucleated giant cells in the vessel wall (12,15). The deposition of immune complexes on vessel walls is observed in small vessel vasculitides (e.g., IgA vasculitis, cutaneous leukocytoclastic angiitis, cryoglobulinemic vasculitis), and in hepatitis B associated-PAN. Those deposits contain immunoglobulins, antigen, and complement and trigger vascular inflammation and damage (11). In ANCA-associated vasculitis, extravascular granulomatous inflammation can be observed in patients with GPA and EGPA, and it is the predominant pathogenic mechanism in localized forms. A mixed inflammatory infiltrate is observed in those lesions and includes neutrophils, macrophages (i.e., epithelioid cells and multinucleated giant cells), CD4+ effector memory T cells, plasma cells, and B cells that are usually organized in germinal center-like structures. In these structures, autoreactive B cells would trigger the production of pathogenic ANCA. Once produced, ANCA, either proteinase 3 (PR3)- or myeloperoxidase (MPO)-ANCA may interact with neutrophils and monocytes previously primed by proinflammatory cytokines to express PR3 and MPO on the cell surface respectively. The Fab portion of ANCA binds to its antigens on the cell surface while the Fc portion interacts with Fc receptors to activate neutrophils and cause adherence to vessel wall and transmigration. The production of reactive oxygen radicals and neutrophil degranulation is also triggered by ANCA with the resulting release of proteolytic enzymes and endothelial damage that results in vasculitis (14,16,17).

APPROACH TO THE PATIENT WITH SUSPECTED VASCULITIS

Systemic vasculitides are rare and heterogeneous diseases that affect different organs and systems with variable severity depending on the size, site, and extent of affected vessels (1). Recognition of manifestations suggestive of systemic vasculitis is important since a delay in diagnosis may impact the outcome significantly. Due to the multisystemic nature of vasculitis, a multidisciplinary approach is usually necessary. The presentation may vary from subacute non-specific complaints to life-threatening features, e.g., alveolar hemorrhage and acute renal failure (1,3). Suspicion of systemic vasculitis is usually raised when constitutional symptoms

(e.g., fever, malaise, or weight loss) are present in association with a combination of different organs and systems involved (Table 3). Thus, an overall clinical assessment is important in the initial evaluation of patients with suspected vasculitis. The evaluation of the systemic inflammatory response with acute phase reactants, specific organ involvement (e.g., glomerulonephritis, pulmonary nodules), and disease extension are part of the initial clinical investigation in systemic vasculitis. However, the diagnosis needs to be confirmed by tissue biopsy, imaging studies, and/or specific serological markers (i.e., PR3- or MPO-ANCA, anti-GBM antibodies, and cryoglobulins). Differential diagnosis with secondary causes of vasculitis (e.g., hepatitis B or hepatitis C virus infection) and with vasculitis mimics must be taken into account in a patient with suspected vasculitis (1-3,5).

ORGANS AND SYSTEMS	MANIFESTATIONS
Constitutional symptoms	Fever Malaise Fatigue Polyarthralgias Weight loss Myalgia Headache
Ear, nose, and throat	Epistaxis Nasal crusts Sinusitis Deafness Hoarse voice Saddle nose
Lungs	Cough Wheezing Hemoptysis Dyspnea
Eyes	Redness Eye pain Visual loss
Gastrointestinal tract	Abdominal angina Abdominal distention
Kidneys	Hematuria Proteinuria Renovascular hypertension
Nervous system	Paresthesis Numbness Weakness
Cardiovascular	Intermittent claudication of extremities Chest pain Decreased peripheral pulses Cardiac murmurs Asymmetric blood pressure
Skin	Palpable purpura Subcutaneous nodules Ulceration Digital necrosis Livedo reticularis

Table 3. Disease manifestations that raise the suspicion of a systemic vasculitis.

ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA)

ANCA are antibodies against antigens present in granules of neutrophils or lysosomes of monocytes. Indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA) are the techniques used to detect ANCA in clinical practice. When IIF with ethanol fixed neutrophils is used, the presence of ANCA is described as two major patterns: cytoplasmic (cANCA) and perinuclear (pANCA). In the former, a diffuse fluorescent staining is observed in the cytoplasm of neutrophils, and it is usually associated with PR3-ANCA whereas, in the case of the latter, the immunofluorescent staining surrounds the nuclei of neutrophils and is associated with MPO-ANCA. However, this association is not always absolute (18,19). Atypical patterns of ANCA may be observed in conditions other than vasculitis (e.g., inflammatory bowel disease or drug-induced vasculitis) and are associated with antibodies to other antigens such as elastase, lactoferrin, or catepsin G. IIF results for ANCA are quantified in dilution titers (19). Commercially available ELISA kits can be used to show whether specific PR3-ANCA or MPO-ANCA are present. The detection of ANCA by the ELISA technique seems to be less sensitive and more specific than the IIF. However, an improved sensitivity was seen in the newly developed capture ELISA for PR3 and MPO ANCA (18).

The use of both techniques is recommended for the detection of ANCA in patients with suspected AAV. A definite diagnosis is more likely when the presence of a cANCA or pANCA pattern on IIF is associated with PR3-ANCA or MPO-ANCA by ELISA respectively (20). Although the use of ANCA testing is well established for the investigation and diagnosis of AAV, serial monitoring of ANCA titers in patients in remission does not seem to be a reliable predictor of disease relapses and should not be used to guide therapy (18).

ANTI-GLOMERULAR BASEMENT MEMBRANE ANTIBODIES

The detection of serum anti-GBM antibodies can be an alternative tool for the diagnosis of anti-GBM antibody (Goodpasture) disease, especially in those who could not undergo a renal biopsy (21). ELISA is the most reproducible and widely used technique to detect anti-GBM antibodies. The use of native or recombinant alpha-3(IV) antigen for detecting anti-GBM antibodies in ELISA kits has yielded high a sensitivity (95-100%) and specificity (91-100%) (21,22).

CRYOGLOBULINS

Cryoglobulins are serum proteins that precipitate at low temperatures and solubilize again upon rewarming. The collection and handling of samples are crucial for a successful test. Blood should be collected, clotted, transported, and centrifuged at 37-40°C in prewarmed syringes and tubes. Then, the serum should be stored at 4°C for up to 7 days in order to observe precipitation of cryoglobulins. Quantification of cryoglobulins can be evaluated either indirectly by measuring the protein content in the cryoprecipitate or

by measuring the cryocrit. Immunofixation is then used to characterize the type of cryoglobulin (23).

TISSUE BIOPSY

In patients with suspected vasculitis, especially when small vessels are involved, a tissue biopsy of an affected organ should be obtained whenever possible. Histological confirmation of the diagnosis is useful and will prevent subsequent confusion regarding the underlying disease and therapy. The following sites are frequently biopsied in patients with suspected vasculitis: skin, kidney, upper airways, muscles, and lungs (24). Histological examination of the temporal artery biopsy (TAB) is recommended as a gold standard method for the diagnosis of GCA (25). Leptomeninges with the underlying brain cortex and sural nerve biopsy are less common procedures but both may be useful in the diagnosis of primary central nervous system angiitis and of systemic vasculitis involving peripheral nerves respectively (24,26). Histopathological signs of vasculitis include the presence of an inflammatory infiltrate within the vessel wall, fibrinoid necrosis, and leukocytoclasia (i.e., accumulation of nuclear remnants from unscavenged polymorphonuclear cells) while in medium sized arteries, the disruption of internal elastic layers can be found. Immunofluorescent staining is the usual way to detect vessel deposits of immunoglobulins such as IgA in IgA vasculitis and IgM and IgG immune complexes in cases of cryoglobulinemic vasculitis (2).

IMAGING STUDIES

Imaging studies can be used for the diagnosis, to evaluate the extension of arterial involvement and for monitoring medium- and large-sized vessel vasculitis when tissue specimens are not usually available for histopathological examination. Stenosis, occlusion, vessel wall dilation, and aneurysms can be shown by imaging studies. Conventional angiography is considered a gold standard method to evaluate vascular involvement since it shows luminal changes and guides vascular interventional procedures. However, angiography is an invasive method, and the patient is exposed to a significant amount of radiation. The use of iodinated contrast medium may result in allergic reactions or in ischemic complications (27,28).

Non-invasive imaging techniques, e.g., computed tomography angiography (CTA) and magnetic resonance angiography (MRA) can be used to detect early changes in vessel walls and are more suitable for serial measurements to monitor disease progression. However, both methods lose resolution in smaller vessels. Vascular ultrasound (US), in turn, is another non-invasive method that can show vessel wall changes, differentiate them from surrounding tissues, and estimate the velocity of blood flow. US can easily differentiate vasculitic features from atherosclerosis in carotid arteries. However, US is operator-dependent and cannot evaluate some vessel segments such as the thoracic aorta and the proximal left subclavian adequately. More recently, positon emission tomography (PET) combined with CT has been shown to be an important tool in the assessment of disease activity in large vessel vasculitis. The increased uptake of 18 fluordeoxyglucose (^{18}FDG) by inflammatory cells in vessel walls may reflect the degree of disease activity. However, the widespread use of this imaging modality to monitor disease activity in large vessel vasculitis still needs further validation (27,28).

DISEASE ACTIVITY ASSESSMENT

Once the diagnosis of a systemic vasculitis is established and therapy is initiated, there is a need for periodic assessment to evaluate response to treatment or to detect a disease relapse after achieving remission (1). Hence, several tools have been developed to evaluate disease activity, disease extension, prognosis, and damage in systemic vasculitis. The Birmingham Vasculitis Activity Score (BVAS) is the most widely used assessment tool for disease activity in vasculitis, and currently, in its third version, the BVAS includes 66 items in 9 different systems. The BVAS is often used to evaluate small vessel vasculitis and PAN while a specific version of BVAS was developed for disease activity in GPA (29). Although not formally validated, the criteria defined by Kerr et al are usually used to assess disease activity in patients with TA. The disease extent index – Takayasu (DEI.TAK), and its new version, the Indian Takayasu arteritis activity index (ITAS) were recently developed to evaluate active disease in TA (30). The OMERACT Vasculitis Working Group is developing a core set of outcome measures in large vessel vasculitis.

The disease extent index (DEI) scores the number of organ and systems involved in GPA. The DEI is complimentary to BVAS and helps to differentiate whether a high BVAS is due to severe manifestation in only one organ/system or if it is due to a multisystem disease (29). The first version of the five factor score (FFS) was developed to assess prognosis in patients with PAN, MPA, and EGPA, and it has been used to guide therapeutic decisions in these diseases (31). Recently, a new version of the FFS has been developed to include patients with GPA also in the evaluation (32). The assessment of permanent damage in systemic vasculitis can be done with the vasculitis damage index (VDI). In this tool, permanent damage is defined by an irreversible scar that lasts more than 3 months (29).

GIANT CELL ARTERITIS

Giant cell arteritis (GCA) is classified as large-vessel vasculitis affecting preferentially medium- and large sized arteries. The extracranial arteries, especially temporal arteries, are preferentially involved in GCA while intracranial branches are often spared. Aging is the most important risk factor for GCA since this disease affects people who are > 50 years almost exclusively. The disease risk is highest among those between 75 and 85. The estimated prevalence in Europe and North America is 200 per 100,000, and the incidence

is 20–30 per 100,000 with an increased frequency in northern latitudes, especially in Scandinavian countries. GCA is uncommon in African or Asian individuals (33). The largest series of GCA from Latin America reports only twenty-two Mexican patients and highlights the rarity of GCA in this population (34). There is an association between the *HLA-DRB1*04* allele and an increased susceptibility to GCA and polymyalgia rheumatica (PMR) (35).

The pathogenesis of GCA is very similar to TA. The histological findings include panarteritis with T cells and macrophages infiltrating the arterial wall associated with intimal hyperplasia. Multinucleated giant cells can be found and are usually close to fragmented internal elastic lamina. A possible bacterial or viral trigger activates dendritic cells (DCs) that migrate to the inflamed wall in GCA to initiate and maintain activation of T cells. CD4+ T cells produce IFNγ and induce macrophages to carry out their effector functions that result in vascular injury. The formation of granulomas within the media is also a T cell dependent phenomenon (36). Recent studies have also highlighted the participation of Th17 cells in the pathophysiology of GCA. Th17 cell population is explicitly corticosteroid-sensitive whereas Th1 cells are corticosteroid-resistant and persist in vascular walls despite corticosteroid-therapy (37).

A new complaint about headaches, especially in the temporal region, affects two thirds of patients and is considered a typical manifestation of GCA although the headache can also be frontal, occipital, or associated with scalp tenderness. An enlarged, pulseless, and tender temporal artery is usually observed. Half of the patients present jaw claudication which is a specific GCA symptom with a strong correlation with a positive TAB (38). Amaurosis fugax affects 10% of patients due to transitory ischemia of the retina, optic nerve, and/or choroid and precedes visual loss in about 50% of cases by 8.5 days on average (39). Anterior ischemic optic neuropathy (AION) accounts for partial or complete visual loss in 10-60% of GCA patients due to posterior ciliary arteries or retinal artery occlusion and is considered an ophthalmological emergency. The involvement of ocular muscles, their nerves, or the brainstem may cause diplopia in 6-12% of GCA patients and can also precede permanent blindness. The ocular findings in ophthalmological examination are arteritic anterior AION, arteritic retinal artery occlusion, cilioretinal artery occlusion, arteritic posterior ischemic optic neuropathy (PION), and ocular ischemia. The major differential diagnosis of arteritic AION is the nonarteritic form of AION which is associated with cardiovascular risk factors such as diabetes mellitus and high cholesterol as well as with elevated intraocular pressure, hypercoagulable states, and fluctuations of blood pressure. Arteritic AION is usually unilateral, but it may be bilateral and simultaneous or the second eye may become affected days, weeks, or even months after the first eye, particularly if treatment is delayed or is withdrawn while the disease is still active. Pupillary disturbances (bilateral dilated pupils or unilateral/bilateral tonic pupils), Horner's syndrome, visual hallucinations, bitemporal visual field defects, and facial and mandibular swellings have also been described in GCA (40).

Other GCA manifestations include tongue, scalp, or lip necrosis, peripheral neuropathy, hoarseness, throat pain, and audiovestibular dis¬turbances. Cerebrovascular ischemic manifestations due to vasculitis of the carotid or vertebrobasilar arteries are rare (41). Around half of the GCA patients present systemic complaints such as fever, fatigue, and weight loss. Sometimes, they are the presenting manifestations of the disease. In retrospective studies, the prevalence of aortitis ranged from 3 to 18%. However, the routine use of new imaging techniques led to an increase in the detection of aortitis for nearly half of GCA patients, and it is typically located in thoracic aorta. Symptoms such as extremity claudication, arterial bruits, difference in blood pressure between limbs, decreased or absent pulses, chest pain, and Raynaud's phenomenon as well as a sustained increase in acute phase reactants should raise the suspicion of aortic involvement in GCA. Due to its asymptomatic nature in several cases, large vessel involvement should be routinely screened in GCA (42). The estimated period from the diagnosis of GCA to the onset of the arterial involvement is 5 to 7 years, and complications such as aneurysmal rupture and aortic dissection might appear. PMR is closely related to GCA, and 40-50% of GCA patients also develop PMR with aching and morning stiffness in the shoulder, hip girdles, and the neck.

An elevated erythrocyte sedimentation rate (ESR) is a typical feature of active GCA even though there are several reports of low or normal ESR in GCA for up to 22.5% of active patients. A normal ESR is associated with a lower chance of a positive TAB (43). C-reactive protein (CRP) is more sensitive than ESR for the early diagnosis and a better indicator of disease activity during corticosteroid therapy. Plasma IL-6 levels are highly elevated in untreated GCA patients, and CRP is more sensitive to disease activity than ESR (44). A biopsy-proven GCA has been found in 0.8% patients with normal ESR and normal CRP and 1.7 % of the patients with normal CRP despite elevated ESR (45).

Color-coded duplex sonography (CCDS) is a useful imaging tool for GCA diagnosis and for guiding TAB. The typical finding is a hypoechoic rim surrounding the arterial lumen, the so-called 'halo sign'. The presence of the halo sign yields 69% sensitivity and 82% specificity compared to TAB and 55% sensitivity and 94% specificity when compared to the ACR criteria for GCA (Table 1). The findings of temporal artery stenosis or occlusion by ultrasound was an almost equally sensitive marker compared to either biopsy (68% sensitivity) or to the ACR criteria for GCA (66% sensitivity) (46). The specificity of the halo sign increases to nearly 100% when the sign is bilateral. The use of 3-Tesla highfield MRA to show cranial arteries in GCA patients has a sensitivity and specificity comparable to CCDS. In the majority of GCA patients, several cranial arteries were affected simultaneously with a predominance of involvement of the frontal branch of the superficial temporal artery. Neverthe-

less, inflammation of the occipital arteries, with sparing of the temporal arteries, has also been encountered (47). The use of ^{18}FDG PET-CT has been shown to be a suitable tool for evaluating large vessel involvement in GCA. A smooth linear or long segmental pattern of ^{18}FDG uptake in the aorta and its main branches is a characteristic sign. Vessel uptake higher than liver uptake was considered an efficient marker for vasculitis (27).

TAB is the gold standard for diagnosing GCA, but a delay in initiating therapy while waiting for the biopsy is not acceptable. Furthermore, a positive TAB can be found up to 2 weeks after starting corticosteroid therapy. Excised specimens should be at least 1.5–2 cm. TAB can be negative in 10–20% of patients with typical manifestations of GCA due to the segmental distribution of inflammation within the vessel wall which can give falsely negative results. ACR classification criteria have been used to differentiate GCA from other vasculitides (Table 4) (48).

GCA patients with active disease but without visual symptoms should be treated initially with 1 mg/kg/day (maximum 60 mg/day) of prednisolone for 1 month. Afterwards, a gradual tapering to as much as 10–15 mg/day at three months is recommended. After reaching 10mg/day, prednisolone should be reduced by 1 mg every 1–2 months if no flare occurs. Alternating day tapering increases the risk of relapses and is not recommended. If visual symptoms are present, pulsed intravenous methylprednisolone (1g daily for three days) followed by the above mentioned glucocorticoid scheme is advisable. Pulsed intravenous therapy usually does not reverse already existent visual loss but may be of benefit in preventing visual loss in the contralateral eye. For patients requiring a high corticosteroid dose for remission maintenance, methotrexate can be used as an adjunctive therapy. The use of TNF-α inhibitors seems to have a limited role in the treatment of GCA and is not recommended (25). The use of the anti-IL-6 receptor (IL-6R) antibody tocilizumab in patients with GCA has shown some efficacy in refractory cases (49,50). The use of low-dose aspirin is recommended for all GCA patients (25).

Age at disease onset > 50 years
Age at disease onset > 50 years Development of symptoms beginning at age 50 years or older
New headache
New headache New onset of or new type of localized pain in the head
Temporal artery abnormality
Temporal artery tenderness to palpation or decreased pulsation unrelated to arteriosclerosis of cervical arteries
Elevated ESR
ESR > 50mm in the 1st hour by the Westergren method
Abnormal artery biopsy
Biopsy specimen with artery showing vasculitis characterized by a predominance of mononuclear cell infiltration or granulomatous inflammation, usually with multinucleated giant cells

Table 4. ACR Classification criteria for GCA. For purposes of classification of GCA, at least 3 criteria must be fulfilled. Sensitivity 93.5% and specificity 91.2%. From Hunder *et al.* (48).

The overall prognosis of patients with GCA is good. The major morbidity is due to ischemic complications, especially partial or complete visual loss occurring in 15-20% of patients. Cerebral ischemic events affect 3-4%, and thoracic artery aneurysms, 7% of patients. Long-term corticosteroid therapy can also contribute to an increased morbidity in GCA (33). The mortality rate in GCA patients is not significantly different from that in the healthy age-matched population (51).

TAKAYASU ARTERITIS

TA is a chronic idiopathic inflammatory disease affecting large vessels, predominantly the aorta and its major branches. Women younger than 40 years of age are most commonly affected, but it has also been observed in both genders and in children (52). In Israel and India, men and women are almost equally represented (53). The peak onset of disease is during the second or third decade of life. However, there are reports of TA in patients over 40 years of age (54). The disease is thought to be more prevalent in Asian countries, but it has also been described at similar rates worldwide with incidence rates of 1–2 per million (33). The association between TA and different human leukocyte antigen (HLA) alleles in different populations suggests the involvement of genetic factors in its pathogenesis. In Japan, TA is associated with HLA-B52 and B39 (55) whereas in Mexican and Colombian patients, there is a higher incidence of HLA-DRB1*1301 and HLA-DRB1*1602 alleles (56,57). However, no association between HLA alleles and TA was found in US (58).

The pathogenesis of TA involves both native and adaptative immune systems. Infiltration of mononuclear cells in vascular walls is thought to be generated in the adventitia through the vasa vasorum. Vascular DCs are activated by a stimulus recognized from toll-like receptors (TLR) and one possible candidate is heat-shock proteins. Vascular injury is then caused by natural killer (NK) cells and γδ T cells. Granuloma and giant cell formation are dependent on IFNγ producing CD4+ T cells in a Th1 pattern. A link between TA and tuberculosis has also been investigated, but it remains controversial. Anti-endothelial cell antibodies and a complement-dependent cell toxicity have also been described in TA. While destruction of the elastic lamina and the muscular media can lead to vascular dilation, intimal proliferation and wall thickening lead to stenotic lesions (15).

TA is considered to have a "triphasic" course characterized by prepulseless inflammation with predominating constitutional symptoms. This is followed by painful ischemic vessels and a "burnt-out disease" with predominance of fibrotic vascular disruptions although the latter is controversial since TA is mostly a chronic and relapsing disease (59).

However, TA patients may present different courses with some of them lacking constitutional symptoms and a few patients with a monophasic course with spontaneous remission (58). The most characteristic findings are decreased or absent pulses in 84–96% of the patients, usually associated with limb claudication and blood pressure discrepancies. Vascular bruits are also frequently seen in carotids, subclavian, and abdominal vessels. Arterial hypertension is observed in 33–83% of the patients and often results from stenosis of renal arteries or abdominal aorta (60). Takayasu retinopathy can be found in up to 37% of patients. It was first described in 1905 by Mikito Takayasu in a 21 year-old woman with characteristic fundus arteriovenous anastomoses due to ischemia (61). Aortic regurgitation is present in 20–24%. Congestive cardiac failure associated with hypertension is an important cause of mortality in these patients (58,62). Pulmonary artery involvement occurs in 14–100% of patients and coronary artery disease in 9% to 10% of the patients with predominant lesions found on the coronary ostia (63). Neurological manifestations are reported to occur in up to 50% of TA patients and include headache, dizziness, visual disturbance, epileptic seizures, ischemic stroke, and reversible posterior encephalopathy syndrome. Other symptoms include dyspnea, carotodynia, chest pain, and erythema nodosum. Constitutional features include fever, night sweats, malaise, weight loss, arthralgia, and myalgias. The diagnosis of TA is based on the clinical and typical angiographic or radiological findings while the ACR Classification Criteria for TA can be used to enroll patients in clinical and epidemiological studies (64). The extent of the arterial involvement in TA can be classified based on angiographic findings (table 5) (65). There are geographic differences in the vascular involvement of TA. In Japanese patients, the ascending aorta and aortic arch with its branches are more frequently involved whereas in patients from Korea, India, and Western countries, the abdominal aorta and renal arteries are most frequently affected. Thus aortic regurgitation is an important complication of TA in Japan while in other countries hypertension, left ventricular hypertrophy, headache, and cerebrovascular accidents are more common. There is no specific serological disease marker for TA since the ESR is elevated in half to three quarters of TA patients and, in some patients, it may not correlate with disease activity (58).

TYPE	VESSEL INVOLVEMENT
I	Branches from the aortic arch
IIa	Ascending aorta, aortic arch, and its branches
IIb	Ascending aorta, aortic arch, and its main branches, thoracic descending aorta
III	Thoracic descending aorta, abdominal aorta, and/or renal arteries
IV	Abdominal aorta and/or renal arteries
V	Combined features of types IIb and IV

Table 5. Angiographic classification of TA from the Takayasu conference 1994. According to this classification system, involvement of the coronary or pulmonary arteries should be designated as C(+) and P(+) respectively. From Moriwaki *et al.* (65).

Conventional angiography is still the gold standard method for the diagnosis of TA as it shows arterial lumen alterations though it does not allow assessment of vascular wall thickening. Imaging studies with CTA and MRA have been used more for the diagnosis and monitoring of TA due to their non-invasive nature and the better assessment of vascular wall changes. Although there is a good correlation between arterial wall uptake of ^{18}FDG and disease activity, the use of this technique for assessing disease activity in TA requires further investigation (27).

The differential diagnoses include other causes of large vessel vasculitis such as inflammatory aortitis (e.g., syphilis, tuberculosis, chronic periaortitis, lupus, rheumatoid arthritis, spondyloarthropathies, Behçet's disease, Kawasaki disease, and giant cell arteritis), developmental abnormalities (coarctation of the aorta and Marfan syndrome), and other aortic pathologies such as ergotism and neurofibromatosis.

The first line therapy for TA includes glucocorticoids and approximately half of the patients will respond to this treatment (66). Prednisolone should be started at 1 mg/kg/day and maintained for at least a month followed by a gradual tapering (25). An adjunctive immunosuppressive therapy is usually necessary due to the relapsing nature of the disease. Methotrexate has been shown to be a useful and well tolerated drug in achieving disease control in patients who are refractory to glucocorticoids (67). Benefits have also been reported with azathioprine, cyclophosphamide, micophenolate mofetil, leflunomide, and TNFα-blocking agents (68). Tocilizumab and rituximab were also evaluated in patients with refractory disease with good results (69,70). Antiplatelet therapy reduces the risk of acute ischemic events, specially cerebrovascular and cardiovascular events in TA patients (71).

Indications for surgical reconstruction or angioplasty included severe vascular claudication, hypertension with critical renal artery stenosis, cerebrovascular ischemia with critical stenosis of cerebral vessels, aortic regurgitation, cardiac ischemia with coronary artery involvement, and critical aortic stenosis or aneurysms. It should be done when disease is in remission because arterial inflammation at the time of revascularization increases the likelihood of complications up to 7 times (72). Arterial bypass and reconstruction procedures in TA have demonstrated a better sustained patency than percutaneous transluminal coronary angioplasty procedures. Complications described are restenosis, thrombosis, hemorrhage, stroke, or infection.

TA patients may develop substantial morbidity with severe claudication, stroke sequelae, loss of vision, or severe cardiac disease that impairs daily activities and employment. Kerr et al reported 74% of TA patients were functionally affected by their disease, and 47% of these patients were fully disabled (58). The 10-year survival rates are 84-90.8%, and the main causes of death are congestive cardiac failure and

stroke (33). A prognostic assessment and the requirement of a more aggressive therapy can be estimated based on the presence of one of the four major complications of TA – retinopathy, secondary hypertension, aortic regurgitation, and aneurysm formation – each graded as mild/moderate or severe at the time of diagnosis. The presence of one or more severe complications was associated with a worse prognosis (73).

POLYARTERITIS NODOSA

PAN is a systemic vasculitis that affects muscular small- and medium-sized arteries but spares venous circulation. PAN can be idiopathic or related to infectious agents, mainly HBV infection. The peak incidence is between 40-60 years of age, but it has been seen in all ages, including children, especially after a streptococcal infection in this population. There is no gender predominance. Annual incidence of PAN varies between 4.6-9.0 cases/1,000,000 but it reaches 77.0 cases/1,000,000 for Eskimos living in an area that is endemic for HBV virus infection in Alaska. After the decline in HBV infection due to vaccination and appropriate antiviral therapy, the prevalence of PAN decreased. Other infectious agents associated with PAN include HIV, cytomegalovirus, parvovirus B19, human T-lymphotropic virus type I (HTLV-I), and HCV (74).

The pathogenesis mechanisms of idiopathic PAN are unknown. HBV-related PAN is an immune complex disease that involves the deposition of surface antigen (HBsAg) and/or HBeAg with their respective antibodies on vessel walls thus activating the complement cascade and attracting neutrophils. Histologically, an inflammatory infiltrate and fibrinoid necrosis are found in the vessel wall. Immunofluorescence and electron microscopic examination show evidence for immune complex deposition only in HBV-related PAN (74,75).

General symptoms such as weight loss, fever, fatigue, weakness, and arthralgias are present in 60-90% of all patients. Neurological disease affects circa 70% of patients, usually as a mononeuritis multiplex or peripheral polyneuropathy. This is typically distal and asymmetric and often has a motor component. Axonal neuropathy with a sensitive or mixed sensorimotor pattern is observed by electroneuromyography. Cutaneous manifestations are found in 25-60% and include palpable purpura, tender subcutaneous erythematous nodules, livedo reticularis, ulcers, digital gangrene, and bullous or vesicular eruption. Histological examination depicts a necrotizing vasculitis with or without leucocytoclastic alterations. The biopsy should also include the dermis with small arteries. Approximately 40-60% of patients with PAN present gastrointestinal symptoms such as abdominal pain, diarrhea, malabsorption, GI hemorrhages, and small intestine perforations. Pancreatitis has also been described as well as the involvement of the liver and spleen. Renal involvement occurs in approximately 40% of the cases, and vascular nephropathy is the usual manifestation. It can be manifested as a flank pain and hematuria or renal or perirenal hematomas resulting from the rupture of microaneurysms. Rapid renal failure may be a consequence of multiple renal infarcts. Hypertension is a common finding in PAN, and it may develop as a result of renal artery involvement with renal ischemia. Myalgias and muscle tenderness are frequent (50-60%) and can be debilitating. A muscle biopsy showing vasculitis in muscular arteries often helps in diagnosing PAN. Orchitis is also a common feature of PAN (74,76). Pulmonary involvement is very rare in PAN. Other uncommon manifestations include coronary involvement with myocardial infarction as well as the breast and uterine involvement of PAN. Ocular manifestations include retinal vasculitis, retinal detachment, cotton-wool spots, iritis, and iridocyclitis. There are three forms of clinical presentation of PAN: systemic idiopathic, HBV-associated PAN, and isolated forms of PAN such as cutaneous and muscular (74).

PAN should be confirmed by histopathology or by an imaging study showing microaneurysms in visceral arteries. However, a renal biopsy in the presence of microaneurysms of renal arteries is contraindicated due to the high risk of hemorrhage. The classification criteria for PAN are sometimes problematic when there are isolated forms or it must be distinguished from MPA. The presence of ANCA positivity, pulmonary hemorrhage, and/or glomerulonephritis should help in this distinction. Laboratory findings indicating inflammation as elevated ESR and CRP are often present (77).

The combination of cyclophosphamide and glucocorticoid leads to better disease control in PAN (78,79). Nevertheless, glucocorticoids alone should be prescribed for patients without prognostic factors. Therapy in HBV-related PAN includes antiviral therapy, plasmapheresis, and a short course of glucocorticoids. Cutaneous PAN can be treated with non-steroidal anti-inflammatory drugs or colchicine. For relapsing patients, glucocorticoids, dapsone, hydroxychloroquine, pentoxifylline , intravenous immunoglobulins, or infliximab could be used. In severe refractory cases of cutaneous PAN, azathioprine, methotrexate, or cyclophosphamide in combination with high-dose glucocorticoids are recommended (74).

The overall survival of patients with PAN, EGPA, and MPA is lower than the healthy population despite the progress in immunosuppressive therapy with a sharp fall in survival rates during the first 18 months (80). The mortality rate is higher in patients with HBV-associated PAN than with non-HBV-associated PAN, but the latter present a higher relapse rate, especially when skin manifestations are present at diagnosis (81). Age > 65 years, renal insufficiency, serum-creatinine >1,58mg/dl, gastrointestinal manifestations, central nervous system manifestations, and/or cardiac involvement and recent-onset hypertension are predictors of poorer prognosis, and those patients require intensive therapy (31).

KAWASAKI DISEASE

KD is an acute self-limited systemic vasculitis that affects primarily medium-sized arteries, mainly in children < 5 years of age. There are 3 stages of progression in KD including the

acute, subacute, and final. The acute phase of the disease may last 12 days on average without therapy. However, long term complications, e.g., coronary artery aneurysms, heart failure, myocardial infarction, arrhythmias, and peripheral artery disease worsen the prognosis leading to an increased morbidity and mortality (82). The etiology of KD is still unknown, but there is some evidence that an infectious agent could play a role in the pathogenesis. It is hypothesized that the possible pathogen could enter the body via the respiratory or the digestive organs and would be processed by lymphoid tissues within those organs. B cells would then differentiate into IgA-secreting plasma cells that spread throughout the body including the coronary arteries and heart muscle. In the acute phase of the disease, monocytes/macrophages and lymphocytes secrete proinflammatory cytokines (e.g., IL-6, TNFα, IFNγ) and chemokines (e.g., monocyte chemoattractant protein-1) which lead to endothelial cell activation and to an inflammatory infiltrate within vessel walls that results in vascular damage, which is especially caused by the production of matrix metalloproteinase (MMP)-9 by activated endothelial cells (82,83).

Clinical features of KD reflect the systemic inflammatory response and include prolonged fever (>5 days), bilateral non-exudative conjunctivitis, intense mucosal inflammation including red, cracked lips and strawberry tongue, polymorphous cutaneous rash, edema on the hands and feet dorsum, extremity desquamation, and linear nail creases (Beau's line). Cervical lymphadenopathy and arthritis are less common findings. Cardiovascular complications are usually not observed in the acute phase of KD, and only patients with severe disease may develop peripheral artery complications due to aneurysms that lead to digital gangrene. Diagnostic criteria have been developed for KD (Table 6) (82,84).

Proper treatment of KD is important since up to 25% of untreated children may develop permanent coronary damage. The administration of intravenous immunoglobulin (2g/kg in 10-12 hours) associated with aspirin (80-100mg/kg/day) within the first 10 days after the onset of fever (i.e., the acute phase of the disease) can reduce the incidence of coronary aneurysms to 3-5%. Therapy with aspirin should be continued until the patient remains without fever for 24-48 hours. However, aspirin needs to be continued for 6-8 weeks at a lower dose (3-5 mg/kg/day) (82,84). Echocardiography should be done in the second, and in the sixth to eighth week after clinical improvement to make sure of the morphological stability in coronary arteries. If any abnormality is found, then aspirin therapy should be continued. Despite clinical improvement, patients with KD should undergo a long-term follow up in order to establish what the risk for the development of cardiac and vascular problems may be. This long-term management is guided by coronary abnormalities observed at echocardiography examination according to the American Heart Association Risk Stratification for KD (Table 7) (85).

CRYOGLOBUILINEMIC VASCULITIS

Cryoglobulinemic vasculitis is a systemic vasculitis caused by the deposition of cryoglobulins on vessel walls. Clinical manifestations of cryoglobulins may arise from the occlusion of blood vessels due to hyperviscosity syndrome or to immune complex deposition (86). Only 7 to 50% of patients with circulating cryoglobulins will develop disease manifestations (87). The most commonly used classification of cryoglobulinemia recognizes three different immunochemical types of cryoglobulins (Table 8). Type I cryoglobulinemia is associated with hematological disorders such as multiple myeloma and Waldeström macroglobulinemia, and when symptomatic, it usually leads to hyperviscosity syndrome. Both type II and III cryoglobulinemia are referred as mixed because they are composed of IgG and IgM immunoglobulins and both are associated with cryoglobulinemic vasculitis (86-88). No underlying cause can be found in up to 10% of patients with mixed cryoglobulinemia, and those cases are regarded as essential cryoglobulinemia. However, chronic hepatitis C virus (HCV) infection is the most common cause of cryoglobulinemia since HCV RNA is recognized in up to 95% of patients with mixed cryoglobulinemia (87). Other infectious diseases that may less frequently lead to the development of cryoglobulinemia include HIV and HBV. Cryoglobulinemia is rarely associated with other infectious agents. Autoimmune diseases are also associated with the development of mixed cryoglobulinemia, especially Sjögren's syndrome. Rheumatoid arthritis, systemic lupus erythematosus (SLE), and other systemic autoimmune diseases are less often associated (86,88).

The most frequently affected organs are skin, joints, peripheral nerves, and kidney. Although constitutional symptoms such as fever, fatigue, myalgias, and arthralgias are common manifestations, palpable purpura in lower limbs is

Fever lasting at least five days without other explanation combined with at least four of the following criteria:
Bilateral bulbar conjunctival injection
Oral mucous membrane changes, including injected or fissured lips, injected pharynx, or strawberry tongue
Peripheral extremity changes, including erythema of palms or soles, edema of hands or feet (acute phase), and periungual desquamation (convalescent phase)
Polymorphous rash
Cervical lymphadenopathy (at least one lymph node >1.5 cm in diameter)

Table 6. Diagnostic criteria for Kawasaki disease. From Scuccimarri (84).

RISK LEVEL	THERAPY	FOLLOW UP	INVASIVE INVESTIGATION
I – no coronary artery changes in all phases of the disease	None after 6-8 weeks	Cardiovascular risk assessment, 5-year intervals	Not recommended
II – transient coronary artery ectasia that reverses in 6-8 weeks	None after 6-8 weeks	Cardiovascular risk assessment, 3 to 5-year intervals	Not recommended
III – one small-to-medium coronary artery aneurysm	Low-dose aspirin until aneurysm regression	Annual follow-up with echocardiography and ECG, cardiovascular risk assessment, biannual stress test and myocardial perfusion scan	Angiography if ischemia is suggested by non-invasive tests
IV - ≥1 large or giant coronary artery aneurysm or multiple or complex aneurysms in the same artery, without obstruction	Long-term antiplatelet therapy with warfarin (INR 2.0-2.5) or LMWH (target factor Xa 0.5-1.0 U/ml)	Biannual follow-up with echocardiography and ECG, annual stress test, and myocardial perfusion scan	First angiography at 6-12 months or before if indicated. Should be repeated if evidence of ischemia
V – coronary artery obstruction	Long-term antiplatelet therapy with warfarin or LMWH if giant aneurysm; consider beta-blockers to reduce myocardial consumption of oxygen	Biannual follow-up with echocardiography and ECG, annual stress test and myocardial perfusion scan	Angiography should be done to evaluate therapeutic options

Table 7. American Heart Association risk stratification for KD. *ECG* – electrocardiogram, *INR* – international normalized ratio, *LMWH* – low molecular weight heparin. Adapted from Gordon *et al.* (85).

TYPES OF CRYOGLOBULINEMIA	DESCRIPTION
Type I	Monoclonal immunoglobulin is the only immunoglobulin component of the cryoglobulin and it can be IgM, IgG, IgA, or free monoclonal light chain immunoglobulins.
Type II	Polyclonal IgG and monoclonal IgM with rheumatoid factor activity against IgG.
Type III	Polyclonal IgG and rheumatoid factor IgM

Table 8. Classification of cryoglobulinemia.

the most typical sign of cryoglobulinemic vasculitis and occurs in nearly all patients. Peripheral neuropathy is a common manifestation of mixed cryoglobulinemia and presents as sensory involvement with lower limb paresthesia. A wide range of renal involvement is observed in patients with cryoglobulinemic vasculitis including proteinuria, glomerular hematuria, red cell casts, hypertension, and variable degrees of renal failure. Gastrointestinal, pulmonary or central nervous system manifestations may be rarely found in patients with cryoglobulinemic vasculitis (89,90).

The diagnosis of cryoglobulinemia is made when serum cryoglobulins are found in patients with typical manifestations. The detection of serum cryoglobulins should be followed by the measurement of cryoglobulin levels and the characterization of cryoglobulins (i.e., mono or polyclonal immunoglobulins or kappa/lambda light chains). High titers of serum rheumatoid factor and low serum complement levels, especially C4, may correlate with clinical symptoms. Other laboratory tests should include renal parameters (e.g., urinalysis, proteinuria, and serum creatinine levels), autoantibody tests to investigate associations with systemic autoimmune diseases, and serology tests for chronic infectious diseases (e.g., HCV, HBV, HIV) (91). Patients with hyperviscosity syndrome together with type one cryoglobulinemia should be investigated for hematological disorders (86,87).

Cryoglobulinemic vasculitis must be confirmed by histopathological examination. Skin and kidney biopsy are the most commonly evaluated sites for histopathological examination in cryoglobulinemic vasculitis. Leukocytoclastic vasculitis and hyaline thrombi occluding small vessels are typical findings. Direct immunofluorescence microscopy reveals IgM, IgG, and C_3 deposits on vessel walls (86,87). Kidney biopsies in patients with active renal involvement usually reveal membranoproliferative glomerulonephritis with endocapillary proliferation, hyaline intraluminal thrombi, and endomembranous immune deposits (92).

The treatment of cryoglobulinemic vasculitis depends on the underlying infection or autoimmune disorder and on the severity of the manifestations. Mild to moderate disease may manifest as purpura, constitutional symptoms, joint complaints, mild neuropathy, and/or mild glomerulonephritis whereas patients with severe disease manifestations may also develop skin ulcers, digital ischemia, severe neuropathy, glomerulonephritis with renal failure, and/or nephrotic syndrome or involvement of the gastrointestinal tract. Life-threatening cryoglobulinemia is considered when patients present any of the following manifestations: rapidly progressive glomerulonephritis, intestinal ischemia, central

nervous system involvement, or alveolar hemorrhage (87,93).

Patients with essential cryoglobulinemic vasculitis and those associated with systemic autoimmune diseases may benefit from glucocorticoids in mild cases. In severe cases, methylprednisolone (0.5 to 1.0 gram per day for 3 days), high dose prednisone (1mg/kg/day), and oral (2mg/kg/day) or intermittent intravenous cyclophosphamide (0.75g/m^2) are usually prescribed to control disease manifestations. Azathioprine (2mg/kg/day) or mycophenolate mofetil (2g/day) are regarded as an alternative to cyclophosphamide or as a maintenance therapy after achieving remission. Plasmapheresis is added to the treatment in life-threatening situations. However, no clinical trial has evaluated this approach in cryoglobulinemic vasculitis (87).

In HCV-associated cryoglobulinemia, antiviral therapy is indicated in all cases regardless of disease severity. The combination of ribavirin with pegylated interferon alfa has shown the best evidence of achieving a virological response and, in turn, helping to control manifestations of cryoglobulinemia. At present, there is some evidence that the use of rituximab in patients with severe manifestations of HCV-associated cryoglobulinemia will lead to a higher rate of complete response in comparison to patients on glucocorticoids and antiviral therapy. Patients with life-threatening manifestations of HCV-associated cryoglobulinemia may also benefit from the use of cyclophosphamide or rituximab and plasmapheresis (94).

ANCA-ASSOCIATED VASCULITIS (AAV)

ANCA-associated vasculitides affect small-vessels and are associated with the production of ANCA while few or no immune deposits are found on vessel walls. AAV includes GPA, MPA, EGPA, and renal limited vasculitis (RLV). AAV shares the same features as renal pathology (i.e., focal necrotizing, often crescentic, pauci-immune glomerulonephritis). However, this classification is not a suitable term because not all patients with AAV develop detectable ANCA in serum. ANCA can be detected in serum by IIF as two main patterns cANCA and pANCA usually associated with PR3-ANCA and MPO-ANCA respectively. IIF ANCA results should be paired with the antigen specificity detected by ELISA or capture ELISA (18).

ANCA positivity is variable in different AAV subtypes and in different clinical presentations. Testing for ANCA in GPA yields a pooled sensitivity of 91% and specificity of 99%. Sensitivity falls significantly (63%) when the disease is in non-acute stages (95). This suggests that ANCA directed to PR3 or to MPO is sensitive for active disease. For example, in a prospective study, the sensitivity of ANCA was 96% in patients with generalized GPA and 83% for those with limited disease 83% (96). However, fluctuations in ANCA titers do not completely follow disease activity, and the utility of serial ANCA testing is not entirely clear. Even though increases in ANCA levels may be associated with relapsing disease in some cases, there are no data justifying treatment decisions based on changes in ANCA titers (18).

Although PR3-ANCA is strongly associated with GPA and MPO-ANCA with MPA, approximately 80% to 95% of ANCA found in GPA patients with activated severe disease are detectable PR3-cANCA while 5% to 20% are MPO-ANCA. In MPA patients, about 40% to 80% present MPO-ANCA while approximately 15% develop PR3-cANCA (97). In EGPA, MPO-ANCA is present in around 40% of patients. However, while ANCA-positive patients predominantly present with small-vessel vasculitis, ANCA-negative patients develop clinical manifestations caused by tissue infiltration with eosinophils (98). The outcome of MPO-ANCA vasculitis differs from that of PR3-ANCA vasculitis. Therefore, it is important to detect ANCA specificity (99).

The pathogenesis of these disorders is not fully elucidated. Yet, clinical findings combined to in vitro and in vivo experimental data suggest a pathogenic role for ANCA. In vitro studies demonstrate that ANCA can induce vasculitis by activating primed neutrophils to produce reactive oxygen species and to release lytic enzymes that will damage endothelial cells. Necrotizing small vessel vasculitis is characterized by neutrophil infiltration and activation within the vessel wall. The ANCA-induced neutrophil activation occurs by its attachment to an endothelial surface. This proposed mechanism may explain necrotizing vasculitis. However, it does not explain granulomatous inflammation which occurs predominantly in airways from PR3-ANCA-associated GPA (100). The granulomatous lesions suggest the involvement of cell immune responses since several observations support a key role for T helper cells in disease pathophysiology. Apart from the expanded population of effector memory T cells contributing to tissue damage, there is a functional impairment of regulatory T cells that have the ability to differentiate into Th17 cells (14). There is an animal model for MPO-ANCA vasculitis but not for PR3-ANCA (100).

GRANULOMATOSIS WITH POLYANGIITIS

GPA, formerly known as Wegener's granulomatosis, is a small-vessel vasculitis characterized by granulomatous and necrotizing inflammation affecting the upper and lower respiratory tracts and, often, the kidneys (10). GPA could be subclassified into generalized and limited disease, based on the presence or absence of renal involvement respectively (101,102). GPA patients usually present systemic manifestations including fever, weight loss, malaise, anorexia, and arthralgia. These symptoms may last for months before the diagnosis. The signs and symptoms of nasal involvement in GPA include nasal crusting, sinusitis, persistent rhinorrhea, purulent/bloody nasal discharge, oral and/or nasal ulcers, nasal obstruction due to mucosal swelling, and the saddle-nose deformity due a progressive loss of nasal septal support. The impairment of blood supply to the cartilaginous portion of the nasal septum can cause septum perforation or collapse of the nasal bridge. It has a significant psychological impact and also results in worsening nasal obstruction and increases the incidence of anosmia. Notably,

erosions of the hard palate differentiate from lymphoproliferative diseases, fungal infections, or cocaine abuse (103). In addition to upper airway features, GPA patients more typically show evidence of orbital masses, otitis media, mastoiditis, and cranial nerve entrapment. Another typical feature is subglottic stenosis as a result of which, the patients may complain about hoarseness, cough, dyspnea, and stridor. This life-threatening manifestation affects 10-20% of patients and can occur regardless of systemic disease activity (103). Epiphora is a common ophthalmological finding in patients with GPA (104).

Pulmonary manifestations of GPA include pulmonary nodules, transient infiltrates, patchy or diffuse opacities, hilar adenopathy, pulmonary consolidation, and/or pleural effusion. Pulmonary nodules often cavitate. Any GPA patient presenting respiratory symptoms must be evaluated for infectious disease, mainly due to immunosuppressive therapy. *Pneumocystis jiroveci* pneumonia is a relatively common opportunistic infection in patients with GPA (105,106). Therefore, prophylaxis with trimethoprim/sulphamethoxazole should be encouraged in all patients being treated with cyclophosphamide (78). Glomerulonephritis may occur at presentation in about 20% of GPA patients and develops in nearly 80% of patients during the disease course (107). It is manifested as asymptomatic glomerular hematuria, or the patient may present proteinuria or even renal insufficiency. In those patients with pulmonary-renal syndrome, antiglomerular basement antibodies and antinuclear antibodies should be considered in order to exclude anti-GBM antibody disease or SLE.

Whenever possible, the diagnosis of GPA should be based on typical histological findings of necrosis, granulomatous inflammation, and vasculitis. Nevertheless, infections should also be ruled out with special stains and cultures. Biopsy sites are chosen based on disease manifestations and the likelihood of a positive biopsy also depends on the organ sampled and the amount of tissue biopsied. The highest positivity rates are found in open lung biopsy in GPA patients with abnormal pulmonary parenchyma. Although transbronchial biopsies are less invasive, the positivity is lower than 10%. Upper airway biopsies yield positive results in about 20% (97). Renal biopsies are usually done on GPA patients with active urine sediment and/or renal failure. Histological findings range from mild focal and segmental glomerulonephritis to a diffuse necrotizing and crescentic glomerulonephritis. Granulomatous changes are rarely found in renal biopsy specimens even among patients with well-documented GPA. The glomerulonephritis in GPA is associated with few or no immune deposits in the glomeruli (pauci-immune glomerulonephritis) on immunofluorescence and electron microscopy. Tubulointerstitial infiltrates are very common. There is a pathological classification assessed by light microscopy proposed to determine whether these lesions have prognostic value, which has been validated on 100 biopsies from European patients (Table 9) (108).

MICROSCOPIC POLYANGIITIS

MPA is a necrotizing vasculitis with few or no immune deposits that affects small vessels without granuloma. Glomerulonephritis occurs in nearly all patients, and pulmonary capillaritis is a common finding. An important life-threatening manifestation of MPA is pulmonary hemorrhage. The differential diagnosis and diagnostic evaluation are similar to GPA (Table 9) (10,76).

EOSINOPHILIC GRANULOMATOSIS WITH POLYANGIITIS

EGPA is a granulomatous vasculitis characterized by eosinophil-rich infiltrate. Asthma and eosinophilia are common. EGPA seems to develop in three phases: prodromal, eosinophilic, and vasculitic. The first phase is characterized by asthma and/or allergic rhinitis with or without polyposis while the eosinophilic phase is characterized by eosinophilia in peripheral blood and eosinophils infiltrating tissues. Features of the vasculitic phase are similar to other vasculitic diseases since they affect multiple organs, e.g., nerves, heart, lungs, kidneys, and gastrointestinal tract (Table 9) (76).

The differential diagnosis of EGPA shares some similarities with GPA and MPA. However, hypereosinophilic syndrome, eosinophilic leukemia, and parasitic diseases must be considered during diagnosis evaluation. A large cohort of EGPA patients confirmed the previously reported differences in clinical disease presentation based on ANCA status. ANCA-positive patients had more frequent peripheral neuropathy and renal involvement but less cardiomyopathy. However, cardiomyopathy still remains a poor-prognosis factor. The FFS is used to optimize treatment since cyclophosphamide should be added to the glucocorticoid regimen in patients with at least one prognostic factor (Table 10) (109).

MANIFESTATIONS	GPA	MPA	EGPA
Granulomatous inflammation	++	-	++
Asthma and eosinophilia	-	-	+++
Ear, nose, and throat involvement	+++	+	++
Pulmonary nodules or infiltrates	+++	-	++
Alveolar hemorrhage	++	+++	+
Glomerulonephritis	+++	+++	++
Peripheral neuropathy	++	+	+++

Table 9. Clinical manifestations of different subsets of ANCA-associated vasculitis.

Renal insufficiency (serum creatinine ≥1.58mg/dl or ≥140 micro mol/l)
Proteinuria >1g/day
Central nervous system involvement
Severe gastrointestinal involvement (gastrointestinal bleeding, infarction, or pancreatitis)

Table 10. The original Five Factor Score (31).

TREATMENT OF AAV

Induction of remission with intermittent intravenous or oral cyclophosphamide and glucocorticoids are the standard therapy for AAV patients with active generalized disease and should be kept up for 3 to 6 months (78). In a randomized trial, oral and intermittent intravenous pulse cyclophosphamide were compared in patients who had newly diagnosed, generalized AAV with renal involvement but without life-threatening disease. No difference was found regarding remission rates. However, the intravenous cyclophosphamide arm achieved remission with a lower cumulative dose (110). In a 4.3-year follow-up, although there was no difference between groups with respect to renal function, end-stage renal disease, or death, the intravenous pulse cyclophosphamide treatment was associated with a higher risk of relapse (111). A combination of methotrexate and glucocorticoid is a less toxic alternative to cyclophosphamide for the induction of remission in non-organ threatening or non-life threatening ANCA-associated vasculitis (112). On the other hand, duration of relapse-free survival was longer among patients treated with cyclophosphamide than among those patients treated with methotrexate during short-term and long-term (6 years) follow-up (113). For patients with crescentic, rapidly progressive renal disease (serum creatinine >500 micromol/litre or 5,65 mg/dl), plasma exchange must be used as an adjunct therapy to cyclophosphamide and prednisolone (114).

In two randomized trials, rituximab was as effective as cyclophosphamide in inducing remission among patients with newly diagnosed or relapsing GPA or MPA. In the RAVE trial, a multicenter non-inferiority trial, rituximab was superior to cyclophosphamide in inducing remission in the relapsing disease. There was no difference in the number of adverse events in either study. A potential limitation is that the duration of follow-up was limited to six to twelve months compared with the extensive long-term experience with cyclophosphamide (115,116). Patients who attain remission receive the maintenance therapy that includes a combination of low-dose glucocorticoid and, either azathioprine, leflunomide, or methotrexate for at least 2 years (78).

Albeit GPA patients with saddle-nose deformity seek surgical reconstruction, this procedure must be reserved for highly selected patients. Because of the vasculitic involvement of mucosal blood vessels, the nasal tissue becomes devascularized owing to worse wound healing. Accordingly, the clinical management must be improved before considering surgery. Apart from the structure correction, the surgical procedure could improve nasal breathing, anosmia, and quality of life. These patients often present difficult to treat bacterial sinusitis due to an inability by antibiotics to penetrate ischemic tissue. Saline irrigations using high-volume, high-flow irrigation devices can improve mucociliary clearance in affected patients (117). Upper airway infection by *Staphylococcus aureus* must be ruled out and cultures should be used to guide antibiotic treatment. The topical antibiotic treatment (e.g., mupirocin) can be useful to reduce the frequency of disease exacerbation (118). Surgical intervention should be reserved for refractory cases. In a large series of 120 patients, only 16% of these patients with upper airway manifestation of granulomatosis with polyangiitis required surgery (104).

IGA VASCULITIS (HENOCH-SCHÖNLEIN PURPURA)

IgA-Vasculitis (IgAV), formerly Henoch-Schönlein Purpura (HSP), is a small-vessel vasculitis associated with the deposition of IgA1 in blood vessels that affects mainly children between 3 and 8 years of age. The annual incidence in children varies between 8.5-21.7 per 100,000 while in adults IgAV is rare and its incidence lies between 0.12-0.8 per 100,000. There is a tendency for IgAV to affect boys more frequently than girls and IgAV is a rare condition in the black race. IgAV is often associated with a triggering factor such as viral and bacterial infections in children, and drugs and toxins in adults. There are several reports about the association between IgAV and previous infection with *β-hemolytic streptococci*. A seasonal increase in IgAV cases in the winter and spring has been observed, which strongly suggests an infectious event triggering disease. The mechanism of disease is thought to be associated with IgA1-containing immune complex deposits in vascular and glomerular sites. These immune complexes activate the complement pathway, induce recruitment of inflammatory cells, and in addition, cause damage to endothelial cells through a membrane-attack complex. Some alterations found in IgA1-molecule (IgA hypogalactosylation and reduced sialic acid content in the Fc segment) have been associated with an impaired clearance of IgA1 molecules and a disturbance in the mesangial filter of these molecules leading them to deposit in those sites (10,119).

The four main manifestations of IgAV are skin lesions, joint, and gastrointestinal and renal involvement. Skin manifestations are present in nearly all patients, usually as palpable purpura. The lesions are usually found on the lower extremities, buttocks, and extension surfaces of the elbows. The face, palms, and soles are usually spared. Skin biopsy of

early lesions shows leukocytoclastic vasculitis of the dermal vessels, predominating in the postcapillary venules. Immunofluorescence deposits of IgA and C_3 are found in dermal capillaries as well as IgM but rarely IgG deposits. Transient arthritis and, more frequently, arthralgia have been observed in 60-70% of IgAV patients, preferentially involving knees and ankles. The frequency of GI symptoms varies from 46% in adults to 66% in children and reflects intestinal vasculitis. Abdominal pain is the most frequent complaint, but patients may also present nausea, vomiting, and GI hemorrhage. Renal manifestations are more frequent in adults (70%) than in children (37%). The renal manifestation varies from isolated microscopic hematuria with or without red cell casts and/or proteinuria. IgA deposits are also found in kidney biopsies especially in mesangial areas. While scrotal involvement frequently occurs in children, cardiac and pulmonary involvements are rare. Neurological manifestations such as headache, behavioral disorders, seizures, mononeuropathies of the peripheral or cranial nerves as well as plexopathies and polyradiculoneuritis have been described. The ACR classification criteria were developed for IgAV and include palpable purpura, age at onset ≤20 years, acute abdominal pain, and biopsy showing granulocytes in the walls of small arterioles and/or venules. Two or more of the criteria are necessary to classify IgAV patients (120).

Supportive treatment with analgesics or non-steroidal anti-inflammatory drugs is often sufficient in most patients with IgAV to control articular/muscle pain and fever. Ranitidine is helpful for improving abdominal pain and subclinical GI hemorrhage in patients with moderate GI involvement. Temporary bed rest should be recommended. Dapsone is efficacious in treating cutaneous, GI, and articular manifestations. Corticosteroids may be useful in reducing the duration of abdominal pain. Intravenous pulse methylprednisolone, immunosuppressive drugs (cyclophosphamide and azathioprine), plasma exchange, and polyclonal immunoglobulins can be used in life-threatening or severe refractory forms of IgAV. The disease in children is an acute mild condition with approximately 60% of patients reaching complete spontaneous remission. In adults, it is a chronic condition for half of the patients, and the risk of chronic nephropathy with renal failure appears to be higher in adults (121).

HYPERSENSITIVITY VASCULITIS

Hypersensitivity vasculitis (HV) is a small vessel vasculitis caused by immune complex formation and deposition (122). Although the term HV was adopted by the ACR classification system, it was changed to cutaneous leukocytoclastic vasculitis by the first CHCC in 1994 and then to immune complex small vessel vasculitis with a probable etiology by the updated CHCC (6,10). HV usually develops 7 to 10 days after a triggering antigen exposure. It is typically caused by drugs (penicillins, cephalosporins, sulfonamides, loop, and thiazide-type diuretics, phenytoin, allopurinol) acting as haptens, but it may also be associated with some infections such as HBV or HCV and HIV (2). In addition to palpable purpura, HV can be manifested as fever, arthralgias, low serum complement levels, and elevated ESR. Classification criteria include: age >16 years, use of a possible offending drug in temporal relation to the symptoms, palpable purpura, maculopapular rash, biopsy of a skin lesion showing neutrophils around an arteriole or venule (122). Discontinuation of eliciting drug or treating the underlying condition is the main target of therapy and is often sufficient. In patients with more severe or refractory disease, antihistamines, colchicine or dapsone can be used. Combination treatment with dapsone and pentoxifylline has been demonstrated to be effective. Immunosuppressive therapy with glucocorticoids should be reserved for severe cases or progressive disease (2).

BEHÇET'S DISEASE

Behçet´s disease (BD) is a idiopathic, multisystem, relapsing vasculitis that affects vessels of all sizes (small, medium, and large) including both arteries and veins. BD is commonly found along the ancient silk road where the prevalence ranges from 80 to 370 cases per 100,000 (123). There is no specific test to diagnose BD, so its diagnosis is based on clinical findings. Several diagnosis criteria were developed for BD but the International Study Group (ISG) criteria remains the most widely used criteria for BD (124) (Table 11).

Oral ulcers are generally the first clinical manifestation in patients with BD but not all patients will have them prior to the other features. Oral ulcers must recur more than three times in one year and are characteristically painful. They can be classified as minor ulcers (< 1 cm in diame-

Major criterion
Recurrent oral ulcerations (apthous or herpetiform) at least three times in one year
Minor criteria
Recurrent genital ulcerations Eye lesions (uveitis or retinal vasculitis) observed by an opthalmologist Skin lesions (erythema nodosum, pseudofolliculitis, papulopustular lesions, acneiform nodules) adult patients not on corticosteroids Positive "pathergy test" read by a physician within 24-48 hours of testing

Table 11. International Study Group Criteria for BD. To classify a patient as presenting BD, a major criterion plus 2 minor criteria are necessary. From Sakane *et al.* (123).

ter), major ulcers (> 1 cm in diameter), and herpetiform (multiple small ulcers). The borders of oral ulcers in BD are well-defined with a white-yellow necrotic base surrounded by erythema. The genital ulcerations resembles oral ulcers but they are more painful, recur less frequently, and usually leave scars. In men, they are most commonly found on the scrotum and scrotal scarring secondary to ulcers strongly suggests BD. Ocular disease (affecting up to two-thirds of patients) in BD may result in significant morbidity with impaired visual acuity or even complete blindness. Uveitis is the most prominent feature, and it is typically bilateral and relapsing. Posterior uveitis, retinal vasculitis, and optic neuritis require systemic immunosuppressive therapy. The pathergy is defined as a pustule-like lesion or papule that appears 48 hours after skin prick with a 20-gauge needle. It represents a skin reaction secondary to a minor trauma.

Although the patient must present oral ulcers to fulfill the ISG criteria for BD, in some cases, not all the criteria are met. Therefore, a high degree of suspicion of a BD diagnosis is necessary when patients present any of the following: mouth or genital ulcers, arthritis or arthralgia, nervous system symptoms, gastrointestinal manifestation, deep vein thrombosis or superficial thrombophlebitis, arterial thrombosis, vascular aneurysm, epididymitis, inflammatory eye disease, skin disease, fatigue, fever, and other systemic manifestations. Male sex and a younger age at onset are prognostic factors in BD (125-127). Large vessel disease is the most important cause of mortality in BD followed by central nervous system involvement (128). Vascular diseases include superficial and deep vein thrombosis, arteritis with occlusion, and aneurysm formation. The primary pathology leading to venous thrombosis in BD seems to be the inflammation of the vessel wall, which is treated with immunosuppressive agents. The pathogenesis of thrombosis in BD has not been fully elucidated. Immunosuppressive agents reduced the incidence of venous thrombosis relapse in BD patients in a large retrospective cohort (129).

There is no evidence for anticoagulant use to treat vascular BD. Nevertheless, some authors recommend anticoagulants in special cases such as major vein thrombosis and the risk of bleeding in patients with associated aneurysms should be taken into account. Among vascular manifestations, the pulmonary artery aneurysm is the main cause of death and is found almost exclusively in the male population. It must be readily recognized to be treated properly. Hemoptysis is the most common presenting symptom, but cough, dyspnea, fever, and pleuritic pain should prompt the diagnosis (130). Saadoun et al found an independent association of mortality with male sex, arterial involvement, and a high number of disease flares in BD (131). Central nervous system involvement is seen in approximately 10% of patients. The neurological involvement manifests as either parenchymal involvement with pyramidal signs and symptoms or as a sinus thrombi. The first one, seen in the majority of patients, is attributed to an inflammatory CNS disease with a focal or multifocal parenchymal involvement presenting mostly as subacute brainstem syndrome and/or corticospinal tract affection (132). The other form has a better prognosis and is attributed to isolated venous sinus thrombosis and intracranial hypertension. It presents as headaches and papilledema. Peripheral neuropathy is uncommon but may develop in a subset of patients (132). Other neurological manifestations include aseptic meningitis, seizures, and psychiatric disorders. Dementia may develop. A controlled study using brain MRI in a Brazilian population suggests that cognitive impairment may exist regardless neurological manifestation (133).

The treatment of BD depends on disease manifestations presented by the patient. Only the treatments for ophthalmic, mucocutaneous or articular manifestations were evidence based. Inflammatory eye disease affecting the posterior segment should be treated with azathioprine and corticosteroids. If there is any sign of severity defined as worsening in visual acuity and/or retinal disease, the combination with either cyclosporine A or infliximab should be used. Although there have been no randomized trials of anti-TNF therapy in BD ophthalmic disease and more controlled data are needed, there is published experience to suggest that it represents an important therapeutic alternative for severe disease or for patients who are intolerant to standard immunosuppressive treatments. IFNα may be an alternative treatment for controlling Behçet's uveitis in patients with refractory disease (134). Rituximab was also shown to be beneficial compared to cyclophosphamide in a small trial in patients with severe ocular manifestations resistant to cytotoxic agents (135). The skin and mucosal involvement should be treated based on severity. Isolated oral and genital ulcers could be treated with topical medication such as corticosteroids, lidocaine gel, and oral hygiene with chlorhexidine (134). Colchicine has had its efficacy for erythema nodosum, pseudofolliculitis, oral and genital lesions proven in a randomized crossover trial (134,136). In resistant cases, other immunosupressors such as azathioprine, IFNα, and TNFα antagonists must be considered. The results of two controlled trials suggest that colchicine is effective for the articular manifestation of Behçet's disease (134).

Recommendations on vascular, neurological, and GI involvement were based on expert opinion and uncontrolled evidence from open trials and observational studies. There is no preference for one immunosuppressive drug over another for the management of acute vein thrombosis. Corticosteroids, azathioprine, cyclophosphamide, or cyclosporine A is recommended. More potent immunosuppressive treatments such as cyclophosphamide are preferred for thrombosis of the superior vena cava and Budd-Chiari syndrome as well as for the management of pulmonary and arterial aneurysms that carry a high rupture risk (134).

COGAN SYNDROME

Cogan syndrome (CS) is a chronic inflammatory disorder that typically presents a progressive inflammatory involve-

ment of ocular and audiovestibular organs that leads to severe hearing loss and blindness in at least 50% of the patients (137). CS affects more females than males, and the mean age of onset varies from 22-38 years (138).

Ocular symptoms in CS are typically due to an interstitial keratitis, but other forms of ophthalmic disease such as conjunctivitis, iridocyclitis, episcleritis/scleritis, posterior uveitis, retinal vasculitis, and vitreitis have been described. Audiovestibular dysfunction typically presents at the beginning with unilateral involvement, gradually progressing to a bilateral sensorineural hearing loss accompanied by vestibular disease with tinnitus, vertigo, nausea, emesis. Approximately 15-20% of patients present a vascular involvement, usually as vasculitis of large- and medium-sized vessels. Aortitis is one of the most common vascular manifestations of CS causing proximal aorta dilation, aortic valvular regurgitation, ostial coronary artery disease, and thoracoabdominal aortic aneurysms. A coronary arteritis causing stenosis has also been described. As already mentioned, CS can be associated with urticarial vasculitis. Systemic manifestations (fever, fatigue, weight loss) are present in a subgroup of patients, as well as myalgia, arthralgia/arthritis. Laboratory findings showing an inflammatory condition such as anemia and elevated ESR/CRP can be seen. Interstitial keratitis is often more responsive to therapy than audiovestibular manifestations and responds well to topical glucocorticoid or cyclosporine eyedrops. Audiovestibular disease requires systemic therapy with prednisone and, in patients with frequent flares, additional corticoid-sparing immunosuppression. Vascular disease when present should be treated with systemic corticosteroids and cyclosporine or cyclophosphamide (139). Cochlear implantation is necessary for end-stage hearing loss in patients. CS generally has a good prognosis, but patients with systemic manifestations, especially vascular diseases, have an increased risk of death. Morbidity of disease is mainly due to hearing loss and adverse effects of immunosuppressive therapy (138).

SINGLE-ORGAN VASCULITIS

SOV refers to the vascular inflammation restricted to a single organ without any evidence of other involvement. It can be focal (testicular, breast, gynecological, aorta) or may be diffuse/multifocal (for example, central nervous system and skin). However, the patient must have no sign of vasculitis beyond this focus over a period of at least six months to be classified as such. The patient will be followed to watch for future changes in disease patterns. The nomenclature used is based on the site involved and histological features (granulomatous inflammation, for example) (4).

Patients with focal SOV can be treated with surgical resection and this is often incidentally diagnosed by biopsy in patients under screening for malignancy or infection. It usually occurs in the absence of constitucional or musculoskeletal symptoms and with normal laboratory exams. Vasculitis of the breast is not an SOV. Some features like anemia and higher erythrocyte sedimentation rates suggest systemic illness, especially in GPA. Histological finding do not help to differentiate SOV from systemic vasculitis. However, granulomatous inflammation in gynecological vasculitis must alert the physician to GCA. SOV with diffuse involvement requires systemic therapy that varies depending on the severity and organ affected. It cannot be treated with surgical resection (4).

VASCULITIS ASSOCIATED WITH SYSTEMIC DISEASE

Vasculitic manifestations may follow the manifestations of some systemic rheumatic diseases. Rheumatoid arthritis associated vasculitis (RAV) is currently a very rare complication of rheumatoid arthritis and affects small- and medium-sized vessels. RAV can present as skin manifestations such as digital microinfarcts, ulcers, livedo reticularis, purpura, or digital gangrene. Mononeuritis multiplex is the most prominent form of RAV neuropathy but sometimes the polyneuropathy is exclusively sensory. Severe gastrointestinal perforations, ischemic colitis, pancreatitis, and mesenteric infarction can also be vasculitis manifestations in rheumatoid arthritis. Ocular manifestations such as episcleritis and scleritis can also be observed. Five-year mortality rates are high between 33 to 43% (140). The prevalence of vasculitis in SLE ranges between 11-20%. In an analysis of a large series of SLE patients, the most frequent type of vasculitis was the small vessel vasculitis, defined as leukocytoclastic vasculitis. This presented mostly as erythematous or violaceous punctate lesions on the fingertips and/or palms. Cryoglobulinemia with purpura, often associated with HCV, was the second cause of associated vasculitis followed by urticarial vasculitis. Medium-vessel vasculitis was also described as visceral vasculitis (most frequently as mononeuritis multiplex and intestinal vasculitis) and cutaneous vasculitis (ulcers or ischemic lesions) in SLE (141). Cutaneous vasculitis can manifest in 10% of patients with Sjögren's syndrome and mostly involves small vessels presenting as palpable purpura. Vasculitis is a rare manifestation in sarcoidosis and can involve small-, medium- or large arteries, the latter being a differential diagnosis of TA (142,143).

VASCULITIS MIMICS

There are some conditions that may be misinterpreted as systemic vasculitis presenting with multiorgan disease or vascular damage. The absence of vascular inflammation in the biopsy is important to elucidate this differential diagnosis. Imaging studies (angiography, MRA, or CTA) can help differentiate those conditions from true vasculitis. Table 12 depicts common conditions that mimic vasculitis (144).

EMBOLIC	THROMBOTIC	VASCULAR CONDITION
Cholesterol crystals	Antiphospholipid syndrome	Drug induced vasospasm (ergotamin, cocaine, anphetamin etc)
Atrial myxoma	Procoagulant states	Fibromuscular dysplasia
Infection (septic)	Calciphylaxis	Neurofibromatosis
	Cumarinic necrosis	Coarctation
	Thrombotic thrombocitopenic purpura	Malignant Atrophic Papulosis (Kohlmeier-Degos' Disease)

Table 12. Diseases that mimic systemic vasculitis.

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37

AUTOIMMUNE UVEITIS

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INTRODUCTION

The uvea represents the vascular organ of the eye and it is composed of the iris, ciliary body and, choroid. Autoimmune uveitis (AU) is an inflammatory process of these uveal components due to an autoimmune reaction to self-antigens or caused by an innate inflammatory reaction secondary to an external stimulus. It can present as an isolated entity or associated with a systemic autoimmune or autoinflammatory disease. Because of its proximity to other parts in the eye, inflammation can cause damage to ocular layers such as the retina and structures like the vitreous body and optic nerve. This compromise is considered one of the principal causes of preventable blindness around the world (1).

Eye inflammation can be the initial presentation in many autoimmune diseases (AD) manifesting as conjunctivitis, episcleritis, or scleritis. Diseases such as rheumatoid arthritis (RA) systemic lupus erythematous (SLE) are commonly associated with these type of manifestations. On the other hand, anterior uveitis typically appears as the initial manifestation in autoinflammatory diseases such as ankylosing spondilitis (AS) (2). There is a clear association described with the HLA-B27 positivity and a higher risk of presenting recurrent anterior uveitis in AS (3). Since AU is the initial presentation of some AD it is important that both ophthalmologists and rheumatologists work together to achieve a faster diagnosis and a more effective treatment for patients. Herein we present a way to address the disease from the point of view of both fields.

CLASSIFICATION

Clinical picture may vary depending on the anatomic location of the inflammation. It is important to understand that the primary site of inflammation defines the type of uveitis. Anterior uveitis is the most commonly seen. It may manifest as iritis, affecting the iris, or iridociclitis affecting the ciliary body as well. Intermediate uveitis or vitritis involves the vitreous cavity and may involve the pars plana. Finally, posterior uveitis is divided in three types: choroiditis, retinochoroiditis, and chorioretinitis (4). This last one is usually associated with infective diseases such as toxoplasmosis. In diffuse involvement or when uveitis affects many areas, it is described as panuveitis. These four anatomical types of uveitis can all be associated with other disorders.

The International Uveitis Study Group (IUSG) Classification is used for classification of the different types of Uveitis (5) (Table 1). The Standardization of Uveitis Nomenclature (SUN) group also defined criteria for the onset, duration, and course of uveitis (6). Categorization by etiological criteria may be appropriate in the field of uveitis as compared to other ophthalmologic subspecialties because of its significant degree of association with systemic disease (7).

APPROACH TO UVEITIS

CLINICAL PRESENTATION

Common symptoms of uveitis are blurred vision, photophobia, eye pain, floaters (floating spots), headache and injected conjunctiva. Usually in children can be asymptomatic. The suspicion of uveitis requires a prompt referral to an ophthalmologist at the emergency room who is in charge of the uveitis pattern definition to rule out other ophthalmologic syndromes and establish initial treatment. The rheumatologist is then in charge of the complete diagnostic workup and should look for additional autoimmune diseases. This will help the patient to acquire prompt treatment, manage the inflammatory process, and reduce the risk of permanent damage.

1. BY LOCATION		
Type	**Primary Site of Inflammation***	**Includes**
Anterior uveitis	Anterior chamber	Iritis Iridocyclitis Anterior cyclitis
Intermediate uveitis	Vitreous	Pars planitis Posterior cyclitis Hyalitis
Posterior uveitis	Retina or choroid	Focal, multifocal, or diffuse choroiditis Chorioretinitis Retinochoroiditis Retinitis Neuroretinitis
Panuveitis	Anterior chamber, vitreous, and retina or choroid	
2. BY CLINICAL COURSE		
Acute		
Chronic		
Recurrent		
3. BY LATERALITY		
Unilateral		
Bilateral		
4. BY ETIOLOGY		
Type	**Etiology**	
Infectious	Bacterial Viral Fungal Parasitic Others	
Noninfectious	Known systemic associations No known systemic associations	
Masquerade	Neoplastic Nonneoplastic	

Table 1. Classification of uveitis.
*As determined clinically. Adapted from Bloch-Michel *et al.* (5), Deschenes *et al.* (7) and The SUN Working Group (6).

MEDICAL HISTORY

A detailed medical history is the key to achieving a proper diagnosis and to assessing the response to therapy and any side effects of medications. The major symptoms should be organized in chronological order along with a description of what makes them better or exacerbates them. The past medical history should include social and sexual history. It is also very important to look for associated AD in the patient and the family. Impressions about the course of the disease and the impact on the patient's quality of life may vary among patients. Some may be troubled by mild conjunctival erythema and request relief of the symptoms. Others might tolerate this mild redness and prefer not to use any kind of treatment. The same happens with the perception of visual loss. The perception of visual distortion secondary to inflammation may differ greatly. The understanding of the patient and his/her relationship to the disease is the key for the physician to explain and put the disease in proper perspective (8).

Uveitis may be the initial symptom of AD and autoinflammatory diseases like Seronegative spodyloarthropathies. Therefore, ophthalmologists and rheumatologists should collaborate on identifying systemic diseases that may be present to avoid unnecessary diagnostic tests and delay of proper treatment. Close collaboration between the two specialties will help achieve a more accurate diagnosis and efficient treatment for patients (9) (Figure 1).

PHYSICAL EXAMINATION

The ocular examination of patients with uveitis is important for diagnosis and to determine the proper therapy. A complete eye examination with the aid of slit-lamp biomicroscopy and other tools should include the following: Visual acuity, complete external examination, extraocular muscles, intraocular pressure (IOP) measurement, pupils, conjunctiva, cornea, anterior chamber and its angle, iris, lens, anterior chamber angle, vitreous, retina, choroid and optic nerve.

External examination has to include the entire body especially skin and articulations to evaluate the presence of lesions or inflammation that may suggest an associated AD. Involvement of intraocular muscles must also be made in

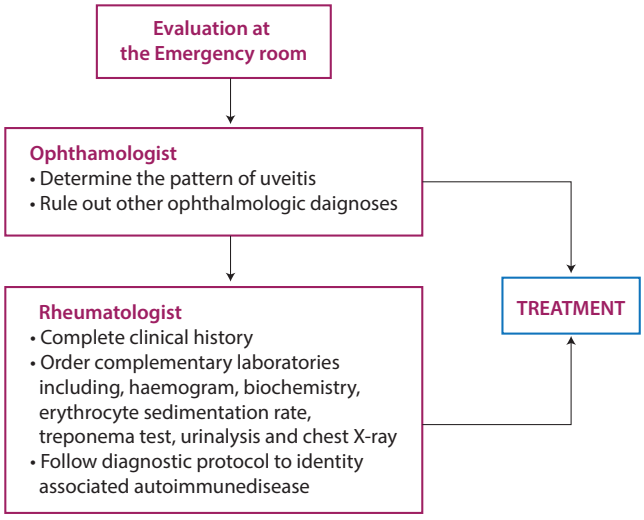


Figure 1. Assistance protocol of Uveitis. Adapted from Muñoz-Fernandez *et al* (9).

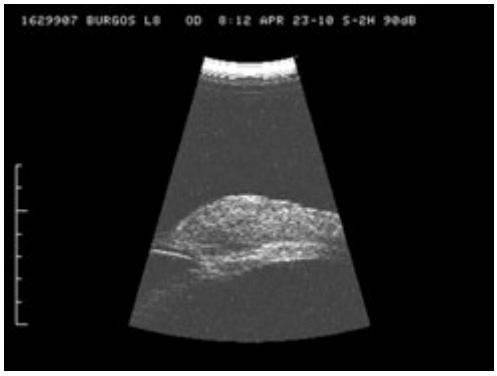


Figure 2. Scleral nodule (Ultrasound). Reprinted by permission from the Escuela Superior de Oftalmologia Clinica Barraquer de America, Bogota, Colombia, copyright (2013).

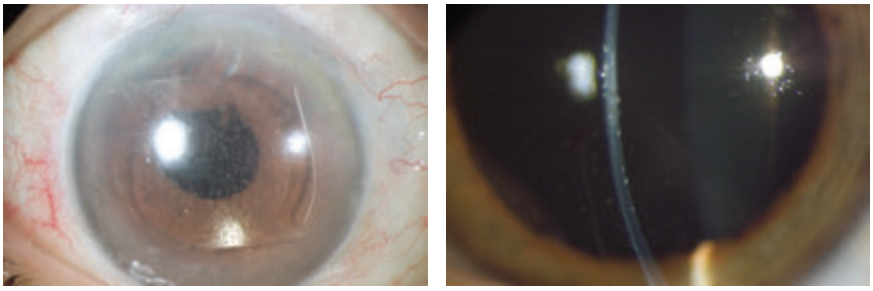
order to discard central nervous system diseases associated with uveitis like multiple sclerosis (MS) and Sarcoidosis.

IOP has to be measured. Initially, Patients with uveitis tend to have low IOP and then it can increase secondary to the use of corticosteroids or inflammation. Conjunctival hyperemia is a common sign and usually presents in the perilimbal region representing ciliary body inflammation. Scleritis (Figure 2) and episcleritis may also occur. Keratic precipitates are the most common corneal finding in uveitis (Figure 3 and 4). They are small aggregates of inflammatory cells that can accumulate on the endothelial surface of the cornea. The presence of these deposits on the cornea help the ophthalmologist to determine the level of inflammatory activity or if it was previously inflamed.

Evaluation of pupils is usually difficult because of synechiae (adhesions) (Figure 5). They can form between the iris and the lens (Figure 6) or between the iris and the cornea. It is also important to check for iris nodules associated with other AD. Many patients with uveitis also develop cataracts because of the inflammation or the chronic use of corticosteroids.

Examination of the retina must include periphery and pars plana. Cystoid macular edema and retinal vascular alterations are common retinal finding in patients with uveitis. Choroidal lesions with or without retinal involvement, are characteristic of posterior inflammatory disease. On the other hand retinal hemorrhages and cotton-wool spots are signs of retinal vasculitis. Finally, optic nerve assessment must be done to search for papilledema, disc hyperemia or papillitis which are commonly seen in uveitis.

The anterior chamber is examined in search of inflammatory cells. The presence of cells or increased protein (flare) in the anterior chamber means spillover from the inflamed iris or ciliary body while in the posterior chamber, they arise from the choroid, retina, and ciliary body (8). Floaters and reduced vision are the two most common complaints of patients with inflammation of the vitreous, retina, and choroid. Other patients complain of blurred or reduced vision. A change in the pattern of floaters or visual impairment often means a change in the underlying ocular disease, e.g., an increase in inflammation, the development of vitreous hemorrhage, or the condensation of the vitreous into a more organized opaque tissue.



Figures 3 and 4. Keratic precipitates. Reprinted by permission of the Escuela Superior de Oftalmaologia Clinica Barraquer de America, Bogota, Colombia, copyright (2013).

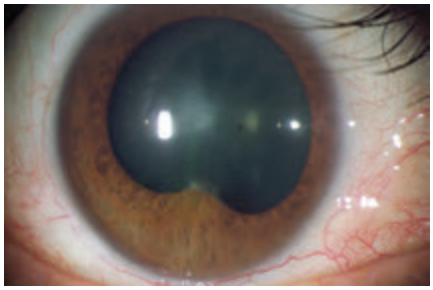


Figure 5. Posterior synechiae attached to anterior lens capsule. Reprinted by permission of the Escuela Superior de Oftalmologia Clinica Barraquer de America, Bogota, Colombia, copyright (2013).

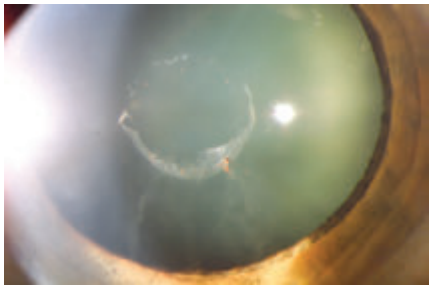


Figure 6. Posterior synechiae leaves marks of iris pigment on anterior lens capsule. Reprinted by permission of the Escuela Superior de Oftalmologia Clinica Barraquer de America, Bogota, Colombia, copyright (2013).

For grading the degree of inflammation in the anterior chamber, two grading schemes have been used for cells and flare (see Tables 2 and 3). For vitreous cells a grading for haze has been established (Table 4). Activity of Uveitis terminology is also shown (Table 5).

EPIDEMIOLOGY

Incidence of uveitis has been reported to range between 17 per 100,000 to 61 per 100,000 (10–14). In one of the largest studies done in the United States, the incidence of uveitis was reported to be 52.4 cases per 100,000 person/year, with a prevalence of 115.3/100,000 persons. Incidence was reported to be lower in children and higher in patients 65 years or older. Prevalence was also higher in women than in men. Primary Inflammatory disorders potentially associated with systemic manifestations are generally the most frequently reported. Bodaghi *et al*. (15) showed a 61.8% prevalence of non-infectious anterior uveitis among 264 patients in his study. Also, 89.2% of 139 patients had noninfectious related intermediate uveitis, 54.5% of 200 patients had noninfectious related posterior uveitis, and 76.2 of 324 had noninfectious related panuveitis. The systemic diseases most frequently associated with noninfectious uveitis were sarcoidosis, Behçet disease, spondylarthropathies, Juvenile idiopathic arthritis (JIA), Vogt-Koyanagi-Harada syndrome (VKH), and MS. All of these will be reviewed in this chapter.

Uveitis is even more common in the developing world. This can be attributed to infections like tuberculosis and trauma. Infectious uveitis can account for up to 50% of cases in developing nations while certain developing countries have a higher prevalence of noninfectious uveitis (16). In Latin America, specifically in Colombia, a retrospective study of 693 patients showed an important prevalence of uveitis secondary to toxoplasmosis. In contrast to most other studies, the most common location of uveitis in this country was posterior (31%) followed by anterior (29%). The high prevalence of posterior uveitis was attributed to the high prevalence of toxoplasmosis and toxocariasis (17).

Anterior uveitis is the most common form of uveitis with an annual incidence of 8 per 100,000 person/year. It represents approximately three-quarters of the cases of uveitis. Most of these cases are considered idiopathic. However HLA-B27 positivity is associated with the disease in up to one third of patients (8). In the general Western population, the prevalence of HLA-B27 positivity is approximately 8–10% whereas the prevalence of HLA-B27 in patients with AU is approximately 50% (9).

HLA-B27 positive anterior uveitis may be considered a different clinical disorder and is associated with systemic diseases such as AS, Reiter's syndrome, inflammatory bowel disease (IBD), and psoriatic arthritis (PA). This does not mean that all patients with the HLA-B27 haplotype and anterior uveitis necessarily manifest a systemic illness. Some

GRADE	CELLS IN FIELD*
0	<1
0.5+	1–5
1+	6–15
2+	16–25
3+	26–50
4+	>50

Table 2. Grading scheme for anterior chamber cells (6). *Field size is a 1x1mm slit beam.

GRADE	DESCRIPTION
0	None
1+	Faint
2+	Moderate (iris and lens details clear)
3+	Marked (iris and lens details hazy)
4+	Intense (fibrin or plastic aqueous)

Table 3. Grading scheme for anterior chamber flare (6)

SCORE	DESCRIPTION	CLINICAL FINDINGS
0	Nil	None
1	Minimal	Posterior pole clearly visible
2	Mild	Posterior pole details slightly hazy
3	Moderate	Posterior pole details very hazy
4	Marked	Posterior pole details barely visible
5	Severe	Fundal details not visible

Table 4. Grading scheme for vitreous haze. From (10).

TERM	DEFINITION
Inactive	Grade 0 cells
Worsening activity	Two-step increase in level of inflammation (e.g. anterior chamber cells, vitreous haze) or increase from grade 3+ to 4+
Improved activity	Two-step decrease in level of inflammation (e.g. anterior chamber cells, vitreous haze) or decrease to grade 0
Remission	Inactive disease for >3 months after discontinuing all treatments for eye disease

Table 5. Activity of uveitis terminology. From (6).

studies show that 50% of patients with anterior uveitis are HLA-B27 positive, and half of these have spondyloarthropathies (18). Zeboulon *et al*.(19) did a systematic review of 126 articles describing 29,877 patients with spondyloarthropathies. They reported a mean uveitis prevalence of 32.7%.

Intermediate uveitis which involves the vitreous cavity and may involve the pars plana can account for approximately 4–8% of cases of uveitis. Case reports have shown presence of pars planitis in the members of the same family suggesting genetic factors involved (20). In some of these cases, patients had the same HLA association or were identical twins (21). Possible association of intermediate uveitis and HLA haplotypes has been described especially with HLA-A28, HLA-DR15, HLA-DR17, and HLA-DR51(8).

ASSOCIATED DISEASES

In AU, systemic AD should be always investigated, and the patient's treatment should be modified if other organs are involved. Some of these conditions may be associated with HLA-B27 positivity.

SERONEGATIVE SPONDYLOARTHROPATHIES

Seronegative spodyloarthropathies (SpA) are a group of diseases that usually involve the axial skeleton, have a negative rheumatoid factor (RF), and have no other serologic marker. Patients are more frequently male and have positive HLA-B27. Some of the diseases in this group are PA, reactive arthritis (ReA) and the most prevalent, AS. Anterior uveitis is a frequent complaint in the follow up of patients with spondyloarthropathies and is most frequently seen in AS. In most of the cases of SpA uveitis is the first clinical sign (22).

ANKYLOSING SPONDYLITIS

AS is characterized by an inflammatory arthritis involving axial skeleton and sacroiliac joints. Anterior uveitis in these patients is usually acute, unilateral and recurrent with a tendency to recur in the contralateral eye. Recurrence may occur as frequently as every 3 weeks. It may affect 10-40% of AS patients during follow up (23). It commonly affects young males and HLA-B27 positivity is seen in almost 80-95% of the patients. Articular manifestations are common and present as low-back pain due to sacroilitis and oligoarthritis related to enthesitis. X-rays of sacroiliac joints show sclerotic changes and total joint obliteration at later stages. The eye involvement usually manifests as acute recurrent attacks of a nongranulomatous, unilateral anterior uveitis (4). Sampaio-Barros *et al*.(23) showed an association in HLA-B27 positive AS patients who had anterior uveitis and HLA-B*2705 haplotype. They also showed an association between uveitis and juvenile-onset AS and the presence of achillean and plantar enthesopathies.

PSORIATIC ARTHRITIS

PA initially manifests as joint pain and stiffness associated with a skin disease secondary to proliferation of the epidermis and scaling. Patients may manifest patchy asymmetric lesions and dactylitis. In contrast to AS, patients with PA tend to have uveitis with an insidious onset, usually with anterior compromise, and more likely bilateral. It presents commonly with conjunctivitis and abundant mucoid secretion (24). In children with idiopathic PA, the anterior uveitis may not show any symptoms at all (25). Approximately 7-25% of patients with PA may develop uveitis and it is associated with axial skeleton and sacroiliac joint involvement. However association have also been found in psoriasis patients without arthritis and distinct clinical characteristics in anterior uveitis (24).

REACTIVE ARTHRITIS

ReA is characterized by the triad of arthritis, conjunctivitis and urethritis. Like AS and PA, ReA is also related to HLA-B27. The presenting symptom may be, in most of the cases, arthritis followed by genitourinary symptoms. Arthritis involves hands, sacroiliac, feet, and knee joints. Nongranulomatosus iritis can occur in 3-12% of the patients, but the most common ocular manifestation is conjunctivitis in about 30-60% of the patients. These manifestations are usually mild (8,26).

OTHER ASSOCIATED DISEASES

INFLAMMATORY BOWEL DISEASES

Ocular manifestations may be found in 2.5% of Crohn's disease patients and 5% of ulcerative colitis patients. Gastrointestinal manifestation may include recurrent and/or bloody diarrhea with abdominal cramping which are highly suggestive of IBD. Extraintestinal manifestations include migratory polyarthritis, erythema nodosum, cholangitis, and uveitis. Usually the uveitis presents as non-granulomatous anterior uveitis. Chronic posterior uveitis has also been described but at a lower frequency (4). Conjunctivitis and episcleritis are also commonly described.

JUVENILE IDIOPATHIC ARTHRITIS

JIA is a term that describes a group of disorders that share the clinical manifestation of chronic joint inflammation from unknown causes that begins before 16 years of age. They usually have a rapid onset of inflammation in multiple joints, especially hands, wrists, elbows, and feet. The systemic type, also called Still's disease, is the most severe form and the least frequent. The presence of HLA-B27 in JIA was negatively associated with long term remission status, possibly because of its association with clinical disease characteristics such as sacroiliitis (27). A subtype called RF-positive polyarthritis, which can present with anti-CCP antibodies, is also associated with the presence of HLA–DR4 alleles and an aggressive disease course (28). Uveitis is rare in children with systemic JIA. It is important to understand the different types of JIA. For example, patients with the pauciarticular form of JIA have a much higher risk of developing uveitis than patients with polyarticular arthritis. Casidy *et al*.(29) showed that women with pauciarticular arthritis and a positive antinuclear antibody (ANA) test result have a higher risk of developing chronic iridocyclitis. This type of patient should be screened every 3-4 months for the development of chronic uveitis (Figure 7). The joint manifestation in patients with acute uveitis tends to start later than in patients with chronic uveitis, and joint inflammation usually precedes ocular disease, but iritis can precede the joint manifestations (8,30).

BEHÇET'S DISEASE

Behçet's is a systemic vasculitic disease characterized by recurrent oral aphthous ulcers, genital ulcers, uveitis, and skin lesions. Usually these manifestations are self-limiting except for the uveitis. Susceptibility to Behçet's disease is associated with the presence of the HLA-B51 haplotype and this genetic marker is associated with ocular involvement (31). The International Study Group (ISG) criteria is the one most widely used to diagnose this type of patient (32). The ocular involvement is estimated to be between 83–95% in men and 67–73% in women and occurs after the onset of other symptoms (33).

KAWASAKI DISEASE

Kawasaki disease is a medium vessel vasculitis that occurs in children under the age of 4. It manifests as a fever with lymphadenitis followed by conjunctivitis, changes in lips and oral mucosa (strawberry tounge) and desquamation. Conjunctivitis is the most common ocular finding but cases of uveitis have been described (34).

VOGT–KOYANAGI–HARADA SYNDROME

VKH characteristically presents as a severe bilateral granulomatous uveitis with signs of meningitis, bilateral neurosensorial dysacusia, and skin alterations. There is a strong relationship with the presence of HLA-DR4. Uveitis may last up to several weeks after a prodromic period. After this, tissues such as the skin, the uvea, and the pigmented retinal epithelium may begin to depigment (35) (Figure 8). Other common findings are optic disc edema (Figure 9) and retinal folds (Figure 10).

SARCOIDOSIS

Sarcoidosis is a multisystemic disease characterized by immune-mediated (CD4) widespread noncaseating granulomas. It is associated with restrictive lung disease, bilateral hilar lymphadenopathy, erythema nodosum, and hypercalcemia. It commonly occurs in African descendants between 20-50 years of age. Patients with systemic disease may present with AU in about 27-40% of the cases. In approximately half of the cases with ocular involvement, the presentation is an acute, self-limited iridociclitis. Patients in whom AU secondary to sarcoidosis is suspected, must be screened for other systemic involvement (4).

TREATMENT

It is very important to distinguish between infectious and AU as the therapeutic approach and treatment is completely different. An infectious etiology always has to be rule out before beginning treatment of AU. Treatment of AU depends on the clinical presentation. On acute uveitis, the therapeutic approach must be more aggressive to achieve fast control of the inflammation. In these cases, corticosteroids are the most frequently used. On the other hand, for chronic uveitis treatment must not be so aggressive and it should planned for the long term in order to reduce side effects (36). Clinical correlation and interaction between rheumatologists and ophthalmologists is the key to achieving the best treatment.

CORTICOSTEROIDS IN ACUTE RECURRENT INFLAMMATION

Corticosteroids are considered the gold standard in management of acute AU. They can only be administered after excluding infectious origin. Corticosteroid eyedrops are especially useful in inflammation of the anterior segment. They are related to a high rate of side effects including an increase in IOP and the development of cataract. Thus, it is sometimes useful to combine with corticosteroid-sparing immunosuppressive agents. Periocular corticosteroids like the Triamcinolone acetonide are injected into the lower exter-

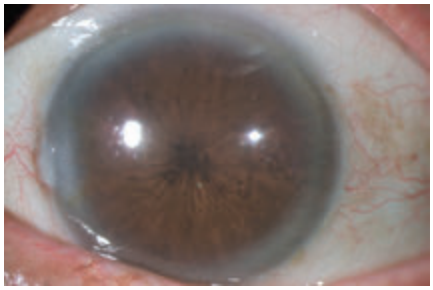


Figure 7. Chronic uveitis sequela. Pupillary seclusion by synechiae. Reprinted by permission of the Escuela Superior de Oftalmologia Clinica Barraquer de America, Bogota, Colombia, copyright (2013).

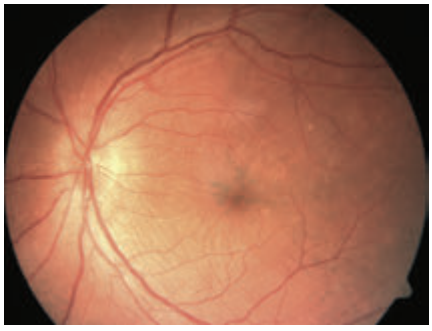


Figure 8. Macular pigment deposits with stellate appearance. Reprinted by permission of the Escuela Superior de Oftalmologia Clinica Barraquer de America, Bogota, Colombia, copyright (2013).

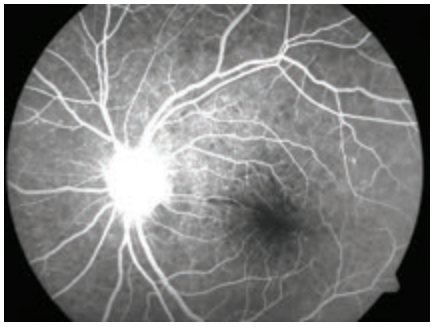


Figure 9. Optic disc edema: Diffuse dye leak with blurred disc edges. Reprinted by permission of the Escuela Superior de Oftalmologia Clinica Barraquer de America, Bogota, Colombia, copyright (2013).

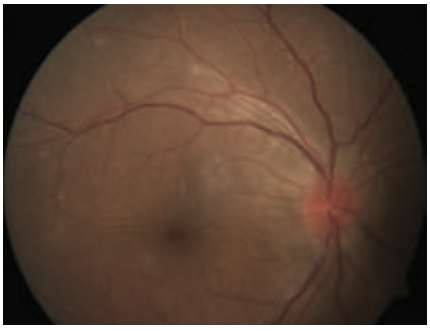


Figure 10. Retinal folds extending through temporal vessels with bilateral serous detachments appearance. Reprinted by permission of the Escuela Superior de Oftalmologia Clinica Barraquer deAmerica, Bogota, Colombia, copyright (2013).

nal region of the periorbital tissue at doses of 40 mg in 1 ml to a maximum of 5-10 every three months (37) (Figure 11). Intraocular injections, in turn, are only applied in severe recurrences when the patient has a sight threatening uveitis. New steroid devices are being used for local drug delivery and are surgically implanted in the intravitreous cavity (38). They can provide slow release of corticosteroids to control inflammation. Systemic corticosteroids are useful for acute outbreaks. Oral prednisone is used at doses of 1-1.5 mg/kg/day. When the inflammation is controlled, prednisone can be progressively decreased. As a maintenance therapy, prednisone is used in doses of 5-10 mg daily (36).

Chronic use of corticosteroids as anti-inflammatory and immunosuppressive therapy is associated with high morbidity for patients. That is why practitioners start using corticosteroid-sparing immunosuppressive drugs.

Based on their mechanism of action, they are divided into alkylating agents (cyclophosphamide and chlorambucil), antimetabolites (methotrexate, azathioprine, and Mycophenolate Mofetil), and calcineurin inhibitors (cyclosporine, tacrolimus and sirolimus). Finally, biological anti-inflammatory agents (i.e., antagonists of tumor necrosis factor alpha like Infliximab and adalimumab) are also showing very promising results.

In many cases, these agents will be seen listed as first choice in some autoimmune diseases, depending on the patient's history, age, sex, type and severity of the inflammatory disease, etc. The interdisciplinary treatment between ophthalmologist, rheumatologist, oncologist and hematologist is essential to initiate and monitor these therapies.

Figure 11. Periocular injection. Reprinted by permission of the Escuela Superior de Oftalmologia Clinica Barraquer de America, Bogota, Colombia, copyright (2013).

CYCLOSPORINE AND TACROLIMUS (FK506)

Cyclosporine and Tacrolimus (FK506) are immunosuppressive drugs that act on T cells. Cyclosporine blocks the differentiation and activation of T cells prevents the production of IL-2. It is used in doses of 3-5 mg/kg/day divided into twice daily doses. After inflammation is controlled the dose may be reduced to 2-3 mg/kg/day (39). Tacrolimus binds to FK-binding proteins and inhibits secretion of IL-2 and other cytokines. The oral dosage administered is between 0.05 and 0.2 mg/kg/day. Tacrolimus is well tolerated and used to control posterior uveitis. It may be considered in cyclosporine resistance or toxicity (40).

MYCOPHENOLATE MOFETIL

Mycophenolate Mofetil selectively inhibits proliferation of T and B lymphocytes. In uveitis, the recommended dose ranges from 500 mg to 2 g daily and may be combined with cyclosporine and corticosteroids (41).

METHOTREXATE

Methotrexate is a folic acid analog that inhibits the dihydrofolate reductase. It may cause myelosuppresion for which folic acid must always be supplied. In uveitis, doses range from 7.5 to 25 mg/weekly in a single dose and it is considered a corticosteroid sparing drug. It is the drug of choice in sarcoidosis. It is also used intravitreally, administered at a dose of 400µg/0.1ml (36). Taylor *et al*. (42) showed in a prospective study that intraocular methotrexate was found to be effective in reducing vitritis and macular edema without raising the IOP in patients with a history of steroid response.

BIOLOGICAL THERAPIES

ANTI-TNF

Infliximab is a chimeric monoclonal antibody against TNF-α. TNFα, which is secreted by monocytes, macrophages, lymphocytes, and mast cells seems to play a key role in ocular inflammatory diseases. High levels of TNF-α and TNF-receptor were observed in patients with uveitis (43). It is administered intravenously at infusions of 5-10 mg/kg every 1-2 months. Once injected into the body, these monoclonal antibodies will, therefore, directly block TNF-α. The effectiveness of anti-TNF-α in uveitis usually lasts up to 15 days to 3 weeks after initiation of treatment (44). Adalimumab is a totally humanized monoclonal antibody and thus has less adverse effects than infliximab which is a chimeric monoclonal antibody. It is administered subcutaneously at doses of 40 mg every 2 weeks (45). In the case of uveitis, only adalimumab and infliximab are used. Etanercept seems to have less efficacy due to lower ocular penetration and is not recommended for this indication. Golimumab is injected subcutaneously at a dose of 50mg every four weeks, and both its route of administration and time are great advantages compared to other anti-TNF (46).

ANAKINRA

Anakinra, a soluble antagonist IL-1Ra, is effective in uveitis. It is administered at 100 mg subcutaneously daily. Unlike anti-TNF, there is no increased risk of tuberculosis (44).

RITUXIMAB

Rituximab is a chimeric monoclonal antibody that binds to B lymphocyte antigen CD20. Rituximab has been approved for the treatment of diffuse large B lymphoma cells in combination with chemotherapy. In rheumatology, it is used for the treatment of RA. Two dosing regimens are used: the regimen in RA is 2 infusions of 1000 mg at 15 day intervals, and in hematological malignancies it is 4 weekly infusions of 375 mg/m2. Evidence suggests the importance of this molecule for peripheral ulcerative keratitis, scleritis, and refractory uveitis (47). It also has been also used for treatment of refractory ocular JIA after failure of one or more immunosuppressor and anti-TNF. Davatchi *et al*.(48) showed that Rituximab was more effective than cyclophosphamide for the control of ocular inflammation.

INTERFERONS

IFN-α is a naturally occurring cytokine secreted in response to viral infections. Only the non-pegylated IFN-α (IFN-α2a and IFN-α2b) and pegylated IFN-α (peginterferon-α2a) are used in ophthalmology. The most frequently used is IFN-α2a at dose ranges from 3 to 6 mIU, 3 times per week. Steroids and immunosuppressive drugs may antagonize the effect of IFN, and thus stopping the cytotoxic agents before initiating the treatment, and reduce corticosteroid dose as low as possible is recommended (44).

FOLLOW-UP

Follow-up visits of subjects with uveitis must be frequent and focused on monitoring the recurrence of acute outbreaks, if any. They should be aimed to determine the evolution of inflammatory signs until their complete resolution, thus ensuring the least possible consequences. After the initial phase of treatment, the duration of use of maintenance medication is directly related to the diagnosis, treatment and control of the underlying disease. This will help to determine if a patient is badly controlled and requires a more aggressive therapeutic plan. Badly controlled is defined as a serious outbreak that limits the patient's visual acuity less than 50% of the initial value (36). A complete physical exam must be done each time the patient sees to the physician in order to monitor ophthalmological complications or development of adverse effects secondary to the treatment.

KEYPOINTS

- Autoimmune uveitis is a prevalent disease that principally affects young people and may lead to significant visual limitation or total blindness.

- To establish a correct diagnosis, clinical course, location, and patient symptoms are essential.
- Autoimmune uveitis presents more frequently as anterior uveitis and is associated with systemic disease. Diagnosis of the underlying condition is the key to treating both uveitis and the systemic disease.
- Close collaboration between ophthalmologists and rheumatologist will serve to provide a more effective diagnosis and patient treatment.
- Corticosteroids remain the gold standard for therapy of autoimmune uveitis. Immunosuppressive drugs may be useful in addition to steroids and biological therapies have shown promising results.

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38

CARDIOVASCULAR INVOLVEMENT IN AUTOIMMUNE DISEASES

Jenny Amaya-Amaya
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INTRODUCTION

Autoimmune diseases (ADs) represent a broad spectrum of chronic conditions that may afflict specific target organs or multiple systems with a significant burden on quality of life. These conditions have common mechanisms including genetic factors, gender disparity, environmental triggers, pathophysiological abnormalities and certain subphenotypes which are represent by the autoimmune tautology (1,2). Atherosclerosis (AT) was once considered to be a degenerative disease that was an inevitable consequence of aging. However, researches in the last three decades have shown that AT is not degenerative or inevitable. It is an autoimmune-inflammatory disease associated with infectious and inflammatory factors, characterized by lipoproteins metabolism alteration that leads to immune system activation with the consequent proliferation of smooth-muscle cells, narrowing arteries and atheroma formation (3). Both humoral and cellular immune mechanisms have been proposed to participate in the onset and/or progression of atheromatous lesions (4). In recent years, many reports have been focused on the immunologic background of AT, and it is no longer in doubt that shares several autoimmune pathways (5). It is not surprising, to find an accelerated AT in quite a lot of ADs.

Several risk factors have been described since The Framingham Heart Study, known as classic risk factors, which over time conduce to endothelial dysfunction, subclinical AT and Cardiovascular (CV) event manifest. Interestingly, the excessive CV events observed in patients with ADs are not fully explained by these factors. Several novel risk factors contribute to development of premature vascular damage. Sarmiento-Monroy *et al.* (6,7) previously proposed a classification for non-traditional risk factors in ADs, which divide them into genetic determinants, AD-related and miscellaneous. Therefore, a complex interaction between traditional and disease-specific traits leads to premature AT process in autoimmunity.

All of these pathways may eventually converge into a shared pro-atherogenic phenotype (8). Cardiovascular Disease (CVD) represent a broad spectrum of subphenotypes: hypertension (HTN); Coronary syndromes: angina, Ischemic Heart Disease (IHD), Acute Coronary Syndrome (ACS), Coronary Artery Disease (CAD), Myocardial Infarction (MI); Congestive Heart Failure (CHF); Peripheral Arterial Disease (PAD); Left Ventricular Diastolic Dysfunction (LVDD); cerebrovascular disease (Cerebrovascular Accidents [CVAs]; Transient Ischemic Attacks [TIAs]); thrombosis: Deep Vein Thrombosis (DVT), Pulmonary Embolism (PE); Peripheral Vascular Disease (PVD); and subclinical AT.

ATHEROSCLEROSIS

Atherosclerosis is a multifactorial, chronic and inflammatory disease that had been traditionally viewed as a lipid-based disorder affecting the vessel walls. Nowadays, this theory had been modified, and it is known that all arms of the immune system take part in atheroma formation. The increased understanding of the mechanisms promoting vascular damage has been recently focus on pro-inflammatory pathways, which appear to play key role in development and propagation of the disease. Thus, some of the mechanisms that drive the atherosclerotic plaque formation, and therefore CVD, are shared with several ADs, although each disease may have particular immunological aberrations that provide specific atherogenic pathways (8). The lesions of AT, the plaque rupture and atherothrombosis resulting in infarction, occur mainly in large and medium sized elastic and muscular arteries and can lead to heart ischemia, brain, or extremities (9–11).

CELLULAR IMMUNITY AND INFLAMMATORY MARKERS

AT is characterized by the accumulation of lipid particles, immune system cells [e.g., monocytes/macrophages (Mϕ) and T lymphocytes], autoantibodies, autoantigens (e.g., vessel walls components), and the multiple production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and Interferon-γ (IFN-γ) in subendothelial regions. All these components leading a gradual thickening of the intima layer, causing a decrease in elasticity, arterial lumen narrowing, blood flow reduction, plaque rupture and finally CV event (9,12). The systemic inflammatory response that characterizes AT also involves acute-phase reactants, such as serum amyloid A, fibrinogen, erythrocyte sedimentation rate (ESR), and C-Reactive Protein (CRP). For instance, CRP can activate complement system and enhance the pro-inflammatory production cytokines, such as interleukin-6 (IL-6) (13). Thus, increased levels of CRP has been shown to be an independent risk factor for CVD (14–16). Endothelial dysfunction is the first step leading to AT and had been associated with both, traditional and non-traditional risk factors related to several ADs. For instance, free radicals caused by cigarette smoking and type 2 diabetes mellitus (T2DM) (17), some of them caused or exacerbated by steroid therapy (18), contribute to this process. Other factors involved are angiotensin II high concentrations, which increase smooth-muscle hypertrophy, peripheral resistance and oxidation of low density lipoprotein cholesterol (LDL), as well as elevates plasma homocysteine concentrations (19,20). Several infectious pathogens have been also related with atherosclerotic process, which include *C. pneumoniae*, *M. pneumoniae*, *H. pylori*, Cytomegalovirus, Epstein-Barr virus and Herpes Simplex Virus type 1.

Early in the development of AT, LDL becomes modified (i.e., oxidation, glycation). During this process, reactive aldehydes are produced, which binding to lysine and histidine residues in the ApolipoproteinB (ApoB) component of LDL, and produce immunogenic neoepitopes (21). It is a major cause of injury to the endothelium and underlying smooth muscle (9,12,22,23). Thus, the different forms of injury increase the endothelium adhesiveness regarding leukocytes or platelets, as well as its permeability, with the expression of multiples vascular cell adhesion molecules (VCAM), intercellular adhesion molecules-1 (ICAM-1), selectins and chemokines. In response to these adhesion molecules, monocytes are recruited and differentiated into Mϕ. Thereby, when LDL particles become trapped in an artery, they can submit progressive oxidation, facilitates the accumulation of cholesterol esters and be internalized by Mϕ through scavenger receptors pathway (24,25), resulting in the formation of foam cells. Besides their differentiation, Mϕ are associated with up-regulation of toll-like receptors (TLRs), which enhance a cascade of Mϕ activation and release of vasoactive molecules such as nitric oxid (NO), reactive oxygen, which increase oxidation and toxicity of lipoproteins, endothelins, eicosanoids, and proteolytic enzymes, all of which lead to the plaque destabilization of and increased risk for rupture (24,25). The foam cells subsequently produce growth factors and cytokines that lead to the proliferation of vascular-smooth-muscle cells and plaques development (8,25). In addition to its ability to injure these cells, modified LDL Cambiar por [i.e., oxidized-LDL (ox-LDL)] is chemotactic for other monocytes and can up-regulate the expression of genes for Mϕ colony stimulating factor (MCSF) and monocyte chemotactic protein (MCP) derived from endothelial cells. Thus, it may help expand the inflammatory response by several ways: stimulating the replication of monocyte-derived Mϕ and the entry of new cells into lesions, increasing the bind of LDL to endothelium, transcription of the LDL-receptor gene and attracting more lipoprotein and lymphocytes within artery (23).

After the process is begun, rolling and adherence of monocytes and T cells occur at these sites as result of up-regulation of adhesion molecules on both endothelium and leukocytes. Chemokines may be responsible for chemotaxis and accumulation of Mϕ in fatty streaks. Activation of monocytes and T cells leads to up-regulation of receptors on their surfaces, such as the mucin-like molecules that bind selectins, integrins that bind adhesion immunoglobulin superfamily molecules and receptors that bind chemoattractant molecules (23,26). T cells, predominantly lymphocyte T helper 1 (Th1) are also recruited to the subendothelial space where they produce cytokines. They appear in the arterial intima as early in 1 year old children in fatty streaks. Th1 are dominating over lymphocytes T helper 2 (Th2) and their anti-inflammatory mediators (i.e., IL-4, 5,10). This kind of reaction is increased in several ADs, which are characterized as being Th1 cell-mediated more than Th2 cell-mediated conditions, with high production of TNF-α, IFN- γ, IL-2, IL-6, IL-17 among others, further to be able to activate T cells, favor smooth muscle cell migration, proliferation and foam cell formation (8,27–29). The continuing entry, survival and replication of mononuclear cells in lesions depend on factors such as MCSF and granulocyte–Mϕ colony-stimulating factor for monocytes and IL-2 for lymphocytes. Therefore, as fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap. The fibrous cap covers a mixture of leukocytes, lipid and debris, which may form a necrotic core, due to inflammatory cytokines such as IFN-γ, which activate Mϕ and under certain circumstances induce them to undergo programmed cell death, increase proteolytic activity, and lipid accumulation. Those are producing cytokines, such as TNF-α, IL-1, transforming growth factor-β (TGF-β), and growth factors such as platelet-derived growth factor (PDGF) (25), enhancing the inflammatory response and thereby the atherosclerotic process.

Furthermore, activated Mϕ express human leukocyte antigen (HLA) II such as *HLA-DR* that allows them to present antigens to T lymphocytes. T cells are activated when they bind antigen processed and presented by Mϕ. Smooth-muscle cells from the lesions also have class II HLA molecules on their surfaces, presumably induced by IFN-γ and can also present antigens to T cells, such as ox-LDL and heat shock proteins (HSP) 60/65 which can be produced by Mϕ

(25,27). The immune regulatory molecule CD40-ligand and its receptor CD40 are expressed by Mϕ, T cells, endothelium and smooth muscle. Both are up-regulated in lesions of AT, providing further evidence of immune activation. Furthermore, CD40-ligand induces the release of IL-1β by vascular cells, potentially enhancing the pro-inflammatory response (23). Rupture of the fibrous cap or ulceration of the fibrous plaque can rapidly lead to thrombosis and usually occurs at sites of thinning of the fibrous cap that covers the advanced lesion. Thinning of the fibrous cap is apparently due to the continuing influx and activation of Mϕ, by activated T cells, which release metalloproteinases and other proteolytic enzymes. These enzymes cause matrix degradation, which promotes plaque instability and can lead to hemorrhage from the vasa vasorum and can result in thrombus formation (30).

HUMORAL IMMUNITY AND AUTOANTIGENS

As ox-LDL is a huge molecule with many potential autoantigens, it is possible that anti-oxidized low-density lipoprotein antibodies (anti-oxLDL) represent a family of auto-antibodies against different autoantigens. In the conventional view, the antigen-antibody reaction is prone to enhance inflammation and results in exacerbation of AT. Thus, the clinical impact of these auto-antibodies might vary. However, reports on elevated anti-oxLDL titers in humans have been detected in patients with early-onset PVD, severe carotid AT, CHF, CAD, MI and death (31,32), suggesting a pro-atherogenic role for this auto-antibodies. Several authors had found elevated levels of these antibodies related to CVD, supporting a key role in the progression of AT (31,33,34).

Beta-2 glycoprotein-1 (β2GPI) is a polypeptide that binds to negatively charged molecules and plays a part in the clearance of apoptotic cells and inhibition of coagulation. It is considered to be the autoantigen in antiphospholipid syndrome (APS). β2GPI is abundantly expressed within the subendothelial regions and in the intima-media layers at the border of human atherosclerotic plaques, and it co-localized with CD4+ T cells (35). Both, IgM and IgG anti-β2GPI levels are elevated in patients with AT and other inflammatory conditions. Immunization with this polypeptide results in accelerated AT in mice (36). Other antibodies with pro-coagulant activity are anti-cardiolipins antibodies (ACLA). β2GPI is the actual autoantigen for most ACLA, as the binding of cardiolipin exposes a cryptic epitope of β2GPI. The association between anti-phospholipid antibodies (APLA), AT and thrombosis can also be seen outside the setting of autoimmunity. Thus, ACLA promote AT by attracting monocytes into the vessel wall, by induction of monocyte adherence to endothelial cells, which is mediated by adhesion molecules such as ICAM-1, VCAM-1 and E-selectin (37). The APLA should be considered as more than a AT marker, as they can enhance AT and are pro-atherogenic (38,39).

HSPs are a family of proteins well conserved across species that may be elevated on endothelial cells and participate in atherosclerotic, inflammatory and autoimmune response. HSP60 is expressed by vascular cells in response to stressful events such as infections, fever, cytokines, oxidative stress and mechanical injury. This and other HSPs perform several functions, including the assembly, intracellular transport, and breakdown of proteins, facilitate refolding of denatured proteins and loading of immunogenic peptides to HLA-I and II. It is assumed that the endogenous HSP60 becomes immunogenic as a consequence of molecular mimicry of HSPs expressed by pathogens such as *C. pneumoniae* and *H. pylori.* Likewise, serum of patients with CVD shows high prevalence of antibodies against HSP60 that shares epitopes with cytomegalovirus. Thereby, these antibodies formed against HSP60/65 mediate lysis of stressed endothelial cells *in-vitro* and are elevated in patients with CVD (35). There are also data showing the existence of oligoclonal T cells within atherosclerotic plaques that can recognize HSP60 specifically, in contrast to T cells more distant from the lesion (21,40).

RHEUMATOID ARTHRITIS

Rheumatoid Arthritis (RA) is the most common autoimmune arthropathy worldwide. The overall prevalence in developed countries ranges from 0.5 to 1.0% (41). In addition to dyarthrodial joints, RA can damage virtually any organ thus leading to potential extra-articular manifestations (EAMs). CVD is consider an EAM, and represents the major predictor of poor prognosis and main cause of death in this population (6,42–44).

There is evidence that vascular damage accrual begins prior to the diagnosis of RA and accelerates as the disease progress. RA patients present with endothelial dysfunction and increased subclinical AT compared to age-matched controls (45). Endothelial function, assessed by brachial artery flow-mediated vasodilation (FMV), also worsens with disease duration (46).

CARDIOVASCULAR BURDEN IN RA

Many research groups have demonstrated that RA patients are at increased risk of fatal and non-fatal CV events, compared with the general population (47). The CV mortality is higher in RA and life expectancy of patients with RA is three to ten years less than general population (48,49). CVD accounts for 30-50% of all deaths in RA patients (43), and it is known that occurs earlier and 3.6 times more frequently than general population (9,44,50). Thus, CVD is the leading cause of death around the world in RA patients (51,52).

Currently, IHD secondary to AT is the most prevalent cause of death associated with CVD in RA patients (53). Almost all mortality studies have been conducted in populations of European origin, and limited information exists in other ethnic groups. A meta-analysis of 24 RA mortality studies, published between 1970 and 2005, reported a weighted combined all-cause standardized mortality ratio (meta-SMR) of 1.50, with similar increases in mortality risk apparent from the ratios for IHD (meta-SMR 1.59) and for CVA (meta-SMR 1.52) (54). RA patients frequently experience 'silent' IHD with

no symptoms before a sudden cardiac death. Indeed, sudden cardiac deaths are almost twice as common in patients with RA as in the general population (55). According with above, the Rochester Epidemiology Project (47) showed RA lends a risk for MI than controls of equivalent age and sex. Nicola *et al.* (56) demonstrated that cumulative incidence of CHF at 30-year follow-up was 34%, compared with 25% in the non-RA cohort.

Recently, Sarmiento-Monroy *et al.* (6) conducted a systematic literature review of CVD in Latin American (LA) population. A wide range of prevalence for CVD has been reported (13.8-80.6%) for this population. The highest prevalence was indicated by Santiago-Casas *et al.* (57) in Puerto Rican patients (55.9%). Cisternas *et al.* (49) evaluated CV risk factors in Chilean RA patients and reported a CVD prevalence of 46.4%. For Brazil (58,59), Colombia (7,42,60,61), and Argentina (62,63), a similar prevalence was reported (47.4, 35.1 and 30.5% respectively). In Mexico, five studies (64–68) reported an overall prevalence of 20.9% for CVD in RA patients. However, the mortality in RA patients had been poorly evaluated in this population. Acosta *et al.* (69) demonstrated a mortality rate of 5.2% in a six-year follow-up. For both, the most frequent cause of death was CVD in 44.7% and 22.2% of the cases, respectively.

TRADITIONAL CVD RISK FACTORS

General population studies have identified a number of risk factors associated with the development of CVD [e.g., obesity, dyslipidemia, advanced age, T2DM, hyperhomocysteinemia, metabolic syndrome (MetS), sedentary lifestyle, male gender and smoking]. These parameters are often referred to as 'traditional' or 'classic' CVD risk factors and have also been associated with CVD in RA patients (70–75). In a large retrospective cohort study of RA patients, Solomon *et al.* (76) showed a two to three-fold increase in the Relative Risk (RR) of MI after adjusting by traditional risk factors. Furthermore, the RR for stroke was 1.5 when compared with control groups. Boyer *et al.* (77), in a recent meta-analysis, confirmed the known association of traditional risk factors for CVD in RA patients. In Colombian population, Amaya-Amaya *et al.* (7), found that the traditional risk factors including male gender, hypercholesterolemia and abnormal body mass index (BMI) were associated with CVD. These factors, along with age, family history of CVD, and smoking habits were widely related to the high prevalence for CVD. Nevertheless, the increased prevalence of CV events in RA is not fully explained by these classic risk factors. Both non-traditional RA risk factors and traditional risk factors act together to develop CVD (Figure 1).

Obesitiy. Obesity is associated with the presence of CVD in RA patients (42,66,67,70,78) as in the general population. In particular, abdominal fat is associated with insulin resistance and the evidence shows that, in RA patients, abdominal fat is distributed differently between the visceral and subcutaneous compartments, with visceral fat being more strongly associated with CVD risk than subcutaneous adiposity. Adipose tissue is metabolically active, and, through a network of adipocytokines, regulates not only energy intake and expenditure but also inflammation (79,80). Insulin resistance has consistently been found to be more frequent in RA than general population, and had been related to increased coronary calcification (81). A direct correlation between disease activity in RA and insulin resistance has been demonstrated, where cytokines, especially TNF-α, can directly impede insulin-mediated glucose uptake by skeletal muscle, thus promoting insulin resistance (80,82).

Dyslipidemia. A meta-analysis confirmed that RA is associated with an abnormal lipid pattern, mainly with low levels of high density lipoprotein cholesterol (HDL), high LDL and triglycerides (TGL) levels (48,56,64–66,68,69,72,74,76,91). This altered lipid profiles had been related with higher probability of IHD by accelerating AT (42,83). In the large apolipoprotein-related mortality risk (AMORIS) study, the risk of MI was 60% higher in people with RA than in those without it. Total cholesterol (TC) and TGL levels were associated with the development of acute MI in individuals without RA, but not in those with RA (84). Levels of oxidized pro-inflammatory HDL are elevated in RA compared to healthy controls, this phenomenon may promote LDL oxidation and foam cell formation and decrease reverse cholesterol transport (85).

Advance age. It is mostly acknowledged that 'normal' or 'healthy' ageing of the CV system is distinct from the increasing incidence and severity of CVD with advancing age. Nevertheless, even in the absence of overt coexisting disease (e.g., RA), advanced age is always accompanied by a general decline in organ function, and specifically by alterations in structure and function of the heart and vasculature that will ultimately affect CV performance (86). For this reason, advanced age is considered a strong traditional risk factor and one of the most closely associated with AT in RA patients (30), especially for women over 55 years and men over 45 according to Framingham study (17). In the same way, the immune system of RA patients is subject to accelerated aging probably because of deficiencies in maintaining telomeres and DNA stability leading to excessive T cell apoptosis and increased proliferative pressure (87). A senescent immune system is normally associated with phenotypical and functional changes in cells characterized by a progressive loss of surface molecules such as CD28 and CD27, and the acquisition of cytokine-mediated pro-inflammatory activity and cytolytic functions (9,12,29,30,87).

Family history of CVD. There are several studies in healthy population and RA patients that had demonstrated the association between CV events and the family history of CVD (42,61). This is a non-modifiable risk factor and highlights the genetic features related to other risk factors that are heritable (e.g., HTN, familial hypercholesterolemia), and sometimes, lifestyles into the family.

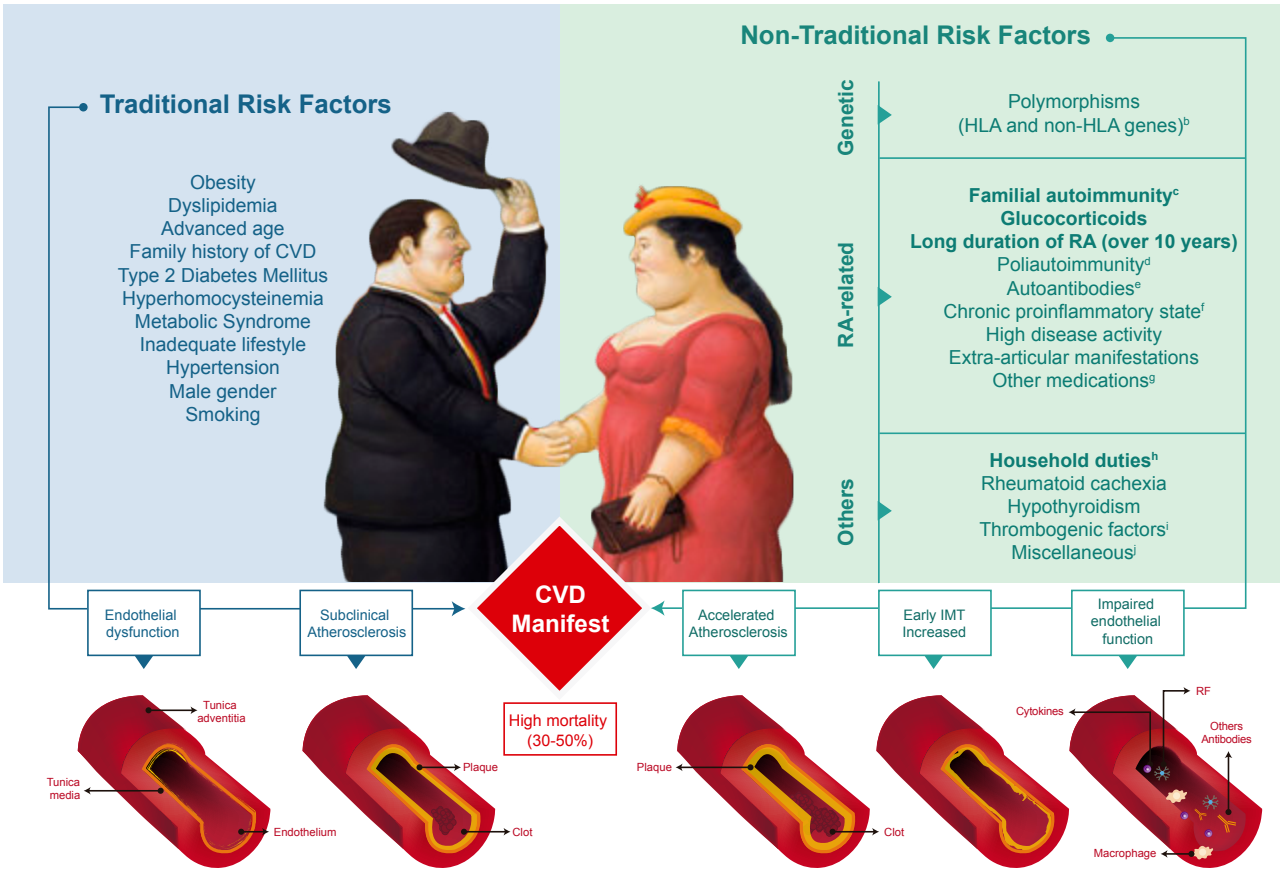

Figure 1. Traditional and non-traditional risk factors for cardiovascular disease in rheumatoid arthritis. [a]CVD include a broad spectrum of subphenotypes: stroke/transient ischemic attack, coronary artery disease, myocardial infarction, angina, congestive heart failure, arrhytmias, ventricular dyastolic dysfunction, hypertension, pulmonary embolism, deep vein thrombosis, and periferic arterial/venous disease. [b]Mainly *HLA-DRB1*0404* shared epitope alleles. [c]The presence of any diagnosed AD in first degree relatives of proband. [d]The presence of two concomitant AD in a single patient on the basis of international criteria. [e]Rheumatoid factor, anti-citrulinated peptides antibodies, anti-oxidated-low density lipoprotein, anti-cardiolipins, anti-phosphorylcholine, anti-modified citrullinated vimentin, anti-apolipoprotein A-1, and anti-cytokeratin 18 antibodies. [f]High levels of C-reactive protein and erythrocyte sedimentation rate. [g]Methotrexate, leflunomide, and nonsteroidal anti-inflammatory drugs. [h]Patients (females and males) with RA working on household duties. [i]von Willebrand factor, plasminogen activator inhibitor-1, and tissue plasminogen activator. [j]Hypothyroidism, periodontal disease, and others markers such as mannose-binding lectin, serum pentraxin 3, osteopontin, osteoprotegerin and seric uric acid. *AD*: autoimmune disease; *CVD*: cardiovascular disease; *IMT*: intima-media thickness; *RA*: Rheumatoid arthritis; *RF*: rheumatoid factor.

T2DM. Defects in glucose metabolism are frequently impaired in patients with active RA and may contribute to CVD risk, at least partly related to inflammation. In fact, patients with RA have a similar risk of developing CVD when compared to the same risk in patients with T2DM (42,59,62,65,69). Unfortunately, when there is a coexistence of both diseases, this risk is increased by three times (88). Abdominal obesity, antihypertensive medication, disease activity and use of glucocorticosteroids (GC) affect glucose metabolism in RA patients (89).

Hyperhomocysteinemia. Homocysteine is considered as a biomarker for AT and a risk factor related with CAD and CAVs (81,90), because it contributes to endothelial dysfunction by reducing the availability of endothelium-derived NO (91). In RA patients with PE/DVT it has been found elevated levels of plasma homocysteine (49,65), especially in those receiving methotrexate (MTX). It remains controversy about whether hyperhomocysteinemia is a factitive agent of CV damage or only an epiphenomenon of inflammation (65).

MetS. The development of accelerated AT and increased risk of CVD disease in patients with RA may be influenced by the occurrence of MetS (42,48,59). MetS is characterized for an alteration in production/secretion of pro-inflammatory adipoquines leads to increased activity of RA and accelerating AT (92,93), thus an association between inflammatory activity of RA and MetS has also been suggested. Nevertheless, the frequency of MetS in RA varies according to the criteria used for the assessment [e.g., National Cholesterol Education Program (NCEP); International Diabetes

Federation (IDF); Group for the Study of Insulin Resistance; World Health Organization (WHO)]. Da Cunha *et al.* (59) in a case-control study found MetS associated with disease activity, increased prevalence of waist circumference (WC), blood pressure, and fasting glucose in this RA population when compared to controls, using the NCEP scale.

Sedentary lifestyle. RA patients are less physically active than controls matched for age and sex, as a consequence of pain, stiffness, deformity and impaired mobility (42,67). Using the Paffenbarger physical exercise index, Mancuso *et al.* (94) found that patients with RA expended fewer kilocalories per week exercising than controls. The difference was mainly attributable to less walking by the RA patients rather than more high-intensity exercise in the controls. Alternatively, a sedentary lifestyle could also be an important cause of impairment of altered lipid pattern (95).

Hypertension. This is common in RA patients and it is known that HTN increases the risk to suffer IHD or CVA with an important impact on mortality (71). HTN is the major determinant of target organ damage in these patients and a link with low-grade inflammation. High CRP can reduce endothelial NO, leading to vasoconstriction, increased endothelin-1, platelet adherence, oxidation and thrombosis; it can also up-regulate angiotensin type 1 receptor expression and thus influence the rennin–angiotensin system (73,96). Multiple other factors may influence blood pressure control in people with RA, including physical inactivity, obesity, specific genetic polymorphisms and several antirheumatic drugs.

Male gender. In general population, CVD is more common in males than females, even a modest increased RR of CVD might translate into a high absolute risk for RA patient (42,60,65,66,68,78).

Smoking. This is a well-known risk factor for the development of CVD in healthy population. In addition, it has been recognized as a potent risk factor for RA development, especially in seropositive patients [i.e., rheumatoid factor (RF) and/or anti-citrullinated peptide antibodies (ACPA)] (42,49,65,67). In a meta-analysis of 4 case–control studies of traditional CVD risk factors in RA, the smoking prevalence was found to be higher in patients than in controls (77). Smokers with RA have a worse prognosis in terms of RF titers, disability, CVD, radiological damage and treatment response (72,87). Interactions are known to occur between smoking, *HLA-DR1* shared epitope (SE) alleles, ACPA production, smoking and premature CVD mortality in RA (97).

NON-TRADITIONAL CVD RISK FACTORS

It is known that RA is an independent factor for developing MI (98). Hence, there is an increasing interest in identifying novel risk factors in order to explain the early development of endothelial dysfunction, increased intima-media thickness (IMT) and finally, accelerated AT. This novel risk factors could be categorized into three groups: genetic (e.g., *HLA-DRB1* SE alleles), RA-associated (e.g., auto-antibodies, inflammatory markers, high disease activity, long-standing disease and medications), and others (e.g., hypothyroidism, thrombogenic factors) (6,7) (Figure 1).

Genetic determinants. The genetic group includes several polymorphisms at the HLA and non-HLA loci. *HLA-DRB1* SE alleles are related to chronic inflammation, more EAMs, high disease activity, endothelial dysfunction, premature death and CVD itself (42,97,99–105). Thereby, association between *HLA-DRB*0404*, and endothelial dysfunction and CV mortality has been described in RA patients (106). Therefore, polymorphisms of *HLA-DRB1* can be considered as a predictor of CV events. In Colombian population, Rojas-Villarraga *et al.* (42) found that being a carrier of a single copy of *HLA-DRB1* SE was significantly associated with an increased risk of atherosclerotic plaque in patients with RA. Other authors (97,99), demonstrated that SE alleles, particularly compound heterozygotes, were associated with death from all causes (including CVD), independently of auto-antibody subphenotype.

The non-HLA group includes polymorphisms in endothelin-1 and methylene tetrahydrofolate reductase (MTHFR) genes, TNF-α rs1800629, *TRAF1/C5* (TNF receptor-associated factor 1), *STAT4* (signal transducer and activator of transcription 4), factor XIIIA, PAI-1 (plasminogen activator inhibitor type-1), *TNFR-II* (tumor necrosis factor receptor II), ACP1 (acid phosphatase locus 1), *VEGFA* (vascular endothelial growth factor A), *LT-A* (lymphotoxin-A), IL-6, *LGALS2* (galectin-2), *TGF-β, GSTT1* (glutathione S-transferase T1, MBL (mannose-binding lectin), and nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (*NFKB1*)-94ATTG ins/del. All of these genes contributes significantly to increased risk of CVD and showed a potential influence on the course and complications in RA patients (107–122).

RA-RELATED FACTORS

The RA-associated risk factors represent a broad spectrum of conditions related with the autoimmune nature of the disease. In fact, RA seems to be an independent risk factor for the development of accelerated AT.

Familial autoimmunity. Familial autoimmunity (FA) is defined as the presence of any AD in first degree relatives (FDRs) of the proband (123,124). This had been associated to presence of atherosclerotic plaque in Colombian patients with RA (42). Amaya-Amaya *et al.* (7) have replicated the same observation indicating that FA confers additional susceptibility to CVD in RA patients. Conditions related to FA and CVD include radiographic progression, high disease activity and persistent inflammation. El-Gabalawy *et al.* (125) indicated that levels of multiple cytokines and high sensitivity-CRP are higher in Amerindian patients with RA and their FDRs as compared to individuals from a non-AD. Saevarsdottir *et al.* (126) showed

that familial RA patients had increased frequency of *HLA-DR4* as compared with the non-familial RA group. In addition, the mean age at onset of RA was significantly lower in the familial than in the sporadic RA patients and the difference still remained when the DR4 positive and negative subgroups were compared separately.

Glucocorticoids. The use of systemic GC in RA patients has a paradoxical effect. Several studies have reported an increased on CV risk and mortality in RA due to a longer duration of disease and subsequently longer use of GC (127). This can be explained by a chronic systemic pro-inflammatory state that enhances physiopathological changes in the endothelium (42) or due to their potentially deleterious effects. In addition, the effect assessment of GC on CV outcomes is complicated by the potential for confounding factors such as indications due to more severe subphenotypes needing more aggressive treatment with this medication (42,49,57,61–63,128). However, GC can reduce the atherosclerotic risk and CVD by suppressing inflammation, which paradoxically may improve glucose tolerance and dyslipidemia (127,129–138).

Long duration of disease. Solomon and Sattar *et al.* (53,139) have found adjusted odds ratios (AOR) of 1.48 for CVD and 2.0 for MI in healthy women, which increases to 3.1 when the disease has more than 10 years of duration. In the same way, disease duration over 10 years was significantly associated with an increased risk of atherosclerotic plaque in Colombian population (42). Other studies found increased carotid atherosclerotic involvement and subclinical AT in patients with long-standing RA when compared with patients of the same age but with shorter disease duration (42,46,61,140–153).

Poliautoimmunity. Polyautoimmunity is defined as the presence of more than one AD in a single patient. It was present in average 14-28% in patients with RA, being autoimmune thyroid disease (AITD) the most frequent AD associated (7). Polyautoimmunity was associated with CVD in Colombian population (154).

Auto-antibodies. There are several auto-antibodies associated with CVD in RA, such as RF and ACPA. Immune complexes from RF can be deposited in the endothelium and through inflammatory reactions generate endothelial dysfunction and AT (42,155–157). In a cohort of older women, the association with the increase mortality appeared to be restricted to those with RF(+) subphenotype. The widening in the mortality gap between RA subjects and the general population is confined to RF (+) RA subjects and largely driven by CVD (158,159). In fact 0 (+) RA may, like diabetes, act as an independent risk factor for CVD, after controlling inflammatory profiles (160). Similar correlations have been made between ACPA and decreased endothelial dysfunction, presence of atherogenic risk factors and increased IMT, independent of other CV risk factors in RA patients (161,162). Another auto-antibodies related to CVD in RA patients are those directed against to ox-LDL (22,24,163), which are associated with subclinical AT (164). The presence of ACLA, APLA and anti-apolipoprotein A-1 antibodies (anti-ApoA-1) are also associated with early atherosclerotic changes in RA patients (165–170). Finally, others auto-antibodies implicated in the atherosclerotic process are anti-phosphorylcholine (anti-PC) and anti-heat shock protein 60/65 (anti-HSP 60/65) (171). Anti-malondialdehyde-modified LDL antibodies (anti-MDA-LDL) may have independent roles in subclinical AT (167,172), meanwhile high levels of anti-modified citrullinated vimentin antibodies (anti-MCV) and LDL-immune complexes are risk factors for increased AT and are associated to inflammation (173).

Chronic pro-inflammatory state. The association of inflammatory pathways with CVD is complex and is composed of several intermediate factors, including dyslipidemia, homocysteinemia, insulin resistance, and endothelial dysfunction (174). However, a pro-inflammatory state is the hallmark of CVD in RA patients (175) since it may accelerate atherogenic processes and microvascular dysfunction (15,176), either by the accentuation of known pathways of plaque formation or by the onset of additional immune mechanisms (177). Markers of chronic inflammation such as CRP, ESR, TNF-α, IL-6, IL-17 and haptoglobin are indicators of endothelial activation and are associated with the increased in carotid IMT the carotid plaque, presence extent of CAD and CV complications (45,61,65,66,105,115,116,171,178–188).

High disease activity. It has also been shown that higher activity index is associated with CV events. Therefore, adequate treatment of the disease can reduce CV mortality (18). The lipid profile in RA depends on disease activity. Higher disease activity leads to depressed levels of TC. For instance, disease activity score-28 (DAS28) was a significant predictor of major adverse CV events and mortality, independent of traditional risk factors (42,63,66,171,189,190). van Halm *et al.* (191) demonstrates that high disease activity, RF (+) and joint destruction conferred, approximately double the risk for CVD.

EAMs. Other non-traditional risk factor related to RA is the presence of EAMs, which are related to the severity, disease activity and endothelial damage and therefore with the risk of developing AT (101). Patients with EAMs are considered to have three times higher risk to develop CVD (61,101). EAMs and long disease duration, even in the absence of traditional clinical CV risk factors, were associated with greater carotid IMT, suggesting an unfavorable CV risk profile (61,101,149). Severe EAMs are associated with an increased risk of CVD events in patients with RA (42,61,63,68,101). In fact, some authors had considered CVD such as a severe EAM of the disease (90,102,192).

Household duties. The association between household duties and CVD is novel and striking and was described re-

cently by Amaya-Amaya *et al.* (7). Nevertheless, there are few studies about the impact of household duties on RA (193). Employed women are somewhat less physically disabled than their unemployed counterpart (including housework) (194). This factor may correspond to an information bias. In fact, we were unable to determine if this working status was a consequence of RA or was the patient´s choice. Habibet *et al.* (193) showed that socio-economic status (SES) are highly predictive of homemaking disability in Arabic women with RA and more predictive than the clinical examination. Pincus *et al.* (195) reported that low educational level (LEL) was a significant risk factor for mortality in RA. In Colombians, household duties are also associated with LEL, low SES and poor access to health services, which could ensure a poor control of the disease, and therefore more systemic involvement.

Rheumatoid cachexia. Rheumatoid cachexia is associated with high levels of LDL, low levels of atheroprotective anti-PC and high frequency of HTN in RA patients (196).

Hypothyroidism. In RA patients, clinical hypothyroidism was associated with a fourfold higher risk of CVD even after adjustment for other traditional CV risk factors (197,198). McCoy *et al.* (197) found that Hashimoto's disease was associated with CVD in patients with RA in a retrospective cohort. Although CVD is linked to the presence of EAMs an increased CV risk is observed within patients with polyautoimmunity. AITD-RA subphenotype were associated when adjusted for potential confounders and variables of clinical interest (199). For more details, see chapter 30.

Thrombogenic factors. The altered levels of von Willebrand factor (66), PAI-1, and tissue type plasminogen (tPA) in patients with RA had also been associated with CVD progression (66,151,170,180,200–208), which indicates a status of hypofibrinolysis in these patients (171,180).

Many biomarkers such as osteoprotegerin (OPG) (209), osteopontin (OPN) (210), serum pentraxin-3 (sPTX-3) (211), periodontal disease (200), hepcidin (212), seric uric acid (SUA) (213,214), para-articular bone loss (215), MBL (122,216), and many others (149,150,169,217–221) has been related to CVD as well.

MANAGEMENT

CV risk screening and management strategies have been developed for the general population and are based on CV risk score calculators, such as the Framingham score and the Systematic Coronary Risk Evaluation (SCORE) model, but the accuracy of these models has not been adequately evaluated in inflammatory arthritis. The major strategies are to achieve healthy life styles, by means to maintain the control of classical risk factors. Regarding to novel risk factors it is necessary reach an adequate management of the disease. The main goal of the treatment should be reduce the disease activity, and therefore decrease the CV burden (127). Both conventional (222) and biologic disease modifying anti-rheumatic drugs (DMARDs) are used for this purpose. Some studies has shown a greater disease control activity with non-conventional DMARDs, such as anti-TNF agents, which lower CRP and IL-6 levels, increase HDL levels and decrease endothelial dysfunction (223).

Statins and Angiotensin-Converting-Enzyme (ACE) inhibitors. Statins can effectively lower total cholesterol in RA patients and significantly improve CV-related and all-cause mortality when used for primary prevention of vascular events (224). In addition, statins had shown a moderate decrease in disease activity and a significant reduction in TC and LDL in treated RA patients (225). Similarly to statins, ACE inhibitors and angiotensin II blockers may also have a favorable effect on inflammatory markers and endothelial function in RA (226). Hence, these agents are preferred, when antihypertensive agents are indicated (127).

DMARDs. Early and effective antirheumatic treatment, such as MTX, has been shown to be independently associated with a lower CV risk (191,227). This protective effect may be secondary, at least in part, to promotion of reverse cholesterol transport, inhibition of foam cell formation, and important anti-inflammatory effects of this drug (228). Effective treatment may also result in improved physical activity, subsequently leading to a decreased risk of hypertension, obesity and diabetes, all important determinants of CV disease. Studies had reported a lower CV mortality in RA patients using methotrexate, which was ascribed to its anti-inflammatory properties (191).

Antimalarials. The antimalarials (AMs) drugs had been associated with a better CV outcome, improved glycated hemoglobin in patients with T2DM, enhanced glycemic control, improve lipid profiles, decrease thrombosis risk and reduced probability of developing T2DM in patients with RA (191,229,230). Other conventional DMARDs, such as sulfasalazine, is also associated with a reduction in the risk of developing CVD, which strengthens the hypothesis that reducing inflammation is of importance to reduce CVD burden (9,12,191).

Glucocorticosteroids. These drugs should be used prudently to minimize CV risk secondary to their effects on metabolic parameters and blood pressure. Altogether, there is no clear evidence that low-dose of GC contribute significantly to the enhanced CV risk in inflammatory arthritis, in contrast to high doses. Corticosteroids rapidly and effectively suppress inflammation in RA and their use might be justified for short-term in the period between initiation and response to DMARD treatment, although the debate appears not to be settled yet. Therefore, a conservative approach was chosen, recommending the use of the lowest dose for the shortest period possible (127,222).

Biological therapy. Some studies have found no benefit in IHD (despite good anti-inflammatory response) in RA, whereas other reports indicate that anti-TNF shows to be independently associated with a lower CV risk, due to reduce CV events in young patients, improving the lipid profile, insulin resistance, endothelial function, aortic compliance and decrease progression rates of subclinical AT (127,231). Patients treated with tocilizumab, an IL-6 neutralizing antibody, have improvements in insulin sensitivity and lower lipoprotein (a) levels, which contribute to decrease CV risk (232). Finally, data about other biologics are conflicting and preliminary; as such, randomized controlled studies are needed to identify their CV risk reduction role (9,12).

SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a complex, multiorganic, and chronic disease, with an autoimmune background. Its heterogeneous nature explains the broad spectrum of clinical manifestations (i.e., subphenotypes). SLE occurs most often in young women of child-bearing age, the same population that is at highest relative risk of AT (233,234). Lupus cohorts have documented differences in health status, disease prevalence, treatment outcomes, and healthcare use among different ethnic groups which suggest that minority influence SLE health disparities (235–240).

Classically, a bimodal mortality pattern among SLE patients, with an early peak in the first 3 years after diagnosis due to active disease, infections and glomerulonephritis, and later deaths, 4–20 years after SLE diagnosis due to CVD, was described by Urowitz *et al.* (241). Although overall mortality for SLE patients has improved over the past 30 years, mortality due to CVD has remained equal (242).

CARDIOVASCULAR BURDEN IN SLE

AT is an accelerated process in SLE patients (50). There is strong epidemiologic evidence that CVD risk among SLE patients compared to the general population is at least doubled (243). Carotid plaque is prevalent in 21% of SLE patients under age 35 and in up to 100% of those over age 65 (244). The increased risk of MI and angina among SLE patients has been well characterized in a number of population-based studies. Most studies reported a 2–10 fold increase in the risk of MI among SLE patients, with a greater increase in RR generally observed in younger patients groups (245–250). MI occurs in 6-20% of premenopausal women with SLE (9,12). CVD may account for 3-25% of total mortality in SLE (103,247).

SLE patients have an increased risk of CVA when compared to control populations, similar to that seen for MI and CHF. Ward *et al.* (247) found the stroke risk was 1.75 times that of age-matched controls. The increased RR diminished with age such that SLE patients over age 65 actually had a somewhat lower overall stroke risk than controls. Mok *et al.* (251) similarly found a 2-fold increase in the risk of CVAs among all SLE patients over an 8-year period at a single institution in Hong Kong. This risk was 22-fold higher for the youngest group of SLE patients. Bengtsson *et al.* (250) further corroborated these results in their population-based Swedish study where they demonstrated that the risk of CVA and/or MI in the total SLE population was 1.27 fold higher than the general population, but among women with SLE aged 40–49 it was 8-fold higher over the 7-year follow-up period.

Several research groups have reported prevalence rates in SLE cohorts. In the Systemic Lupus International Collaborating Clinics-Registry for Atherosclerosis (SLICC-RAS) cohort, there were 8 cases of PVD among 1,249 patients during a 2-year period (252). In the Lupus in Minorities: Nature vs. Nurture study (LUMINA), a large multicenter, multiethnic inception cohort, 5.3% of 637 patients developed PVD over a mean follow-up of 4.4 years (253). The average age of this population was young (36.5 years) and PVD predicts either more severe SLE activity or more widespread AT.

In a recent meta-analysis, Schoenfeld *et al.* (243) showed that epidemiologic data strongly support that SLE patients are at elevated relative risk of CVD. The risks of MI, CHF, CVAs and CVD mortality are all increased among SLE patients compared to general population risks. The variability regarding the relative importance of risk factors for CVD among SLE patients in past epidemiologic studies is likely due in part to different design methods and different patient and comparison groups. Independent predictive risk factors (from multivariate analysis) for CV events had been assessed in five large prospective cohorts of patients with SLE, including Baltimore (254), Pittsburg (246), LUMINA (255), Toronto (256), and SLICC-RAS (252) cohort. The main results are discussed below.

TRADITIONAL CVD RISK FACTORS

Diverse SLE cohorts had shown the influence of advanced age, dyslipidemia, obesity, HTN, and hyperhomocysteinemia, as classical risk factors for CVD in lupus population (257–259). Younger patients with SLE have the greatest RR compared to their healthy counterparts, but the absolute risk of CVD among SLE patients increases with advancing age (243). There is strong epidemiologic evidence that traditional CVD risk factors also elevate CVD risk among SLE patients (Figure 2).

Amaya-Amaya *et al.* (260) recently adds further evidence of the high frequency of CVD in 310 consecutive patients with SLE. Their traditional risk factors (i.e., dyslipidemia, smoking), and highlights coffee consumption as a confirmed factor for such a complication in LA population. In addition, they evaluated the state of the art regarding traditional and non-traditional risk factors, as well as CV subphenotypes, and mechanisms underlying accelerated AT in Colombian lupus patients. Out of total of 53 articles that fulfilled eligibility criteria, 40 had interpretable data regarding CVD prevalence, which accounts approximately for 39.2% in Hispanics. This prevalence was similar to that encounter in the analysis of this cohort (36.5%). Several classic CV risk factors such as MetS, obesity, dyslipidemia, HTN, T2DM, sedentary lifestyle, male gender, smoking,

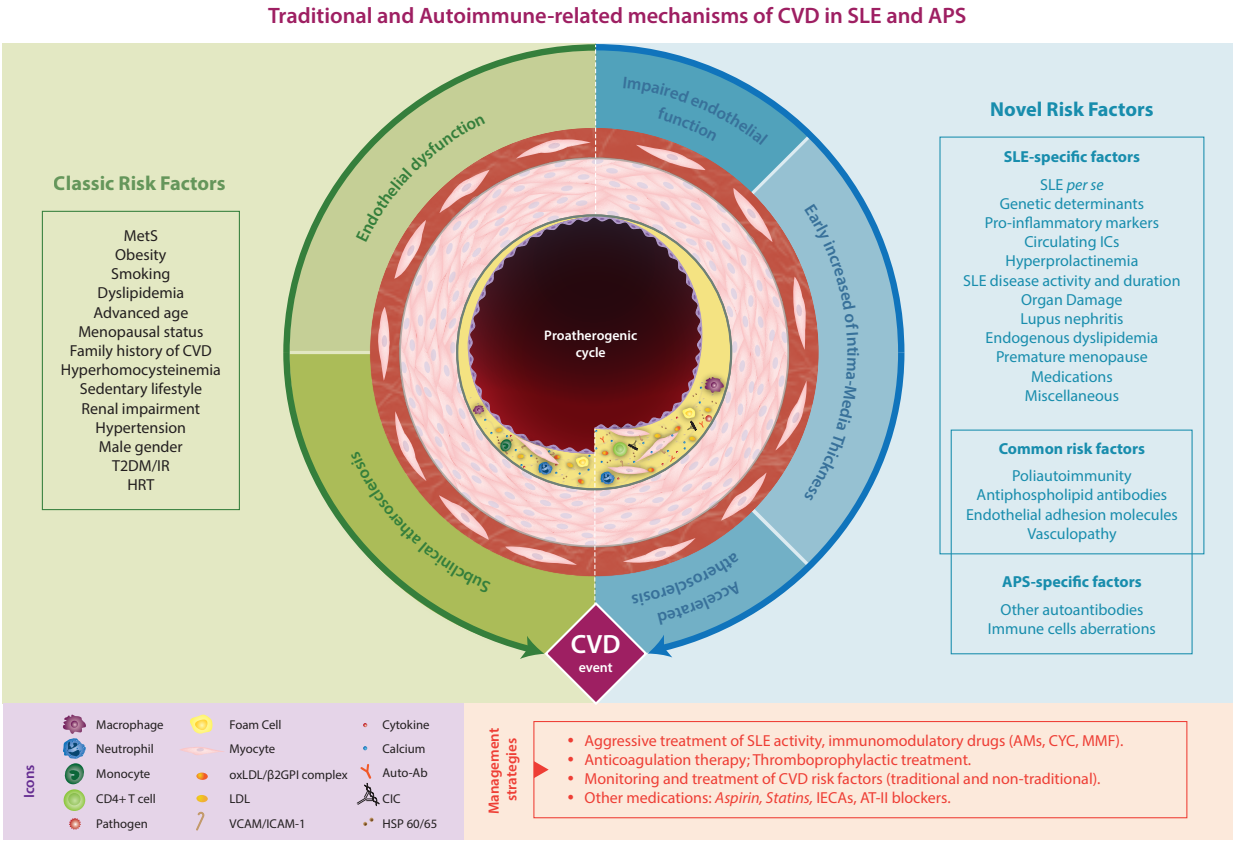

Figure 2. Traditional and autoimmune-related mechanisms of cardiovascular disease in systemic lupus erythematosus and antiphospholipid syndrome. A complex interaction between traditional and disease-specific traits leads to premature atherosclerosis process. Several risk factors (left) have been described since The Framingham Heart Study, known as classic risk factors, which over time conduce to endothelial dysfunction, subclinical atherosclerosis and CV event manifest. In the autoimmune setting (right), several novel risk factors contribute to development of premature vascular damage. This damage is represented by impaired endothelial function and early increased of Intima-Media Thickness which are surrogates of the accelerated atherosclerosis process. These associations are even more pronounced in this case of Polyautoimmunity (SLE and APS in the same individual), where risk factors have additive effects and atherosclerosis develop earlier. The cornerstone of management of CV risk include an aggressive treatment of disease activity, the continuous monitoring and treatment of modifiable CV risk factors, and the use of other medications in order to diminish de CV burden. *CVD*: cardiovascular disease, *SLE*: systemic lupus erythematosus, *APS*: antiphospholipid syndrome, *MetS*: metabolic syndrome, *T2DM*: type 2 diabetes m ellitus, *IR*: insulin resistance, *HRT*: hormone replacement therapy, *CIC*: circulating immune complex, *oxLDL/β2GPI complex*: oxidized-low density lipoprotein/β2 glycoprotein I, *HDL*: high density lipoprotein, *Auto-Ab*: auto-antibodies, *AMs*: antimalarials, *CYC*: cyclophosphamide, *AZA*: azathioprine, *MMF*: mycophenolate mofetil, *ACE-I*: angiotensin-converting enzyme inhibitors, *AT-II blockers*: angiotensin II receptor blockers.

advanced age, hyperhomocysteinemia, renal impairment, family history of CVD, and menopausal status were reported. Moreover, several studies were associated with novel risk factors, including ancestry, certain SNPs, SLE *per se*, poliautoimmunity (154), autoantibodies (e.g., APLA), markers of systemic inflammation (e.g., CRP), SLE disease activity and duration, organ damage, immune cells aberrations, medication (e.g., GC), vasculopathy, lupus nephritis, endogenous dyslipidemia, bone mineral density (BMD), education level, and monthly income. A broad spectrum of CV subphenotypes including HTN, IHD, CAD, ACS, MI, CHF, CVA, thrombosis, PVD, subclinical AT, and mortality due to CVD, were described in LA individuals with SLE.

Obesity. Obesity has not been frequently examined in relation to CVD risk in SLE populations (246,261,262). Only one study in the early 1990s has reported that obesity was correlated with an increased risk of CVD events (RR not reported) (254).

Smoking. Several studies have assessed smoking as an independent risk factor for CV atherosclerotic disease (255,257,261,263–265). Gustafsson *et al.* (264) found that smoking may be the main traditional risk factor promoting increased CV risk in 208 SLE patients. Previously, the same group found that smoking was predictive of MI, stroke, PVD or CV mortality among the same patient population (257). Toloza *et al.* (255) prospectively followed SLE patients over a median follow-up of 73.8 months and compared those who had a CVD event to those who did not as part of the LUMINA study. Current cigarette use was significantly associated with a 3.7-times increased risk of having a CVD event. In the PROFILE population, another multicenter, multiethnic study population, Bertoli

et al. (265) found that smoking acted as an independent risk factor associated with a 2-fold decrease in time to a CV event.

Dyslipidemia. The inflammatory milieu of SLE leads to dysregulation of lipid metabolism pathways, which contributes to the increased risk of atherosclerotic disease among SLE patients (266,267). Five large cohort studies have shown hypercholesterolemia to be a significant risk factor for CVD in SLE patients (246,254,268–270).

Advanced age. Older age is a relatively consistent independent predictor of CVD events among SLE patients. Gustafsson *et al.* (257) found the strongest correlation between age and CV events, with age predicting a 2 to 3-fold increase in CVD events and death.

Hyperhomocysteinemia: Elevated homocysteine levels may act as an independent risk factor for atherosclerotic CVD in general population (91) and among those with SLE. Petri *et al.* (271) compared homocysteine levels in those patients who had a stroke or thrombotic event versus those who did not over a mean follow-up period of 4.8 years. After adjusting for age, sex, race, obesity, hypercholesterolemia, HTN, T2DM, renal insufficiency and presence of the lupus anticoagulant, homocysteine remained an independent stroke predictor and arterial thrombotic events.

Hypertension. In the Toronto Lupus Cohort, 33% SLE patients were hypertensive compared to 13% of age-matched controls (272). In the Hopkins Lupus cohort, the presence of hypertension or use of antihypertensive medications were independent risk factors for CVD (254). Two recent studies using data from the Toronto Lupus Cohort found that hypertension was associated with 1 to 2-fold risk of CAD among SLE patients (268,270).

Male gender. While most SLE cohorts are comprised predominantly of women, the risk of CVD events, as in the general population, appears higher among men. In each of the 2 large cohort studies, SLICC and LUMINA, male SLE patients had a nearly 4-fold increased risk of experiencing a CVD event compared to females (252,270). Although both studies were comprised of large databases and were well conducted, it should be noted that nearly 90% of the patients were females in both studies. Similarly, in a population-based study by Nikpour *et al.* (270) males had a nearly 2-fold increased risk of CV events, although again the cohort was comprised of only 10% males.

T2DM. Many of the cohort studies have not examined diabetes as a risk factor (262,265). Those studies that did include this condition had small numbers of patients (261,273).

Hormone replacement therapy. Rojas-Villarraga *et al.* (274) showed in a recent meta-analysis an association between hormone replacement therapy (HRT) exposure and SLE. This study highlight that identifying individual risk factors that predispose healthy individuals to develop an AD such as SLE, have to be considered with special attention in those who are planning to begin HRT.

NON-TRADITIONAL CVD RISK FACTORS:

It is well known that while traditional CVD risk factors are undoubtedly important in increasing the CVD risk among SLE patients, these do not fully accounts for the elevated risk of CVD in this population. Esdaile *et al.* (275), evaluate risk factors for CAD in two Canadian lupus cohorts by means of the Framingham multiple logistic regression model, and found a high risk of developing CAD after removing the influence of these risk factors. Thereby, SLE-associated factors play an important role in the premature AT process characteristic of those patients (276,277). Evidence strongly suggests that AT is largely driven by inflammation, active immunological response (278,279), and points to SLE itself as an independent risk factor (280,281) (Figure 2). Several SLE-specific factors, including disease activity and duration, and possibly specific manifestations and therapies (243). Hence, there is an increasing interest in identifying novel risk factors in order to explain the development of accelerated AT in these population.

Genetic determinants. Family and twin studies have repeatedly supported a role for heredity in CAD, particularly when it occurs in relatively young individuals. Several genetic markers have been proposed as predisposing factors for CVD in SLE patients (282–284).

SLE-specific factors. The SLE-associated risk factors represent a broad spectrum of conditions related with the autoimmune nature of the disease. In fact, lupus per se seems to be an independent risk factor for the development of accelerated AT (275).

Poliautoimmunity. In patients with SLE and APS, CV risk is even higher, and APLA-induced arterial events are most pronounced, where traditional and non-traditional risk factors are multiplied and AT occurs more prematurely (30,285).

Auto-antibodies. Many studies have tried to determine whether the presence of APLA may be an independent risk factor for CVD among SLE patients. In the LUMINA study, the presence of a positive APLA was significantly associated with 4-times increased risk of having a CVD event over a median of approximately 6 years (255). The presence of APLA was also associated with a greater than 4-fold increased risk of first time MI, stroke or PVD (257,264). Bengtsson *et al.* (250) found that ACLA IgG predicted a 3-fold increased risk of stroke, but not MI. In a Spanish study by Ruiz-Irastorza *et al.* (286) the presence of a positive APA was associated with a nearly 3-fold risk of thrombosis or death.

Systemic inflammation. Unlike in the general population, where high sensitivity CRP has clearly been shown to be

associated with increased risk of CVD (287), this finding is less consistent among SLE patients. In the LUMINA study (255,273), elevated CRP levels were associated with anywhere between a 1.5 and 3.3 increased CVD risk. However, most of the other large SLE cohorts have not examined CRP in their analyses (246,252,254,261).

SLE disease activity and duration. Several studies have found that disease activity is an important predictor of CVD events. In a recent study, Bengtsson *et al.* (250) found that a higher Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score predicted both stroke and MI among SLE patients followed over 7 years. Manzi *et al.* (288) found an inverse relationship between SLE activity and plaque size, and noted that longer disease duration was independently associated with carotid plaque. Nikpour *et al.* (270) and Touma *et al.* (268) similarly found that disease activity, as measured by Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) was predictive of a modest increase risk of CAD during a 6 and 37 year follow-up period, respectively. Mikdashi *et al.* (96) reported that baseline SLEDAI-2K scores were associated with a 2-fold increased risk of stroke within 8 years. Nevertheless, measures of SLE disease activity, including the SLEDAI, British Isles Lupus Assessment Group (BILAG) and Systemic Lupus Activity Measure (SLAM), used in many of these studies, have been criticized as being insensitive and may not accurately capture systemic inflammation that could drive AT. Disease duration may be of outstanding importance with regard to atherogenesis (103). Von Feldt *et al.* (289) found disease duration was significantly associated with coronary calcium scores in a cross-sectional cohort. Similarly, Roman *et al.* (290) found, in multivariate analysis, that longer disease duration and higher Systemic Lupus International Collaborative Clinics damage index (SDI) were independent predictors of carotid plaque in both a cross-sectional and a longitudinal study (291).

Glucocorticoids. Petri *et al.* (254) demonstrated that longer duration of GC use was independently associated with incident CV events. Nikpour *et al.* (270) also found that GC use independently predicted a 2-fold increase in the risk of MI, angina and sudden cardiac death, but conflicting data exists regarding the overall risk of GC therapy (246,252,255,261,262,273). Examination of medications and CVD risk is problematic because of the risk of confounding by indication bias. More severely ill phenotypes are more likely to receive GC and other immunosuppressants, while those with milder disease may be more likely to receive hydroxychloroquine (HCQ) alone. In addition, a controversy arises from the dual action of GC, as they are atherogenic (related to exacerbation of multiple traditional risk factors), but, on the other hand, also anti-inflammatory (292–294).

Azathioprine. Two studies have reported that azathioprine (AZA) use is an independent risk factor for CVD among SLE patients, predicting 3-fold PVD (253), MI and angina (295). In a study by Touma *et al.* (268), immunosuppressive medications including AZA were associated with CAD.

Renal disease. The prevalence of CV morbidity and mortality is higher in patients in the general population with chronic kidney disease, and is even more strongly associated with end-stage renal disease (296). Factors that may contribute to this increased risk include HTN (297) and dyslipidemia (298), both of which are frequently seen in patients with proteinuria. Patients with proteinuria also have an increased risk of thrombosis (299). Both proteinuria (300,301) and elevated serum creatinine (302) have been associated with early AT in patients with SLE, and a history of nephritis has been associated with subclinical AT in some studies (289,302).

Endogenous dyslipidemia. Lupus hyperlipidemia pattern include elevated TGL and Very Low-Density Lipoprotein-cholesterol (VLDL), and decreased HDL and ApoA-1 (103). Lupus nephritis could result in HTN, and the nephrotic syndrome could aggravate this hyperlipidemia (5).

Neuropsychiatric disease. A number of studies have demonstrated a correlation between neuropsychiatric SLE and an increased risk of CV events. In particular, Urowitz *et al.* (261) found that neuropsychiatric disease predicted an early 4-fold increased risk of CV events. In another large study, Bertoli *et al.* (265) found that both psychosis and seizure were independent predictors of time to a CVD event.

Miscellaneous. A number of other risk factors have been shown to be predictors of CVD among SLE patients. These include number of years of education, hypovitaminosis D, osteoporosis, absence of thrombocytopenia, some biomarkers (e.g., cystatin C, soluble-VCAM levels), and others (243).

MANAGEMENT

It has been proposed that SLE should be treated as a "CVD equivalent" such as T2DM is, with lower lipid goals, more aggressive aspirin use and potentially more aggressive monitoring (292,303). Recent studies have started to address whether traditional treatment regimens may prevent or slow AT in SLE patients (234).

Antimalarials. There are several new mechanisms of action described for AMs, many of them with beneficial effects in the management of CV risk in patients with SLE (230,304). There is evidence that AMs drugs reduce LDL levels, elevate HDL, and when taken concomitantly with steroids can reduce TC (305). Rekedal *et al.* (306) showed that HCQ initiation was associated with a significantly greater reduction in HbA1c as compared to MTX initiation among diabetic patients with rheumatic diseases. In addition, beneficial effects of HCQ on thrombosis formation have also been described. Multiple retrospective cohort studies have shown a reduced incidence of thrombotic events and improved overall survival in patients with SLE treated with AMs (307–310). Nikpour *et al.*

and Ruiz-Irastorza *et al.* (270,286), both found that HCQ use conferred a 50–60% decrease in risk of CVD.

Statins. The recent randomized controlled Lupus Atherosclerosis Prevention Study by Petri *et al.* (311) suggests that atorvastatin did not in fact slow progression of subclinical AT in 200 SLE patients over 2 years. It has been further demonstrated that statins also reduce CD40 levels in vivo and in vitro, and therefore interfere with CD40–CD40 ligand interactions, both in SLE and AT (293).

Other immunosuppressants. As inflammation is one of the targets of therapy in SLE, the several other immunosuppressants and immunomodulatory drugs currently employed in SLE could also be considered such as potential new anti-atherogenic agents. Some data are available for cyclophosphamide (cyclophosphamide) therapy in atheroma. Therefore, multivariable analyses from a recent study revealed that only a few factors are predictors of AT in SLE patients including the use of lesser immunosuppressants such as cyclophosphamide (312). Mycophenolate mofetil (MMF) is able to diminish the plaque formation, as well as the cholesterol arterial content in animal models (313).

ANTIPHOSPHOLIPID SYNDROME

The APS is a pro-thrombotic state that can affect both the venous and arterial circulations. The deep veins of the lower limbs and the cerebral arterial circulation are the most common sites of venous and arterial thrombosis, respectively (314). It is characterized by recurrent thrombotic events, pregnancy loss, and the presence of circulating APLA.

The prevalence of APS ranges from 1.7-6%, and that of APLA reaches to 14% among patients with PVD defined on the basis of clinical outcomes. On the other hand, the prevalence of asymptomatic AT, defined in terms of plaques in ultrasonography, reaches to 15% of patients with APS compared to 9% of SLE patients and 3% of normal controls (315). In the Euro-Phospholipid cohort, that includes 1,000 European patients with APS, MI was the presenting manifestation in 2.8% of the patients, and it appeared during the evolution of the disease in 5.5% of the cohort (316). Therefore, when compared with age and sex matched controls, these patients present an increased risk and a higher prevalence of CVD (317). Cardiac manifestations may be found in up to 40% of patients with APS, but significant morbidity appears in only 4-6% of these patients. Most of these manifestations are explicable on the basis of thrombotic lesions either in the coronary circulation or on the valves (318).

CARDIOVASCULAR BURDEN IN APS

The clinical spectrum of CVD includes features such as arterial and venous thrombotic events: PAD, valve abnormalities, intracardiac thrombus formation, pulmonary hypertension, ventricular hypertrophy and dysfunction, dilated cardiomyopathy, and myxomas. Therefore, patients with APS could have a significant involvement of the CV system. The heterogeneity of APS clinical manifestations is likely linked to the varied effects that APLA can induce on endothelial cells (319).

Thrombosis. Thrombotic events are the clinical hallmark of APS, occurring in the venous and arterial circulations, with a high recurrence rate (3). Arterial involvement in APS can be expressed as CVA, CAD, and PVD, due to thrombus formation or to AT. Involvement of larger vessels manifests in the form of recurrent thromboembolism, including DVT, cerebral venous thrombosis, PE, whereas involvement of small vessels manifests as thrombotic microangiopathy (320).

Coronary syndromes. AT is one of the main features of APS, and can be the first finding of this entity (3,321). The association between APS and AT probably was suggested for the first time by Shortell *et al.* (322). Vaarala *et al.* (285) provided the first evidence that APLA may be involved in AT. As some of thrombotic manifestations occur in the coronary arteries or in the carotid arteries, it is of interest to examine whether APLA are also associated with AT in addition to their relation with thrombosis (5). With regard to recurrent coronary events in post-infarction patients, Bili *et al.* (323) demonstrated that elevated IgG and IgM APLA at any level are independent risk factors for recurrent cardiac events. Furthermore, patients with both elevated IgG and IgM APLA at any level have the highest risk. This risk was comparable to other known risk factors for recurrent coronary events.

Valvular disease. Apart from AT and CVD, other cardiac manifestations may also be present in these patients. It can include irregular thickening of the valve leaflets due to deposition of immune complexes that may lead to vegetations, and valve dysfunction. APLA are involved in the pathogenesis of Libman–Sacks endocarditis usually associated with SLE (324). These lesions are frequent and may be a significant risk factor for stroke (325). Several studies have demonstrated a positive correlation between the APLA titers and valvular heart disease severity (326,327).

TRADITIONAL CVD RISK FACTORS

A number of traditional CV risk factors, such as hyperlipidemia, T2DM, smoking, obesity, HTN and sedentary lifestyle have been assessed in APS patients. None of these Framingham risk factors showed any difference between APS patients and the general population (4). The prevalence of traditional risk factors for atheroma among patients with APS and PVD has not been extensively studied (Figure 2).

MetS. Medina *et al.* (328) showed a high prevalence of MetS in APS patients, similar to that in the general population and other ADs. This study found the most frequent CV risk factors were hypertriglyceridemia, low HDL levels, and visceral

obesity. The prevalence of MetS was 17.2% according to WHO, and 37.9% according to IDF criteria.

NON-TRADITIONAL CVD RISK FACTORS

Immuno-inflammatory mechanisms, primarily APLA, have an outstanding role in APS-related vasculopathies (103). APLA includes antibodies directed towards phospholipid moieties, such as cardiolipin or its cofactor β2GPI (anti-β2GPI), as well as lupus anticoagulant (329). These antibodies may induce a pro-inflammatory endothelial phenotype and interfere with pro and anticoagulant reactions by cross-linking membrane-bound proteins, and by blocking different proteins interactions (293) (Figure 2). Increasing evidence suggests that a subset of APLA can also be detected in patients with AT. In fact, patients having APLA and AT may have greater risk for ischemic events than patients with the same degree of AT but without APLA. These autoantibodies have probably been elicited as a consequence of the hypercholesterolemia in AT-prone individuals and autoantibody production could be an epiphenomenon of the disease, simply reflecting the abnormalities in lipid metabolism. However, several studies suggest that APLA can participate in the disease process, in fact, some studies have been associated APLA with mortality in CAD (330,331). Several studies have shown correlation between serum levels of ACLA and anti-β2GPI ACLA and anti-B2GPI, with the incidence and severity of CV events (285,329).

It has been demonstrated that atherosclerotic lesions possesses β2GPI in abundance. β2GPI was distributed both intracellularly and extracellularly and was most commonly present in the subendothelial regions of the atherosclerotic plaque. Therefore, the β2GPI within atherosclerotic regions could be a target for anti-β2GPI that aggravate the atherosclerotic process (332). ACLA and anti-β2GPI may be involved in a number of vascular diseases including CAD and stroke. Veres *et al.* (329) assessed the presence of APLA in ACS, and found that anti-β2GPI in ACS were associated with previous stroke, but not with HTN or previous MI. Thus, anti-β2GPI may be involved in the thrombotic events underlying ACS.

Soltész *et al.* (333) in a retrospective analysis of 1,519 APLA positive patients, among them 637 with clinical APS, detected venous thrombotic events more frequently in patients having circulating lupus anti-coagulant in comparison to patients with other types of APLA. In contrast, coronary, carotid and peripheral arterial thrombosis occurred more often in patients with elevated serum levels of IgG or IgM APLA, including ACLA or anti-β2GPI.

MANAGEMENT

The presence of early atheroma in APS underscores the necessity of new therapies for this disorder (293). Although the presence of early atheroma in APS is still a matter of debate, there is increasing evidence for the usefulness of several therapies indicated in AT, which further strengthens this hypothesis (293).

Although the thromboembolic potential of APLA has been well documented, there is still no general consensus on the prophylactic treatment of APLA carriers who have never developed vascular/obstetric manifestations (320). Early diagnosis of APS, thorough examination of the heart, examination od the heart and aggressive control of all traditional risk factors should be performed by lifestyle modifications and pharmacotherapy; probably anti-inflammatory treatment and close follow-up of APS patients may help to minimize CV risk in these individuals (30,324). The APS coagulopathy in these patients requires the careful and judicious use of appropriate anti-aggregant and anti-coagulant therapy (318). There has been a consensus report on the management of cardiac disease in APS (334).

Specifically targeted therapies, exerting anti-inflammatory or immunomodulatory effects become important therapeutic tools in APS. In order to achieve beneficial effects, these drugs should primarily antagonize the pathogenic effects of APLA. Moreover, these treatments should also control atheroma, which is one of the major causes of CV mortality in this pathology (293).

Antimalarials. AMs drugs, may exert evident anti-atherogenic properties (292). In vitro studies suggest that AMs may inhibit platelet aggregation and the thrombogenic effects of APLA (335). Despite these potential benefits, the anti-atherogenic effects of these drugs need to be clinically confirmed. The HCQ also has been associated with decreased levels of TC, TGL, glucose, and also the reduction of blood pressure, with anti-platelet effects as mentioned before.

Statins. These have pleiotropic characteristics, which include anti-atherosclerotic, anti-inflammatory, anti-oxidant, immunomodulatory and anti-thrombotic effects (336). Statins represent a powerful potential therapeutic tool in APS. It has been demonstrated that they prevent endothelial cell activation induced by APLA (337), induce modifications in the cellular and protein composition of atherosclerotic plaques (338), and have various beneficial effects on the endothelium, including improved NO availability and the stimulation of endothelial progenitors (339). Statin therapy significantly reduces the risk of CVD (340), by reducing CRP levels, and preventing endothelial dysfunction (341).

Aspirin. For a number of years, aspirin has been used in secondary prevention in APS patients particularly for its inhibitory effects on platelet aggregation. Aspirin may also have additional effects on the vascular wall (342). Moreover, aspirin may interfere with endothelial NO synthase and stimulate NO production, and is also shown to have an inhibitory effect on endothelial cell activation from inflammatory cytokines by preventing the activation and nuclear translocation of NFkB (343).

Heparin. In addition to their anticoagulant effects, unfractionated heparins and low molecular weight heparins also have anti-inflammatory properties. Thus, heparins may represent another anti-inflammatory therapeutic tool, however, the mechanisms of action responsible for their anti-inflammatory effects are not yet fully understood (344).

CVD IN OTHERS ADS

It has become evident over the last years that some ADs are characterized by common pathogenetic mechanisms and high rates of morbidity and mortality, mainly CVD-related. The increased CV mortality in the 3 most investigated rheumatic disorders (i.e., RA, SLE, and APS), appears to be sustained by vascular damage secondary to accelerated AT. However, the burden of CV involvement in other ADs appears to be lower and it is characterized by specific risk factors in addition to those shared with the general population.

SJÖGREN'S SYNDROME

This is an autoimmune epithelitis that affects the exocrine glands, with a functional impairment that usually presents as persistent dryness of the eyes and mouth (345,346). While sicca complaints in the setting of SS are quiet common, a relatively few number of studies have estimated the population prevalence of SS and its association with CVD. The clinical spectrum extends from an autoimmune exocrinopathy to a systemic involvement with vasculitis and diverse extra-glandular systemic manifestations (40-50%), including CVD, although with lower prevalence (347,348). Chronic systemic inflammation is a risk factor for developing AT, developing AT, but on the contrary to what is expected, the prevalence of CVD associated with AT, such as IHD, PAD and CVA, are not appreciably increased in patients with SS. This probably is characterized by chronic but milder inflammation (349) as Ramos-Casals *et al.* showed (345).

A recent prospective evaluation showed that CV events occurred in 7.7% of SS patients (350). Recently, Pasoto *et al.* (351) reported a prevalence of 5%, being the most frequent CVD associated MI, CVA and DVT. On the other hand, Vassiliou *et al.* (352) evaluated 107 patients with SS by echocardiography, without heart disease and found mainly tricuspid regurgitation, mitral and aortic valves, pulmonary hypertension and increased left ventricular mass. Dyslipidemia has been found as the most significant traditional risk factor linked with SS (353–357). Lodde *et al.* (354) showed a significant difference in the lipid profile (i.e., low HDL) not associated with acute phase reactants, by means with high level of inflammation. Furthermore, he found association between anti-Ro/SSA antibodies (SS-A) and anti-La/SSB antibodies (SS-B) and altered TC levels. In another study, Kang *et al.* (355) recorded the presence of dyslipidemia as CV compromise and arrhythmias. It has also been reported first-degree heart block, mainly related to the presence of SS-B and low HDL levels (354). It should be noted that the CV risk in patients with SS is increasing with the population affected by the disease (i.e., postmenopausal women) (354,358). Regarding non-traditional risk factors, Perez-De-Lis *et al.* (358) added to the list of risk factors for CVD in patients with SS the longer duration of the disease, steroid use, elevated CRP, SS-A, thrombocytopenia and severity of the disease (i.e., higher percentage in peripheral neuropathy, gastrointestinal and CNS involvement). By contrast, he also found a protective role of AMs in CVD, since these drugs shown association with lower frequency of HTN, T2DM and dyslipidemia. Vaudo *et al.* (349) found a high rate of subclinical AT due to changes of the carotid arterial wall, studied by femoral and carotid ultrasonography. This rate is mainly associated with the presence of leukopenia and SS-A antibodies, suggesting that the lack of immune regulation may play an important role in the early development of AT. In the same way, Gofinet *et al.* (351) observed an relation of APLA with thrombotic events, being the most frequent the lupus anticoagulant, which was significantly associated with APS. By contrast, two studies demonstrated that the APLA in SS are predominantly of the IgA isotype, which are not associated with thromboembolic events in SS (359,360). Increased serum levels of IgG, reflecting B cell hyperactivity of these subjects, were predicted by HDL in Lodde's study (361). Gerli (356) found that mean levels of serum IgG in SS patients with high-risk HDL levels were higher than the levels found in patients with normal HDL. Both observations suggest an association between hypergammaglobulinaemia and low HDL levels, and may be intriguing to test the hypothesis of the presence of circulating anti-HDL antibodies.

Gerli *et al.* (357) shown a slight loss of FMV, and lower nitrate mediated vasodilation (NMV) in SS subjects than controls. Patient NMV values were inversely correlated with soluble-VCAM-1 levels. An NMV decrease was confirmed in SS patient subsets with evidence of leukopenia, RF, SS-B and joint involvement. However, patients with joint involvement or parotid enlargement, two of the sites mainly affected by chronic inflammation in SS, had an FMV lower than controls and patients without these clinical features. All these findings suggesting that a functional impairment of the arterial wall may sustain early phases of atherosclerotic damage in SS. A combined effect of disease-related chronic inflammatory and immunologic factors appears to support dysfunction of endothelium and vascular smooth muscle cells, respectively. Finally, the management of CVD in SS patients must be directed toward rigorous intervention of modifiable risk factors as well as non-traditional risk factors, warranting a routine evaluation of autoantibodies and others factors SS-related. So in the future, it is necessary to analyze prospectively the incidence of thrombotic complications and the role of the different risk factors listed in this series for the development of such complications (Table 1).

SYSTEMIC SCLEROSIS

Systemic sclerosis (SSc) is a chronic disease, characterized by skin fibrosis, microvascular abnormalities and involve-

	SS	REF.	SSc	REF.
Cardiovascular burden	7/90 (7.7%) 5/100 (5%)	(350) (351)	CV symptoms 10% Mortality 20-30%	(387) (370)
CVD subphenotypes	MI, Stroke, DVT	(350, 351)	MI, PAD, CVA, CAD,	(366, 387)
	Arrhythmias	(353, 354)	Arrythmias, Coronary spasm	(377, 378)
	Valvular disease	(352)	LVDD	(367, 368, 379)
Traditional risk factors	Dyslipidemia T2DM	(353-358)	Dyslipidemia T2DM	(382) (388)
Non-traditional risk factors	SS-Related: Articular, renal, liver, peripheral neuropathy, CNS and gastrointestinal; parotid enlargement,	(353, 357, 358)	--	--
	Polyautoimmunity (SS and APS)	(351)	--	-
Non-traditional risk factors	Duration of the disease	(356, 358)	--	--
	Anti-Ro/SSA Anti-La/SSB APLA, Lupus anticoagulant RF anti-HDL	(349, 353, 358) (353, 357) (351) (357) (356)	anti-centromere anti-HSP65/60, APLA, anti-ox-LDL, antibodies	(383) (380, 384)
	Inflammatory markers	(354, 358)	--	--
	GC use	(358)	--	--
	Others: leukopenia, thrombocytopenia s-VCAM-1 Hypergammaglobulinemia	(349, 358) (357) (353, 356)	--	--
Subclinical atherosclerosis	Increase cIMT measure	(349)	CAC, increase cIMT	(365, 371, 387, 389)

Table 1. Common and specific mechanisms of CVD in SS and SSc. *anti-HDL*: anti-high density lipoprotein antibodies; *anti-HSP60/65*: anti-heat shock protein antibodies 60/65; *anti-oxLDL*: anti-oxidized low-density lipoprotein antibodies; *APLA*: anti-phospholipid antibodies; *APS*: antiphospholipid syndrome; *CAC*: coronary artery calcification; *CAD*: coronary artery disease; *cIMT*: carotid intima-media thickness; *CNS*: central nervous system; *CV*: cardiovascular; *CVA*: cerebrovascular accident; *CVD*: cardiovascular disease; *DVT*: deep venous thrombosis; *GC*: glucocorticosteroids; *LVDD*: left ventricular diastolic dysfunction; *MI*: myocardial infarction; *PAD*: peripheral artery disease; *Ref*: reference; *RF*: rheumatoid factor; *SS*: Sjögren´s syndrome; *SSc*: Systemic sclerosis; *s-VCAM*: soluble vascular cellular adhesion molecules; *T2DM*: type 2 diabetes mellitus.

ment of multiple internal organs. The incidence is approximately 10 cases per one millon persons. There are two major disease presentations: 1) diffuse cutaneous, associated with high risk of early pulmonary fibrosis and acute renal involvement; and 2) limited cutaneous SSc, associated with calcinosis cutis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly and telangiestasia. Diffuse cutaneous SSc is usually associated with a worse overall prognosis compared with limited cutaneous SSc (362).

It is thought that the vascular involvement is the result of an immune/inflammatory response that activates and injures the vascular endothelium which may increase the risk of coronary AT. The vasculopathy of SSc typically affects the small arteries and capillaries (i.e., microvascular occlusive disease with vasospasm and intimal proliferation in conjunction with cutaneous and parenchymal fibrosis), although macrovascular disease has been demonstrated by carotid ultrasonography, ankle-brachial blood pressure index and peripheral angiography (363,364). Recently, increasing evidence has demonstrated increased atherosclerosis, including coronary artery calcification (CAC), higher prevalence of subclinical CAD and thicker carotid IMT in SSc patients compared with healthy controls, suggesting SSc patients may be exposed to more CV risks (365,366). Patchy fibrosis is the most important feature in the myocardium, especially localized in subendocardial regions, which usually accompanies LVDD (367,368) but it is symptomatic in 10% of the cases (369). Mortality in patients with SE given by CVD is between 20-30% and, despite being similar to general population, it occurs a decade earlier (370). In asymptomatic patients, coronary calcifications were found in approximately 33.3% in diffuse SSc and 40% in limited SSc (371).

There have been reported MI or myocardial perfusion defects with patent coronary arteries, suggesting that the etiology of infarction may be due to microvascular disease rather than coronary AT, while recognizing that the latter is increased in patients with SSc (371,372). Patients with SSc have a reduced coronary flow reserve (373,374), which is associated with higher coronary events (375,376). Other authors have reported ectasia, spasm and coronary artery stenosis (377,378). Arrhythmias and conduction disturbances are characteristic of cardiac involvement in SSc, as hypertrophy and heart failure contractility (368,379) had been reported.

Ultrasonography evaluation is also used to evaluate the

carotid arteries, which has proven to be a useful marker for the assessment of subclinical AT and a strong predictor of subsequent MI and CVA (20,373,380). By performing Doppler it has been reported that 64% of the patients with SSc have carotid stenosis, compared with 35% presented in control patients (381).

Regarding the risk factors, it has been described the alteration of lipid profile, given by the increased levels of LDL and lipoprotein A, with a reduction in the fibrinolysis (382). In addition, it has also been found that decreased levels of HDL are related to anti-centromere antibodies positivity (383). According to the presence of antibodies that predispose to develop thrombotic events, there are no conclusive studies on the presence of anti-HSPs65/60. Some authors have found anti-ox-LDL antibodies elevated in limited SSc and cutaneous diffuse SSc. APLA were more found regarding capillary severe compromise (380,384), while anti-HSP 70 have been suggested as a protective factor for CAD (385). Among the non-cardiac alterations, there is the macrovascular involvement, observed by angiography and clinically performed with intermittent claudication (362). Histologically it can be observe fibrosis, thickening and chronic proliferation of the intimal layer as well as transmural lymphocytic infiltrate without evidence of atherosclerotic plaque (362,386) (Table 1).

CONCLUSIONS

Atherosclerosis and ADs shares several mechanisms. The excessive CV events observ ed in patients with ADs are not fully explained by classic risk factors. Several novel risk factors contribute to development of premature vascular damage. Therefore, a complex interaction between traditional and disease-specific traits converges into a shared pro-atherogenic phenotype in this population. Until additional research and disease-specific risk prediction tools are available, current evidence supports aggressive treatment of disease activity, and careful screening for and management of modifiable traditional risk factors in patients with ADs. The finding and understanding of complex interactions between predisposing factors will allow us to better describe and assess the broad spectrum of CV subphenotypes in ADs.

ABREVIATIONS

- **ACE:** angiotensin-converting-enzyme
- **ACLA:** anti-cardiolipins antibodies
- **ACP1:** acid phosphatase locus 1
- **ACPA:** anti-cyclic citrullinated peptide antibodies
- **ACS:** acute coronary syndrome
- **AD:** autoimmune diseases
- **AITD:** autoimmune thyroid disease
- **AMs:** antimalarials
- **anti-ApoA-1:** anti-apolipoprotein A-1 antibodies
- **anti-β2GPI:** anti-beta2 glycoprotein 1 antibodies
- **anti-MCV:** anti-modified citrullinatedvimentin antibodies
- **anti-MDA-LDL:** anti-malondialdehyde-modified LDL antibodies
- **anti-HSP 60/65/70:** anti-heat shock protein 60/65/70
- **anti-oxLDL:** anti-oxidized low-density lipoprotein antibodies
- **anti-PC:** anti-phosphorylcholine antibodies
- **AOR:** adjusted odds ratios
- **APLA:** anti-phospholipid antibodies
- **APS:** antiphospholipid syndrome
- **ApoB:** apolipoprotein B
- **AT:** atherosclerosis
- **AZA:** azathioprine
- **β2GPI:** beta-2 glycoprotein-1
- **BILAG:** British Isles Lupus Assessment Group
- **BMD:** bone mineral density
- **BMI:** body mass index
- **CAC:** coronary artery calcification
- **CAD:** coronary artery disease
- **CHF:** congestive heart failure
- **CRP:** C-reactive protein
- **CV:** cardiovascular
- **CVA:** cerebrovascular accident
- **CVD:** cardiovascular disease
- **CYC:** cyclophosphamide
- **DAS28:** Disease activity score-28
- **DMARDs:** disease modifying anti-rheumatic drugs
- **DVT:** deep vein thrombosis
- **EAMs:** extra-articular manifestations
- **ESR:** erythrocyte sedimentation rate
- **FA:** familial autoimmunity
- **FDRs:** first degree relatives
- **FMV:** flow-mediated vasodilation
- **GC:** glucocorticosteroids
- **GSTT1:** Glutathione S-transferase T1
- **HCQ:** hydroxychloroquine
- **HDL:** high density lipoprotein cholesterol
- **HLA:** human leukocyte antigen
- **HRT:** hormone replacement therapy
- **HSP:** heat shock proteins
- **HTN:** hypertension
- **ICAM-1:** intercellular adhesion molecules

- **IDF:** International Diabetes Federation
- **IFN-γ:** Interferon- γ
- **IHD:** ischemic heart disease
- **IL:** interleukin
- **IMT:** intima-media thickness
- **LA:** Latin American
- **LDL:** low density lipoprotein cholesterol
- **LEL:** low educational level
- **LGALS2:** Galectin-2
- **LT-A:** Lymphotoxin-A
- **LUMINA:** Lupus in Minorities: Nature vs. Nurture
- **LVDD:** left ventricular diastolic dysfunction
- **Mϕ:** macrophages
- **MBL:** mannose-binding lectin
- **MCP:** monocyte chemotactic protein
- **MCSF:** macrophage colony stimulating factor
- **MetS:** metabolic syndrome
- **MI:** myocardial infarction
- **MMF:** mycophenolate mofetil
- **MTHFR:** methylene tetrahydrofolatereductase
- **MTX:** methotrexate
- **NCEP:** National Cholesterol Education Program
- **NFKB1:** nuclear factor kappa-light-chain-enhancer of activated B cells
- **NMV:** Nitrate mediated vasodilation
- **NO:** nitric oxid
- **OPG:** osteoprotegerin
- **OPN:** osteopontin
- **ox-LDL:** oxidized-LDL
- **PAD:** peripheral arterial disease
- **PAI:** plasminogen activator inhibitor type-1
- **PDGF:** platelet-derived growth factor
- **PE:** pulmonary embolism
- **PVD:** peripheral vascular disease
- **RA:** rheumatoid arthritis
- **RF:** rheumatoid factor
- **RR:** relative risk
- **SDI:** SLICC damage index
- **SE:** shared epitope
- **SES:** socioeconomic status
- **SLAM:** systemic lupus activity measure
- **SLE:** systemic lupus erythematosus
- **SLEDAI:** Systemic lupus erythematosus disease activity index
- **SLEDAI-2K:** Systemic lupus erythematosus disease activity index 2000
- **SLICC:** Systemic lupus international collaborating clinics score
- **SLICC-RAS:** Systemic Lupus International Collaborating Clinics-Registry for Atherosclerosis
- **SMR:** standardized mortality ratio
- **sPTX-3:** serum Pentraxin-3
- **SS:** Sjögren's Syndrome
- **SS-A:** anti Ro/SSA antibodies
- **SS-B:** anti La/SSB antibodies
- **SSc:** systemic sclerosis
- **SSZ:** sulfasalazine
- **STAT4 :** signal transducer and activator of transcription 4
- **SUA:** serum uric acid
- **SCORE:** Systematic Coronary Risk Evaluation
- **T2DM:** type 2 diabetes mellitus
- **TC:** total cholesterol
- **TGF-β:** transforming growth factor -β
- **TGL:** triglycerides
- **Th1:** lymphocyte T helper 1
- **Th2:** lymphocyte T helper 2
- **TIA:** transient ischemic attacks
- **TLR:** toll-like receptors
- **TNF-α :** tumor necrosis factor-α
- **TNFR-II:** tumor necrosis factor receptor II
- **TRAF1:** TNF receptor-associated factor 1
- **t-PA:** tissue type plasminogen
- **VCAM:** vascular cell adhesion molecules
- **VEGFA:** vascular endothelial growth factor A
- **VLDL:** very low density lipoprotein cholesterol
- **WC:** waist circumference
- **WHO:** World Health Organization

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39 AUTOIMMUNE DISEASES AND CANCER

Michael Ehrenfeld

INTRODUCTION

Autoimmune diseases are often associated with malignancies, and, in contrast, some malignant diseases are also associated with an increased risk of developing autoimmune disorders.

This review will update the recent literature which relates to these issues with regard to the most prevalent autoimmune diseases such as Rheumatoid Arthritis (RA), Systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), Idiopathic inflammatory myopathies (DM/PM), Scleroderma and Systemic Vasculitis.

RHEUMATOID ARTHRITIS AND CANCER

Various early studies have already suggested an increased risk of malignancies among patients with RA (1-2). It has also been shown that patients with severe RA, and particularly Felty's syndrome, have a clinically significant increased risk for the development of cancers. Most of the earlier reports described the development of solid and lymphoproliferative malignancies such as non-Hodgkin's lymphoma, acute and chronic leukemias, and multiple myeloma which were all said to be associated with rheumatoid arthritis (3-6). These early studies were later confirmed by large population-based studies demonstrating the increased risk for the development of lymphoproliferative diseases, and particularly non-Hodgkin's lymphoma (7-9). Later studies have reviewed the rate of cancer among large cohorts of RA patients. A standardized incidence ratio (SIR) of 2.4 for lymphoma was described in a population based study of more than 20 000 Danish patients, and a closely related increased risk of 1.9 has been shown in 18,572 US patients (10-11). In a more recent large scale study, attempting to link RA to data drawn from the cancer registry in California over a period of more than 10 years with data of approximately 400,000 person/years an increased risk of developing lymphoproiferative cancers was found among both women and men with RA (12). In this study, women were found to be at a decreased risk for several cancers, e.g., cancers of the breast, ovary, cervix, uterus, and melanoma, with a risk reduction of 15-57% compared to the general population. The findings in the group of the men, showed however a significantly higher risk of lung, esophagus and liver cancer though a lower risk for prostate cancer was noted. A recent large Swedish study examined the site-specific cancer risk in 42,262 hospitalized RA patients and found again that the highest risks were noted for Hodgkin's disease (SIR 4.05), non-Hodgkin's lymphoma (SIR 2.34), squamous cell skin cancer (SIR 1.89), and lung cancer (SIR 1.73) (13). With respect to solid tumors, increased risks of lung cancer and melanoma have been reported. A recent large scale study from the Veteran Affairs database confirmed the significant association between RA and lung cancer in this population with 43% (odds ratio 1.43) likelihood of developping lung cancer, compared with non-RA population, when adjusted for covariates (14). The contribution of various DMARDs, including cytotoxic and immunosuppressive agents to the overall risk of cancer and specifically to the risk of lymphoproliferative tumors was also reported (15-18). Cytotoxic agents may have a direct oncogenic effect. Both in vitro and in vivo studies have shown that methotrexate was associated with chromosomal aberrations as well as cellular morphological aberrations. Whether the development of malignancies in RA patients is the result of the inflammatory process, the immunological imbalances or the use of cytotoxic and immunosuppressive agents remains controversial. The introduction of the new biological disease modifying anti-rheumatic drugs for the treatment of RA and other inflammatory rheumatic

diseases over 10 years ago raised the issue of the risk of malignancies in RA again. A Swedish study analyzed the occurrence of cancer among a national cohort of 6,366 patients with RA who first started anti-TNF therapy between January 1999 and July 2006, and compared them to the national biologics-naïve RA cohort of 61,160 patients. During this study-period (25 693 person years), 240 first cancers occurred yielding an RR of 1.0. The relative risk did not increase over time after initiating therapy with anti-TNF agents during a follow up period of 6 years, thus this large study did not reveal an overall elevation for a risk of cancer (19). Similar studies yielded similar results from the German biologics register RABBIT and the British register BSRBR (20-21). These studies also provided limited evidence regarding the risk of patients with a prior malignancy treated with anti-TNF agents. A systematic review and meta-analysis of European data drawn from registries and prospective observational studies, also confirmed the lack of any increase in the risk of malignancies, particularly lymphoma (RR 1.11). The study however did point to an increase in the risk of non-melanoma skin cancer (RR 1.45) as well as melanoma (RR 1.79) (22). Another Swedish nationwide population based prospective cohort study recently looked specifically at the risk of malignant melanoma in RA patients who are being treated with TNF inhibitors (23). RA patients who have not been treated with biological drugs were not found to be at an increased risk of invasive melanoma compared to the general population. Those treated with anti-TNF agents were not found to be at an increased overall risk for cancer, but had a moderately increased relative risk of invasive melanoma (23). A meta-analysis of short-term randomized controlled trials of the newer anti-TNF agents Certolizumab and Golimumab did not find any increased risk of total malignancy and non-melanoma skin cancer (24). Similarly, the incidence ratio of total malignancy (excluding non-melanoma skin cancer), breast, colorectal, lung cancers, and lymphoma in the abatacept clinical development program, were consistent with those in a comparable RA population (25).

SJOGREN'S SYNDROME (SS) AND CANCER

Studies have shown that patients with primary SS have a higher incidence of non-Hodgkin's lymphoma compared to the general population with an SIR ranging from 8.7 to 48.1 (26-28). A meta-analysis of cohort studies reported a pooled SIR of 18.8 (29). In most cases, when lymphoma develops, it is a low grade B cell lymphoma of the mucosa associated lymphoid tissue (MALT) or large B-cell lymphoma (27, 30). It was further shown that the detection of germinal center-like structures by light microscopy in primary Sjogren's syndrome diagnostic minor salivary gland biopsies suggested a highly predictive and easy to obtain marker for the development of non-Hodgkin's lymphoma (31). The authors suggest that such a finding on histology will allow for risk stratification of the patients with the possible initiation of preventive B-cell directed therapy. Other suggested risk factors for non-Hodgkin's lymphoma include high levels of rheumatoid factor, high levels of anti Ro (SSA) and anti La (SSB), low levels of complement C3 and C4, leucopenia, mixed cryglobulinemia, splenomegaly, lymphadenopathy, and salivary gland swelling, vasculitis, purpura, and pulmonary infiltrates (27, 32). Patients with any of these risk factors should probably be followed much more carefully and closely. In a recent study, authors from Taiwan assessed the incidence of cancer in a nationwide population cohort of 7852 patients with primary Sjogren's syndrome, and calculated the SIRs for various sites (33). Among the SS cohort, 2.9% developed cancer. The SIR for cancer was 1.04 among patients of all ages, and 2.19 for patients aged 25-44. Female patients with primary SS had a higher risk of non-Hodgkin's lymphoma (SIR 7.1), multiple myeloma (SIR 6.1), and thyroid cancer (SIR 2.6). A decreased risk for colon cancer (SIR 0.22) was found in this cohort. In contrast, male patients with primary SS were not found to be at a higher risk of developing cancer in particular sites.

SLE AND CANCER

Earlier studies had shown the frequency of cancer in patients with SLE to be between 2.5% and 13.8%, compared to the general population. However the literature yielded contradictory results with a few studies which did not find any increased risk for the development of overall cancers, as opposed to others who identified a significant risk with a SIR of 1.3 (34-37). Similarly, the risk of lymphoproliferative diseases has been looked into specifically and multiple large scale series of SLE patients have shown a definite increased risk over the years, whereas others were unable to confirm these findings (38-40). A large retrospective cohort study attempting to examine the risk of cancer was done few years ago in California (41). The overall cancer risk amongst 30,478 SLE patients, 1,273 of whom developed a malignancy was significantly elevated (SIR 1.14). Lupus patients had higher risks of vagina/vulva (SIR 3.27) and liver cancers (SIR 2.7). Elevated risks were also found for lung, kidney and thyroid cancers as opposed to a significantly reduced risk of breast, cervix, and prostate cancers (41). A few recent studies confirmed the reduced risk of breast cancer in SLE patients. A group of investigators from the US studied the risk of breast cancer amongst a large group of elderly women suffering from RA (n=5,238), SLE (n=340), SS (n=374), systemic sclerosis (n=128), and dermatomyositis (n=31), and compared them with an equal number of age-matched cancer-free controls (42). Women with RA were less likely to develop breast cancer (OR=0.87). Breast cancer risk was not associated with any of the other systemic autoimmune diseases except for a risk reduction of estrogen receptor-negative among women with SLE (OR=0.49). Another multinational study tried to explore whether certain single nucleotide polymorphisms (SNPs) are of importance in SLE and might be protective against breast cancer

(43). The authors were unable to demonstrate any important associations with 10-lupus-associated SNPs in a large breast cancer cohort. They had, therefore, speculated that if the decreased breast cancer risk is influenced by a genetic profile, this may be due to complex interactions and or epigenetic factors. Since non-Hodgkin's lymphoma is not the only hematological malignancy associated with SLE, and there were reports of associated myeloid leukemia, a Swedish group assessed the risk factors for leukemic transformation and myeloid leukemia in a nested case-control study based on Swedish registers (44). Leucopenia with MDS, which was seen frequently, was found to be a risk factor whereas low-dose chemotherapy was not found to be a major cause of myeloid leukemia in this study. The authors suggest doing a bone marrow study on in SLE patients with long-standing leucopenia and anemia. A group of researches from Italy examined the prevalence and features of thyroid cancer in 153 SLE patients in a prospective study, and compared them to two population-based, gender, and age matched control groups (45). The prevalence of papillary thyroid cancer in SLE patients was found to be higher than in the control groups, particularly in patients with thyroid autoimmunity. Among SLE patients with confirmed thyroid cancer, 80% showed evidence of thyroid autoimmunity whereas only 31% of SLE patients without thyroid cancer exhibited evidence of thyroid autoimmunity. Another recent nationwide Swedish study examined the risk of cancer among a cohort of 3,663 patients with cutaneous lupus erythematosus (46) and found them to have about a fourfold risk increase for buccal cancer, lymphomas, respiratory cancer, and non-melanoma skin cancer. Hazard ratios for all cancers were 1.8. A group of researches from Taiwan recently evaluated the risk of cancer among a cohort of 2,150 SLE patients they were following (47). Each patient was randomly age and sex matched to 8 patients without SLE. In this cohort, the risk of developing overall cancer was marginally significantly higher (HR 1.26) and was found to be significantly higher only for developing prostate cancer (HR 3.78). A more recent multisite international study attempted to update cancer risk estimates in SLE relative to the general population by linking the various cohorts to the regional tumor registries (48). Incidence ratios (SIRs) were calculated as the ratio of observed to expected cancers. Across 30 centers, 16,409 patients were observed for 121,283 (average 7.4) person-years. A total of 644 cancers occurred. Of these, hematological malignancies were substantially increased (SIR 3.02), mainly due to non-Hodgkin's lymphoma (SIR 4.39 and leukemia. Increased risks were also found for cancer of the vulva (SIR 3.78), lung (SIR 1.3), thyroid (SIR 1.76), and possibly liver (SIR 1.87). In contrast, a decreased risk was estimated for breast cancer (SIR 0.73) endometrial (SIR 0.44) and possibly ovarian cancers (SIR 0.64). The overall risk across all cancers compared with the general population, was only slightly increased (SIR 1.14). However, a definitly increased risk was found for some of the cancers, but the etiology of the risk reduction for others is unclear.

POLYMYOSITIS (PM) / DERMATOMYOSITIS (DM) AND CANCER

The association between idiopathic inflammatory myopathies and malignancy has been extensively reported in the literature over the years (49-50). The incidence of cancer in patients with DM has been reported in the past to be 3-40% (51-54). A recent study in Taiwan examined 1,012 patients with DM and 643 with PM in a nationwide cohort study, for the risks of cancer in PM and DM (55). None of the included patients had any prior history of a malignancy. The overall calculated cancer risk in DM was significantly elevated (SIR 5.11), but somewhat less in PM (SIR 2.15). Most cancers were detected in the first year of follow up. DM patients were at the greatest risk of cancer of the nasopharynx, lungs, and hematopoietic malignancies. A Japanese group of researchers published their experience with a large group of 136 DM / PM patients and tried to look for predictive factors for malignancies among this group using multivariate analysis by logistic regression (56). Malignancies were found in 17 of 70 patients with DM (24%), 3 of 15 patients with clinically amyopathic DM (CADM) (20%), and 3 of 51 patients with PM (6%). Gastric cancer was the most common malignancy among this cohort. Compared to the general population, the SIR for malignancies was 13.8 (range 9.0-21.1). Patients who developed malignancies were older, presented more often with dysphagia, and were less likely to have interstitial lung disease. The authors conclude that their study confirms that CADM should be added as a myopathy complicating with malignancy. A group of French investigators tried to do a similar study of the predictive variables of hematological malignancies in PM/DM as well as to assess the characteristics of these malignancies (57). They had retrospectively reviewed the records of 32 patients (14 PM, 18 DM) who developed hematological malignancies. Hematological malignancy was concurrently identified (18.8%) or occurred during the course of PM/ DM (31.2%) although PM/ DM more often preceded the hematological malignancy onset (50%). Types of malignancies were B-cell lymphoma (n=20), T-cell lymphoma (n=4), Hodgkin's disease (n=2), MDS without excess of blasts (n=3), and single cases of myeloma, hairy cell leukemia and acute lymphocytic leukemia. The authors had also observed that patients with PM/DM-associated hematological malignancies had a poor prognosis were older than those without malignancy. However, these patients less commonly exhibited joint disease, interstitial lung disease and anti-Jo1 antibody. Thus anti-synthetase syndrome may be a protective factor for hematological malignancies in PM/DM. Many groups have attempted to find predictive clinical or serological markers of malignancy development in idiopathic inflammatory myopathies as described earlier. Few investigators described 2 new and similar myositis specific autoantibodies reacting to a 155-kDa nuclear protein and with two 155/140-kDa nuclear proteins, both exhibiting a significant association with cancer-associated malignancy (58-59). A recent study attempted to determine the occurrence and types of cancers occurring in Spanish cohorts of patients

with PM/DM as well as analyzing the value of the anti-p155 autoantibody as a serologic marker of cancer-associated myositis (60). Among this study group, cancer-associated myositis was detected in 16 patients (19%). The shawl sign was significantly more frequent in patients with cancer than in those without. Adenocarcinoma was the most frequent type of cancer (87.5%). The serologic marker anti-p155 autoantibody was found in 1 of 20 patients (5%) with PM as opposed to 15 of the 65 (23%) patients with DM thus making this autoantibody a reliable marker of cancer in patients with DM. Based on the above data, malignancy screening is now recommended in patients with DM/PM, but there is no consensus as to how to screen and how often patients should be screened. In a recent prospective study of 55 patients with inflammatory myopathy, the authors concluded that both FDG-PET/CT and the conventional screening used so far (thoraco-abdominal CT, mammography, gynecological examination, ultrasonography, and tumor markers) are similarly effective for the search and detection of an occult malignancy (61). Thus the authors suggest that FDG-PET/CT might replace conventional screening using only a single non-invasive imaging procedure.

SCLERODERMA AND CANCER

The association between scleroderma and malignancy has been a focus of numerous studies for many years (62-63). In a large study, a group of investigators calculated the risk of cancer in a group of 769 patients with scleroderma who were followed between 1987 and 2002 (64). Ninety malignancies were diagnosed in this cohort, 62 of which were diagnosed after the diagnosis of scleroderma, and 28 malignancies preceded the diagnosis of the scleroderma. The SIR for all cancers diagnosed after diagnosis of the scleroderma was 1.55. The SIR for esophageal cancer was 15.9 while that for oropharyngeal cancer was 9.63. Other groups suggested that lung and breast cancer seemed to be the most frequent cancer among scleroderma patients (62, 64-66). An Italian group had recently evaluated the prevalence of lung cancer in their 318 scleroderma patients retrospectively. They had also looked for serological markers of potential associations (67). Lung cancer was found to complicate 5% of the scleroderma cohort which was about 2.5 in males and more than 5 times higher in females when compared to an age and sex matched population from the same geographical area. The presence of lung cancer significantly correlated with the presence of anti-Scl70 antibodies. Recent reviews of the literature on the association of cancer and scleroderma identified female gender, old age, and diffuse skin involvement as the major risk factors (68-69). Another recent study evaluated whether clinical characteristics, including the temporal relationship between scleroderma and malignancy onset, differed based on autoantibody status among those patients (70). Patients' sera were tested for autoantibodies against topoisomerase I, centromere and RNA polymerase I/III. The authors concluded that this close temporal relationship between scleroderma and cancer was also related to those who had antibodies to RNA polymerase I/III, which is distinct from scleroderma patients with other autoantibody specificities. They therefore suggest that malignancy may initiate the scleroderma-specific immune response and drive disease in a subset of scleroderma patients.

SYSTEMIC VASCULITIS AND CANCER

Though vasculitis may be a manifestation of paraneoplastic syndrome, it was believed that there was no major evidence to support an association between systemic vaculitis and cancer. Two recent studies done of patients with giant cell arteritis (GCA) confirm these findings in this most common vasculitis in people over the age of 50 (71-72). Even though no correlation was found in GCA, the European Vasculitis Study Group (EUVAS) recently analyzed their patients who were included in four recent multicentre clinical trials to assess for subsequent cancer risk (73). Patients included in the study were those diagnosed with ANCA-associated vasculitis (AAV) including both granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA). The analysis concerned 535 patients with newly diagnosed AAV from 15 countries. During the study period, 50 cancers were diagnosed in 46 patients. SIR for cancers at all sites was 1.58, for cancers at all sites excluding non-melanoma skin cancer 1.3, for bladder cancer 2.41, for leukemia 3.23, for lymphoma 1.11, and 2.78 for non-melanoma skin cancer. Subgroup SIR for cancers at all sites was 1.92 for GPA and 1.2 for MPA. The authors concluded that cancer rates in these two groups of AAV exceeded those expected in the general population. Some of this could also be related to the use of cyclophosphamide which, however, has been used in reduced doses in current protocols. The current understanding and inconsistencies in data relating to AAV and malignancy have been dealt with thoroughly in a review published this year (74).

There is thus considerable evidence that autoimmune diseases are associated with the development of malignancy, especially lymphoproliferative cancer. This relationship implies the need for awareness of the cancer risk and probably surveillance of patients with autoimmune diseases who are on immunomodulatory therapies for the potential of developing a secondary malignancy. The actual risk of such a secondary malignancy is probably related to the autoimmune disease itself, to the therapeutic agents which are being used, and to the traditional cancer risk factors.

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40 THE EARLY AUTOIMMUNE DISEASE CLINIC. THE CASE OF RHEUMATOID ARTHRITIS

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INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects not only joints but could also involve other major organs. RA patients without an early and appropriate treatment will evolve to joint damage and disability. Early Arthritis Clinics (EACs) have become the best option for treating newly diagnosed RA patients. This model is also implemented for other autoimmune diseases. Herein, we present the experience achieved at the "Clínica de Artritis Temprana" ("CAT", "early arthritis clinic"), in Cali, Colombia.

EAC organization has been described elsewhere (1). At first sight there are no significant differences between EAC and other routine outpatient centers. So briefly, EAC is essentially a "concept of service" in which the center develops several steps to promote early referral of patients with signs or symptoms probably related to RA or undifferentiated arthritis.

At the very first level, there is an increased need for educational material to make the general population aware of the early symptoms and the need for early treatment of RA. This step should be followed by an approach to primary care physicians to improve their knowledge about semiology in rheumatologic diseases and the importance of early referral of patients to EAC (2). Further feedback at regular basis is needed to achieve the best results.

It is also crucial to maintain good communication with health authorities and representatives of the insurance companies. This is essential to reduce barriers, so that patients can get access to the health system (3). Cost-effectiveness is key to achieving this goal (4).

Different ways of communication (besides telephone) such as email, website, fax, and even social networks, must be implemented. Additionally, all the patients should be scheduled for their next visit immediately at the end of the current one in order to improve adherence to follow-up. A simple strategy to assure free openings for medical appointments is to reserve some slots on a regular daily basis to make rapid referral of patients and priority rescheduling possible.

At the time of the visit, there will be a lot of information collected from these patients that is not normally collected at other routine outpatient clinics. The length of the visit may be longer than usual in order to do clinimetric analysis, obtain an extended medical history, and register information into the database. Every patient with an autoimmune or inflammatory rheumatic condition is asked to fill out a consent form to get into the program in order to allow the activities for a close follow-up to be carried out. Tight control and intensive treatment are essential for achieving the best results in RA as reported elsewhere (5-7).

Follow-up activities are essentially done by nurses and primary care physicians. They are responsible for educating the patient about his/her disease, self-care and making sure the patient fully understands the importance of adherence to treatment, warning signs, and when to request a new medical appointment. This is done after rheumatological consultation. They are also responsible for follow-up phone calls. This task was divided into routine and intensive follow-up. Routine follow-up is intended for all the registered patients in our program. In this group, the goal is to have at least one follow-up activity per month. Intensive follow-up is reserved for those patients with clinical conditions such as high disease activity, receiving biological therapy, suspected poor adherence to treatment, intercurrent surgery, recent need to visit emergency service and/or hospitalization, and the presence of significant comorbidities or infections. These patients will be contacted at least two times per month. The first contact should be made during the first week after the initial visit. As expected, the follow-up varies

from patient to patient due to the characteristics of each patient (i.e., tailored follow-up).

The CAT is located in Santiago de Cali (known as Cali), a city in the southwestern part of Colombia. It is approximately 1,000 meters (3.300 ft.) above sea level, and its surface area is 546 km2. It is the third largest city in the country and has a population of 2.2 million, with a high African Colombian ancestry (26%). This makes Cali one of the places with the largest black population in Latin America. The average temperature is 26° C (78.8° F) and its coordinates are 3°25'14"N 76°31'20"W (Figure 1).

Prevalence of RA in Latin American is estimated to be 0.5% with a remarkably high female to male ratio 6-8:1 (8-10), which clearly contrasts with the ratios reported in North America and Europe. The main characteristics of patients with RA assessed at the CAT, presenting duration of symptoms < 1 year will be next presented. All of the patients fulfilled the 2010 ACR / EULAR criteria (11) and were referred from their health insurance companies, mostly by referral of the primary care physicians and other specialists such as orthopedists, physiatrists, and internists.

RESULTS

At the CAT three rheumatologists are working on a full time basis. Primary care physicians and nurses are available to lend support for close follow-up. More than 2,000 patients have been assessed, met the international criteria for any autoimmune or inflammatory rheumatologic disease and were accepted into our special program for tight control. As expected, RA has been the main diagnosis with 1,008 patients (48.36%; 914 established RA and 94 early RA patients). From these patients, we found 94 patients with RA and symptoms with a duration of <1 year (Table 1). Systemic lupus erythematosus, undifferentiated arthritis, spondyloarthritis, and other autoimmune diseases were also present corresponding to 15.35%, 9.85%, 6.75%, and 18.44% respectively.

Among patients with early RA, a female predominance (82.98%), female to male ratio of 4.8:1, and a mean age at diagnosis of 49.42 years were observed. A Mestizo predominance (87.5%), followed by Afro-Colombian, and Caucasian (10.0% and 2.5%, respectively) ancestry were registered. There were no Amerindian patients in this group. Mean duration of disease before entering CAT was 219 days (14-364). Polyarticular involvement was the most frequent pattern of presentation (76.4%). Rheumatoid nodules were found in 4.26% of the patients. Positive rheumatoid factor was found in 80.49% and cyclic citrullinated peptide antibodies (anti-CCP) were also found in 60.71% of the patients. Mean DAS 28-ESR at the first visit was 3.31 and 2.42 at the last visit. Erosions seen by x-ray were found in 10.0%. Current smoking was detected in 4.26% of the patients. Presence of any concomitant autoimmune disease was found in only 1 patient (i.e., immune thrombocytopenic purpura). Antinuclear antibodies were positive in 18.36% of the patients. Other non-autoimmune comorbidities found were arterial hypertension, dyslipidemia, osteoporosis, knee osteoarthritis, and hypothyroidism. Even though most hypothyroidism is considered to be autoimmune, no systematic search was done for the presence of specific autoantibodies.

Previous use of some disease-modifying antirheumatic drugs (DMARD) before the first visit at CAT was observed in 50.0% of the patients. Methotrexate was the most frequent DMARD used, followed by chloroquine, sulfasalazine, and leflunomide (86.2%, 60.6%, 16%, and 12.8% respectively).

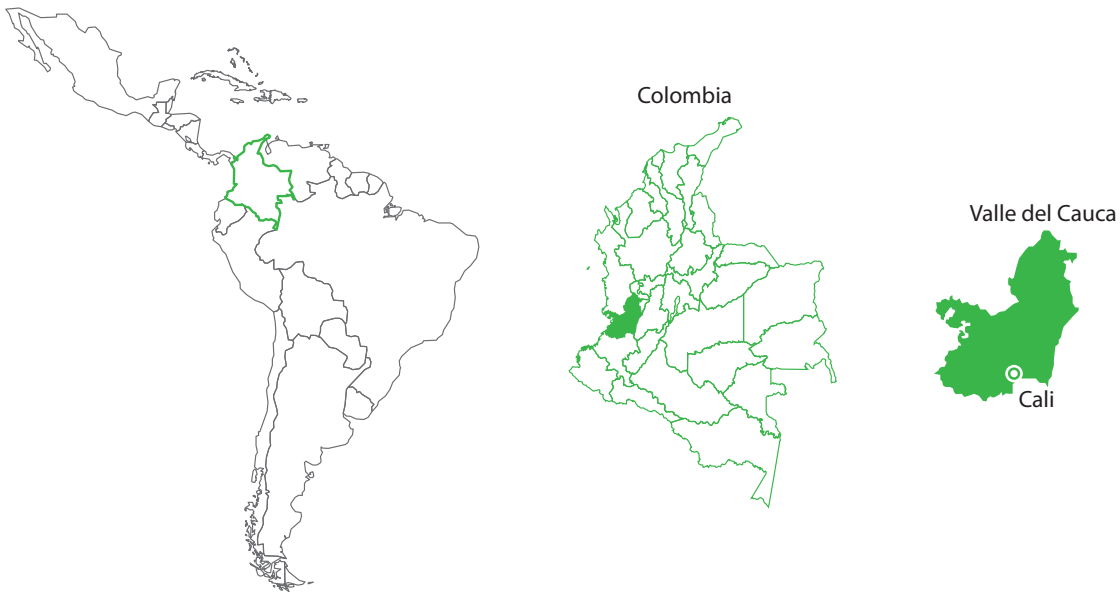

Figure 1. Geographical position of Cali, Colombia, South America.

VARIABLE	RESULT
Age at diagnosis	49.42 (y)
Gender	
Female	82.98
F:M ratio	4.8:1
Ethnicity	
Mestizo	87.5
Afro-Colombian	10.0
Caucasian	2.5
Amerindian	0
Articular involvement	
Monoarticular	5.62
Pauciarticular	19.1
Polyarticular	76.4
Seropositivity	
Rheumatoid factor	80.49
Anti-CCP	60.71
Other features	
Erosive disease	10.0
Rheumatoid nodules	4.26
Current smoking	4.26
Overt polyautoimmunity	1.06
DMARD, previous use	50.0
DMARD, current use	100
Biological therapy	0

Table 1. Early RA. Characteristics of 94 patients. Otherwise indicated, data are percentages. *RA*: rheumatoid arthritis. *F*: female. *M*: male. *Anti-CCP*: cyclic citrullinated peptide antibodies. *DMARD*: Disease modifying anti-rheumatic drug.

No patient with early RA to date has been given a prescription for biological therapy (versus 116 of 914 patients with established RA (> 1 year), 12.69%).

A higher proportion of patients with early RA had achieved remission as compared to those with established RA (Table 2).

DISCUSSION

The analysis of clinical and demographic features from RA patients in Latin America is complex due to the multiethnic origin of the populations (12). Socioeconomic status and access to health systems are also variables that influence the course of RA (9).

The proportion of gender, with a higher female to male ratio 6.3:1 seen in our patients, is similar to those reported by other studies published in Latin America (9, 13). As for the ethnic groups, the Mestizo population in our cohort was predominant, which is similar to the GLADAR study (8, 9). However, it differs slightly due to the fact that the Afro-Colombian population was second in predominance. This could be explained by Cali having a large population of Afro-Colombians. In other cities in our country, RA in Afro-Colombians is less frequent (14). However, it is very difficult to classify the ethnic group of a patient from a single phenotypical analysis. Molecular analysis by using ancestry informative markers will make it possible to further analyze the influence of ancestry on RA and other chronic conditions.

The mean duration of articular symptoms before entering CAT was 219 days (7.2 months) which is higher than that reported by the GLADAR study (6.8 ± 4.4 months) (15). This may be partially due to the fact that our center is relatively new, and because of a lack of knowledge about the need for patients with RA to receive an early referral to rheumatologists. However, this perception is changing, and people involved in the social security system (e.g., physicians, payers, health authorities) are increasingly aware of the need for an urgent referral in the case of rheumatological and systemic autoimmune conditions.

The type of joint involvement our group had was polyarticular in most of the cases (76.4%) which is slightly lower than that reported by the GLADAR (15). The presence of rheumatoid nodules and erosions in hands or feet seen through x-rays in the initial evaluation was low, which is similar to other studies (15). Seropositivity for rheumatoid factor and/or anti-CCP antibodies was similar to what was observed in other studies published on early RA (16).

Previous use of DMARD therapy, especially with methotrexate in patients referred by specialists in internal medicine, was observed at a higher rate than expected. Methotrexate use is increasing in clinical care (17). This is encouraging because we saw a good correlation between criteria used for diagnosis and initial treatment in RA. This is in contrast with the fact that patients presenting with early RA do not receive DMARD promptly (the ERAN cohort) (18). All of these patients treated with DMARD had had polyarticular involvement. As seen in other series, none of the patients has been treated with biological therapy thereby reinforcing the concept that an early diagnosis can overcome most of the adverse prognosis factors and contribute to achieving a sustained remission in RA. Thus, they avoid, at least initially, these expensive medications (19). Previous reports from GLADAR showed that 1-4% (46/1093) of early RA received biological therapy (20). It is noteworthy that remission and a low disease activity were more frequently observed in early RA than in established patients (Table 2). This finding is in agreement with the worldwide concept that there is a window of opportunity and early treatment is the best option for RA.

CONCLUSIONS AND PERSPECTIVES

The demographic and clinical characteristics of the patients in our group from a southwestern Colombian population were similar to those described in other Latin American populations, especially a higher female to male ratio, mean age at diagnosis, and a higher predominance of a polyarticular pattern. A different genetic background and environmental and

socioeconomical status are the main contributing factors that explain differences in demographic and clinical characteristics among cohorts (21). In our case, the higher presence of an Afro-Colombian population could be a key factor.

The daily experience in working as an EAC has developed a rhythm, and expectations are increasingly high, especially for the motivation to carry out many activities aimed at different levels in order to improve clinical outcomes of patients with autoimmune diseases. Furthermore, this will make it possible to improve our knowledge about the behavior of autoimmune and rheumatic diseases. Therefore, EAC will serve to not only improve clinical care but also do clinical research aimed at evaluating, predicting and preventing autoimmune diseases such as RA.

CHARACTERISTIC	ESTABLISHED RA N=914		RA < 1 YEAR N=94	
DAS 28	First (%)	Last (%)*	First (%)	Last (%)*
Remission	12	36	35.1	58.4
Low disease activity	17	31	19.1	22.5
Moderate disease activity	38	23	29.8	18.0
High disease activity	33	10	16.0	1.1

Table 2. RA activity based on DAS 28 (ESR) at first and last visit. *RA*: rheumatoid arthritis. *CAT*: "Clínica de Artritis Temprana" (Early Arthritis Clinic). DAS 28: Disease Activity Score 28 joints. *ESR*: erythrosedimentation rate. *p<0.01.

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Section IV

Innovative therapies

41

INTRAVENOUS IMMUNOGLOBULIN-CUSTOMIZED THERAPY

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INTRODUCTION

Intravenous immunoglobulin (IVIG) is a biological agent though developed for the therapy of immunodeficiency states, is now utilized for many types of diseases including infections, inflammatory and autoimmune diseases. While IVIG is beneficial in organ-specific diseases, especially of the skin and nervous system, it is also efficient for systemic autoimmune diseases including systemic lupus erythematosus (SLE) and vasculitis. The mechanisms of the positive of IVIG effects are manifold. The realistic goal of such therapy is to normalize the immunoregulatory system that has been compromised.

In this update, we will discuss customizing IVIG therapy for the individual patient with an off-label indication, based on updated relevant clinical and research data. We will relate to the factors to consider before initializing IVIG therapy, and thoughts about further application of a risk assessment score for each patient.

MECHANISMS

The various mechanisms for IVIG in the modulation of the immune system are beyond the scope of this review and are summarized in Table 1 (1). Briefly, IVIG has multiple effects on the innate and adaptive immune systems. Mechanisms of action include Fc-receptor blocking by binding inhibitory Fc receptors (FcgR2b) and activating Fc receptor (FcgR1 and FcgR3), anti-cytokine effects, inhibition of complement activation, complement system regulation, enhanced suppressor activity, down-regulation of B and T cell function, idiotype network regulation, enhanced clearance of endogenous pathogenic autoantibodies, neutralization of autoantibodies, neutralization of superantigens, and reticuloendothelial clearance.The therapeutic effects of IVIG in antibody-mediated diseases such as SLE include direct effects on B cells. IVIG suppresses the expansion of autoreactive B lymphocytes through signaling of the Fcg-RIIB, idiotype-mediated inhibition of B cell receptors, and neutralization of B cell survival factors (BAFF and APRIL). (2).

Recently, it was suggested that restoration of levels of sialic acid-rich IGG by therapy with IVIG can dampen inflammatory effects twofold: by inhibition of Fcgamma RIIB expression and by suppression of effector autoantibody function (3).

Much investigation has been applied to the effect of IVIG on T cell activity. IVIG modulates T regulator (Treg) cells, and cytokine activity directly. Treg regulation is altered following IVIG therapy in SLE patients (4). In addition, transforming growth factor beta (TGFβ) may contribute to the immune regulation of IVIG therapy.

If IVIG has an effect on the T arm of the immune system, then in this sense, it may be beneficial for a group of diseases characterized by a cytokine storm. These diseases include adult onset Still's disease (AOSD) and hemophagocytic syndrome (HPS). In AOSD, IVIG was partially effective in a study of 62 patients (5)

IVIG SOURCES AND EXTRACTION

As a biological agent, scrutiny is of utmost importance to prevent contaminants entering the product which is pooled from 1,000-60,000 donors depending on the manufacturer. IVIG extraction processes are necessary, but may alter the structure of the immunoglobulin and hence its biologic activity. Different methods of extraction may generate an immunoglobulin that is suitable for intramuscular or subcutaneous administration. Fractionation leads to a product that is at least 90% pure IgG. Ion exchange chromatography may further enhance the purity of IgG to 98%. The monomeric immunoglobulin is purported to be similar to natural IgG but the form of IgG may differ among products, as it may be monomeric, dimeric, or multimeric.

MECHANISM	DESCRIPTION
Direct neutralization of pathogenic immunoglobulins	Direct interference with epitope binding sites of antibodies
Fc receptor blockade	Interference with Fc-Fc receptor binding
IVIg dimers	Fc receptor blockade by IVIg dimers results in enhanced affinity due to FcγR's clustering
Sialylated IgG	IVIg rich in α-2,6 sialic acid glycosylation bears most of the anti-inflammatory activity present in IVIg.
B Cell regulation	IgG Fc-mediated downlregulation of B-cells by binding of inhibitory FcγRIIB
T Cell regulation	By expansion and activation of FoxP3+ (forkhead box P3) TRegs.
Macrophage regulation	By upregulation of inhibitory FcγRIIB and down regulation of FcγRIII
Neutrophils and eosinophils regulation	By antibodies against siglec-8 and siglec-9 lectins in the surface of neutrophils and eosinophils.
Dendritic cells regulation	By down regulation of HLA molecules and costimulatory CD80/CD86 blockade resulting in the abrogation of the differentiation and maturation of dendritic cells
Complement activation inhibition	By competitive inhibition of effector complement molecules thus preventing complement activation.
Superantigen neutralization	By binding superantigen epitopes/Vβ regions on the TCR thus preventing direct of polyclonal T cell activation by superantigens.
Increased IgG clearance	Huge IgG load results in decreased half life of IgG (including pathological IgG).
Cytokine production modulation	Modulation of cytokines among them IL-1
Competitive inhibition by soluble products in IVIg	Soluble amounts of CD4, CD8 and HLA molecules in IVIg "dilute" effects of pathological antibodies in cells through their membranal counterparts.
Apoptosis modulation	Apoptosis may be activated or prevented by IVIg.

Table 1. IVIg Mechanisms of action. Adapted from Katz *et al.* (1).

Labile viruses (Hepatitis B and C) are removed by cold ethanol processes. Their transmission has been rarely reported. HIV transmission has never been reported.

IVIG does not contain preservatives because of the potential risk of causing denaturation of the proteins, as well as the large amount of preservatives that would be needed in the product that could be hazardous to the patient. Current purification methods are manifold and utilized to reduce viral and prion contamination (6).

While IVIg does not contain preservatives, many stabilizers are utilized among different IVIG products. The stabilizers include dextrose, sucrose, maltose, glucose, L-proline, D-sorbitol, glycine, albumin and polyethylene glycol (PEG). Trace amounts of detergents or solvents are identified in the final product. Sucrose containing IVIG products in the past were implicated in the development of renal failure as an adverse event to therapy. Since most manufacturers have removed sucrose from their products, renal failure has become a rare serious adverse event.

The osmolarity may vary (192-1250 mOsm/L). IVIG are available in the freeze-dried form or as ready to use standardized liquid formulations that contain 5% or 10% globulin (6).

DOSE AND ROUTE

Intravenous administration is the generally accepted method of therapy. However, subcutaneous treatment with IVIG is an accepted method for the treatment of immunodeficiencies. This route has not been investigated for efficacy or safety in other diseases.

Very low doses of IVIG (0/3-0.6 g/kg once monthly) are usually sufficient for treatment of immunodeficiency (7).

Low-dose IVIG protocols (400 mg/kg once monthly) are beneficial for organ-specific diseases especially neurological disorders. Some open trials have shown beneficial effects for low- moderate dose IVIG therapy for Alzheimer's disease (8).

Low-dose IVIG (500 mg/kg once monthly) was beneficial in a study of 62 patients with SLE. There was some improvement in skin, serositis, and hematological manifestations, but not for involvement of the joints (9).

More often high doses are required for SLE and other autoimmune diseases. A currently accepted protocol is high-dose IVIG protocol (2 g/kg/course divided into 5 days or 400 mg/kg/day for 5 days).

Since no definite guidelines exist, some protocols include 1-3 g/kg/cycle. Furthermore, a cycle may be 3-5 days. In our opinion, 2 g/kg/course divided into 5 days is the protocol that should be universally employed (10, 11).

Caution should be used for overweight patients where 400 mg/kg/day for 5 days may produce protein overload. Hence, we recommend that the maximum dose should not exceed 160 grams or 30 grams per day (1).

Furthermore, the rate of infusion should be monitored. For the first timers, a slow rate should be initialized (0.5-1/0 mg/kg/minute), after 20 minutes increased to 1.5-

2.5 mg/kg/min, and then increased if no adverse events are encountered.

The total time of the daily infusions on average are 5-6 hours. IVIG is to be administered in a hospital (or equivalent) day center where experienced health care providers are present throughout administration (6).

Subcutaneous administration can be done at home. The dose is 0.1g/kg/week. The infusion rate is 10-40 ml/hr, with a maximum of 20-30 ml per treatment (6).

If an adverse event occurs, the infusion rate may be decreased or discontinued according to the judgment of the experienced medical staff.

Our protocol includes premedication prior to the administration of IVIG on the first day only. Premedication includes hydrocortisone 50-200 mg intravenously (10,11).

Prophylaxis with low molecular weight heparin - enoxaprin 1 mg/kg sc as a single injection is indicated for patients with an increased risk of developing a thromboembolic event such as in patients with overlap lupus and antiphospholipid syndrome (personal communication).

ADVERSE EVENTS

Each patient should be screened for potential risk factors for the development of serious adverse events from IVIG therapy. We suggest using Table 2 for risk assessment, and approaching IVIG therapy cautiously if patients have more than 3 risk factors.

Many IVIG preparations are available and hence may be customized to the patient that is at risk for adverse events.

Since the era of acute renal failure secondary to IVIG, sucrose (leading to 90% of cases of renal failure) has been removed from most IVIG products. Acute renal failure has become rare in the past years. Sucrose may cause damage to the tubular epithelium and cause osmotic nephrosis. Renal insufficiency develops rapidly within 1-10 days after initiation of IVIG therapy and leads to oliguric renal failure and increasing creatinine levels within 5 days. In most cases, renal failure is reversible upon discontinuation of therapy. However, 30% of patients may need hemodialysis. Mean renal function recovery time is 10 days. Hence, to date, if a sucrose containing IVIG preparation is utilized, the infusion rate should be slower than indicated for non-sucrose containing agents. Follow-up of renal function is mandatory (6, 12),

Another serious adverse effect is the increased risk of thromboembolic events (TE). The incidence of all TE events following IVIG is reported to be 0.6-3% per patient and 0.15-1.2% per treatment course (13). These are data from 8 years ago at the time when physicians were less aware of risk factors for events. Possible explanatory mechanisms include the increase in plasma viscosity, platelet and endothelial activation, and arterial spasms. A recent large scale study found the rate to be lower. Through a retrospective claims-based cohort study of individuals exposed to immunoglobulin products from 2008- 2010, using HealthCore's Integrated Research Database, a longitudinal health care database, the prevalence of thromboembolic events secondary to IVIG treatment was assessed. Of 11,785 individuals exposed to IG products in the study period, 1% had TE events recorded on the same day as immunoglobulin administration. TE rates per 1000 persons exposed ranged from 6.1 to 20.5 for different IG product groups. An increased TE risk was also found with older age (≥ 45 years), prior TE(s), and hypercoagulable state(s) (14).

The FDA guidelines for the prevention of TE events following IVIG include a restricted concentration of the product (no more than 5% IG), a monitored infusion rate of 0.5 mg/kg/hour and not exceeding 4 mg/kg/hour. A risk-benefit assessment should be done on every patient.

The prevalence of aseptic meningitis is up to 11% and remains an uncommon adverse event. Special considerations are necessary for patients with a history of migraine headaches including a slow infusion (no more than 6 g/hour) and premedication with acetaminophen and antihistamines are warranted. Meningitis may be a result of the formation of macroaggregates, crossing of the blood-brain barrier by the immunoglobulin, or stimulation by antigenic determinants of the endothelial meningeal arteries.

IVIG is contraindicated in patients with IgA deficiency. However, if absolutely necessary, one may choose an IVIG product that has minimal IgA concentration.

Other high-risk patients for IVIG therapy include: patients with renal insufficiency, diabetes mellitus, following exposure to nephrotoxic drugs, hypertension, hypercholesterolemia, and polycythemia vera.

BENEFIT

IVIG is indicated as a first –line therapy in a few instances such as primary immunodeficiencies, Kawasaki's disease, Guillain-Barre syndrome, and idiopathic immune thrombocytopenia (ITP). For other off-label indications, IVIG is particularly beneficial for neurological, cutaneous, and hematological organ-specific diseases. Hence, it is not surprising that IVIG is advantageous for these organ manifestations in other systemic diseases (1).

Score
Disease
Antibody burden
Comorbidities
Renal failure
Chronic heart failure
Prior immunosuppression
Renal disease biopsy proven other than membranous disease
IgA deficiency
Paraproteinemia

Table 2. Risk assessment score for IVIG therapy. More than 3 findings, we recommend that IVIG therapy should be administered cautiously.

IVIG is not a first-line therapy. Rather, IVIG is indicated as adjunct therapy to conventional immunosuppressive treatment, or as a substitution for immunosuppression due to failure of response but more often unacceptable side effects. For example, the lupus afflicted population is predominantly young, female, and in the reproductive phase of life. These women may often refuse conservative therapy including steroids and immunosuppression due to the disfiguring side effects of steroids including obesity, hirsutism, acne, moon facies and emotional lability, or possible infertility due to side effects of potent immunosuppression. In addition, SLE patients are more prone to develop infections, either due to the inherent diseases or secondary to immunosuppression. In these cases, IVIG may have an extra advantage. Furthermore, IVIG has a steroid-sparing effect. This is important because often either high doses of steroids are necessary for therapy, or patients develop unacceptable side effects from prolonged steroid consumption (10).

Although no randomized controlled trials were performed for SLE, there is evidence through some open trials and many case reports in over 100 patients that IVIG is beneficial. In most cases, IVIG was given by the high dose protocol (11,15). There is some evidence that low-dose protocol may be beneficial for mild lupus (9).

IVIG is useful for many manifestations of SLE including cutaneous, joint, serositis, hematological, diffuse neuropsychiatric, and salvage for certain types of lupus nephritis. In addition, IVIG is beneficial for patients with catastrophic antiphospholipid syndrome (16).

The protocol for lupus patients at our center includes high-dose IVIG 2g/kg/course divided over 5 days, monthly, for 6 months. Some patients had a remarkable recovery and needed fewer courses (patients with myocarditis). Some patients continued therapy with IVIG every 2-3 months for up to 5 years. Long-term benefit was maintained and no serious side effects were discovered upon long-term use (11). Most of the patients had diffuse neuropsychiatric diseases with features of cognitive impairment or mood swings. Cognitive impairment was diagnosed based on the diagnosis of SLE by the ACR criteria, active SLE disease at least serologically and with inflammatory markers (elevated ESR), neuropsychological testing, and MRI and PET scan. Because the neuropsychiatric manifestations were not life-threatening, the patients were successfully treated with IVIG (personal communication).

Controversy exists upon whether IVIG is an effective agent for Alzheimer's disease based on the finding of naturally occurring antibodies to Aβ amyloid in the product. While there was no a significant change in antibody titers, there was an improvement in cognitive function based on the mini-mental score (8). Recently, a phase II study on Alzheimer patients enrolled to receive different doses and intervals of IVIG did not find an advantage to 6 month therapy. The treatment protocols were safe, but did not show significant benefit. Factors to consider are that a larger population is required for further studies, a longer exposure is needed, IVIG administration for early mild disease, differentiation between familial genetic, vascular, and other types of Alzheimer's disease, and high dose IVIG protocol should be assessed (17). Lastly, perhaps, specific anti- Aβ amyloid IVIG therapy should be considered.

Many other off-label indications exist for organ-specific and systemic autoimmune diseases. This topic is reviewed extensively elsewhere (1,18).

Worldwide, guidelines are being established for the indications of IVIG therapy in autoimmune diseases.

Some of the diseases being evaluated by the European CEDIT for possible IVIG indications include neuromuscular disease including: inclusion body myositis, non multiple sclerosis demyelinating central nervous system disease, cortico-resistant polymyositis, and autoimmune encephalitis. Other diseases include hemolytic anemia, antiphospholipid syndrome, AOSD, ANCA associated vasculitis, and pemphigus vulgaris. Similar guidelines are being established in the United Kingdom, Canada, and Australia (19).

The National Advisory Committee on Blood and Blood Products of Canada (NAC) and Canadian Blood Services convened a panel of national experts to develop an evidence-based practice guideline on the use of IVIG for hematologic conditions. Specific recommendations for routine use of IVIG were made for 7 conditions: 1) acquired red cell aplasia, 2) acquired hypogammaglobulinemia (secondary to malignancy), 3) fetal-neonatal alloimmune thrombocytopenia, 4) hemolytic disease of the newborn, 5) HIV-associated thrombocytopenia, 6) ITP, and 7) post-transfusion purpura. Intravenous immune globulin was not recommended for use, except under certain life-threatening circumstances, for 8 conditions including acquired hemophilia; acquired von Willebrand disease, autoimmune hemolytic anemia, autoimmune neutropenia, hemolytic transfusion reaction, hemolytic transfusion reaction associated with sickle cell disease, hemolytic uremic syndrome/thrombotic thrombocytopenic purpura, and viral-associated hemophagocytic syndrome (20).

The question remains if randomized controlled trials (RCTs) are necessary to prove efficacy and safety for these rare autoimmune diseases. Furthermore, it will be even more difficult to assess the benefit of IVIG when evaluated for specific manifestations of the systemic autoimmune disease in such rigid trials. We suggest that open trials or case series for explicit manifestations of systemic autoimmune diseases are suitable for the evaluation of the efficacy of IVIG.

The RCT and open trials for ANCA associated vasculitis emphasized the need for a standard protocol. Without standard protocols, it will be difficult to evaluate response and safety. Proper protocols should include the total dose, over which period, repeated course after which time period, total length of protocol (how many months). One such consensus exists for the autoimmune mucocutaneous blistering diseases (AMBD). Their consensus statement suggests the use of high-dose IVIG (2 g/kg/cycle over 5 days), given monthly until clinical control with a progressive increase in intervals between cycles thereafter, to 6, 8,10, 12, and 14 weeks. The last cycle is given after a 16 week interval, and is

considered the end of therapy. Using this protocol, patients with AMBD responded well, and other immunosuppressive drugs including steroids were gradually discontinued. After the discontinuation of the IVIG therapy, patients had sustained clinical and serologic remission for a 2 year follow-up (19). Our protocol is similar, but we give IVIG at fixed intervals, initially monthly, thereafter every 2-3 months (11).

To date, the treatment of AOSD has been largely empirical. A study was conducted to investigate the response to therapy and prognostic factors of AOSD in 54 Korean patients. Forty-two patients were treated with non-steroidal anti-inflammatory drugs. However, they also needed corticosteroids and IVIG. Among 42% of patients resistant to corticosteroids, add-on therapy with IVIG or anti-tumor necrosis factor (TNF) agents was administered. Of the 23 patients medicated with IVIG, the prognosis was better in IVIG-responsive patients, indicating a therapeutic effect (5). In AOSD patients that developed HPS, a high dose IVIG protocol (2 gr/kg/2-5 days) was beneficial (21).

In another study on secondary HPS patients, the beneficial effects of IVIG are controversial and may depend on the cause. In those patients with severe infections/sepsis as the casue of HPS, IVIG therapy was not beneficial (23); however, the IVIG dose and protocol used were not mentioned. In another study, HPS secondary to autoimmune disease in a pediatric population treated with high dose IVIG (1 gr/kg/day for 2 days) together with steroids or other immunosuppression showed to be beneficial in some cases (24). Although not many patients have received IVIG for HPS, one should consider its utilization if the HPS is secondary to autoimmune disease or AOSD.

Evidence suggests that IVIG may be beneficial for transplantation patients with preformed anti-HLA antibodies (who are at increased risk for acute rejection) and for HLA sensitized cardiac and renal allograft recipients.

Recently, the concept of dual therapy with IVIG and rituximab has been suggested. In a study of the beneficial effects of IVIG for ANCA positive vasculitis, patients were grouped by the level of response to IVIG therapy. While many were good responders, some were partial or non-responders. Those with partial response received IVIG together with rituximab and complete response was achieved. Those that needed some further treatment following cessation of this regimen could be successfully treated with mild immunosuppression including low dose glucocorticoids.

Similar results were reported for patients with AMBD, a severe form of autoimmune skin disease. Rituximab was added to the non-responder group. Remission was achieved and the combination therapy did not result in increased adverse events (20). These studies suggest that IVIG together with a B cell depletory agent is advantageous.

SPECIFIC IVIG FOR DIFFERENT DISEASES

IVIG may be beneficial for patients with sepsis. Although the studies were not performed in patients with autoimmune diseases, IVIG should be strongly considered in the patients with sepsis and possibly an add-on treatment to standard fluid resuscitation and antibiotic therapy.

Patients with sepsis are a subgroup that may benefit from specific high dose IVIG which is IgM and IgA enriched. The use of polyclonal IVIG in septic patients was beneficial and led to a major reduction in mortality (21).

Specific autoimmune diseases could possibly be treated with antibody restricted IVIG. In murine experimental models, IVIG enriched with a specific antibody that would counteract the pathological antibody responsible for disease could be effective. The rationale would be to produce an antibody enriched concentrate that would be very potent, specific, and reduce the amount of IVIG introduced, and hence possibly reduce the risk for adverse events. The specific IVIG protocol has been prepared for SLE and vasculitis murine models and found to be beneficial. The specific IVIG when compared to the regular IVIG was 200 times more potent in preventing disease (24-27).

IVIG, though a biological agent, is not more expensive than other available biological agents. The cost on average is 75$/gram but may vary among countries (28).

It is yet to be determined if specific IVIG is feasible clinically and economically for patients, but could be an excellent example for tailored therapy in patients with rare diseases.

CONCLUSIONS

IVIG is a biological agent with good efficacy and safety record over the years. While it is formally indicated for only a few diseases, it has been used for therapy for many off-label indications. Guidelines are being assessed for IVIG therapy among the different specialties. IVIG products are different and hence have the advantage that they can be customized therapy according to the patient's comorbidities. A standard dose and duration protocol should be agreed upon so that the beneficial effect can be properly assessed. RCTs will probably not occur, and we need to depend on case series or open trials. Before considering treatment with IVIG, a risk assessment checklist should be done. No formal checklists exist, but we recommend establishing such a checklist so as to prevent serious adverse events. Some evidence is arising that IVIG can be effective and safely administered with a B cell depletion biological agent such as rituximab. This trend still needs to be evaluated.

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42

AUTOLOGOUS PERIPHERAL HEMATOPOIETIC STEM CELL TRANSPLANTATION

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INTRODUCTION

Autoimmunity is an inappropriate response of the immune system directed against self-tissues. Autoimmune disease is defined as a clinical syndrome caused by the activation of T cells and/or B cells in the absence of an ongoing infection or other discernible cause (1). The concept of "autoimmune disease" includes a wide spectrum of pathologies that vary in biological and clinical features.

Autoimmune diseases (ADs) are among the most prevalent diseases in the United States, affecting approximately 7% of the population (2). ADs disproportionally occur more in women. In some conditions, 85% or more of the patients are female (3). The knowledge of the etiology of ADs remains limited. Epidemiologic studies have shown that genetic factors are major determinants of susceptibility to ADs (4, 5). However, a trigger like an environmental exposure is needed to initiate frank autoreactivity in a genetically predisposed individual (2, 6).

The majority of patients achieve short-term disease control with immunosuppressive therapy including biological therapies, but long-term remission or a definitive cure remains unattainable (7). There is a group of patients with refractory and life-threatening states for whom the results of conventional therapy have been considered unsatisfactory. In this context, hematopoietic stem cell transplantation (HSCT) has emerged as an alternative therapy for severe and refractory ADs.

HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation has been the standard therapy for several oncological and hematological disorders since the early 70's (8). The use of HSCT for ADs evolved in animal models, where experimental investigations showed that inherited and induced autoimmune disease (AD) in laboratory animals could be cured by stem cell transplantation (9, 10). The possibility of HSCT as a treatment for ADs was reinforced by reports of patients with coincident ADs and hematological malignancy who remained in long-term remission after allogeneic transplantation (11). The first hematopoietic stem cell transplants performed specifically for ADs were done in the 90's. In 1996, the European League Against Rheumatism (EULAR) and the European Group for Blood and Marrow Transplantation (EBMT) supported the utilization of HSCT in patients with ADs as an experimental procedure. In their consensus, it was established that methods had to follow a standardized protocol, and all the patients should be reported to the EBMT–EULAR Autoimmune Disease Data Registry (12). At present, it is estimated that around 3,000 AD patients have been treated by HSTC (7).

TYPES OF HSCT

Hematopoietic stem cell transplantation can be divided into subtypes based on the relationship between the patient and the donor and by the source of the stem cells. The subtype of HSTC influences the short and long-term results of the procedure. Based on the origin of the transplanted cells, HSCT may be:

Autologous transplantation: The progenitor cells are collected from the patient's own tissues. Then, they are processed, stored, and subsequently transfused into the patient after the conditioning regimen.

Syngeneic transplantation: The couple-donor is an identical twin. The main advantages of this type of HSCT are that it provides a graft free of autoreactive cells and the minimal risk of graft-versus-host disease (GVHD).

Allogeneic transplantation: The progenitor cells are col-

lected from a donor who is not genetically identical. The graft is free of autoreactive cells, but the risk of complications, e.g., GVHD, is significantly increased.

The anatomic source of the progenitor cells may be: 1) peripheral blood – the hematopoietic cells are collected using growth factors, 2) bone marrow – the hematopoietic cells are usually harvested directly from the pelvic bones, 3) umbilical cord blood – unrelated umbilical cord blood is administrated in single or double units. It is associated with a lower risk of GVHD (13). Autologous HSCT (AHSCT) is currently the most frequent transplant procedure worldwide (7).

AUTOLOGOUS PERIPHERAL HEMATOPOIETIC STEM CELL TRANSPLANTATION (AHSCT)

The transplant procedure consists of: 1) stem cell collection, and 2) transplantation phase: conditioning regimen, aplastic phase, and engraftment. In autologous transplantation, hematopoietic stem cells can be obtained from the patient's bone marrow or peripheral blood. Peripheral blood stem cells (PBSC) are the main source for AHSCT due to the ease of procurement and the fact that engraftment is faster and more stable (7, 14). The main steps of AHSCT in ADs are shown in Figure 1.

STEM CELL COLLECTION

Peripheral blood stem cells are prepared by administration of growth factors such as granulocyte colony stimulating factors (G-CSF) and chemotherapy. This process is called mobilization. The drug that is used the most as mobilization chemotherapy in AD is cyclophosphamide (CYC). The recommended mobilization regimen is CYC at 2–4 g/m^2 with MESNA (2-Mercaptoethanesulfonic Acid Sodium Salt) and cautious hyperhydration followed by G-CSF 5–10 microgram (mcg)/kg (7). Aside from steroid therapy, additional immunosuppressive medications should be discontinued if possible before mobilization (7). Once the mobilization regimen is administrated, PBSC are collected by 1 – 3 leukapheresis for storage and subsequently given to the patient by transfusion.

Currently, the use of lymphocyte depletion of autologous PBSC has been implemented. However, the results are controversial and, for the present, the evidence is insufficient to support the routine use of graft manipulation (15).

CONDITIONING REGIMENS

Ablation of the immune system is the goal of the conditioning regimen. Immunoablative conditioning regimens are preferred over their myeloablative counterparts, and some form of in vivo and/or ex vivo T-cell depletion is generally adopted (16, 17).

Conditioning regimens are divided into: a) high intensity – includes regimens based on total body irradiation (TBI) or high-doses of busulphan; b) intermediate intensity - includes combinations such as BEAM (bischloroethylnitrosourea (BCNU), etoposide, cytarabine (Ara-C), and melphalan) and the combined use of antithymocyte globulin (ATG) with high-dose CYC or other chemotherapy; and, c) low intensity – consists of CYC alone, melphalan alone, or fludarabine-based regimens (18, 19). Intermediate intensity conditioning regimens have been associated with significantly improved outcomes compared to the other regimen types since they achieve a balance between efficacy and safety (20).

In its most recent guidelines, the EBMT has recommended using an intermediate regimen in patients who are being treated under the category "clinical option (CO)" (See Table 1) (7). The following recommended conditioning regimens have a balance between safety and efficacy:

Cyclophosphamide 200 mg/kg with polyclonal or monoclonal anti-T-cell serotherapy (ATG or alemtuzumab respectively). In pediatrics, the recommended combination is CYC 120 mg/kg, fludarabine 150 mg/kg, and ATG (or other anti-T-cell serotherapy).

For multiple sclerosis (MS) specifically, BEAM regimen (BCNU 300 mg/m^2 on day -6, etoposide 200 mg/m^2 on day -5 to -2, Ara-C 200 mg/m^2, and melphalan 140 mg/m^2 on day -1) and ATG (or other anti-T cell serotherapy) are recommended. BEAM conditioning has been found to be attractive due to (a) the positive lympholytic effect and (b) the possibility of BCNU and Ara-C exerting their effect across the blood–brain barrier (21).

The use of TBI in AD has been done less frequently in the recent years but has shown good results in the treatment of juvenile idiopathic arthritis (JIA) (22).

APLASTIC PHASE AND ENGRAFTMENT

The aplastic phase lasts approximately 10 – 15 days. Transplantation of more than 2.5 x 10^6 CD34+ cells per kilogram leads to rapid and sustained engraftment. Three consecutive days with a neutrophil count of over 500 cell/mm^3 and platelets over 20,000/mm^3 are required to define the engraftment (23). A rapid recovery diminishes the morbidity rate of HSCT.

CANDIDATE SELECTION

Autologous hematopoietic stem cell transplantation has become an emerging therapy for patients with severe or rapidly progressive AD refractory to conventional therapy (20). The selection criteria should be individualized based on the type of AD and the medical status of the patient. A balance between the risks of toxicity and the clinical benefits should be pursued (Table 1). The decision should be made by an appropriate inter-disciplinary team that includes a hematologist and AD specialist or a rheumatologist.

Pre-transplant evaluation includes a detailed history, physical examination, laboratory studies, imaging tests, and disease-specific studies (24). The detailed history should emphasize the presence of co-morbidities, prior therapies, in-

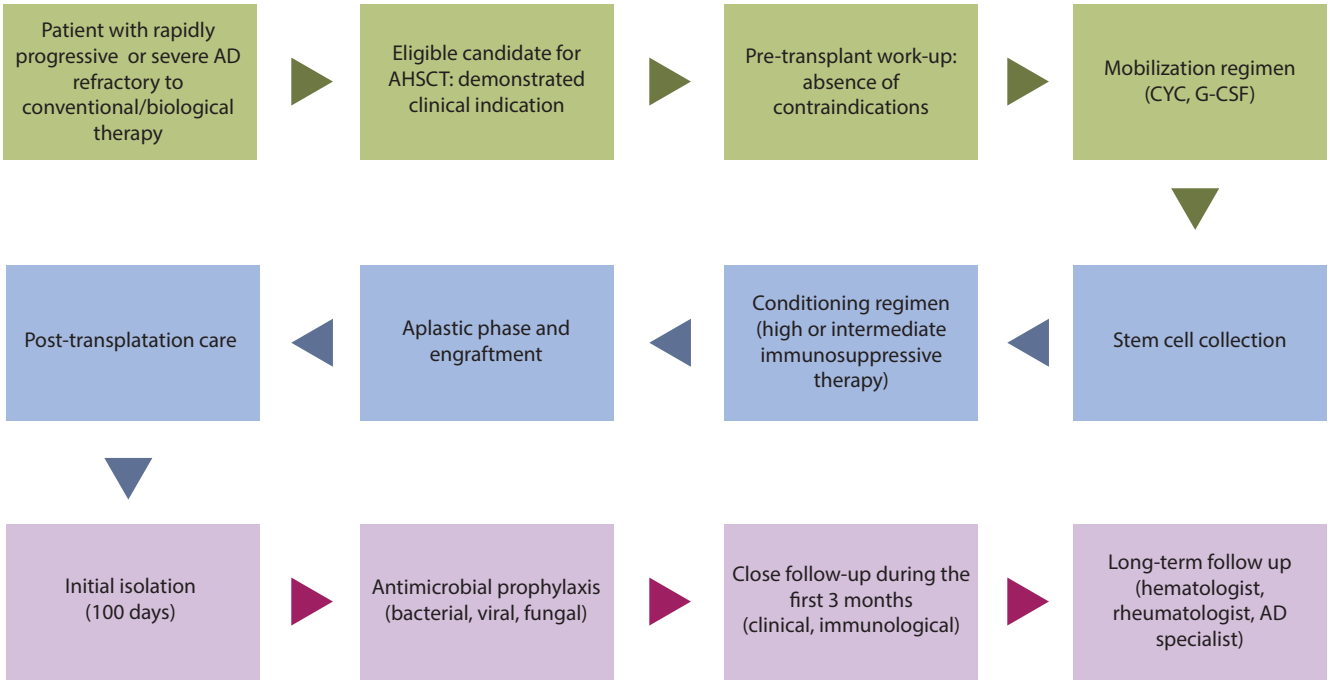

Figure 1. Steps of AHSCT in autoimmune diseases. *AD*: autoimmune disease *AHSCT*: autologous hematopoietic stem cell transplantation. *CYC*: cyclophosphamide. *G-CSF*: granulocyte colony stimulating factors.

fections, and drug allergies. Physical examination should give special attention to evaluation of heart, lung, kidney, and gastrointestinal function. Laboratory studies and imaging tests complement the evaluation of the mentioned organs. Tests in addition to screening for the presence of infections and pregnancy at the moment of transplantation should also be done (24). Table 2 summarizes the pre-transplant work-up.

The most updated guidelines of the European Group for Blood and Marrow Transplantation recommend the following exclusion criteria when considering HSCT (7):

Organ dysfunction: 1) Advanced cardiac disease, defined as left ventricular ejection fraction < 50% in patients with systemic sclerosis (SSc), < 40% in other indications, ventricular arrhythmias or pericardial effusions > 1 cm; 2) renal insufficiency with a creatinine clearance < 40 ml/m^2 in SSc or < 30 ml/m^2 in other indications; 3) respiratory disease – diffusing ability of the lung for carbon monoxide (DLCO) < 40% predicted, mean pulmonary artery pressure > 50 mmHg or ventilator impairment due to respiratory muscle involvement in multiple sclerosis; and, 4) gastrointestinal bleeding.

Uncontrolled infection: any uncontrolled acute or chronic infection is considered a contraindication.

Pregnancy: it should be evaluated 7 days prior to mobilization regimen administration.

POST-TRANSPLANT CARE

After the procedure, the patient should be placed in an isolation facility under the Joint Accreditation Committee-ISCT (International Stem Cell Therapy) & EBMT (JACIE) accreditation standards (25, 26). Patients with AD and HSCT have a significant risk of developing infections due to profound immunosuppression (20). Therefore, antimicrobial prophylaxis is mandatory in all the patients.

Antimicrobial prophylaxis consists of broad-spectrum antibiotics and antifungal and antiviral prophylaxis. The patients should also receive trimethoprim/sulfamethoxazole 3 times per week for prophylaxis against ***Pneumocystis jiroveci*** and ***Toxoplasma gondii***. Antimicrobial therapy is administered for at least 100 days post-transplant. Cytomegalovirus (CMV) disease and Epstein - Barr Virus (EBV) –associated post-transplant lymphoproliferative disorder have been commonly associated with HSCT (27, 28).

Recipients should attend follow-up clinical sessions frequently during the first 3 - 4 months post-transplant or until the recipient is clinically stable. The development of autoimmunity after transplantation is a recognized phenomenon that has to be differentiated from relapse, toxicity, infection, and GVHD (29). Subsequently, the long-term management remains under the supervision of the transplant specialist and AD specialist/rheumatologist. An annual follow-up appointment is recommended as a minimum (7).

RATIONAL USE AND THERAPEUTIC MECHANISMS OF HSCT IN AD

The rationale for HSCT in ADs is the ablation ability of the self-reactive immune system by using chemotherapy and

DISEASE	SIBLING DONOR	E	WELL-MATCHED UNRELATED*	E	AUTOLOGOUS	E
Adults						
MS	D	III	GNR	III	CO	II
SSc	D	III	GNR	III	CO	II
SLE	D	III	GNR	III	CO	II
Crohn´s	GNR	III	GNR	III	CO	II
RA	GNR	III	GNR	III	CO	II
Vasculitis	GNR	III	GNR	III	CO	II
PM/DM	GNR	III	GNR	III	CO	II
CIPD	GNR	III	GNR	III	CO	II
NMO	GNR	III	GNR	III	CO	II
Cytopenia	CO	III	D	III	CO	II
T1D	GNR	III	GNR	III	D	III
RCD II	GNR	III	GNR	III	D	III
Pediatrics						
JIA	D	III	GNR	III	CO	II
JSSc	D	III	GNR	III	CO	II
JSLE	D	III	GNR	III	CO	II
Crohn´s	GNR	III	GNR	III	CO	II
Vasculitis	GNR	III	GNR	III	CO	II
PM/DM-	GNR	III	GNR	III	CO	II
Cytopenia	CO	II	CO	II	CO	II
T1D	GNR	III	GNR	III	D	III

Table 1. EBMT indications, grade of recommendations and evidence for HSCT in AD for adults and pediatrics. Abbreviations: *EBMT*= European Group for Blood and Marrow Transplantation; *CIPD*= chronic inflammatory demyelinating polyradiculoneuropathy; *JIA*=juvenile idiopathic arthritis; *JSLE*= juvenile systemic lupus erythematosus; *JSSc*= juvenile systemic sclerosis; *MS*= multiple sclerosis; *NMO*= neuromyelitis optica; *T1D*= type 1 diabetes; RA= rheumatoid arthritis; *RCD* II= refractory type II celiac disease; *SSc*= systemic sclerosis; *SLE*= systemic lupus erythematosus, *PM/DM*= Polymyositis/dermatomyositis. *A well-matched unrelated donor is defined as a 9/10 or 10/10 identical donor based on HLA high-resolution typing for class I (HLA-A, HLA-B, HLA-C) and II (HLA-DRB1, DQB1). EBMT grades of evidence: *I*= evidence from at least one well-executed randomized trial; *II*= evidence from at least one well-designed clinical trial without randomization: cohort or case-controlled analytical studies (preferably from more than one center), multiple time-series studies: or dramatic results from uncontrolled experiments; *III*= evidence from opinions of respected authorities based on clinical experience, descriptive studies or reports from expert committee. EBMT grades of recommendation: *CO*= Clinical Option: can be carried out after careful assessment of risks and benefits; *D*= developmental; *GNR*= generally not recommended; *S*= standard: generally indicated in suitable patients; *E* = grades of evidence. Adapted from reference (7).

ORGANS/CONDITIONS	TESTING STUDIES
Detailed history and physical examination	Emphasis in the presence of co-morbidities, potential complications: prior therapies, infections and drug allergies. Evaluation of heart, lung, kidney and gastrointestinal function
Heart	Electrocardiogram Echocardiogram (LVEF; PAP in mmHg). Especially in SSc
Lung	Chest X-ray, DLCO
Kidney	Creatinine clearance, uroanalysis
Immunological tests	Disease specific
Infections	Serology for EBV, CMV, HIV
Pregnancy	Blood based β-HCG assay
Previous/current immunosuppressive therapy	Medications checklist Aside of steroids, immunosuppressants should be withdrawn if possible
Prophylaxis	Antimicrobial, antifungal, antiviral

Table 2. Pre-transplant work-up. *LVEF*: left ventricular ejection fraction. *PAP*: mean pulmonary artery pressure. *SSc*: systemic sclerosis. *DLCO*: diffusing capacity of the lung for carbon monoxide. *HCG*: human chorionic gonadotrophin *EBV*: Epstein - Barr Virus. *CMV*: cytomegalovirus. *HIV*: human immunodeficiency virus.

then, regeneration of a new self-tolerant immune system from hematopoietic stem cells (HSCs).

Hematopoietic stem cells have the potential to stop ADs by resetting the immune system, establishing a new immune repertoire, replacing the existing one, and achieving a re-instatement of immune regulation (30).

With respect to the concept of resetting the immune balance, 3 main mechanisms for immune resetting have been identified: 1) influx of naïve cells from the thymus; 2) debulking the mature memory lymphocyte repertoire; and, 3) boosting the number of regulatory cells.

Hematopoietic stem cells represent the best characterized type of adult stem cells. They reside in the bone marrow and generate progenitors that become progressively restricted to different lineages (31). HSCs are a group of cells with several developmental potentials based on intrinsic networks driven by transcription factors and input from the local microenvironment in which they reside. HSCs have two defining properties: 1) capacity for self-renewal, and 2) ability to differentiate into mature blood cell lineages (see Table 3).

Hematopoietic stem cells are defined functionally by their ability to reconstitute the entire blood system of a recipient. HSCs and hematopoietic stem progenitor cells (HSPCs) in humans are enriched within the subset of CD34+ cells. CD34 is a type 1 transmembrane protein expressed on HSPCs with the ability to repopulate bone marrow for all lineages. In the bone marrow, HSPCs preferentially reside in two microenvironments: in association with osteoblasts near the trabecular bone and adjacent to blood vessels (32). HSPCs exist in a relatively quiescent state in the bone marrow microenvironment but can be activated to enter the cell cycle and thus drive hematopoiesis as physiological demands dictate (33). Autologous hematopoietic cell transplantation has been evaluated as a treatment for severe forms of immune-mediated disorders including multiple sclerosis (MS), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and rheumatoid arthritis (RA) or juvenile idiopathic arthritis (JIA). Current concepts of the pathogenesis of autoimmune disorders attribute a crucial role to T and B cells inappropriately recognizing self-antigens and initiating a cell-mediated or humoral reaction, or both, resulting in inflammatory tissue and vascular damage. Autoimmune diseases represent a failure of normal immune regulatory processes as they are characterized by activation and expansion of immune cell subsets in response to non-pathogenic stimuli. Autoimmune diseases result from a failure of normal mechanisms to suppress proliferation in the presence of innocuous or self-antigens (34).

Studies on immune reconstitution following autologous transplant for both ADs and cancer showed a profound lymphopenia in the first year after transplantation. The cytopenia was observed to affect the lymphocyte subsets differently. It is likely that the kinetics of reconstitution depended on different timing of recovery for each cell type. B cells, natural killer (NK) cells, and CD8+ T cells display a rapid and complete reconstitution to pretransplantation levels, whereas the recovery of CD4+ T cells has consistently been observed to be delayed and often incomplete. By extending longitudinal follow-up of patients, recent studies have shown a recovery of the number of CD4+ T cells after a 2-year follow-up in young adults treated for MS (35) and RA (36) and after 12 months in children with JIA (37).

The observation that quantitative recovery of lymphocytes was not correlated to inflammatory activity or disease relapse revealed that numeric immune deficit is an insufficient explanation for a prolonged absence of autoimmune disease activity after AHSCT. A detailed analysis of the T cell receptor (TCR) repertoire showed the regeneration of a different and more diverse TCR repertoire posttransplant. Thymic reactivation, expansion of naïve T cells following autografting, and improved repertoire diversity were subsequently demonstrated in individuals with SLE (30).

It is reasonable to postulate that the normalization of immune regulatory mechanisms could play a role in the suppression of autoimmunity following AHSCT. The CD4+ CD25+ expressing forkhead transcription factor 3 (FoxP3) cells are potent suppressors of immune responses, which are generated in the thymus in both rodents (38) and humans (39). CD25+ FoxP3+ CD4+ T cells were reported to be more resistant to irradiation than effector cells and mediated the amelioration of experimental graft-versus-host disease (GVHD) (40). In experimental autoimmune encephalomyelitis (EAE) rats, there was an increase of CD4+ CD25+ T cells after syngeneic BMT and this was seen in connection with attenuation of active disease and protection from induction of relapses. Longitudinal enumeration of CD4+ CD25+ T cells in children with JIA, was studied following AHSCT, showing recovery of the pretreatment frequency at 6 months post-transplant and a continued increase for the remaining 12-month follow-up. Their frequency were correlated directly with clinical remission (41).

Therefore, re-instatement of immune regulation could be involved in long-term post-transplant tolerance.

In post-transplant patients, both CD4+ CD25+ FoxP3+ and an unusual CD8+ FoxP3+ Treg subset return to levels

Ablation of self-reactive immune system
Suppression of the maximum number of aberrant autoreactive cells
Regeneration of a new self-tolerant immune system from HSCs
Achieving a post-transplant remission state, or at least, return to a state disease more treatable with conventional therapy

Table 3. Objectives of HSCT in autoimmune diseases.

seen in normal subjects. This is accompanied by almost complete inhibition of pathogenic T cell response to critical peptide autoepitopes from histones in nucleosomes, the major lupus autoantigen from apoptotic cells. Therefore, unlike conventional drug therapy, HSCT generates a newly differentiated population of LAP+ CD103+ CD8TGF-b Treg cells, which repairs the Treg deficiency in human lupus to keep patients in true immunological remission (42). Likewise, responders exhibited normalization of the previously disturbed B-cell homeostasis with numeric recovery of the naïve B-cell compartment within 1 year after AHSCT. These data are the first to demonstrate that both depletion of the autoreactive immunologic memory and a profound resetting of the adaptive immune system are required to reestablish self-tolerance in SLE (30).

PRECLINICAL DATA

Preclinical data in HSCT are derived from animal models of ADs. Transplant studies in animals with ADs are divided in genetically determined and inducible models. While mice or rats with lupus-like syndrome, transgenic HLA-B27 expression, nonobese diabetes (NOD), and interleukin-1 receptor antagonist (IL-1Ra) deficiency belong to the first category, those with collagen-induced arthritis or EAE as models of RA and MS respectively belong to the second category. Different results were obtained in these models (34). Conditioning followed by syngeneic HSCT resulted in the cure of induced AD, but not of genetically determined AD. In autologous HSCT, and to a lesser extent in allogeneic HSCT, the outcome depended on the stage of the disease at the time of transplant. In inducible disease models, protective as well as therapeutic effects of HSCT were observed: both syngeneic and allogeneic HSCTs in EAE-susceptible mice protected animals from disease when carried out close to immunization, but only allogeneic HSCT with high-grade chimerism was effective in protecting from EAE when the time lag was longer. In another EAE study, HSCT prevented glial scarring and ameliorated disease severity after immunization but was ineffective as a treatment of established disease (43). In established genetic AD such as in lupus-prone mice, allogeneic, but not syngeneic, HSCT reversed both acute and chronic symptoms (44). In the early animal HSCT studies, myeloablative conditioning was employed prior to allogeneic HSCT to achieve full donor chimerism and eradicate autoreactive lymphocytes. More recent studies, however, have shown that nonmyeloablative conditioning is equally effective in inducing stable chimerism while maintaining efficacy (45). It is of note that no GVHD was observed and thus indicates that the putative graft-versus-autoimmunity effect and GVHD are dissociated. Whereas full donor chimerism was needed in the SLE and EAE models, the induction of mixed chimerism was sufficient to ameliorate chronic inflammatory arthritis in IL-1Ra-deficient mice (44, 46). In the latter, no significant relationship between the arthritis score and the ratio of donor to recipient cell populations in mice with mixed chimerism could be found after allogeneic HSCT.

In collagen-induced arthritis, nonmyeloablative conditioning followed by both syngeneic and allogeneic HSCT (the latter yielding a stable donor chimerism over 95%) had a significant therapeutic effect compared with conditioning alone (47) . In this study, allogeneic HSCT was more effective than syngeneic HSCT in suppressing pathogenic autoantibodies. In HLA-B27 transgenic rats, TBI followed by HSCT from nontransgenic mice led to a prompt and sustained remission of symptoms. In contrast, all rats who received a syngeneic transplant died from exacerbation of colitis (48).

The heterogeneity of results obtained in different transplant settings and disease models implies that extrapolation to the clinical setting in human AD is difficult. Nevertheless, the data suggest that HSCT may be more effective (and probably less toxic) in patients with active progressive disease than end-stage advanced disease (49).

CLINICAL DATA

Autologous HSCT is the most widely used form of HSCT. In hemato-oncological conditions, it is a relatively safe procedure with a transplant-related mortality (TRM) that is typically below 3%. Toxicities and transplant-related causes of death include sepsis, cytomegalovirus infection, and hemorrhage. The overall TRM for autologous HSCT in AD now is approximately 7% although it was as high as 23% in one of the first pilot studies (50). In AD, diagnosis, extent of organ involvement, age, and comorbidity are patient-related determinants of toxicity and TRM. TRM and toxicity also depend on the conditioning regimen and whether or not TBI is done. With adaptation of eligibility criteria (e.g. exclusion of patients with severe pulmonary hypertension) and modification of transplant regimens (e.g. lung shielding with TBI), complications from HSCT can usually be managed in experienced hands, and TRM has dropped as a consequence. It was less than 1% for non-TBI nonmyeloablative, less than 2% for low-intensity myeloablative, and 13% for high-intensity myeloablative regimens (51). Compared to TRM, efficacy seems less influenced by intensity and type of conditioning although this may be confounded by the severity of the underlying disease.

INDICATIONS FOR AUTOLOGOUS HSCT IN ADs

Immunoablative therapy followed by HSCT has evolved from an experimental treatment to a salvage therapy for patients with severe ADs not responding to proven conventional therapy and/or biologicals.

Autologous HSCT has been evaluated as a treatment for severe forms of immune-mediated disorders including multiple sclerosis (MS), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), rheumatoid arthritis (RA), or juvenile idiopathic arthritis (JIA). The goal of this therapy is

to induce medication-free remission of disease activity by correcting the immune aberrations that promote the attack against self-tissue ("immune repair"), (see Table 1).

RESULTS OF HSCT IN ADs

The success of HSCT has been measured in many ways. Clinical endpoints include the evaluation of not only the efficacy but also the safety of the procedure. Disease-free survival, sustained clinical remission, progression-free survival (PFS), overall survival, and TRM are the most common examples of clinical endpoints. Better percentages of response to HSCT have been achieved over time. Treatment-related morbidity and mortality have improved due to better patient selection and modifications of transplant regimens. However, disease specific measurements of activity or quality of life should be clearly defined for each AD.

MULTIPLE SCLEROSIS (MS)

The objective of AHSCT for MS is to reduce inflammation and progression of the disease for a prolonged period of time (52). Multiple sclerosis is the main indication of HSCT in ADs. There are reported benefits from HSCT in clinical disease activity and magnetic resonance imaging-detectable inflammation (53), stabilization of disease, and immunological response to therapy. Safety profiles of HSCT in MS are improving since myeloablative regimens have been changed to less intense immunoablative regimens (54).

The use of intense immune suppression by a TBI-based regimen and HSCT is not effective for patients with progressive MS and high pretransplantation disability scores (55). Dissociation of inflammation parameters and functional disability findings have been demonstrated. This raises questions regarding the future use of HSCT as an optimal strategy for this disease (56). However, the rate of brain tissue loss in patients with MS declines dramatically for the first 2 years after HSCT (57). The results of HSCT in MS are summarized in Table 4 (18, 54, 58-68).

SYSTEMIC SCLEROSIS (SSc)

Systemic sclerosis has a higher mortality when vital organs are affected. No treatment has been shown to influence the outcome or significantly improve the skin score though many forms of immunosuppression have been used. The developments in HSCT have allowed the use of profound immunosuppression followed by transplants in SSc. In trials, the predicted procedure-related mortality has been less than 10% since the elimination of TBI (69). Improvement of skin sclerosis has been observed for the first 3 years in most of the recipients. Autologous HSCT using purified CD34+ cells was effective in controlling the activity in SSc. The Th1/Th2 ratio was significantly increased for at least 3 years after AHSTC (70). In another trial, the population of B and T lymphocytes remained disturbed for at least 1 year post-transplant in SSc patients. This may reflect the persistence of an underlying disease mechanism (71). The results of HSCT in SSc are summarized in Table 5 (69, 72-76).

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

According to experts' consensus, patients with severe SLE refractory to conventional immunosuppressive treatments can achieve sustained clinical remission by undergoing AHSCT. Clinical remission has ranged from 50% to 70% at 5 years accompanied with qualitative immunological changes not seen in other types of therapy. Unfortunately, an increase in short-term mortality has been described in the majority of studies (77).

Severe cases of SLE treated with AHSCT have achieved a better prognosis. TRM has been relatively low over the long-term, and prolonged clinical remissions are reachable. "Re-education" and Treg normalization has been established as immune resetting. As a result, AHSCT for refractory SLE has become a major target (78). A true immunological remission can be obtained with HSCT in human lupus by the generation of a newly differentiated population of Treg cells (42). Transplant-related mortality of 2% and an overall 5-year survival of 84% in patients with SLE treated with AHSCT have been reported. The probability of disease-free survival at 5 years following HSCT was 50%, and secondary analysis demonstrated stabilization of renal function, significant improvement in lung function, SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) score, ANA (antinuclear antibodies), and anti-dsDNA levels (17).

In a single-center study of long-term immune reconstitution, clinical remission associated with the depletion of autoreactive immunologic memory was observed in 7 patients with SLE who underwent AHSCT. These data demonstrate that depletion of autoreactive immunological memory and profound resetting of the adaptive immune system are required to reestablish self-tolerance in SLE (30). The results of HSCT in SLE are summarized in Table 6 (17, 79, 80).

RHEUMATOID ARTHRITIS (RA)

Approximately a decade ago, phase I trials in RA patients treated with AHSCT showed substantial remission of the disease in the majority of patients who had failed to see improvement from all of the available therapies at the moment (81, 82). Additional studies showed that high-dose chemotherapy (HDC) plus HSCT increased the functionality and health status of patients with severe, refractory RA with a low TRM (1.3%) (83). The use of HDC followed by AHSCT is feasible and safe and results in long-term improvement of disease activity for patients unresponsive to conventional antirheumatic drugs or TNF blocking agents. The lack of response in some patients may reflect the heterogeneity of the underlying disease process (84). However, the emergence of biological DMARD therapy in refractory RA has replaced the utilization of HSCT in RA patients. Likewise, an

AUTHOR, YEAR	PATIENTS, (N)	CONDITIONING REGIMEN	RESULTS	FOLLOW-UP (RANGE)	MORTALITY
Burt RK, 1998 (58)	3	CYC/ MTP/ TBI	Improvement: 100%	8 months (6–10)	TRM: 0%
Kozák T, 2000 (59)	8	BEAM	Improvement/ stabilization: 87.5%	8.5 months (1-16)	TRM: 0%
Fassas A, 2000 (60)	24	BEAM/ ATG	Improvement/ stabilization: 78% PFS: 79%	40 months (21-51)	TRM: 4%
Fassas A, 2002 (61)	85	HDIT +/- ATG +/- TBI	Improvement: 21% PFS: 74%	16 months (3-59)	Overall: 8.2% TRM: 6%
Nash RA, 2003 (62)	26	CYC/ ATG/ TBI	Improvement/ stabilization: 76% Estimated survival: 91%	24 months (3-36)	Overall: 7.6% TRM: 3.8%
Saccardi R, 2006 (63)	178	HDIT +/- ATG +/- TBI	Improvement/ stabilization: 63%	41.7 months	Overall: 8.8% TRM: 5.3%
Shevchenko YL, 2008 (64)	45	BEAM/ATG	Improvement: 62.2% PFS: 72%		Overall: 2.2% TRM: 0%
Burt RK, 2009 (65)	21	CYC/ alemtuzumab or ATG	Improvement: 81% PFS: 100%	37 months (24-48)	TRM: 0%
Hamerschlak N, 2010 (18)	41	BEAM/horse ATG versus CYC/rabbit ATG	CY/ rabbit ATG: similar outcome results, but less toxicity	2 – 3 years	TRM: 7.3% (all in BEAM regimen)
Fassas A, 2011 (66)	35	HDIT	PFS: 25% (44% for patients with active CNS disease)	11 years (2-15)	TRM: 5.7%
Bowen JD, 2012 (67)	26	HDIT: TBI, CYC, ATG	EDSS-failure-free survival: 44%	48 months (3-72)	TRM: 7.6%
Mancardi GL, 2012 (68)	74	BEAM/ATG	Improvement/ stabilization: 66%	48.3 months (0.8-126)	TRM: 2.7%
Shevchenko JL, 2012 (54)	95	HDIT	Improvement/stabilization: 80% PFS: 92% (early AHSCT) vs 73% (conventional/salvage AHSCT)	46 months	TRM: 0%

Table 4. Summary of reports of HSCT in multiple sclerosis. *HDIT*: high-dose immunosuppressive therapy. *BEAM*: BCNU, etoposide, cytosine-arabinoside, melphalan. *CYC*: cyclophosphamide. *MTP*: methylprednisolone. *TBI*: total body irradiation. *ATG*: antithymocyte globulin. *PFS*: progression-free survival. *EDSS*: Expanded Disability Status Scale. TRM: transplant-related mortality.

AUTHOR, YEAR	PATIENTS (N)	CONDITIONING REGIMEN	RESULTS	FOLLOW-UP (RANGE)	MORTALITY
Binks M, 2001 (69)	41	CYC +/- ATG +/- TBI	Improvement (skin): 69%	12 months (3-55)	Overall: 27% TRM: 17%
Farge D, 2002 (72)	11	CYC or melphalan	Major/ partial response: 72.7%	18 months (1-26)	TRM: 9.0%
Farge D, 2004 (73)	57	CYC +/- TBI +/- ATG +/- alemtuzumab	Partial/ complete response: 92%	20 months (0.3-81.1)	Overall: 23% TRM: 8.7%
Vonk MC, 2008 (74)	26	CYC	Response: 81% Event-free survival: 64.3% at 5 years, and 57.1% at 7 years.	5.3 years (1-7.5)	Overall: 3.8% at 5 years, and 15.2% at 7 years
Oyama Y, 2007 (75)	10	CYC, rabbit ATG	Improvement in skin score. PFS: 70%.	25.5 months	Overall 10% TRM: 0%
Burt RK, 2011 (76)	19	10: CYC/ rabbit ATG + HSCT; 9: CYC alone	Improvement (HSCT group): 100%	2 years	TRM: 0%

Table 5. Summary of reports of HSCT in systemic sclerosis. *CYC*: cyclophosphamide. *TBI*: total body irradiation. *ATG*: antithymocyte globulin. *PFS*: progression-free survival. *TRM*: transplant-related mortality. *HSCT*: hematopoietic stem cell transplantation.

AUTHOR, YEAR	PATIENTS (N)	CONDITIONING REGIMEN	RESULTS	FOLLOW-UP (RANGE)	MORTALITY
Traynor AE, 2000 (79)	7	CYC, MTP, equine ATG	Remission: 100%	25 months (12-40)	TRM: 0%
Jayne D, 2004 (80)	53	CYC +/- ATG +/- lymphoid irradiation	Remission: 66%, at six months	26 months (0-78)	Overall 22.6% TRM: 12%
Burt RK, 2006 (17)	50	CYC/ equine ATG	Disease-free survival: 50%	29 months (6-90)	Overall: 4% TRM: 2%

Table 6. Summary of reports of HSCT in systemic lupus erythematosus. *CYC*: cyclophosphamide. *MTP*: methylprednisolone. *ATG*: antithymocyte globulin. *TRM*: transplant-related mortality.

early diagnosis and appropriate treatment have changed the prognosis of the disease, making it less likely that RA patients will be enrolled in a HSCT study.

JUVENILE IDIOPATHIC ARTHRITIS (JIA)

In this severely ill patient group, AHSCT induces a very significant and drug-free remission of the disease in the majority of the patients. However, it carries a significant morbidity and mortality risk, especially associated with macrophage activation syndrome (MAS) (85, 86). After fatal complications due to MAS, the protocol has been modified to ensure less profound depletion of T cells, better control of systemic disease before transplantation, antiviral prophylaxis after transplantation, and slow tapering of corticosteroids (22). With the recent availability of anti-TNF therapy for the treatment of JIA, failure to respond to this type of therapy should be an additional inclusion criterion for future studies of AHSCT in JIA.

TYPE 1 DIABETES MELLITUS (T1D)

Promising results have been demonstrated in patients with T1D. A study showed that 20 out of 23 T1D patients became insulin-free following AHSCT treatment. Of these, 12 individuals remained insulin-independent during the entire follow-up period, while 8 subjects relapsed after an average of 18 months and had to resume insulin medication at low doses. There was no mortality reported. (87, 88).

OTHER ADs

Autoimmune cytopenias like autoimmune hemolytic anemia (AIHA), immune thrombocytopenic purpura (ITP), autoimmune Evans syndrome, and pure red cell aplasia (89, 90) have been a common indication for AHSCT. This type of treatment has been used less in other severe and refractory ADs, where it could be a clinical option in an experimental setting, but several reports have shown clinical response in ADs such as systemic vasculitis (91, 92), polymyositis and dermatomyositis (93, 94), and antiphospholipid syndrome (APS) associated with SLE (95).

COMPLICATIONS OF HSCT

Mortality is the major complication in patients who undergo AHSCT, but infections are the most common complications related to AHSCT (86). In a case series study, bacterial infections were reported in 3 of 14 (21%) patients with AD and AHSCT, and viral counterpart in 11 of 14 (78%) including CMV and adenovirus infection. As late infectious complications, 7 patients (50%) developed dermatomal varicella zoster virus infection. No infection-related mortality was seen in this report (28). Recently, the frequency of infections has diminished through the use of less intensive immunosuppressive regimens and better protocol designs.

A new onset of organ-specific (96), systemic (97), or multiple ADs after AHSCT has been reported (98). A multi-center retrospective EBMT study showed that 29 of 363 (8%) patients who received a transplant for an AD developed at least one new AD after HSCT with a cumulative incidence of 7.7% after 3 years and 9.8% after 5 years (99).

The engraftment syndrome has also been reported and consists of non-infectious fever, rash, and fatigue after AHSCT and has been identified in 26% of the patients (100). Currently, HVGD is a rare complication in AHSCT, but it is common in allogeneic HSCT. Finally, treatment-associated toxicity in patients with AHSCT is also described. Cardiac and renal injuries are more common in SSc patients, ATG can cause allergic reactions, and the administration of G-CSF can induce flares of the underlying AD (101).

CONCLUSIONS

Autologous hematopoietic stem cell transplantation is a feasible treatment option for ADs, based on the concept of ablation of a self-reactive immune system and resetting auto-tolerance by regeneration of HSCs. Growing evidence supporting its clinical usefulness is available. Safety profiles of the interventions have improved with the modifications of protocols and the use of a less intensive immunosuppression. The future research agenda should be to push for a definition for/of better inclusion criteria for AD patients and evaluate the efficacy and cost-benefit of AHSCT compared to the best current available therapy for any specific AD.

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43

RNAi AND AUTOIMMUNITY

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INTRODUCTION

Transcription, the process by which messenger ribonucleic nucleic acid (mRNA) is assembled from a strand of deoxyribonucleic acid (DNA) is highly regulated, both in prokaryotes and eukaryotes, by a large group of proteins. For over 50 years, it has been hypothesized that there were "suppressive and/or enhancing" steps beyond the mRNA level (i.e., some form of "post-transcriptional" regulation). However, its nature was unknown. The most straight forward approach was to investigate proteins that carry out this process. So, in the last few decades, several families of proteins have been implicated in the degradation of mRNA. One example of such proteins is Tristhetraproline which degrades the mRNA of Tumor Necrosis Factor-α (TNF-α) (1). Nonetheless recently, other types of RNA able to regulate the mRNAs have been described. Among these a veritable burst has occurred in the study of a type of RNA known as "RNA interference" (RNAi). In 1989, Richard Jorgensen described a form of regulation in which an effort was made to modify the color of petunias by introducing an additional copy of the gene of the pigment. As a result, they obtained partially or completely white flowers, a condition called co-suppression. At the same time, other researchers were using complementary RNA strands to reduce the expression of genes in order to produce phenotypic changes by achieving similar responses in this process of co-suppression (2).

It was in 1998 when Andrew Fire and Craig Mello published the mechanisms of the RNAi (3) system in Nature showing that the observed inhibition was given by the joint action of the "sense" and "antisense" strand precursors of double stranded RNA (dsRNA) molecules. These discoveries were innovative and deemed worthy of the Nobel Prize in Physiology or Medicine in 2006.

TYPES OF RNAi

RNAis molecules described to date have been classified into three major families depending on their origin, structure, associated effector proteins, and biological roles:

1. Short-interfering RNAs (siRNAs) (Hamilton AJ et al. 1999) (4),
2. microRNAs (miRNAs) (Ambros V. et al. 1993) (5,6), and
3. Piwi associated RNAs (piRNA) (AA Aravin et al. 2001) (7).

The siRNAs and miRNAs are molecules that are approximately 22 nucleotides (nt) long which are phylogenetically and widely distributed in both plants and animals. They act on both somatic and germinal cell lines and are characterized by double-stranded precursor molecules. In contrast, piRNAs are made up of approximately 25 to 30 nt distributed mainly in animals and exert their functions in germinal cell lines. Their precursors are probably derived from a single chain (8).

miRNAs mainly regulate endogenous genes, and siRNA regulates exogenous genes. The latter are processed as a defense mechanism of genome integrity in response to viral infections, transposons, or transgenes. Over 800 human miRNAs have been identified so far, and the RNAi group has been studied the most in medicine (9).

RNAi PRECURSOR BIOGENESIS

The precursors of siRNA and miRNA are large dsRNA molecules with several tens of thousands of nucleotides forming a hairpin-shaped structure. This is a terminal hair pin loop with an average length of ~33pb imperfectly formed by two complementary paired arms (10) (Figure 1). siRNA and miR-

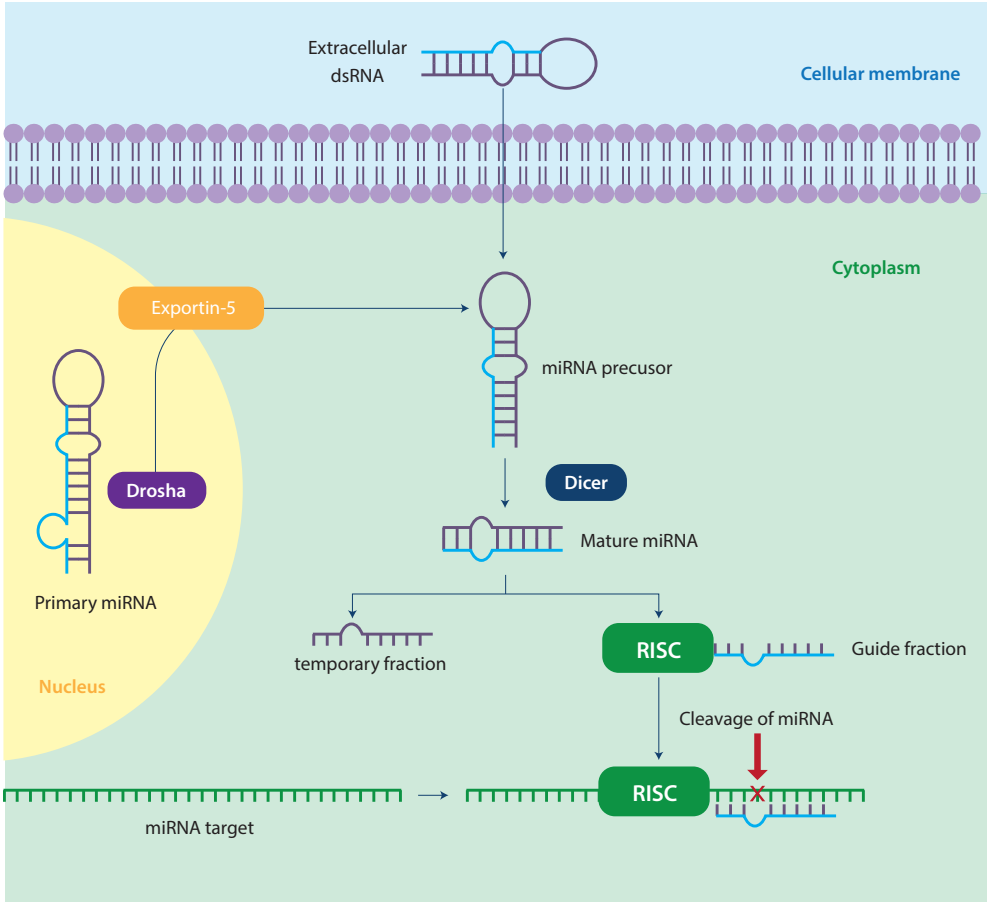

Figure 1. miRNA biogenesis and mechanism of action. The miRNA nuclear precursors are dsRNA "hairpin-shaped" molecules called "primary miRNA." At the nuclear level (i.e., Drosha) the dsRNA becomes "miRNA precursor" by the action of RNase. miRNA precursor passes into the cytoplasm by effect of the Exportin-5. Exogenous sources of miRNA precursors (e.g., viral) enter the cytoplasm. The miRNA precursor becomes a "mature miRNA" by Dicer action after which it then opens into two fractions of RNA, a so-called "temporary," which was degraded, and another called "Guide," which will be part of the molecular complex "RISC" (RNA-induced silencing complex). The guide portion binds to the complementary sequence of target mRNA, and it is degraded.

NA differ in that the precursor of the first is exogenous and is introduced into the cytoplasm from the extracellular medium as part of viral dsRNA. While the siRNA targets the centromeres and/or transposons (11), the precursor of miRNA is endogenous and encoded in the DNA of the individual. The miRNA precursor is called primary mi-RNA or pri-miRNA, has a cap at the 5′ end, and a 3′ polyadenylated tail. This type of precursor is generally transcribed by RNA polymerase type II (12-14) by means of different forms of transcription:

- From classical transcription units, i.e, a "gene" specific for each miRNA.

- From "transcriptional units" that make a product or clusters, in which a transcript may encode different miRNAs.

- From the sequence which is located at an intron.

The pri-miRNA can contain multiple miRNAs. The transcribed pri-miRNAs are processed in the nucleus where they become an intermediate form called preliminary miRNA or "pre-miRNA." In order to unfold and cleave the hair loop complementary structure, action by an RNase III type called "Drosha" is needed (the name was derived from the fly Drosophila melanogaster where it was first described) (Figure 1). Drosha is associated with a cofactor containing a double-stranded RNA-binding (dsRBD) domain thereby forming a complex called "microprocessor" (15). An alternative way to produce pre-miRNA is by using the splicing of miRNA primary transcripts to release introns that precisely mimic the structural properties of the pre-miRNA. These "mintrones" enter miRNA processing without the action of the microprocessor.

DEVELOPMENT OF RNAis ACTIVE FORMS FROM THEIR PRE-RNAI PRECURSOR

Pre-miRNA that originates from the same cell nucleus the cytoplasm requires a carrier protein known as exportin-5 (15). Once in the cytoplasm, the precursor enters the final cleavage to reach the active form of RNAi. This process requires the action of a second enzyme called "Dicer," which is the other form of type III RNases. The dsRBD is also re-

quired to allow the recognition of specific domains of the dsRNAs. The next step is to achieve the separation into two single RNA strands, a so-called "temporary" state followed by another one called "Guide," which will produce a molecular complex called RISC (RNA-induced silencing complex) (Figure 1) called miRISC or siRISC for miRNAs or siRNAs respectively. This complex is composed of the chain "guide" and the proteins dicer and ago. The last protein belongs to the Argonaut protein family, characterized by four domains:

- The PAZ, shared with Dicer enzymes, which specializes in linking the 3 'ends of the RNAs allowing accurate cleavage and formation of miRNA and siRNA from their precursors. Drosha RNase II, in turn, lacks the PAZ domain.

- The PIWI
- The domains Mid (Middle) and
- The N (N-terminal) (16).

Humans have four Ago proteins: Ago1 to Ago 4 and 4 PIWI proteins: Hili, HIWI1, HIWI2, and HIWI3. The Ago proteins interact with both the siRNAs and miRNAs (17). These proteins are the effector RNAi system to cleave the target mRNA.

GENE REGULATION OF THE RNAi

The regulation of RNAi synthesis has not been widely studied. However, recent studies suggest that regulation occurs by a double negative feedback mechanism. For example, the transcription of miR-7 (a gene of Drosophila ssp.) is repressed by a transcription factor called Yan which, at the same time, is negatively regulated by miR-7. It is believed that this type of regulation is crucial due to the fact that aberrant expression of miRNA would often mimic a loss of phenotypical function of the target. The former is prevented if the biogenesis of the RNAi is strictly controlled by its upstream targets (18).

MECHANISMS OF ACTION OF miRNA AND siRNA

When the RISC complex comes into contact with the target mRNA by means of the "guide" chain, either endogenous or exogenous RNAi (miRNA or siRNA) recognizes the target mRNA. The level of complementarity between the RNAi and the target mRNA is crucial. If the complementarity is not significant, silencing mechanisms are activated. However, if complementarity is complete, then the degradation of the target mRNA is induced at the cytoplasmic level. In this last condition, the cleavage process is initiated at the phosphodiester bond between the 10 and 11 nt that are matched with the siRNA or miRNA guide counting from the 5 'end of RNAi. This process is mediated by the PIWI domain of the protein Ago. The cleaved products are then degraded by exonucleases. The RNAi RISC is released. When mRNA targets are partially complementary or recognized by RISC, the mRNAs are not cleaved initially but may still be silenced on a post-transcriptional level using several methods: blocking elongation, co-translational protein degradation, deadenilation, etc. (19).

The foregoing mechanisms mediated both by siRNA and miRNA occur mainly in the cytoplasm in subcellular bodies called "P bodies" that are enriched by mRNA degradation factors (20). These bodies are also called GW because they contain one mRNA binding protein rich in Glycine/Tryptophan (G/W). Other proteins identified in these bodies include proteins with a "decapping" function (i.e., removing the cap at the 5'), e.g., hDcp1, hDcp2, Lsm1-7, Hedls, exonucleases such as hXrn1 or hCcr4, which also have a deadenilation activity (i.e., that removes the 3' poly-As) and a eukaryotic translation initiatin factor (eIF4E) plus its binding protein eIF4E-T, RISC proteins, etc. Based on studies of fluorescence and spectroscopy, different places on these P bodies have been identified as inducers of mRNA target inhibition. The suggested mechanisms are the result of intense debate from observations extracted from different techniques (21).

Table 1 summarizes the main features of the most important molecules implicated in the RNA interference system.

RNAi AND THE IMMUNE SYSTEM

Recently, miRNAs have become key targets for research due to their crucial role in regulating the immune response and cell development. Such regulation is vital to ensure normal and controlled physiology in order to prevent pathological phenomena including autoimmune diseases and cancer.

At the innate response level, miRNAs respond to stimulation of Toll-like receptors (TLR). Molecules such as miR-146a, miR-155, and miR-132 expressed in human monocytes are stimulated by lipopolysaccharide (LPS) (22). Moreover, two targets have been confirmed for miR-146: TNF receptor associated factor 6 (TRAF6) and IL-1 receptor associated kinase 1 (IRAK1), key components of the TLR4 pathway. Note that, expression of miRNA-146a is only induced by TLR2, TLR4, TLR5 stimulation, thus indicating its role in regulating the innate immune response to bacterial pathogens but not to virus. The expression of this miRNA is associated with reduced pro-inflammatory proteins such as IL-8 and RANTES (23), which suggests that miR-146a regulates the inflammatory response triggered for bacteria.

It has been shown that miR-125b is able to recognize the 3' UTR of TNF-α mRNA by reducing its expression in mouse macrophages, which attenuates the LPS response. It is believed that this miRNA must be inhibited in order to enable the production of LPS-induced TNF-α production (24).

In mice models, the expression of miR-155 in macrophages has been stimulated by IFN-β as well as a multitude of TLR ligands to promote the translation of TNF-α, which indicates a plausible regulatory role in the innate immune response against viruses and bacteria. However, miR-155 functions go far beyond that since it is important in the expression of cancer and hematological malignancies. This miRNA has been detected in other cell lines such as activat-

MOLECULE	DESCRIPTION	PRECURSOR	FUNCTION
miRNA	Small double stranded RNA. One of them is selected as the chain guide. Has endogenous source	Pre-miRNA	Recognition sequences of the RNA by means of the chain guide
Pri-miRNA	RNA transcript of the DNA of the individual. It can have multiple miRNAs	DNA, primary transcript, clusters or intron	Processing by Drosha in the nucleus to form the pre-miRNA
Pre-miRNA	Double-stranded RNA, hairpin-shaped with a ring terminal and a double-stranded end	Production by Drosha way. Can also be formed by splicing	Joining siRNA shared cycle, where it becomes miRNA for action of DICER
siRNA	Small double stranded RNA. One of them is selected as guide	Long double-stranded RNA, exogenous	Recognition, via the chain guide
Long double-stranded RNA with terminal ring, "hairpin-shaped"	Exogenous source molecule.	Viral genome component, transposons or transgenes	Serves as substrate to produce siRNA
MOLECULE	**DESCRIPTION**	**FUNCTION**	
Type III RNAase	Specific to double-stranded RNA. Requires ds-RBD Ex. DICER, DROSHA RNase type III. Nuclear. Also called microprocessor. Its inactivation has allowed the study of miRNA system in experimental models. Its dsRBDn is DGCR8.	See DICER y DROSHA	
dsRBD	Recognition protein of double stranded RNA. Complement of type III RNAase	For DICER: TRBP For DROSHA: DGCR8	
Argonaute protein family	There are two relevant ones: Ago and PIWI. The Ago work with miRNAs and siRNAs. PIWI with piRNAs	Effector proteins. Cleave mRNA target.	
DICER	RNase type III. Cytoplasmic. Its inactivation generalized or tissue-specific, allows the study of RNAi system in experimental models. Its dsRBD is TRBP	Its substrate is the long double-stranded RNA to produce siRNA	
DROSHA	Its dsRBDn is DGCR8. Type III RNase. Nuclear. Also called a microprocessor. Its inactivation has allowed the study of miRNA system in experimental models. Its DGCR8 is dsRBDn	Its substrate is the pri – miRNA to form pre – miRNA	
P or GW bodies	Subcellular foci where mRNA is degraded.	Facilitate RNAi system action. • "Deccaping": remove the cap 5' • Exonucleases: degrade RNA • Deandenilases active proteins: remove the poly A	

Table 1. Relevant molecular components of RNAi system.

ed macrophages, and B and T cells acting on the adaptive immune response. In experimental studies, Rodriguez et al. (25) found that bic/miR-155 deficient mice had an impaired adaptive immune response and failed to develop immunity against Salmonella typhimurium due to abnormal functioning of the B and T cells as well as defective antigen presentation by dendritic cells. Furthermore, the miR-155 regulates the response of the germinal centers. A role in the regulation of immunoglobulin class switching to IgG in plasma cells has also been described (26). In this study, mice deficient in miR-155 failed to generate high affinity IgG1.

Different miRNAs have been investigated in immune system development. One of the first described in this field is the miR-181a, which is highly expressed in the thymus. miR-181a has a cyclic expression during B-cell maturation and selection of certain cell lines in the bone marrow. It has also been reported that its signal modulates T-cell receptor expression, affecting the affinity to different antigens. Recently, its role in regulating class switching in B cells has also been described (Table 2) (20).

RNAi AND AUTOIMMUNE DISEASES

miRNAs have been involved in the pathogenesis, relapse, and specific-organ development of diverse autoimmune diseases (27).

miRNAs can be aberrantly expressed in different stages of disease progression, which opens up the field for miRNAs as potential biomarkers in rheumatic diseases (27). Increased knowledge of miRNAs has led to the development of mouse models for studying therapeutic approaches in vivo using specific miRNAs.

The discovery of GW or P bodies (see above for description) was made using the serum of a patient with motor

miRNA	TARGET	FUNCTION
miR-146[a]	TRAF6, IRAK-1	Innate immune response, signaling of TLR (Toll-like receptor)
miR-125b	TNF-α	Innate immune response, signaling of TLR
miR-155	Pu.1	Innate and acquired immune response, IgG "switching"
miR-181[a]	Not determined	Development of B-cells, signaling of T cell receptor
miR-181b	AID (activation-induced cytidine deaminase)	"Switching" of active B-cells
miR-223	Not determined	Granulopoiesis
miR-150	Not determined	Differentiation of B cells

Table 2. miRNAs in normal immune function. Adapted from Pauley *et al.* (20).

and sensory neuropathy which recognized intracellular autoantigens for these cell bodies (28). Subsequently, these autoantibodies were identified in patients with other neurological autoimmune diseases (33%), Sjögren's syndrome (31%), systemic lupus erythematosus (SLE) (12%), rheumatoid arthritis (RA) (7%), and primary biliary cirrhosis (10 %) (29). Satoh et al. (30) characterized these self-antigens in proteins of 100, 102, and 200 kDa, and the autoantibodies were called anti-Su. These autoantibodies were detected in the serum of up to 20% of SLE patients, scleroderma, and overlap syndromes. Later, Jakymiv et al. (31) reported that anti-Su antibodies of patients with rheumatic diseases and a model of autoimmunity in mice recognized the Ago1, Ago2, Ago3, and Ago4 as well as Dicer. Additionally, by immunofluorescence, the authors showed that anti-Su autoantibodies recognized the P bodies. The most common self-antigens were Helds (58%), GW182 (40%), and Ago2 (16%). Approximately 18% of antigens are undiscovered (31).

Since certain miRNAs play critical roles in regulating the immune response and the development of cell populations, it is not surprising that recent studies have revealed their connection with autoimmunity and the pathogenesis of autoimmune diseases (27).

Concerning T-cell response, two studies have shown the role of miRNA in immune system regulation regarding T-cell lineage using animal models to generate conditional knockout mice for Dicer (32,33). Although T cells grew normally in thymic regions, cells showed a peripheral dysfunction and impairment in their differentiation. In addition, Dicer-deficient T cells lose their in vivo suppressive activity and develop rapidly fatal systemic autoimmune disease. Additional data from these studies suggest that the function preserves stable miRNA T cells under inflammatory conditions.

In another study, Yu et al. (34) found a new pathway in the Roquin gene mutant mice that protects from the development of autoimmunity. This regulates the expression of inducible T cell co-stimulator ICOS by promoting the degradation of mRNA.

Another miRNA implicated in immune tolerance and the development of autoimmunity is the miR-146a. Lu et al. (35) demonstrated that miR-146a is critical for the suppressor functions of regulatory T (Treg) cells. In fact, a miR-146a knockout mouse showed some loss of immunological tolerance, which is responsible for fatal IFNγ-dependent immune-mediated lesions in different organs. In another study, Curtale et al. (36) showed that miR-146a is involved in T-cell activation and is highly expressed in mature human memory T cells. miR-146a can modulate activation-induced cell death processes thus acting as an anti-apoptotic factor in T cells. In addition, it is also able to reduce the expression of pro inflammatory cytokines.

The previously described miR-155 promotes the development of inflammatory T cells including the T helper (Th)17 and Th1 cell subset (37). It has also been implicated in the pathogenesis of the mouse model (MRL/lpr) of systemic lupus erythematosus (38) and mouse models of collagen-induced arthritis (39).

miRNA AND RA

RA is a chronic inflammatory disease characterized by synovitis, systemic inflammation, and the development of autoantibodies (e.g., rheumatoid factor and anti-citrullinated peptide antibodies). Several cytokines are implicated in RA pathogenesis, particularly tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6. Some miRNAs have been described as implicated in the pathogenesis of this disease. For example, it has been postulated that miR-146a is up-regulated but unable to properly regulate TRAF6/IRAK1 by initiating a prolonged TNF-α production in RA patients (40). In addition, abnormal expression of this miRNA in T cells contributes to increased severity (41). In research done by Pauley et al. (42), the repression of TRAF6 and/or IRAK-1 by miR-146a in Human acute monocytic leukemia cell line-1 (THP-1) cells resulted in an up to 86% reduction in TNF-α production. This suggests that normal miR-146a function is critical to regulating the production of TNF-α.

Other miRNAs such as miR-223, which is up-regulated in the CD4+ naïve T cells of RA patients, have been detected at high levels in RA (43). Overexpression of miR-346 has been reported in RA fibroblast-like (RASF) synoviocytes thus showing that it is an indirect regulator of the pro-inflammatory cytokine IL-18 release (44). An increased expression of miR-155 in the synovial membrane, synovial fluid, and in monocytes of the synovium compared to periphery monocytes has also been reported (45). One of the potential targets of miR-155 is the matrix metalloproteinase-3 (MMP-3), which suggests that inhibition of this protein can modulate tissue damage (46).

miRNA IN SLE

Since 2007, different groups have reported altered miRNA expression patterns in tissues and peripheral blood mononuclear cells (PBMCs) from SLE patients. Dai et al.(47) evaluated the miRNA expression in 23 patients with SLE compared to 10 healthy subjects and found seven miRNAs (miR-196a, miR-17-5p, miR-409-3p, miR-141, miR-383, miR-112, and miR-184) that were down-regulated while nine (miR-189, miR-61, miR-78, miR-21, miR-142-3p, miR-342, miR-299-3p, miR-198, and miR-289) were up-regulated compared to healthy controls. In 2008, this same group reported the profile of miRNAs in renal biopsies of patients with lupus nephritis (47). It has been shown that down-regulation of miR-146a in SLE contributes to the abnormal activation of the type I interferon pathway (IFN-signature described in SLE) (48). miR-125a level is reduced in PBMCs from SLE patients, and the expression of the predicted target of miR-125a, KLF13, is increased (49). miR-21 and miR-148 are over-expressed in PBMCs of SLE patients, and it has been demonstrated that they can target the DNA-methylation pathway, causing DNA hypomethylation and over-expression of autoimmune-associated methylation-sensitive genes such as CD70 and LFA-1 (CD11a) (50). The same targets are also influenced by another miRNA, miR-126 (51).

Recently, several autoantibodies that act against proteins involved in RNAi mediated regulation of proinflammatory cytokines have been described in different autoimmune diseases (52).

In summary, these studies demonstrate that miRNA expression is altered in RA and SLE. The next step is to identify the targets of these miRNAs and determine the mechanisms by which the regulation or deregulation of miRNAs may contribute to the pathogenesis of these diseases. Some progress has been made, e.g., the relationship between mIR-155/MMP-3 and miRNA 146a/TRAF6-IRAK-1, but more studies are needed to clarify these relationships and dissect the pathways in which they are involved (Table 3).

OTHER AUTOIMMUNE DISEASES

In Sjögren´s syndrome (SS), miRNA signatures from minor salivary glands distinguished between subsets of SS patients with low-grade or high-grade inflammation (53). miRNAs have been also studied in saliva, which suggests that it may be possible to obtain information from these target organs without the need for invasive methods such as salivary gland biopsies (54). In polymyositis/dermatomyositis, the plausible influence of miR-146b, miR-221, miR-155, miR-214, and miR-222 on the NF-kB pathway leading to muscle inflammation has been reported (55).

CONCLUSION

The recently discovered RNAi system has given researchers the opportunity to encourage the advocacy of several lines of research, including phylogeny approaches, mechanisms of production and function as well as their implications for human pathologies. Their specificity for nucleic acid strands opens a new chapter in cell biology, human pathology, diagnostics, and also therapeutics. This system has been proven to be biologically active from the embryonic stage to the adult, and ubiquitously biological processes are involved in intra functions - cell inter, intra - and inter systemic tissue.

According to descriptions from different observations, its dysfunction, which may be due to hereditary and / or acquired processes, plays an important role in the pathophysiology of disease (e.g., autoimmune diseases). Such dysfunctions may occur at the level of the first coding - RNAi, coding associated proteins such as Dicer and Drosha system, as well as at the level of the mRNA target. A therapeutic potential in the future is obvious.

miRNA	PATHOGENIC ROLE	DISEASE
miR-101	Required for ICOS (inducible T-cell co-stimulator) mRNA degradation	"Lupus-like"
Not determined specific miRNA	Stability and function of regulatory T cells	Fatal disease
miR-146a	TRAF6/IRAK-1 regulation, required for the action of TNF-alpha and interleukin-1	RA
miR-155	Targets MMP-3, regulates inflammatory response	RA
miR-132	Not yet determined	RA
miR-16	Not yet determined	RA
Several miRNAs	Not yet determined	SLE

Table 3. miRNAs in autoimmune diseases. *RA*: rheumatoid arthritis, *SLE*: systemic lupus erythematosus. Adapted from Pauley et al. (20).

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44

NANOTECHNOLOGY AND AUTOIMMUNITY

Arley Gómez López

INTRODUCTION

The idea of nanotechnology was first presented by the physicist Richard Feynman in a lecture entitled "There is plenty of Room at the Bottom", given on December 29th, 1959, Annual Meeting of the American Physical Society, California Institute of Technology (1). In this meeting, Feynman unveiled the possibilities that are available in the molecular world. The term "nano-technology" was coined later by Norio Taniguchi in 1974 to describe the precision in the manufacture of materials with nanometer tolerances(2). These so called nanostructures occur also in nature; for example, one of the smallest and highly complex living structures; viruses, are about 20 to 200nm in size (Figure 1), and proteins which carry out intracellular functions range between 3 to 20nm in size. Other examples in nanoscale dimensions with functional properties in nature involve receptors, channel receptors in cells, ligands, effectors proteins, and nucleic acids as constituents of the DNA.

Moreover, nanotechnology can also be defined as the branch of science that studies and designs materials from 1–100 nm in size. Another description encompasses a physicochemical point of view as the ability to build and shape matter one atom at a time.

The construction of a functional nano system can be achieved by different techniques: top-down, bottom-up or self-assembly. In the top-down process, technologists start with a bulk material and carve from it a smaller structure (3). The top-down approach uses advanced lithographic techniques such as optical lithography and electron beam lithography to create structures as small as 100 and 20 nm. A major advantage of the top-down approach implies the development and building of integrated circuits in which the parts are both patterned and built in place so that no assembly step is needed (4).

On the other hand, the bottom up method is based upon the design and integration of the smaller building blocks or basic units into complex structures. Inspiration of the bottom up approach comes from biological systems where nature has harnessed chemical forces to create all the structures needed in life.

Nowadays, chemists, biologists, physicists, mathematicians and translational researchers from different disciplines are engaged in replicating nature's ability to produce clusters of specific atoms which can self assemble into much more complex structures. A number of bottom up or self-assembly approaches have been developed to produce size-selected nanoparticles (NPs), such as liposomes, micelles, nanoshells including quantum dots (QDs) , carbon-based particles (including nanotubes and fullerenes), nanoemulsions, nanocrystals, and polymer-based (including dendrimers), used for nanotech medical applications (Figure 2).

For the purpose of this chapter, the term nanobiotechnology will be widely used and will be defined as a subset of nanotechnology in which atom-level engineering and manufacturing is performed using biological precedents for guidance. Moreover, nanobiotech applications in prevention, diagnosis and treatment of human diseases will be referred as to nanomedicine. Both nanobiotechnology and nanomedicine are new and rapidly developing areas applied to different fields such as computing, electronics, biology, medicine, and pharmacology. In fact, recent advances in diagnostic and therapeutics based on a molecular and multidisciplinary approach have lead to the introduction of *theragnostics*; *a term* which combines both processes (5,6). This chapter will illustrate how nanotechnology and other disciplines of nanosciences are contributing to the development of new insights and translating them into useful tools to improve screening, diagnose and treatment of autoimmune diseases (Figure 3).

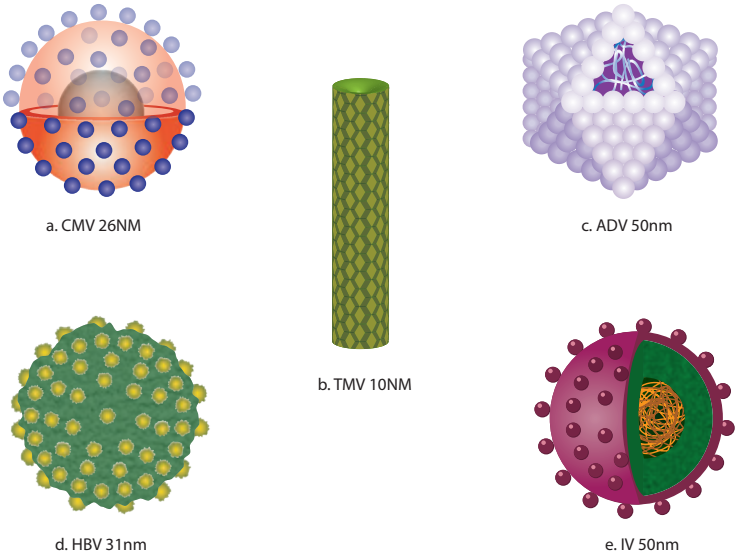

Figure 1. Size and shapes of viruses: **a**. cytomegalovirus (CMV), **b**. tobacco mosaic virus (TMV), **c**. adenovirus (ADV), **d**. hepatitis B virus (HBV), **e**. influenza virus (IV).

NANOTECHNOLOGY IN AUTOIMMUNE DISEASES

Autoimmune diseases (ADs) are chronic conditions initiated by the loss of immunological tolerance to self-antigens. The diagnosis of ADs depends on the identification of disease-associated clinical signs and symptoms as well as the detection of autoantibodies.

Development of new tests to detect multiple antibodies, in combination with genetics, epigenetics and immunological anomalies may allow to predict ADs (9). Techniques based on nanomaterials can support several in vitro diagnostic assays, where the overarching goal is the detection of the disease at an early stage. In the last two decades, the "top-down" and "bottom-up" techniques have allowed the production of a large number of nanomaterials potentially useful in diagnosis, therapy and prevention of human diseases.

The advent of biological agents have dramatically altered therapeutic strategy in medicine (10). On the other hand, a great number of nanodrugs and nanodiagnostics have been developed to increase the drug's safety profile in terms of bioavailability, thus enabling the administration of lower doses and minimizing adverse reactions. In regards to ADs, ideal therapeutics must focus on personalized medicine where early diagnosis and treatment can be combined with single NPs. That is, *theragnostics*.

DIAGNOSTIC APPROACHES

NPs may enhance disease screening by improving sensitivity, specificity, prompt time in diagnosis, and the availability of testing equipment. High diagnostic sensitivity can facilitate assays to be quicker, more flexible and will enable them to use lower amounts of a target biomarker. Furthermore, due to the fact that many of these techniques measure molecules and cells without any intermediate reactions and sample processing, these nanoparticle tests are considerably more rapid and could be conducted by the primary-care physician of the patient at home. In addition, a cost reduction can be obtained from reagents and lower time analysis since these systems require smaller sample volumes. Bulky materials have constant physical properties regardless of size; however NPs exhibit different characteristics for two main reasons: First, because of their size and big surface area to volume ratio, NPs have a tremendous driving force for diffusion, especially at high temperatures. Second, the quantum effect that arises in a nano scale regime due to the overall dimensions of objects is comparable to the typical wavelengths for fundamental excitation in materials. In terms of biological function at a nano scale level: as particle size decreases, (and surface area increases) the biological activity of NPs increases too. A summary of the main NPs properties is shown in Table 1.

IN VITRO DIAGNOSTIC TECHNIQUES

Biosensors are chemical sensors, in which recognition processes rely on the use of biochemical mechanisms; they can be classified based on signal transduction (mechanical, electrical, optical or thermal), composition (Nucleic acids, proteins, antibodies, etc.,) or a combination of the two. Techniques based on biosensors are available for *in vitro* and *in vivo* screening, i.e., nano-mechanical biosensors, which could have applications in genomics, proteomics, medical diagnostics and label-free protein analysis.

A distinctive hallmark of ADs implies the production of high-affinity autoantibodies; and protein signature patterns that can reflect different stages of the disease and can be promptly detected using immunosensor chips based on multiple antibody-functionalized transducers (11). Andrew et al., developed a porous silicon biosensor for detection of La protein; this protein with antigenic properties is associ-

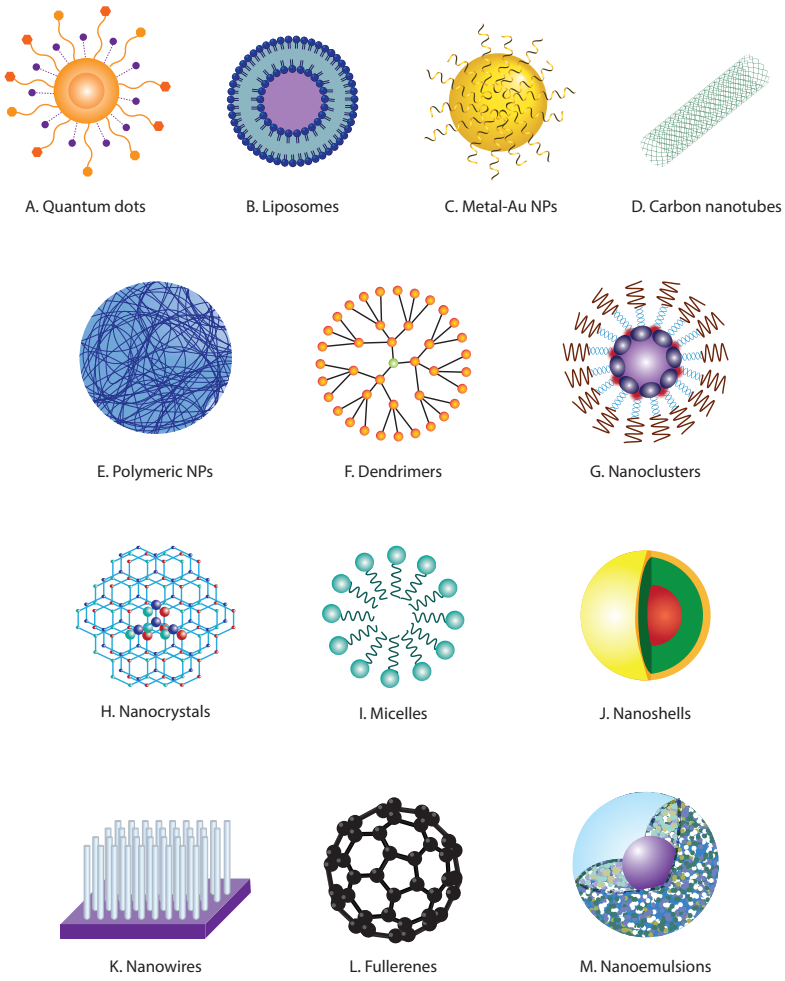

Figure 2. Types of nanoparticles. **A**. quantum dots, **B**. liposomes, C. metal Au NPs, **D**. carbon nanotubes, **E**. polymeric NPs, **F**. dendrimers, **G**. nanoclusters, **H**. nanocrystals, **I**. Micelles, **J**. Nanoshells, **K**. Nanowires, **L**. Fullerenes, **M**. Nanoemulsions.

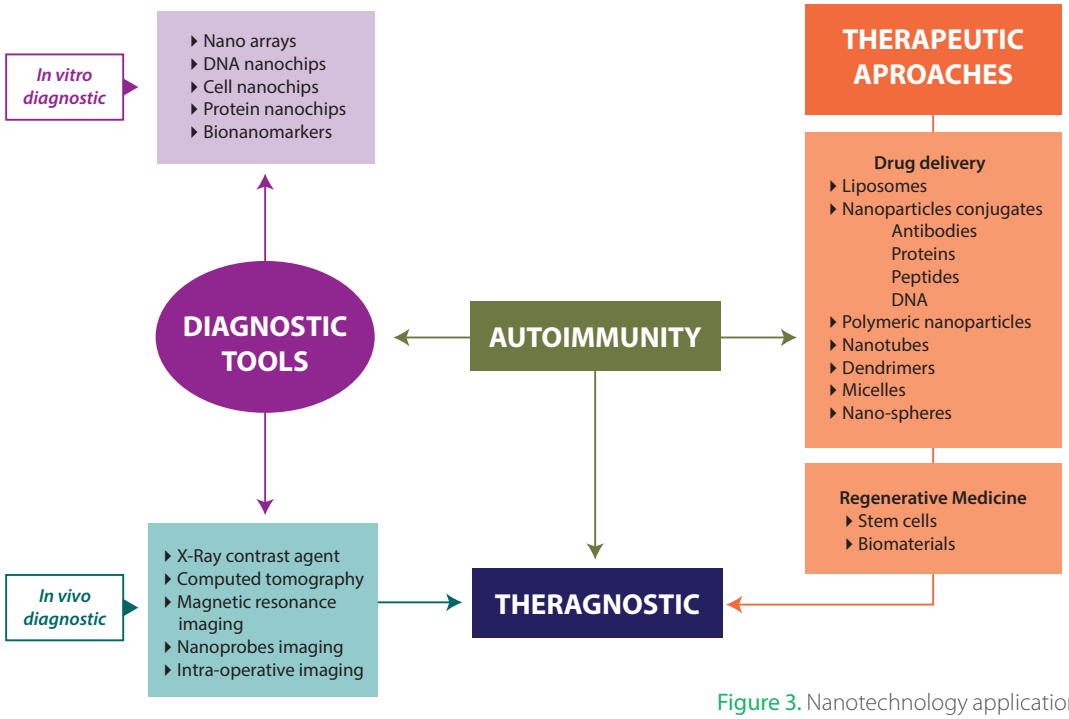

Figure 3. Nanotechnology applications to autoimmunity.

PROPERTIES	CHARACTERISTICS	NANOPARTICLE
Electrical	Can hold considerably more energy than conventional sized materials because of their large surface area Semiconductor and superconductors Magnetism, paramagnetism and superparamagnetism	CNts Fe3O4, Cu, Co, Ni
Optical	Linear and nonlinear optical properties can be finely tailored by controlling the crystal dimensions and surface chemistry Surface plasmon resonance Optical transmission Optical absorption and photoluminescence	Qd, Au, Ag, Cu and IONPs
Chemical	Enhanced chemical activity: particle-particle interactions are either dominated by weak Van der Waals forces, stronger polar and electrostatic interactions or covalent interactions Great ability to form suspensions Higher catalyst activity	AgNPs, dendrimers, liposomes
Mechanical	Increased hardness, fracture toughness, scratch resistance, fatigue and strength	CNTs, fullerenes

Table 1. Main nanoparticles properties. *CNTs*: Carbon nanotubes; *IONPs*: Iron oxide nanoparticles; *AgNPs*: Silver nanoparticles.

ated with ADs including rheumatic disorders, such as systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) (12). In the silicon biosensor device, immobilization of the La protein into porous silicon films gave a protein receptor-decorated sensor matrix where immunological reactions were carried out on the functionalized surface; detecting the anti-La antibody at a concentration of 500 - 125 ng/ml. Another *in vitro* diagnostic approach involves the use of AuNPs as a DNA-carrier based on a biobarcode assay. This technique is skilled to amplify and quantify a connecting molecular loop at ultralow concentration for signal detection, transduction (recording), and signal documentation at molecular scale (13). This device has shown robust advances in the diagnosis of multiple sclerosis (MS), since radio diagnoses magnetic resonance imaging (MRI), Eco-Doppler are at present the only available gold techniques for early detection of MS and other brain pathologies.

Electrical biosensors can be useful in clinical diagnosis of ADs, since disease-related proteins; protein-protein interaction

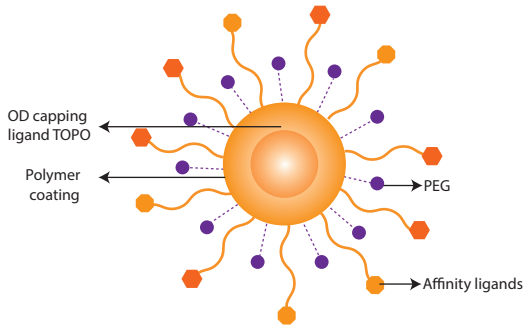

Figure 4. Quantum dots used in autoimmune disorders: A single QD is large enough for conjugation to multiple ligands such as biomarker-specific antibody or PEG and can be modified on the surface with a capping ligand TOPO which keeps QDs from aggregating in solution.

such as antigen-antibody binding can be detected by different readout signals such as piezoresistive response, conductance change and voltammetry. Other targets for marker detection are DNA and RNA; some researchers have shown that CdS, ZnS, CuS, and PbS NPs or QDs can be used to differentiate the signals of four different proteins or DNA targets along with stripping voltammetry of the corresponding metals (14, 15).

Drovulakis and coworkers (16) developed a label-free detection method using as platforms to immobilize peptides for the detection at the femtomol range of rheumatoid arthritis (RA) specific (citric-citrulline-containing) peptides directly from crude human serum.

On the other hand, a study by Chen et al. (17), utilized SWNTs as multicolor Raman labels for sensitive, multiplex detection of anti-proteinase 3, a useful biomarker for Wegener's granulomatosis disease; another example of an AD. He et al. (18) reported a method for cell recognition in SLE patients that using photostable luminescent silica NPs as biological labels. Another successful example of the use of nanotechnology in ADs is the use of an ultrasensitive protein nanoprobe system capable to detect a specific antibody [65 kDa glutamate decarboxylase (GAD65)-specific autoantibody, i.e., the early marker of Type I diabetes (T1DM)], in the sera from T1DM patients at a 3 attomolar concentration (19).

The most widely used tool for imaging biological events in biological systems is optical detection. This is due to the fact that optical biosensors can be used for different types of spectroscopy (luminescence, absorption, polarization, fluorescence, etc.). NPs or QDs, used as tags or labels, can increase sensitivity, rate, and flexibility of detecting or measuring the presence or activity of selected proteins or DNA.

QDs are NPs composed of inorganic semiconductor molecules. Chemical and optical properties of QDs and their stability in biological fluids and tissues may be controlled during nanoprobe production through variation of their chemical composition, size and quality of shells as well as conjugation with bio-recognition molecules such as aptamers, peptides, antibodies, nucleic acids or ligands for application as fluores-

cent probes (Figure 4). QDs NPs emit strong fluorescent light under ultraviolet (UV) illumination, and the wavelength (color) of the fluorescent light emitted depends on particle size. An essential part of using QDs in biological and medical fields is to coat them with a thin layer of a water-soluble material, since they are insoluble in aqueous solutions. QDs can be targeted to specific organs within the body by coating the QD surface with appropriate molecules; for example through the use of peptide translocation domains attached to QDs, internalization into the cell occurs readily (20).

The latest development in optical biosensing includes the conjugation of QDs to Carbon Nanotubes (CNTs). This hybrid nanocompound display unique electrochemical luminescent properties with major application for intracellular fluorescent imaging (Figure 5B) (21). The most commonly used methods to verify the conjugation of QDs to CNTs are atomic force microscopy and transmission electronic microscopy (22).

The miniaturization and integration of different functions in a single device, based on nanotechnology-derived techniques, have led to a new generation of devices that are smaller, faster and cheaper, require no special skills and give accurate readings. *In vitro* diagnostic devices include nanobiosensors, microarrays, biochips of different elements (DNA, proteins or cells) and lab-on-a-chip devices (23). Lokate et al. (24), have shown the potency of a surface plasmon resonance (SPR) imaging analysis system by automatically monitoring the interactions between citrullinated peptides and serum autoantibodies of 50 RA patients and 29 controls in a single step.

IN VIVO DIAGNOSTICS TECHNIQUES

The main benefits of nano-imaging or molecular imaging for *in vivo* diagnosis of ADs involve early detection, assess of the disease and severity (monitoring of disease stage) and follow up of the therapeutic efficacy. An ideal imaging modality should be non-invasive, sensitive, and provide unbiased information on cell survival, function and localization. Unlike classic imaging diagnosis with computed tomography (CT), magnetic resonance imaging (MRI) or ultrasounds, nano-imaging or molecular imaging includes techniques designed to

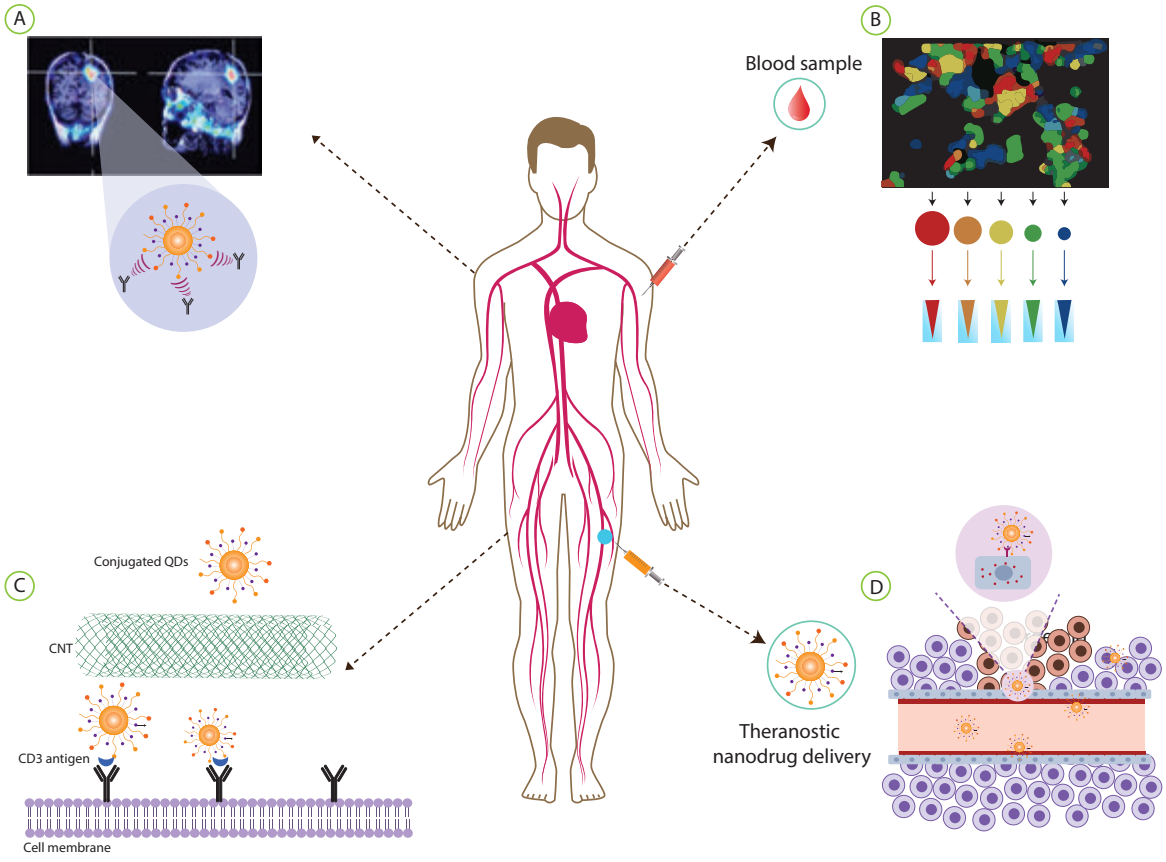

Figure 5. Multifunctional nanoparticles applications in diagnosis, therapeutic and theranostic approaches for autoimmune diseases. A. Contrast agents can be loaded onto nanoparticles to be used for *in vivo* diagnosis procedures such computed tomography and NMR imaging B.NPs bioconjugates coated with hetero-bifunctional PEG and monoclonal antibodies, aptamers, peptides and other small molecules can be used to label sections of healthy and abnormal tissues for *in vitro* diagnosis C. QDs can be used to label drug molecules or nanocarriers, D. Multifunctional NPs can be used as a theranostic agents to deliver the diagnostic/therapeutic probe with high molecular specificity.

obtain molecular data to identify the causes of the disease *in vivo* rather than its eventual consequences (Figure 5A) (25).

ADs can be organ specific (e.g., T1DM) or systemic (e.g., SLE). Therefore, an important group of targets are disease-related membrane antigens. These antigens can act as biomarkers and could help define the phenotype of the disease and sometimes identify therapeutic targets. Recent technological advances on the generation of diverse types of NPs exemplify the importance of them in imaging on ADs. For instance, gold NPs have the potential biocompatibility, relatively low short-term toxicity, high absorption coefficient and physical density compared with other metal NPs (26). Other important NPs are iron NPs, which have been used for more than two decades as contrast agents for MRI. These particles can be organized according to their hydrodynamic diameter into several categories: standard superparamagnetic iron oxide particles (SPIOs) (50 to 180 nm), ultrasmall superparamagnetic iron oxide particles (USPIOs) (10 to 50 nm), and very small superparamagnetic iron oxide particles (VSPIOs) (< 10 nm) (27). Tourdias et al reported that combination of gadolinium and USPIO in patients with MS can help identify additional active lesions compared with the current standard, the gadolinium-only approach, even in progressive forms of MS (28).

Magnetic resonance contrast agents accelerate the rate of T1 and T2 relaxation; however, the natural differences in relaxation times between regions of interest (such as normal versus scar tissue) are small, requiring the use of contrast agents. Several NP-based contrast agents have been developed to overcome issues that plague conventional contrast agents (Table 2). The ideal NP agent must fulfill a number of stringent requirements: it should be easily dispersible and stable in a variety of local in-vivo environments and not be affected by differences in solvent polarity, ionic strength, pH, or temperature when these conditions are not intended for measurement; it should exhibit limited nonspecific binding and be resistant to reticuloendothelial system (RES) uptake, and have programmed clearance mechanisms. Ideally, a NP agent should also have high sensitivity and selectivity for the target (i.e., antigen, cell, tissue) with good contrast quality (high signal- to-noise ratio, SNR) and sufficiently long circulation times in the blood if administered intravenously. Ideally Preferably, these materials must be suitable for long-term quantitative imaging at low doses and be safely cleared from the body after imaging is complete (29).

Contrast agents are typically paramagnetic molecules that can alter the relaxation times of selected regions or types of tissue or fluid within the body. Paramagnetic agents principally accelerate longitudinal T1 relaxation, producing 'bright' contrast in T1 weighted images (i.e., gadolinium based). Thurman et al developed a MRI-based method for noninvasive detection of the activation of the immunological complement system; essential in the physiopathology of some ADs.

This method uses iron-oxide NPs that are targeted to sites of complement activation with a recombinant protein that contains the C3d-binding region of complement receptor 2. Iron-oxide NPs darken (negatively enhance) images obtained by T2-weighted MRI (30). Due to its unique ability to directly image myocardial necrosis, fibrosis and edema, cardiac magnetic resonance (CMR) is now considered the primary tool for noninvasive assessment of patients with suspected myocarditis. Moon et al has described a CMR imaging with magneto-fluorescent NP that allows visualization of myocardial inflammation cellular infiltrates and distinction of the extent of the inflammation compared with conventional CMR in a preclinical model of experimental autoimmune myocarditis in rats (31).

Recently, Gaglia et al. (32) developed a noninvasive method to visualize T1DM at the target organ (pancreas) in patients with active insulinitis; using magnetic resonance imaging of magnetic NPs. The authors visualized islet inflammation, manifested by microvascular changes and monocyte/macrophage recruitment and activation. Positron Emission tomography (PET), Single Photon Emission Computed Tomography (SPECT) technologies in combination with radiolabeled immunoglobulin derived targeting probes could be used for tracking inflammatory cells in vivo. Dearling et al. (33) described the radiolabeling of an anti-β7 integrin antibody with the positron-emitting radionuclide 64Cu and its use in detecting acute colitis in an experimental murine model using microPET. Higher uptake of the radiolabeled antibody in the intestine of mice with acute colitis compared with controls was found by both microPET imaging and ex vivo tissue assay, suggesting that the β7 integrin monomer could be a viable target for colitis imaging and that the radiolabeled antibody, targeting a subset of lymphocytes, could serve as a specific imaging agent.

Nanobodies are the smallest antigen-binding antibody-fragments, that shows fast and specific targeting *in vivo* and have low immunogenicity due to their large sequence identity with human VH genes of the VH III family (34). Recently, Put et al (35) reported the use of SPECT/micro-CT imaging with ^{99m}Tc-labeled Nanobodies directed against the macrophage mannose receptor for monitoring and quantifying joint inflammation in collagen-induced arthritis , a mouse model for RA. The authors showed that macrophage mannose receptor is expressed on macrophages *in vitro* and *in vivo* in synovial fluid of inflamed paws, whereas expression is relatively low in other tissues.

THERAPEUTIC APPROACHES

Current therapeutic strategies against ADs can be classified into three main categories: (1) therapies to improve signs and symptoms, i.e., nonsteroidal anti-inflammatory drugs (NSAIDs); (2) therapies to modify the natural course of the disease, including biological and nonbiological disease-modifying antirheumatic drugs (DMARDs); and (3) therapies directed to the complications from the result of disease-associated organ damage (36).

At present, nanotechnology-based drug delivery systems (NDDS) based on NPs (Liposomes, polymers, dendrimers, nanocapsules and nanocrystals) for clinical use

TECHNIQUE	LABEL NP	SIGNAL MEASURED	RESOLUTION	DEPTH	MAIN LIMITATION
NIRF	QDs, dye-doped NPs, upconverting NPs, SWNTs and other carbon-based nanomaterials	Light, particularly in the near-infrared	1–3 mm	<1 cm	Low Poor depth penetration
MRI	Iron oxide NPs, Gd(III)-doped NPs, NP-based CEST and hyperpolarized probes (e.g., 129Xe)	Alterations in magnetic fields	50 μm	No limit	Low sensitivity, cannot follow many labels
PET	NPs incorporating radioisotopes (e.g., 18F, 11C, 64Cu, 124I)	Positron from radionuclides	1–2 mm	No limit	Can detect only one radionuclide, requires radioactivity
SPECT	NPs incorporating radioisotopes (e.g., 99mTc, 111In)	γ-rays	1–2 mm	No limit	Requires radioactivity
CT	Iodinated NPs, gold NPs, iron oxide-doped nanomaterials	X-rays	50 μm	No limit	Poor resolution of soft tissues
US	Microbubbles, nanoemulsions, silica NPs, polystyrene NPs	Sound	50 μm	Several cm	Poor image contrast, works poorly in air-containing organs
PAI	Gold nanoshells, gold nanocages, gold nanorods, gold NPs, SWNTs, dye-doped NPs	Sound	50 μm	<5 cm	Information processing and machines still being optimized

Table 2. Comparison of commonly used bioimaging techniques. Abbreviations: *NIRF*: Near-infrared fluorescence imaging; *MRI*: Magnetic Resonance Imaging; *PET*: Positron emission tomography; *CT*: Computed tomography; *US*: Ultrasound; and *PAI*: Photo acoustic imaging. Adapted from Hahn *et al.* (29).

have been reported (Figure 5C). Modulation of the particle size and elastic properties and particle uptake by phagocytes can be tailored at the single-cell or whole or whole organ level providing the means to optimize drug delivery to a particular tissue and/or organ (37).

On the other hand, synthetic NPs can be functionally designed to mimic antigen presenting cells (APCs); therefore enabling them to induce T-cell activation and differentiation. This approach is useful for cell therapy and vaccine development. Recently, Steenblock et al. (38) mimicked physiological antigen presentation on a biodegradable microparticle constructed from poly(lactideco-glycolide) (PLGA), a polymer system whose safety has been established for use in humans. The shape and the mechanical properties of the engineering NPs influence the particle-phagocyte interaction as has been demonstrated by Mitragotri and colleagues(39). These authors used a polymeric core with biconcave geometry as a template, upon which layer by layer polymers coating containing drug cargos were applied, followed by chemical crosslinking to stabilize the shell. After template core dissolution, microcapsules mimicked live mouse red blood cells. They demonstrated three preliminary examples: surface-absorbed hemoglobin for oxygen delivering, encapsulated iron oxide nanocrystals as imaging contrast agents, and encapsulated heparin as an anticoagulant.

Another therapeutic strategy to prevent pathological self-reactivity (autoimmunity) is to target the regulatory mechanisms arisen from T cell-APC junction. Stephan et al. (40) have shown that synthetic nanocarriers can be linked to the surface of T-cells and are actively carried to the immunological synapse by the membrane proteins by which they anchor. T-cell linked synthetic NPs could be used as efficient drug delivery vehicles into the immunological synapse as well as signal event regulators at the T-cell/APC interface.

New strategies to deliver anti-inflammatory drugs to innate immune cells selectively and inflamed tissues and reverse their pathological phenotypes are of great interest as a therapeutic tools for ADs. Nanodelivery systems are capable to reduce drug dose and administration frequency by extending circulation time and increasing the metabolic stability of small molecules. Also, as a result of the enhanced vascular permeability in inflammation sites, nanocarriers can preferentially accumulate in arthritic joints where they are subsequently phagocytozed by recruited monocytes/macrophages, activated and eventually inducing apoptosis (41).

Liposomes are spherical nanoparticles (NPs) made of lipid bilayer membranes with an aqueous interior (Figure 2B). Liposomes can be used as drug delivery systems, loading highly toxic drugs as antineoplastic and antimicotics, either in the aqueous compartment or in the lipid membranes producing much better efficacy and safety. One of the disadvantages of liposomes is the accumulation of these colloidal systems into the reticular connective tissue (RES) (i.e., liver and spleen) (42, 43). Nevertheless, PEGylation techniques have allowed a reduction of uptake from the RES. The use of PEG-poly-DL-lactic/glycolic acid-poly-DL-lactic acid (PEG-PLGA-PLA) micelles to encapsulate glucocorticoids in the treatment of an adjuvant-induced arthritis rat model has been also tested.

Furthermore, Wang et al. (45) developed a novel pH-sensitive drug delivery system of dexamethasone (Dex) based on an N-(2-hydroxypropyl)methacrylamide copolymer (P-Dex) and demonstrated that the delivery system specifically accumulates in inflamed joints in an animal model of arthritis (44). Recently, the same authors confirmed that monthly administration of P-Dex provided superior prevention of lupus nephritis and reduced toxicity in (NZB-NZW)F1 mice when compared with daily administration of

dose-equivalent Dex. In addition to the previously mentioned approaches, there are other nanoparticle-based methods such as new delivery systems for NSAIDs and glucocorticoids, anti-angiogenic nanotherapy, induction of immune tolerance, gene therapy as single or combination therapy that has been proven to be effective in animal models and it is awaiting for clinical trials (46-50).

Several therapeutic strategies based on NPs to improve T1DM have been reported to date. These strategies are mainly based on insulin delivery systems, gene therapy and islet cell-targeting molecular therapeutics. Chalasani et al. (51) developed a VB12-coated dextran NP-loaded insulin system, which exhibited a profound (70–75% blood glucose reductions) and prolonged (54-h) anti-diabetic effects with biphasic behavior in STZ diabetic rats. A polymeric gene delivery system for insulin has attracted considerable interest since the insulin gene was successfully cloned and expressed in cultured cells during the late 1970s (52). Niu et al. (53), have shown that the human insulin gene can be transfected successfully by chitosan NPs in vitro and in vivo, since they succeed to transfect and express the human insulin gene wrapped with chitosan NPs in NIH3T3 cells and diabetic rats. AuNPs-DNA functionalized conjugates used as an islet-targeting gene therapy have shown to be an efficient and nontoxic transfection vehicle for islet cells by both *in vitro* and *in vivo* studies (54, 55).

Although pancreatic islet transplantation is considered the most promising treatment strategy for T1DM patients, the transplanted islet is always challenged by immunoreactive cells and antibodies (56). Therefore, surface camouflage of pancreatic islets using biocompatible polymers has been proposed as an alternative approach to protect transplanted islets against immune reactions. Utilization of polymeric semipermeable membranes, like Poly Ethylene Glycol (PEG) hydrogel, to encapsulate the islet and islet-PEGylation technology, has been evaluated for protecting islet graft (57) but several therapeutic immunosuppressive medications must be administered simultaneously to protect transplanted islets in the long-term, and these expose patients to the risk of serious complications. Thus, we developed chemically modified islets with a protective poly(ethylene glycol. However, lately Jeong et al. (58), determined that PEGylation alone was not an affordable immunoprotective method, but the combination of Cyclosporine and anti-CD4 monoclonal antibody (OX-38) along with PEGylation showed a highly improved synergistic effects on the inhibition of sensitized host immune reactions.

Gallego-Perez et al. (59) combined micro and nanofabrication techniques to assemble a biodegradable platform capable of supporting the formation of islet-like structures from pancreatic precursors. The implementation of this fully biodegradable platform could potentially go beyond *in vitro* applications and in the future be adapted as an implantable cell cluster carrier to assist anti-diabetic cell-based therapies.

Axonal degeneration is a hallmark of neurological disability in MS patients. Therefore an alternative therapeutic option is promoting neuroprotection and remyelination (60). Basso et al developed a NPs system based on a water-soluble fullerene derivative (ABS-75) that posses neuroprotective effects against excitotoxic, apoptotic, and metabolic insults in cortical cell culture and *in vivo* models (61). For MS therapy, PEGylated IFN-b1a (BIIB017) has undergone Phase III clinical trials (62). PEGylated anti- TNF-b Fab' fragment (Certolizumab pegol), used for the treatment of RA and Crohn's disease (CD), has also been approved for MS therapy (62, 63).

Nowadays, treatment for inflammatory bowel disease (IBD) such as CD and ulcerative colitis (UC) lacks specificity to the inflammatory sites in the gastrointestinal tract, thus resulting in side effects. In addition, oral macroscopic delivery systems often have a decreased therapeutic efficacy in many cases related to diarrhea, which can accelerate the elimination of the drug from the body (64).

Today, a wide range of potent drugs is available for IBD therapy and can be incorporated into appropriate nanoscale drug delivery systems often complicated by serious adverse effects. Thus, a carrier system that delivers the drug specifically to the inflamed intestinal regions and shows prolonged drug release would be desirable. The advent of TNF-\u03b1 antibodies and other biopharmaceuticals as potent and specific immune modulators in recent years has broadened the treatment options in IBD, but further increases the necessity for adequate drug delivery, as integrity and bioactivity of the biological active have to be ensured. Exploiting the pathophysiological idiosyncrasies of IBD such as increased mucus production, changes in the structure of the intestinal epithelium and invasion of activated macrophages, different colloidal drug carrier systems have been designed to passively or actively target the site of inflammation. This review introduces different micro- or nanoparticulate drug delivery systems for oral application in IBD therapy for the delivery of small molecular compounds and next generation therapeutics from the group of biological (i.e. peptide and nucleotide based (65). A variety of materials like liposomes, solid NPs, and nanocrystals are available for nanocarrier preparation allowing the modulation of oral drug release in particular a reduction in burst release and systemic absorption. The use of liposomes for local delivery in the gastrointestinal tract has hardly been evaluated due to issues of stability and coalescence in the low pH and enzyme rich environment of the gut.

Comparison between Infliximab and Certolizumab pegol in Phase III trials have shown lower immunogenicity (as shown by anti-drug antibodies, absence of infusion reactions, and low rate of antinuclear antibodies); and a maintained clinical response and remission in CD patients (66). Which eventuates in a T-cell driven process, characterized by a T-helper cell 1 type cytokine profile. Several new treatments now focus on suppressing T-cell differentiation or T-cell inflammation. Since inflammatory bowel disease (IBD). Similar to liposomes, solid lipid NPs (SLN) are preferentially employed in parenteral and topical drug administration. A cyclosporin A-loaded SLN formulation has been shown to reduce the undesired plasma peak and to achieve a similarly high reproducible oral bioavailability in the therapeutic window in, *in vivo* stud-

ies done in pigs and healthy human volunteers. However to date, no studies specific for local delivery to the gut have been conducted so far for this delivery platform (67, 68). Recently, Serpe et al reported that Dex loaded SLN determined a more than 90% decrease in the IL-1β and TNF-α mRNA expression; therefore enhancing the anti-inflammatory efficacy of the drug in a human inflammatory bowel disease whole-blood model (69). In a study by Bhol et al. (70), silver nanocrystals were administered intracolonically at a dose as low as 4 mg/kg, and were effective to decrease the signs of colitis in a rat model of UC and was as effective as 100 mg/kg sulfasalazine.

While several *in vitro* and *in vivo* studies have demonstrated the potential use of nanotechnology to improve prevention, diagnosis and treatment/follow up of ADs, some questions still need to be addressed to allow the application of these approaches in the clinical practice.

THERANOSTIC APPROACH

The term theranostics was originally coined to describe a treatment platform that combined a diagnostic test with targeted therapy based on the test results (71). The current nanotheranostics utilize controlled drug vehicles and contain cargo, targeting ligands, and imaging labels for delivery to specific tissues, cells, or subcellular components (Figure 5D) (72). The integration of diagnostic imaging along with therapeutic interventions is critical to address the challenges of autoimmune disorders. For instance, NPs are able to target multiple tissue and cell markers, and deliver multiple agents (peptides, nanobodies, antibodies and nanodrugs) simultaneously for synergy in addressing the challenges of organ and systemic autoimmune involvement.

Most studies on the CNT conjugated QDs have been focused on cancer; however physical and biological properties of these hybrid nanocomposites can be used as a theranostic tool in ADs. For instance QDs-CNT complex can be used in fluorescent intracellular imaging due to the electrochemical luminescent properties of this nanocomposite. Furthermore QDs-CNT complex can also function as biosensors and biological nanoprobes as well as tools for drug delivery into cells (73, 74).

The larger inner volume of CNT allows them to be filled with a drug cargo load and their surface can be modified with a nanoprobe (antibody or a peptide) in order to direct these materials to the site of action. Recently Villa et al demonstrated the selectivity of the immunosensor response and its sensitivity for the detection of anti-chimeric fibrin-filaggrin synthetic peptide (CCFFCP-1) antibodies, present in RA Patients (75). On the other hand, CNTs can be loaded with antirheumatic drugs like methotrexate as have been shown by tethering MTX to multi-walled CNTs (76).

Finally, is important to highlight that networking interaction of interdisciplinary research teams able to integrate the outcomes of studies of multifunctional and theranostic NPs and translate their potential application to autoimmune disorders into clinical advances a reality.

BENEFITS AND DISADVANTAGES OF NPS AND ENGINEERED NANOMATERIALS

Nanotechnology is a fast-growing field of activity that will allow development of materials with brand-new properties. The current knowledge of the toxic effects of NPs is relatively limited. Nonetheless, the available data indicate that some insoluble NPs can pass through the different protective barriers, can be distributed in the body and accumulate in several tissues and organs. Indeed toxic effects have already been documented at the pulmonary, cardiac, reproductive, renal, cutaneous and cellular levels. NPs can be distributed throughout the body, including the interior of cells (77).

Some researchers have shown that most of the NPs can release active oxygen and cause oxidative stress and inflammation by the RES (78). In fact, NPs activate the immune complement system which is a normal response of the to external materials and antigens (79). Therefore, they must be hidden with synthetic materials such as PEG or natural materials such as collagen or chitosan to enable them to be used as NPs in medical applications. v illustrates the main benefits and disadvantages associated with NPs and engineered nanomaterials.

ETHICAL CONSIDERATIONS

Most technologies have the potential of doing good or bad. For many years, ADs have been one of the biggest challenges in medicine, but the seeds of basic and clinical research have begun to bear fruit. In addition, the emergence of new tools such as genomics, proteomics and metabolomics has significantly contributed to the understanding of these pathologies. Recently advances in nanomedicine applied to ADs could allow prevention, early diagnosis, treatment and monitoring of these pathologies. Although there has been more than 30 years of research on ADs, to date no therapies have ever been approved using nanoparticle carriers of molecular recognition agents such as antibodies, aptamers, or peptides. Nonetheless, several clinical trials assessing these "active targeting" strategies are currently ongoing.

At present, the most significant concerns involve the risk/benefit balance from manufacturing mishaps assessment, management of engineered nanomaterials and negative environmental impact.

As the technology has developed, the regulatory challenges have become clearer: defining biocompatibility, biodistribution, manufacturing standards and environmental protection regulations, regulatory classifications, and new regulatory pathways for approval of multifunctional nanotechnologies (80). The development of standard tools for *in vivo* and *in vitro* imaging diagnostics, drug deliver and theragnostic, through proactive translational research in nanomedicine, has opened new pathways to solve unmet clinical needs in autoimmunity.

However it is fundamental to create simultaneously a regulatory framework that allows the protection of public health and the establishment of accurate procedures for the analysis of risk/benefit balance among all parties (i.e., pharmaceutical companies, physicians and patients).

BENEFITS	DISADVANTAGES
Enhance *in vitro* and *in vivo* disease screening by improving sensitivity, selectivity and time to diagnosis	Toxicological risks that are unique to nanomaterials
Development of noninvasive imaging methods	There is a growing set of materials of which the properties are largely unknown
Higher target specificity of drug delivery Significant reduction of local and systemic toxicity	Detection of NPs and nanocompounds adverse effects is complex; since they depends upon the routes of administration, doses, size,
Improved bioavailability of the active compounds	Some nanoparticles have a toxic effect on cerebral endothelium cells
Enable administration of lower doses and minimize the drug adverse effects	NPs can be uptake and accumulate into the reticulo endothelial system
A new generation of devices that are smaller, faster and cheaper	Lacking of *in vivo* of long term about NPs and nanomaterials effects on immune system
Integration of diagnostic imaging capability with therapeutic interventions (theranostics)	

Table 3. Benefits and disadvantages of nanoparticles and engineered nanomaterials. *NPs*: nanoparticles.

ABBREVIATION LIST

- **ADs:** Autoimmune diseases
- **APCs:** Antigen presenting cells
- **AuNPs:** Golden nanoparticles
- **CD:** Crohn's disease
- **CMR:** Cardiac magnetic resonance
- **CNTs:** Carbon nanotubes
- **Dex:** Dexamethasone
- **DMARDs:** Disease-modifying antirheumatic drugs
- **IBD:** Inflammatory bowel diseases
- **MRI:** Magnetic resonance imaging
- **MS:** Multiple sclerosis
- **NDDS:** Nanotechnology-based drug delivery systems
- **NPs:** Nanoparticles
- **NSAIDs:** Nonsteroidal anti-inflammatory drugs
- **PEG:** Poly ethylene glycol
- **PET:** Positron emission tomography
- **QDs:** Quantum dots
- **RA:** Rheumatoid arthritis
- **RES:** Reticuloendothelial system
- **SLN:** Solid Lipid NP
- **SPECT:** Single photon emission computed tomography
- **SPIOs:** Superparamagnetic iron oxide particles
- **SPR:** Surface plasmon resonance
- **SWNTs:** Single Wall Nanotubes
- **UC:** Ulcerative colitis
- **USPIOs:** Ultrasmall superparamagnetic iron oxide particles
- **UV:** Ultraviolet
- **VSPIOs:** Very small superparamagnetic iron oxide particle

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Section V

Basic principles of laboratory

45 CELL CULTURE AND CELL ANALYSIS

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INTRODUCTION

In vitro cell culture is a method used for studying the behavior of animal cells in a controlled environment, free of systemic variations. Currently, different types of cell cultures have been adapted and developed. Animal cell cultures have been applied for studying basic cell biology, interactions of drugs and other chemicals with cells, production of vaccines and proteins, etc. This chapter covers a brief summary of the main features, types, requirements and applications of cell culture methodology. Also, we describe applications and principles for cell separation techniques, ranging from basic to more advanced applications.

DEVELOPMENT OF CELL CULTURE

Cell culture was developed in the early twentieth century as a method to study the behavior of animal cells in an environment free of the systemic changes that can be found in an animal during the normal homeostasis and stress of an experiment. The first model chosen for cell culture were amphibian cells, presumably for being exothermic animals and given that their cells would not require successive incubations. Later, medical science breakthroughs led to an interest in endothermic animals, where the normal and pathological development is similar to that of humans. The advent of mouse strains genetically pure brought mammals to the research laboratories. While the embryos provided a wide range of cell types in primary culture, mouse models had the advantage of continuous cell lines and a substantial tumor cell repertoire.

The progression of cell culture as a modern and sophisticated technique is based mostly on the needs of two major medical branches: virology and oncology. Cell culture has also been welcomed in many medicine and manufacturing routine applications. Cytogenetically analysis of amniocentesis derived-cells has the ability to disclose genetic disorders in the fetus. Likewise, viral infections can be evaluated quantitatively and qualitatively in host-cultured cells. Toxic effects of potential pharmaceutical compounds and contaminants can be evaluated also by using cell cultures.

CELL CULTURE APPLICATIONS

The main uses of cell culture systems include:

Experimental model systems in basic and medical sciences. Cell culture offers certain advantages over the environmental and biological variability of other models. In addition, the use of genetically defined and characterized cell lines can simplify the analysis of experimental data. On the other hand, results obtained with specific cellular systems may not be representative of a wide range of other types of cells.

Study of physiological requirements for certain cell types. These include studies on positive effects of growth factors, growth-promoting substances, negative effects of cytotoxic compounds or xenobiotics and events related to programmed cell death (apoptosis), as well as cell proliferation, cell activation, cell signaling or any other cellular process.

Studies of cell development and differentiation. these include aspects of cell cycle and gene expression.

Pathological studies. these are enclosed in the characterization of cells using karyotyping to determine their genetic status.

Genetic Manipulation. cell culture techniques have played an essential role in the development of molecular biology, through the development of methods such as transfection.

Biotechnology. based on the manufacturing and industrial production of therapeutic proteins, vaccines and monoclonal antibodies.

ANIMAL CELL CULTURE SYSTEMS

Cellular systems can be established from whole organisms (e.g., chicken embryo), discrete organs (e.g., mouse liver), or blood cells (e.g., lymphocytes). While, in theory, it is possible to grow nucleated cells from any source, in practice, the highest probability of success is achieved with active young cells. The main considerations for the development of cell cultures are:

Biosafety. It is important to be aware of the potential infection risks when culturing animal cells. Although animal cells have a reduced risk of disease transmission compared to human cells, cultures should always be handled as a potential source of pathogenic microorganisms.

Using primary culture or continuous cell lines. freshly isolated cells more easily reflect the biochemical dynamics of the cells *in vivo*, although having a limited life span. Continuous cell lines are easy to use and offer the advantage of a priori knowledge of their specific growth requirements.

Culture media requirements. These include the provision of inorganic ions (such as a balanced salt solution), a carbon source, organic nutrients and other supplements that include antimicrobial agents to counteract the risk of contamination. For growth support, usually a basal medium supplemented with serum (e.g., fetal bovine serum) or a homologous serum supplement with a set mixture composition of proteins, polypeptides, hormones, lipids, and trace elements. Levels of CO_2 and O_2 should be considered; many cultures are buffered with bicarbonate and should be kept in a CO_2 rich atmosphere.

ADVANTAGES AND DISADVANTAGES OF CELL CULTURE

Clearly, the study of cellular activity *in vitro* has several advantages and disadvantages. The main advantage is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. Cell cultures have a highly control of the physicochemical environment (i.e., pH, temperature, osmotic pressure, oxygen, and carbon dioxide tension) which can be controlled very accurately, and the control of physiological conditions, which can be constantly examined.

The disadvantages of cell culture are: highly skilled personnel, techniques must be performed using strict asepsis techniques because animal cells grow slower than many of the common contaminants (e.g., bacteria, viruses and fungi). Additionally, animal cells may not survive when isolated and therefore are not capable of an independent sustainable existence without providing a complex environment. One of the main limitations of cell culture is the expense and effort that has to be applied to obtain a relatively low amount of cells.

In addition, tissue composition is variable and heterogeneous. Replicas from the same sample have various constituents. To replicate an experimental result, cell lines must be manipulated many times in serial passages. For instance, every culture is going to be different from the original and less uniform in its constitution. In order to resolve this issue, the replicas are randomly mixed in each passage and the selective pressure of growing conditions tends to produce an optimal prevalent phenotype.

MAIN TYPES OF CELL CULTURE

Cell culture is a collection of techniques and resources in which cells that were part of an organism are growth in an artificial controlled environment. Usually, tissue must be previously treated to disrupt it by mechanical or enzymatic processes depending on the origin of the tissue and the purpose of the cell culture. However, some cells can be culture without this treatment such as in the case of liquid samples.

During tissue culture, tissue characteristics and architecture must be retained, at least partially. For instance, growth is slow and restricted to the use of embryonic tissue or 3D cell culture procedures. The 3D cell culture mimics the microenvironment, architectural design and functionality of a normal tissue under control conditions. In some cases, cell migration is observed through the solid phase when a piece of tissue is placed on a solid liquid-interface. Thus generating primary explants that grow outside the original tissue

The Primary Culture is the first culture that grows successfully after the cell isolation from a tissue. As mentioned above, cell should be subculture in a series of passages in order to keep the best condition for cell growth. As result, these cell lines go into senescence after the thirtieth division cycle. For this reason, cells are storage in a cell bank system to maintain them for long periods of time. In some cases, the cells can be immortalized, e.g., B cell lymphocyte can be immortalized with the Epstein- Barr virus to confer them the ability to proliferate indefinitely. These transformed cells have the advantage of unlimited availability, but they have the disadvantage of losing its initial characteristics. Figure 1 shows the main ways to start cell cultures (for further information please see recommended readings).

PRELIMINARY PREPARATION AND SAMPLING

Cell samples are obtained and prepared from organs, tissues and biological fluids depending on the target cell type, type of genetic material and type of analysis to be performed (Table 1). In all cases one must follow processes to ensure the quality of the sample. Generally, the preparation is performed after obtaining the sample, however, if this is not possible, conditions should be provided to ensure that the sample does not undergo degradation.

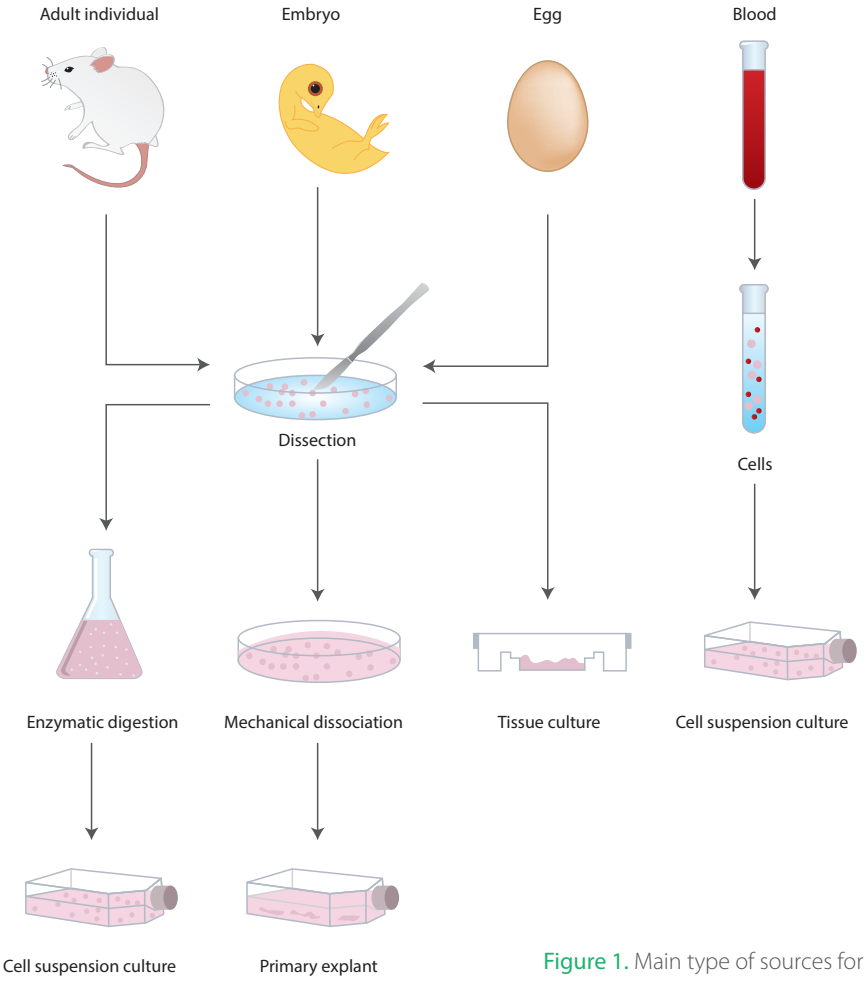

Figure 1. Main type of sources for cell cultures. Adapted from (1).

The success of each technique depends on the proper acquisition, processing and preservation of the sample.

CONSIDERATIONS FOR BLOOD SAMPLES

Blood-drawing is a critical step for the experimental design. There are many factors that influence the conditions of blood components such as nutritional status, hormone levels and the circadian rhythms. If an experiment or project depends on the analysis and comparison of multiple parameters in blood samples collected from different individuals, it must be ensured that blood drawing should be carried out under the same conditions. For example, nutritional state and circulating hormones and cytokines may have important effect on the performance and behavior of the cells. Thus, in fasting state, the leptin levels in the blood are diminished and as a result, there are differences in T cell processes such as differentiation and increasing IFNy secretion and decrease IL-4 production. In addition, differences in leptin levels have been shown to interfere with the T-regulatory cells proliferation.

Another important factor for evaluating immune functions in blood samples is the time when the sample is collected as circadian rhythms play an important role. One example is the response to the toxoid of *Clostridium tetani*. Typically, a higher inflammatory response is seen around 3 a.m (represented high IFNy/IL-10 ratio) and the lowest point during the day is late morning and evening (10 a.m and 8 p.m). The highest values correspond to the peak of plasma melatonin, while the lowest values are related with higher levels of cortisol. These two hormones have opposing immune effects; cortisol naturally inhibits the pro-inflammatory response while melatonin stimulates it. As a consequence, the levels of these hormones and the way that cells respond to stimulation depend on the level of hormones and cytokines that are also influenced by the circadian rhythm.

Regarding the anticoagulant used for getting blood samples, it is very important to have in mind the effect that it may have on the cells. As for the more common anticoagulant the ethylenediamine tetraacetic acid (EDTA), one of its properties is to chelate metals, especially calcium which is extremely important for the cell activation through the calcium channels. For this reason it is recommended using other anticoagulants such as acid citrate-dextrose (ACD), sodium heparin, lithium heparin or sodium citrate. Also, the time that cells are ex-

SAMPLES TYPE	CELL TYPE	ANALYSIS
Solid tissue	Tumoral	Nucleous, cytoplasm and membrane markers
	Hepatic	Metabolic diseases
	Muscular	Enzymopathies
	Intestinal	Enzymopathies
	Bone	Tooth or pulp marrow or other bones
Cellular suspension	Embryonic cells	Pluripotent cells
	Amniotic liquid	Amniocentesis. Prenatal diagnosis
	Blood or bone marrow	Prenatal diagnosis. Cytological observation, genetic and histological studies
	Buccal swaps	DNA isolation
	Semen	Fertility markers. Forensic investigation
	Cultured cells	Cell line characterization
Extravascular biological fluids	Cerebrospinal	Lumbar punction. Nervous system disease detection and some cancer markers
	Synovial or articular liquid	Inflammatory and pathological mechanical process

Table 1. Some types of cell cultures and their applications.

posed to the anticoagulant must be very short, to ensure proper cell recovery, viability and function. Temperature for blood storage is also critical. Therefore, it is recommended to storage the blood at room temperature to avoid abrupt changes that may have effects on the cells.

CELL SEPARATION METHODS

To obtain tissue samples suitable for laboratory analysis, several procedures like separation, fractionation and characterization are usually performed and they are applied based on the properties of each cell type in the sample. The choice of each protocol depends on the desired degree of separation, preservation of viability, and technical analyses that would be studied.

Cell separation techniques have the advantage to allow high yield and recovery in a shorter time. Some examples are separation by density sedimentation and/or flow cytometry. In general, high performance techniques used for cell separations rely on differences like: 1. Cell size, 2. Cell density (specific gravity), 3. Cell load, 4. Cell surface chemistry, 5. cellular complexity and 6. fluorescence emission of two or more cellular constituents or adsorbed antibody.

CELL SEPARATION BY SEDIMENTATION AND CENTRIFUGATION METHODS

Centrifugation depends not only on the centrifugal force but also on other factors which modify sedimentation and are dependent on the cell characteristics.

Differential centrifugation

This process is normally the most simple in practice, given that it only can separate cells showing large differences in size (at least 10 times) (Figure 2a). In the case of blood with anticoagulant, at 200 g (g = gravities) erythrocytes sediment in the lower zone, leukocytes appear at the interface and at the supernatant there will be a platelet rich plasma phase. It is possible to obtain plasma with less platelets using higher speed (3.000g) (Figure 2b).

Density gradient centrifugation

Barrier methods (centrifugation through a continuous centrifugation media). To achieve more effective separations

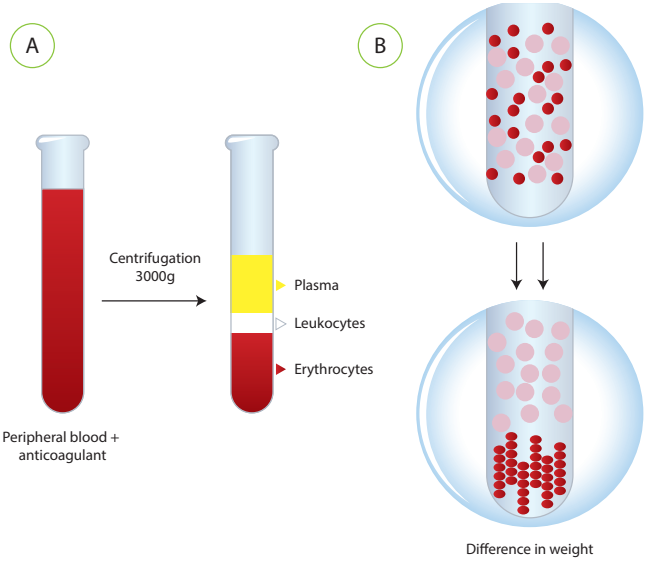

Figure 2. Differential centrifugation. Separation is done according to size and the sedimentation coefficient, which is dependent on the mass. **A.** Peripheral blood separation. **B.** Differential centrifugation by size.

each sample is centrifuged on a bed of density intermediate between the two cell types that are to be separated.

Zonal centrifugation or sedimentation rate. This method is used to separate cell types whose sedimentation coefficient differs. To be able to perform this separation it is necessary to form a density gradient, which favors the concentration of each cell type in a narrow band or zone. The density gradient is generated before adding the sample, using several different solutions with suitable concentration of a compound (Ficoll, sucrose, albumin, fetal bovine serum).

After the gradient is formed, a small volume of sample is deposited upon it and centrifuged for a short time. Typically, for this process a gradient less than the maximum density of cells is used and therefore it may not reach the sedimentation equilibrium. Always shorter times are used, if cells are centrifuged for longer times they will leak and end up in the background, this is how the result is dependent on the time of centrifugation. This technique can separate all blood cell types, viable sperm, viable and non-viable cells from disaggregated tissues and suspension cell samples (Figure 3a).

Peripheral mononuclear blood cells separation. The peripheral blood mononuclear cells (PBMCs) include lymphocytes, monocytes and macrophages. They share different characteristics as the presence of a circular nucleus and their density. As a consequence of these characteristics they can be isolated by different techniques (see above-Cell separation methods) and also by the use of special type of tubes as the BDVacutainer® CPT™ which is a vacuum-driven drawing tube containing anti-coagulant and a cell separation medium. The procedure to isolate the cells may also affect them, as for the use of different components to the separation and the serial washes to clean them.

Using Ficoll-Hypaque, which has a density of 1.077 g/mL, density identical to that of lymphocytes and monocytes, it is possible to recover PBMCs. The Ficoll-Hypaque is a combination of a polymer of high molecular weight sucrose (Ficoll) and an organic compound (sodium diatrizoate: 3-5 bis acetylamino-2, 4, 6 tri-iodobenzoic acid). Granulocytes and erythrocytes have a higher density and when peripheral blood is centrifuged in a Ficoll-Hypaque gradient, it passes through a package formed on the bottom of the tube. Platelets have a lesser density and remain in the plasma and the mononuclear fraction located at the interface (Figure 3b).

There are other solutions for the separation of mononuclear cells using density gradients, the most effective one is the use of Percoll. To separate mononuclear, the commercial solutions most commonly used is Lymphoprep, Hystopaque and Lymphopure. Several media solutions are available with suitable densities for the separation of other specific cell types: Nycoprep -1.077 for mononuclear cells, Nycoprep -1.068 for monocytes, Polymorphoprep for polymorphonuclear cells and Nycoprep -1.063 for platelets.

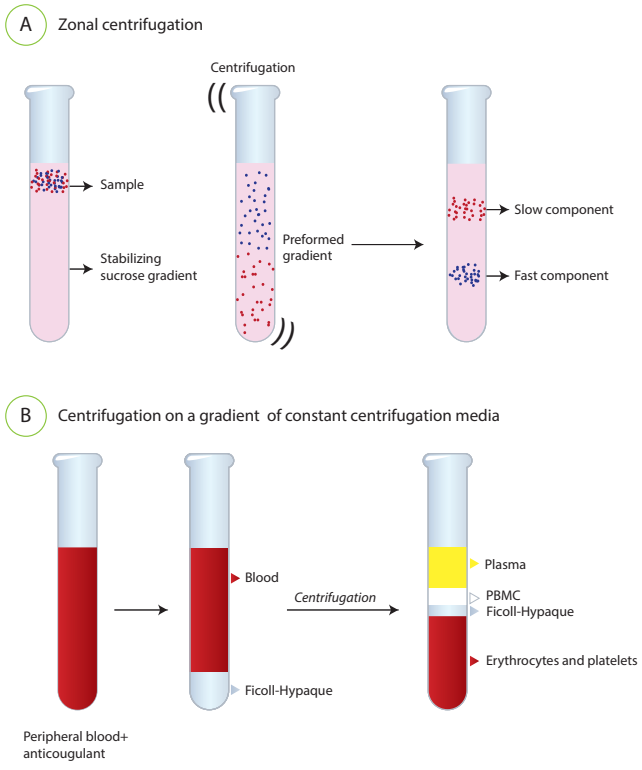

Figure 3. **A.** Zonal centrifugation, separation depending on the coefficient of sedimentation. Centrifugation stops before the sedimentation equilibrium is reached. **B.** Centrifugation on a gradient of constant centrifugation media, separation media have constant density between both cell types (see text). PBMC: Peripheral blood mononuclear cells.

Isopycnic centrifugation or sedimentation equilibrium. This is a method in which cells are separated solely according to its density using another centrifugation variant, called isopycnic (der. Greek. Similar density). It is also performed on a density gradient, but in this case the centrifugation time is sufficiently long to reach the sedimentation equilibrium. To achieve the sedimentation equilibrium continuous gradients are used to cover the entire range of cell densities: at the bottom of the tube has to have the greater density than the denser cells. Thus, independent of the time of centrifugation, the cells will never sediment at the bottom but instead will reach a stable intermediate position in the gradient.

Centrifugal elutriation. This process involves the separation of cells according to their sedimentation rate. The original process consists on performing successive cycles of sedimentation and decanting provided by the incorporation into a system of liquid flow suspension. Finally, when it is combined with the effect of the centrifugal force it results in centrifugal elutriation. Here the cells are exposed to two opposing forces; firstly by centrifugal force and the drag force by the continuous flow of the medium counter force (Figure 4a & b).

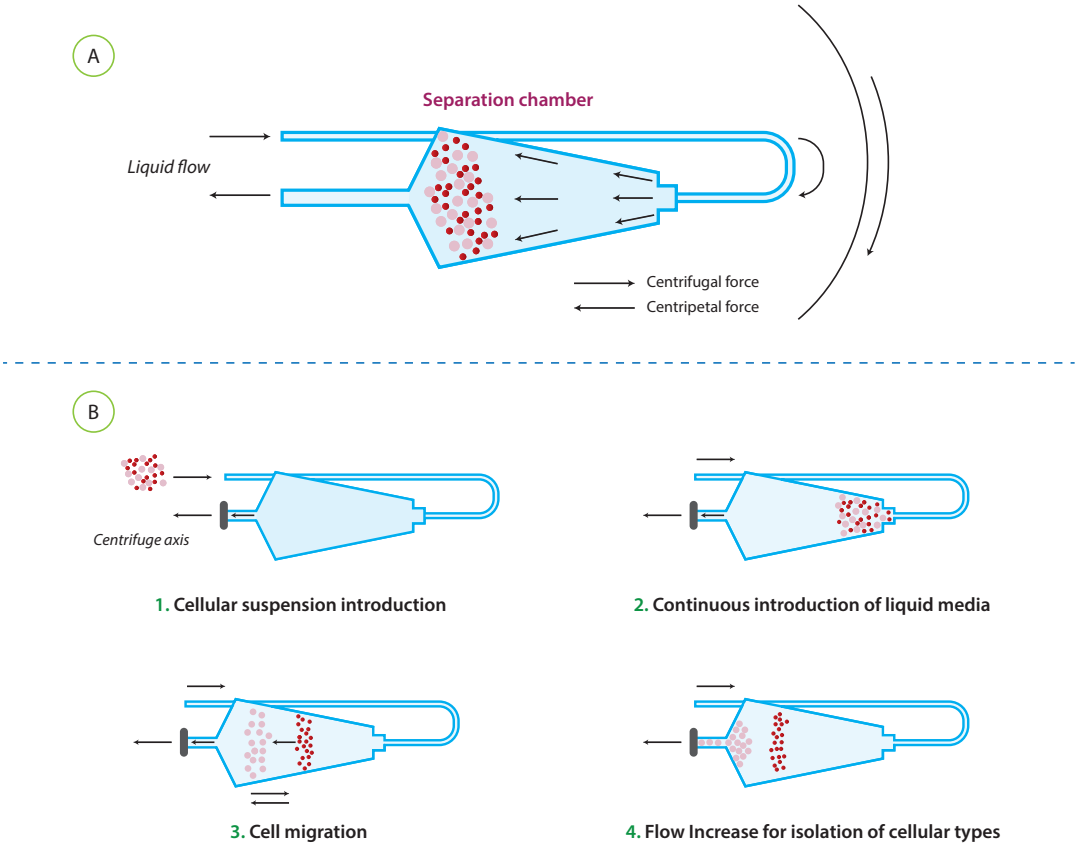

Figure 4. Elutriation. **A.** Elutriation separation chamber. **B.** Elutriation process. 1. Introduction of the cell suspension. 2. The centrifugal force pushes the cells to the bottom of the chamber, continuously introducing a liquid medium, which opposes the centrifugal force. 3. Cell migration in the flow and achieving of a dynamic equilibrium. 4. Increased recovery rate for separate cell populations.

The above method yields a versatile, rapid and effective form of separation for cell subpopulations according to their size from a mixture. Moreover this process, admits high cell concentrations and even allows for a greater recovery rate of viable cells compared to the original cell.

BLOOD CELL STORAGE: CRYOPRESERVATION AND THAWING PROCESS

Sometimes cells are not necessarily cultured immediately after the blood drawing, thus it is necessary to keep and storage them for future experiments. Freezing is the best way to storage cells. However, freezing affects the proliferation and cytokine secretion as well as the protein production and mRNA expression. Other consequences of the freeze-thaw process are mechanical injuries produced by crystal formation, alteration of physical properties and shape of cellular structures. That is why protocols for freezing cells include cryoprotectant substances in the freezing media that prevent crystal formation and avoid osmotic injury. Some examples are the dymethylsulphoxide (DMSO), glycerol, ethylene glycol or hydroxyethyl.

Hence, it is necessary to take into account many considerations when freeze-thawing cells. Firstly, it is extremely important to process and store the cells within a period between 8 to 24 hours after samples are collected and they should be conserved in especial freezing media. Secondly, it could be helpful to include in the freezing media caspase inhibitors to avoid the apoptosis of the cells as a consequence of the stress. And finally, the rate by which the temperature will decrease should be slow enough to avoid the rupture of the cells. This is done using special containers that have alcohols (i.e Isopropanol) which surround the sample tubes. Thus, the rate of temperature decreasing is gradually, and close to 1°C/min to -70°C. After this process frozen samples should be transferred to liquid nitrogen promptly within the next 24 – 72 hours.

As for the thawing process it is recommended to thaw the cells rapidly by transferring the cryovials directly from liquid nitrogen to 37°C. Immediately after the samples are thawed, they should be diluted and washed to eliminate the cryopreservant that could be toxic for the cells. The rapid change of temperature and media diminish the osmotic variation and protects the integrity of the cells. However, despite all these considerations there may be some problems, for example, cell clumping is frequent following the thawing process as a consequence of the release of DNA of dead cells. In that case, DNase can be used to avoid the aggregation of cells. Finally,

SAMPLES TYPE	ANALYSIS
Blood drawing	Fasting State
	Same time for all samples
	Anticoagulant: Sodium citrate or lithium heparin
	Minimum delay in processing (within 8 to 12 hours)
PBMC isolation	Ficoll
	CPT tubes
	Washes with media containing inactivated serum
PBMC freezing	Cell concentration per tube < 3, 107 cell/ ml
	Freezing rate 1°C/ min to -70°C.
	Within 24 to 72 h transfer samples to liquid nitrogen for storage
PBMC long preservation	Preferably, storage in liquid nitrogen for less than 6 months
PBMC thawing	Fast, transfer to 37°C for complete thawing
	High volumes for thawing process (15 to 50 ml), using media with serum.
	Low speed and 5 to 10 min of centrifugation for washing
	1 to 12 h of cell resting for culture and functional analysis
	Note: DNase treatment is required when there is cell clumping
PBMC assays	Human serum-supplemented media
	Use of high quality protein or peptide antigens

Table 2. General guidelines for sample blood collection and cell processing.

it is recommended giving the cells a resting period before the experiment so they can eliminate the components that they were producing before the freezing process. This resting period allows them also to get used to the new conditions and favors to normalize the conditions for future comparisons. General recommendations about the treatment of blood samples for cell culture are summarized in table 2.

CHARACTERIZATION AND SEPARATION OF CELLS BY CELLULAR MARKERS

FLOW CYTOMETRY

Today, flow cytometry is an important method in biomedical research and clinical laboratories, especially for its ability to analyze automatically different cell suspensions. Flow cytometry is based on the transportation of a cell suspension (e.g., blood cells, bone marrow aspiration and dissociated tissues) driven by the flow of an isotonic solution to the measuring point or flow chamber. Flow cytometry uses include analysis (biomarker detection) and separation (sorting) of cells previously labeled with fluorochromes (Figure 5). Some flow cytometers only perform the first, while others carry out both.

Characterization of a cell population. Briefly, the cell suspension arrives in laminar flow conditions, forming a very fine line containing individual cells in succession. These cells pass one by one through a laser whose wavelength allows excitation of previously incorporated fluorescent markers. The light emerging from each cell is analyzed for scattering and fluorescence intensity.

Cell characterization is accomplished by measuring multiple physical characteristics of the cells, as they flow to a beam of light. The properties measured include the relative size, relative granularity or internal complexity, and relative fluorescence intensity given by the use of fluorocromes. In order to make these measurements, the cytometer has three main systems: fluidics, optics, and electronics. The first one transports the cells to the interrogation point where the laser bean pass through. The second one consists of the different lasers that illuminate the cells in the interrogation point, and directs the light to the filters and detectors. And finally the third one is the electronic system that helps to convert the changes in light signals from the detectors to values that can be interpreted by the computer.

This technique has multiple functional applications. study different cell surface markers and intracellular signaling by using monoclonal antibodies, assessment of DNA and RNA content of the cell and the determination of its shape and size.

CHARACTERIZATION OF A CELL POPULATION BY FLOW CYTOMETRY

The first characteristics than can be determined by the simplest cytometer are the shape and the internal complexity of the cells. These characteristics are measured by the changes in the light scattering. It occurs when a cell deflects incident laser light. Hence there are many cellular factors that affect light scattering such as cell's membrane, nucle-

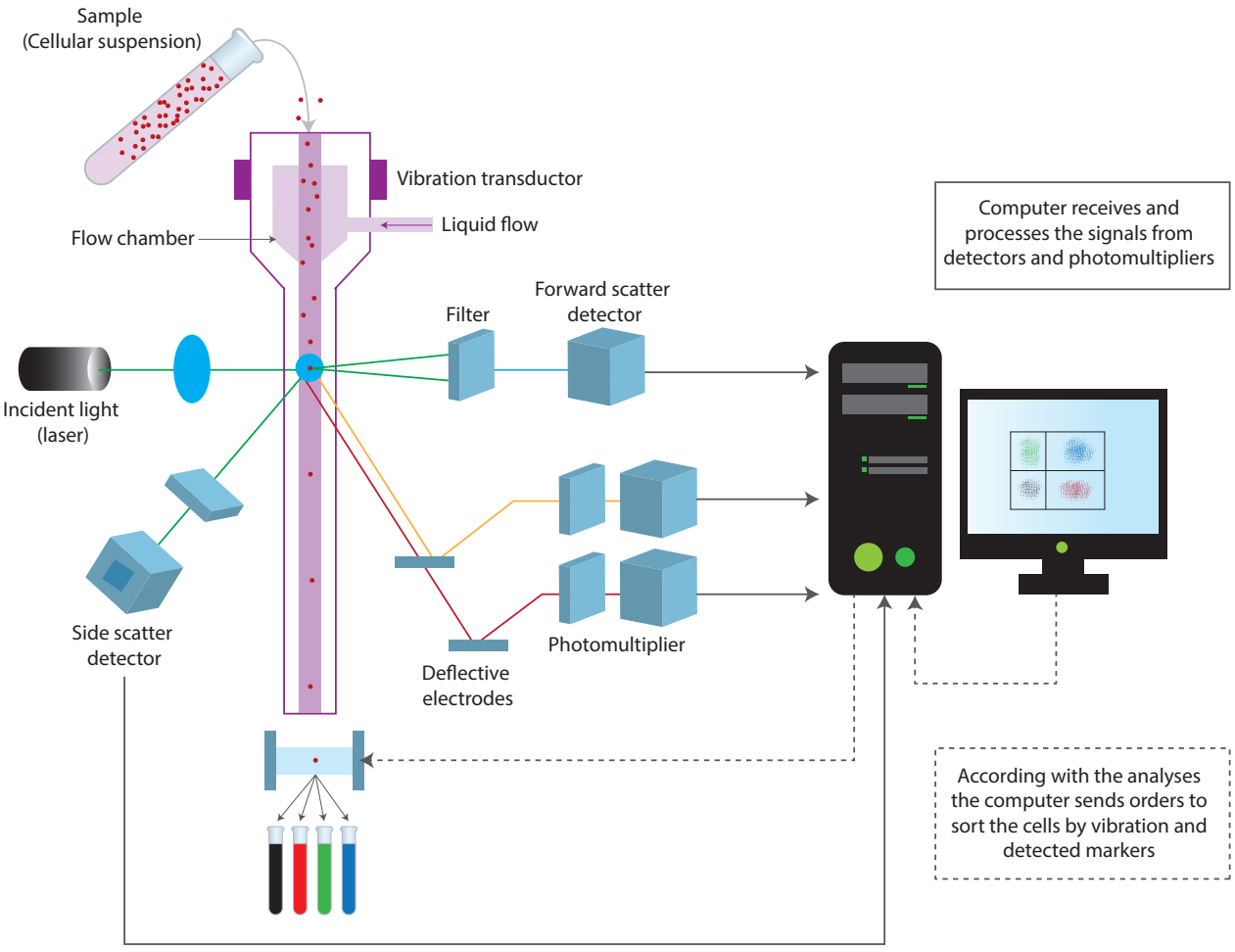

Figure 5. Flow cytometry. Principles of operation of the flow cytometer (see text). Adapted from (1).

us, and any granular material inside the cell. Also, the cell shape and surface topography can contribute to the total light scatter.

There are two ways to measure the scattered light. First, the forward-scattered light (FSC) is proportional to cell-surface area or size. As a consequence, FSC can be interpreted as the shadow projected by the cell, finally the detection of FSC is done parallel to the lasers. On the other hand, the side-scattered light (SSC) is proportional to cell granularity or internal complexity. For instance, SSC is the measurement of mostly refracted and reflected light that occurs at any interface within the cell, it means that SSC indicated how much the light is diverted from the original source as a consequence of the content of the cells. As results, SSC is collected at approximately 90 degrees to the laser beam (figure 6).

In addition, superficial and internal cell markers can be detected by flow cytometry and they allow a better characterization of the cell population within a sample. In order to do these analyses it is necessary to use antibodies that bind to the markers of a specific cell population, but there is not enough binding of the antibodies to identify the cell. As a result, the antibodies should be labeled with different flurochromes. As an example, a cytometer with three lasers can detect 8 colors. It means that it can be used to find 8 different cell markers plus SSC and FSC.

FLUOROCHROMES

Typically, a fluorescent compound absorbs light energy over a range of wavelengths that is characteristic for that compound. The absorbed light causes excitement of electrons in the compound raising them to a higher energy level. Finally, when the source energy finishes the excited electrons quickly decay to their basal state, emitting the excess energy as photon light. This process is called fluorescence.The range over which a fluorescent compound is excited is known absorption spectrum. On the other hand, the range of emitted

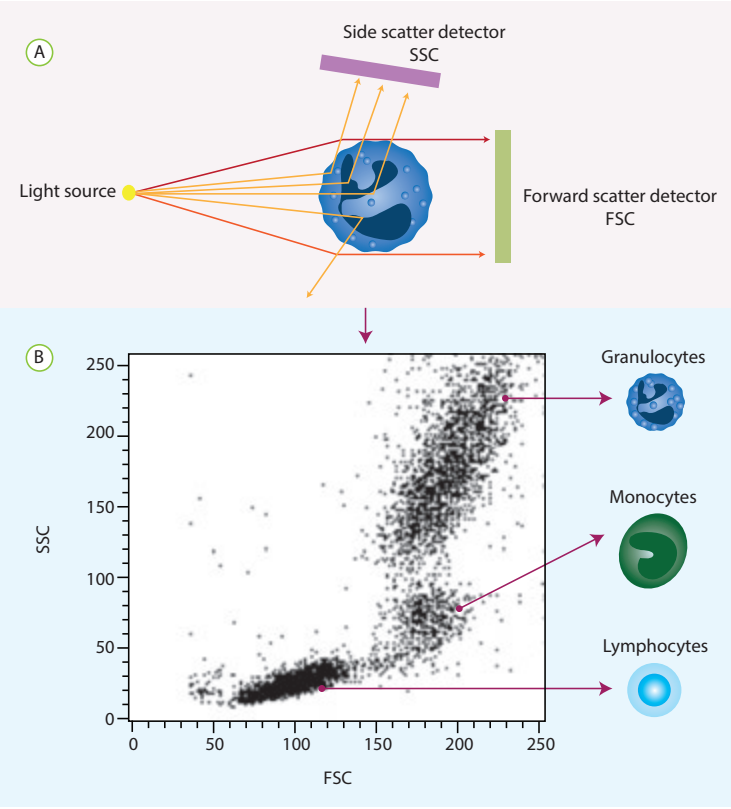

Figure 6. **A.** Schematic representation of side and forward scatter light (SSC and FSC). **B.** Results of FSC vs SSC analysis of leucocytes from whole blood.

A

Fluorochrome	Excitation nm	Emission nm
Hoechst	350	461
Brilliant Violet ™ 421	407	421
Brilliant Violet ™ 421	404	448
Pacific Blue	401	452
BD Horizon ™ V500	415	500
AmCyan	457	491
Alexa Fluor ® 488	495	519
FITC	494	519
PE	496	578
Brilliant Violet ™ 605	407	602
Horizon ™ PE-CF594	496	612
PI	351	617
7-AAD	543	647
APC	650	660
Alexa Fluor ® 647	650	668
PE-Cy™5	496	667
PerCP	482	678
PerCP-Cy™5.5	482	695
Alexa Fluor ™ 700	696	719
PE-Cy™7	496	785
APC-Cy7	650	785
APC-H7	650	785

B

Fluorochrome	Relative Brightness
Brilliant Violet ™ 421	Brightest
PE	
Brilliant Violet ™ 605	
Horizon ™ PE-CF594	
PE-Cy™5	
APC	
PE-Cy7	Bright
Alexa Fluor ® 647	
PerCP-Cy™5.5	
Alexa Fluor ® 488	Moderate
FITC	
BD Horizon ™ V450	
Pacific Blue	
Alexa Fluor ™ 700	Dim
PerCP	
APC-Cy7	
AmCyam	
BD Horizon ™ V500	
APC-H7	

Figure 7. Fluorochrome characteristics. **A.** Specific wavelength values of excitation and emission. (Colors represented the fluorescence emission visible color for each fluorochrome). **B.** Relative brightness scale of common fluorochromes. It is recommended to use dim fluorochromes for cell surface markers highly expressed. Meanwhile, for intracellular markers or low expressed surface markers it is better to use bright fluorochromes.

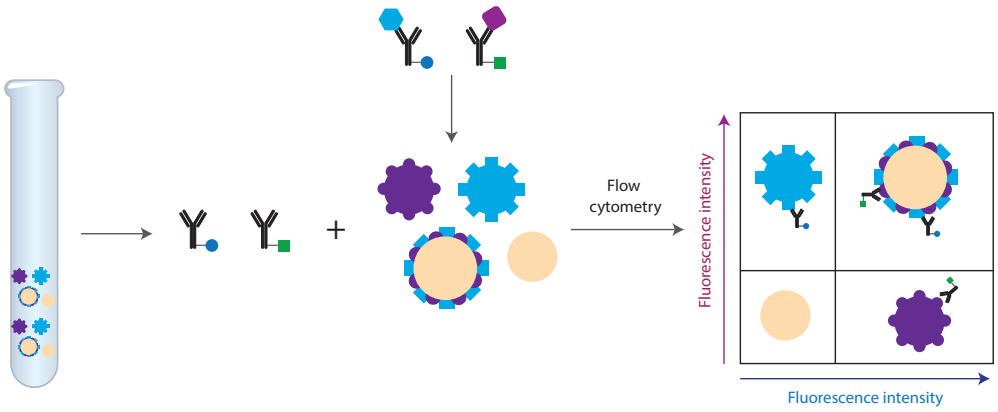

Figure 8. Antibodies labeled with fluorochromes against specific cell markers allow the characterization of a cell population within a sample.

wavelengths for a particular compound is known as emission spectrum.

Fluorochromes are fluorescent components that are excited by different wavelength, and they emit light in specific wavelengths in the visual spectra that can be detected by an instrument (Figure 7). Hence, this property is used for labeling antibodies; commonly, a fluorescent dye is conjugated to a monoclonal antibody, then it can be used to identify a particular cell type based on the characteristic cell markers (Figure 8).

The correct detection and characterization of cell populations depends on the right choice of fluorochromes and the correct calibration of the Flow cytometry. In the first case, it is important to know which cells are going to be analyzed and how is the expression pattern of the marker that is going to be used for their characterization. For example, a marker which has a low expression in the cell surface should be labeled with a strong fluorochrome in order to intensify the signal. On the contrary, a marker highly expressed can be labeled with a fluorochrome with mild intensity (Figure 7).

Secondly, the cytometer should be calibrated in order to provide good quality results. One of these calibration processes is called compensation and it should be done every time that an experiment is run. Compensation is the correction for the spectral overlap during multicolor flow cytometry experiments. The goal of color compensation is to correctly quantify each dye with which a particular cell is labeled. This is done by subtracting the portion of the signal overlapping between fluorochromes (Figure 9. For further information please go to the recommended lectures).

All this together, allows the characterization of a mixed population of cells by using different fluorochromes. This method leads to distinguishing and characterizing the subpopulations within the sample in combination with FCS and SSC. The combination of different markers, FCS or SSC enables the definition and sub-analysis of populations known as "gating'. (Figure 10)

CELL SEPARATION BY FLOW CYTOMETRY OR FLUORESCENCE ACTIVATED CELL SORTING (FACS)

This process allows the separation of cell samples fractions according to their morphologic and fluorescent characteristics. A regular flow cytometry analysis must be done before to define which populations will be sorted. Then, cells are separated in different fractions when the flow passing through the detection point is transformed into small droplets. These droplets contain a single cell (as a result of an

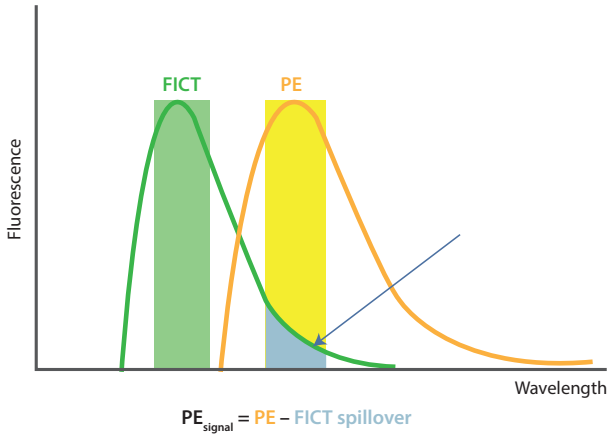

Figure 9. Compensation. In an experiment with more than two fluorochromes there can be spillover. Spillover takes place when the presence of the other fluorescent reagent can contribute significantly to optical background in proportion to the brightness of a specific fluorochrome (In this case FITC is affecting PE signal). This is the result of the physical overlap among the emission spectra of certain commonly used fluorochromes. Consequently, spillover occurs whenever the fluorescence emission of one fluorochrome (FITC) is detected in a detection window designed to measure signal from another fluorochrome (PE). This phenomenon is corrected by the compensation process, which basically subtracts the signal generated by FITC in the detection window of PE in order to have the exclusively PE signal.

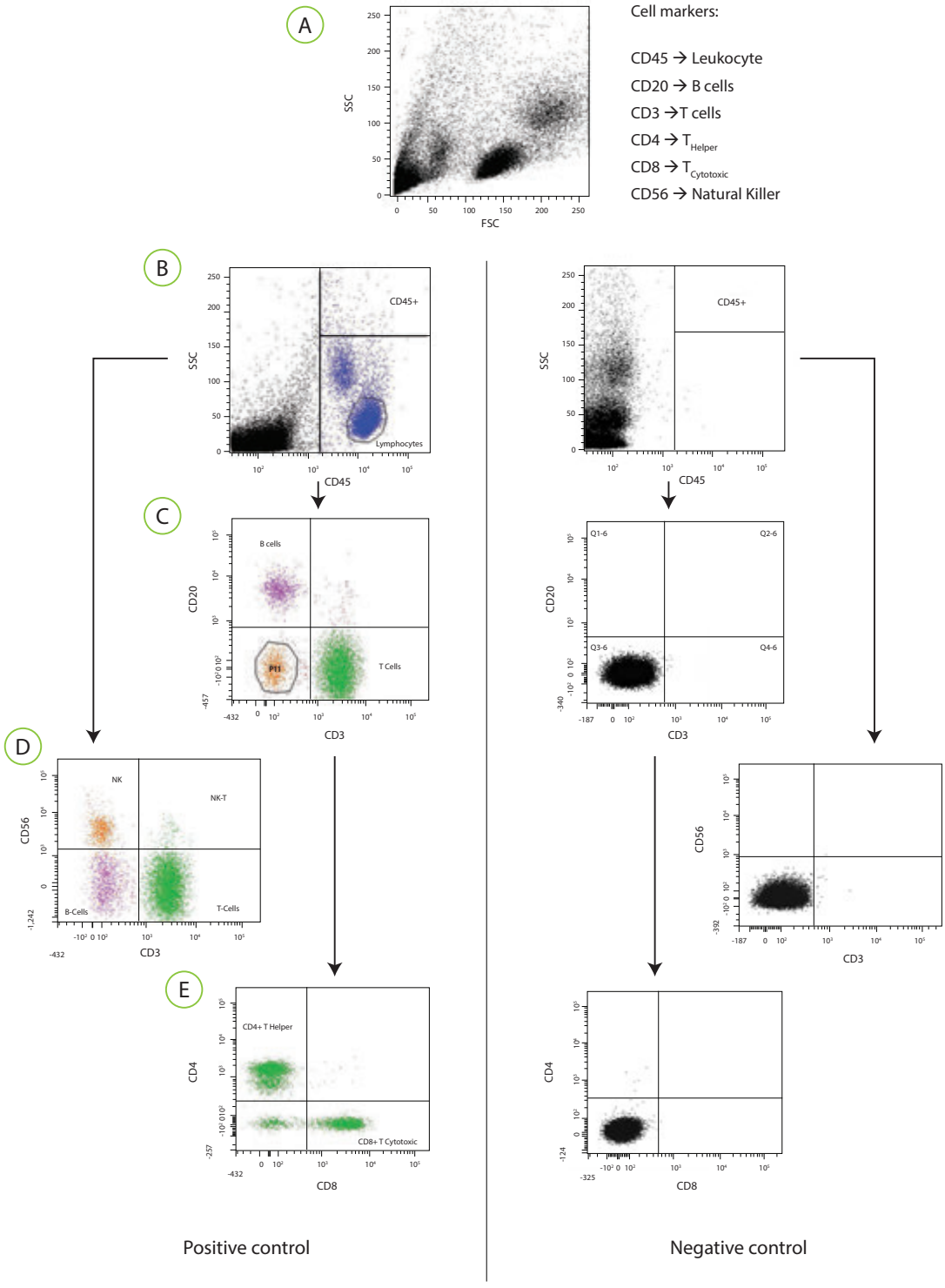

Figure 10. Gating approach to analyze peripheral blood mononuclear cells (PBMC). Left panel shows the results for the stained control and right panel shows the results for the unstained/negative control, which includes all the events registered in panel A. **Panel A.** Normal distribution of PMBC by complexity and size. **Panel B.** Strategy is based on the natural differentiation process of the CD45 positive leukocytes. CD45 is universal common leukocyte antigen (Blue). Next in the gating strategy, it is possible to identify monocytes from lymphocytes by their internal complexity and CD45 marker presence. **Panel C.** Gate perform to gate lymphocytes by the presence of CD20 and CD3 markers, leading to the examination of B- and T- Cells. D. Lymphocyte gate is screened for CD56 and CD3 markers, this will confirm the distribution and identity of T- and B Cell relative to panel C. Moreover, NK and NKT cells are observable. **Panel E.** The T cell gate is analyzed for the presence of CD4 and CD8 markers leading to the identification of T Helper and T cytotoxic cells.

ultrasonic vibration). Finally, a voltage pulse which provides an electrical charge is applied according with the cell characteristics. This pulse allows the cells separation when the droplet passes through an electrical field. Then, cells are deflected according to their charge, thus falling into different sample collection tubes (Figure 5).

AFFINITY SEPARATION BY MAGNETIC PARTICLES

This process is of great application. Currently in use are commercially available microspheres (beads) in several sizes (0.5 - 10 microns) for different applications and formed by a super-paramagnetic material (iron oxide) coated with a thin layer of plastic polymer which allows the absorption and/or covalently binding of different molecules. Often an antibody is bound to magnetic or immunomagnetic microspheres antibodies (Figure 11).

COUNT AND CELL VIABILITY

The accurate determination of the amount and viability of the cells is very important for correct standardization of reagents and conditions for cell culture experiments. Counting is performed using a hemacytometer counting chamber consisting of a central chamber (double counting chamber) which is divided into two parts by a transverse slit of 1mm. Each chamber consists of a silver film etched in a grid of 3x3 mm. Each rack is divided into nine side frames, each of 1x1 mm. Boxes in the corners are divided into 16 squares and center box in 25. The hemacytometer is accompanied by a thin cover glass slide with a weight that determines the exact depth when placed on the chamber (Figure 12). Alternatively, cells can be counted by using flow cytometry according to the number of events registered in the analysis or the use of special tubes which allows the determination of absolute cell counts (Further information can be found in recommended readings or in the Becton Dickinson website http://www.bdbiosciences.com/research/).

Cell viability refers to the ability of a cell to perform its biochemical and physiological processes, particularly in regards to its metabolism and ability to divide. However, in practice the term is relative as it is used with different criteria; viability is commonly spoken of when referring to cell integrity or metabolic activity or their proliferative capacity. In fact, with the use of a vital dye exclusion is possible to determine cell viability base on the integrity of the membrane from living cells. This membrane excludes certain dyes such as trypan blue, eosin, 7-AAD or propidium iodide, whilst dead cells allow their passage into the cytoplasm (Figure 13). For this method, the cell suspension is mixed with a known volume of dye and examined visually to determine the dead and live cells. In the case of trypan blue, cytoplasm is seen in refractive (clear) when the cell is alive while the cytoplasm of dead cells is seen blue. Propidium iodide stains dead cells red when observed in the fluorescence microscope. Alternative, flow cytometry can be used instead of the microscope with the colorants 7-AAD or propidium iodide (Figure 14).

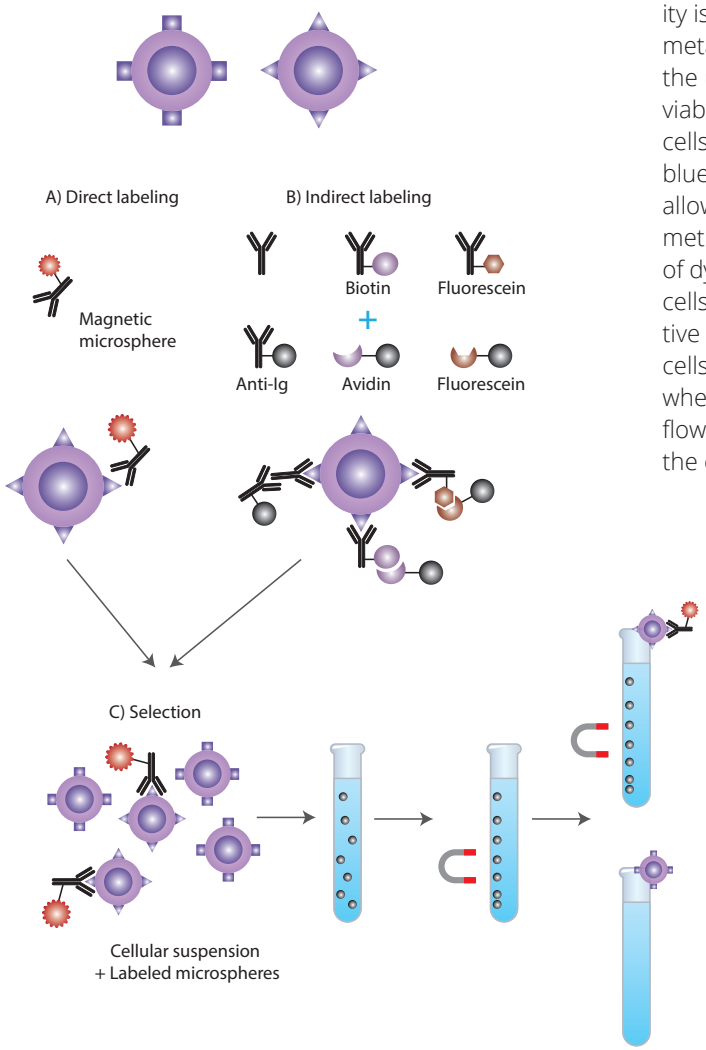

Figure 11. Affinity separation using magnetic beads. **A.** Direct labeling: microsphere covalently linked to the Fc domain specific antibody for the target cells. **B.** Indirect labeling: uses two types of reagents, an unmodified primary antibody covalently linked to a marker (biotin, fluorescein) and a magnetic microsphere bound respectively with a secondary antibody (e.g., anti-Ig, avidin, anti-fluorescein antibody). In either case the target cells remain surrounded by magnetic microspheres. **C.** Selection: Suspension labeled with magnetic particles. Retention in the tube or plate culture of the magnetic complexes formed. Positive selection: binding of the microspheres which want to be separated. Negative selection: the microspheres are labeled with all cell types except the target cells of interest.

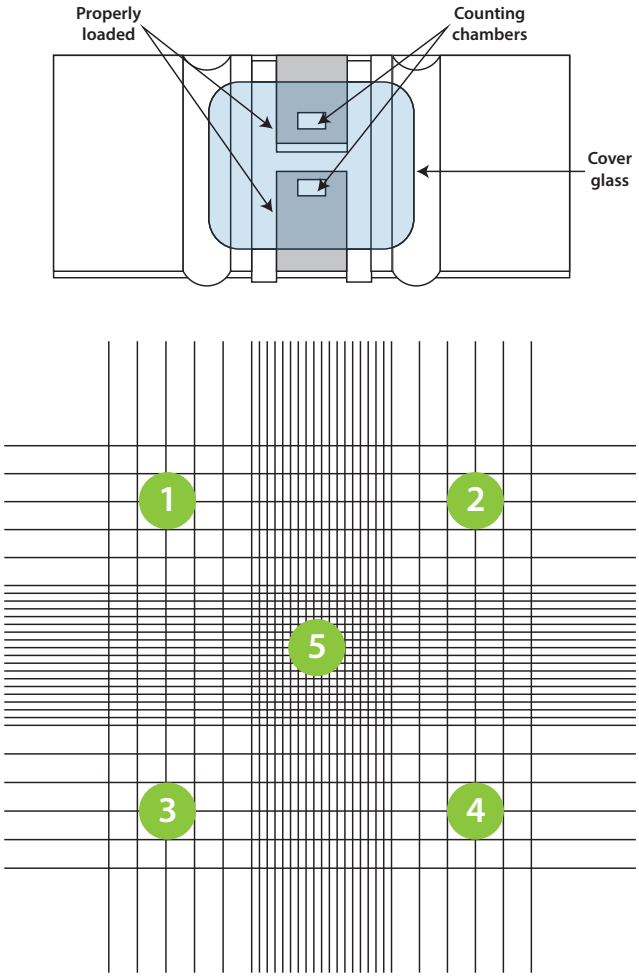

Figure 12. Hemocytometer. The coverslip is 0.1 mm above the grid, and the lines etched on the grid are at preset dimensions. The four outer squares, marked 1-4, each cover a volume of 10-4 mL. The inner square, marked as 5, also covers a volume of 10-4 mL, but is further subdivided into 25 smaller squares. The volume over each of the 25 smaller squares is 4.0 x 10-6 mL. Each of the 25 smaller squares is further divided into 16 squares, which are the smallest gradations on the hemocytometer.

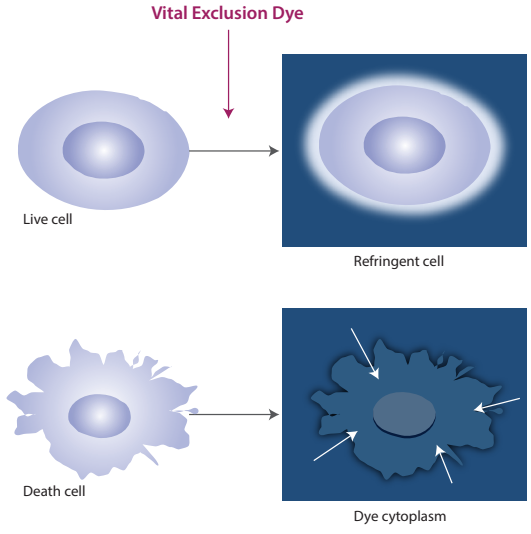

Figure 13. Cell viability - Exclusion assays These tests determine the number of viable cells present in a cell suspension. Live cells possess intact cell membranes that exclude certain dyes like trypan blue. If the cell membrane is damaged (dead cells) trypan blue will penetrate and stain the cell blue. Viable cells will have a clear cytoplasm.

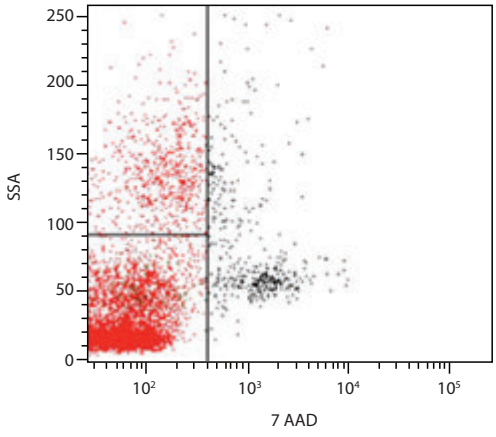

Figure 14. Cell viability - Exclusion assays in flow cytometry. The 7-AminoActinomycin D (7-AAD) has the ability of inserting itself between the tops of successive CG bases of the DNA double strand. This occurs when the interior of the cell and the nuclear chromatin are accessible as a consequence of membrane damage, necrotic or apoptotic process. Red dots in the figure represent live cells that are been analyzed by flow citometry, where the 7-AAD could not penetrate.

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46 ANALYSIS OF NUCLEIC ACIDS

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ISOLATION OF DNA AND RNA FROM EUKARYOTIC CELLS

ISOLATION OF GENOMIC DNA (gDNA)

Protocols for isolation and preparation of gDNA are comprised of two basic steps: an initial cell lysis, followed by one or several chemical or enzymatic methods which precipitate, remove proteins, RNA and other macromolecules.

The double-stranded DNA is chemically inert with reactive groups within its central helix, forming hydrogen bonds to hold the complete double helix structure. Nucleobases are held in their position by support consisting of phosphates and sugars that are internally reinforced by strong covalent molecular bonds. This conformation allows DNA to be more durable than other intracellular components. Despite their chemical stability, the double-stranded DNA is physically fragile. Very lengthy and tangled with miniature lateral stability, the high molecular weight gDNA is vulnerable to hydrodynamic forces. The molecule in solution behaves as a random coil hardened by the accumulation of interactions between pairs of bases and the electrostatic repulsion between charged phosphate groups in the backbone of the molecule. gDNA is relatively easy to obtain in a fragmented form but is progressively more difficult to obtain as its molecular weight increases. gDNA molecules with lengths greater than 150 kb are prone to break up by the forces generated in the procedures commonly used for obtaining genomic DNA.

Many different methods and techniques are available for the isolation of gDNA. In general, all methods involve disruption and lysis of the starting material followed by the removal of proteins and other contaminants and finally recovery of the DNA. Removal of proteins is typically achieved by digestion with proteinase K, followed by salting-out, organic extraction, or binding of the DNA to a solid-phase support (either anion-exchange or silica technology). DNA is usually recovered by precipitation using ethanol or isopropanol. The choice of a method depends on many factors: the required quantity and molecular weight of the DNA, the purity required for downstream applications, and the time and expense (For detailed information see: http://www.qiagen.com).

Cell lysis

Generally, cell lysis is performed in a hypertonic medium and can be applied to both cells growing in suspension or adhering to culture dishes. In preparations from whole blood cells, the use of differential lysis reagents is commonly used. For example, to study leukocytes, erythrocytes are lysed in an acid environment or in a solution of magnesium chloride. Generally for tissues or cell samples containing large amount of debris (i.e., extracellular matrix), it is often difficult to obtain gDNA with high efficiency. This can be increased if the tissue is pretreated by mechanical or enzymatic means before the homogenization in lysis buffer. Following cell lysis, the lysate may be subjected to treatments that prevent the destruction and elimination of the DNA molecules.

Preparation of high molecular weight DNA using proteinase K

This method comprises digestion of the cellular suspension or tissue with proteinase K in the presence of EDTA (ethylenediaminetetraacetic acid) and SDS (sodium dodecyl sulfate). EDTA chelates divalent cations and therefore inhibits the action of DNase while SDS is an anionic detergent used for membrane solubilization and protein denaturation. RNA contamination is removed by RNase digestion; proteins are then removed and nucleic acids are precipitated. Most commonly used methods for isolation of gDNA are

described below, (For detailed information see: http://www.qiagen.com).

- Salting-out method: This is a process based on the differences in solubility of DNA and RNA depending on salt concentration. Both DNA and RNA are soluble in concentrated salt solutions, while in low salt concentrations only RNA is soluble. The precipitates are removed by centrifugation, and the DNA is recovered by alcohol precipitation. RNase treatment, dialysis, and/or repeated alcohol precipitation are often necessary before the DNA can be used in downstream applications. DNA yield and purity are highly variable using this method (Figure 1).
- Organic extraction method: This is a conventional technique that uses organic solvents to extract contaminants from cell lysates. The cells are lysed using a detergent, and then mixed with phenol, chloroform, and isoamyl alcohol. The correct salt concentration and pH must be used during extraction to ensure that contaminants are separated into the organic phase and that DNA remains in the aqueous phase. DNA is usually recovered from the aqueous phase by alcohol precipitation. DNA isolated using this method may contain residual phenol and/or chloroform, which can inhibit enzyme reactions in downstream applications, and therefore may not be sufficiently pure for sensitive downstream applications such as PCR (Figure 1).
- Anion-exchange methods: Solid-phase anion-exchange chromatography is based on the interaction between the negatively charged phosphates of the nucleic acids and positively charged surface molecules on the substrate. DNA binds to the substrate under low-salt conditions, impurities such as RNA, cellular proteins, and metabolites are washed away using medium-salt buffers, and high-quality DNA is eluted using a high-salt buffer. The eluted DNA is recovered by alcohol precipitation, and is suitable for all downstream applications. Anion-exchange

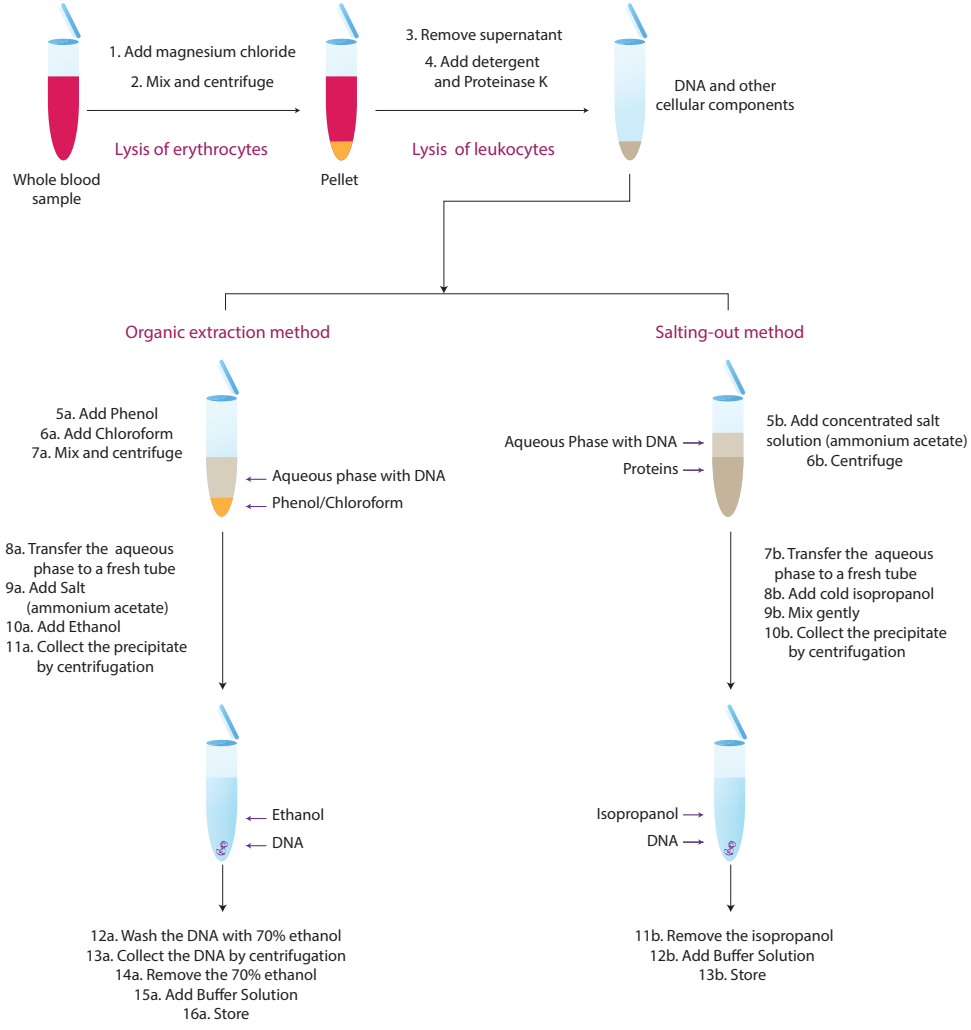


Figure 1. Process for obtaining DNA by salting out or organic extraction methods.

technology completely avoids the use of toxic substances, and can be used for different throughput requirements as well as for different scales of purification.

Nowadays, extraction of gDNA is becoming more frequent and efficient by using commercially available kits, which are more specific, easier, safer and faster.

Storage of gDNA

After extraction, the gDNA must be stored appropriately in buffer (usually TE [Tris-EDTA]) at 4°C for subsequent analysis. Long-term storage of DNA should be at -20°C.

ISOLATION OF RNA

Each mammalian cell contains about 10-30 pg of RNA, made up of ribosomal RNA (rRNA), (80-85%), transfer RNA (tRNA) (10-15%) and messenger RNA (mRNA) (1-5%). While rRNA and tRNA components are of discrete sizes, mRNA is heterogeneous and varies in length from several hundred to several thousand nucleotides. Most eukaryotic mRNAs carry at their 3′ end a tail of polyadenylic acid residues that is generally long enough to allow selective isolation of mRNA. Because ribose residues carry hydroxyl groups both the 2′ and 3′ positions, RNA is chemically much more reactive than DNA and due to its biological function; RNA is degraded easier by common RNases.

The key to successful purification of intact RNA from cells and tissues is speed. Once cellular RNases are inactivated, the RNA integrity can be guaranteed. Many methods for the isolation of RNA use strong denaturants such as guanidinium hydrochloride or guanidinium thiocyanate to disrupt the cells, solubilize their components and denature endogenous RNases simultaneously.

Purification of RNA based on Guanidinium isothiocyanate (GITC)/phenol/chloroform extraction

The use of guanidinium isothiocyanate in RNA extraction was developed by Chomczynski and Sacchi in 1987. The principal reagent used is sold under different names such as TRI Reagent, TRIzol, Trisure or STAT-60. This reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which facilitate the isolation of a variety of RNA species of large or small molecular size. It maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization. After homogenizing the sample with the reagent, chloroform is added, and the homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and proteins). RNA is then precipitated from the aqueous layer with isopropanol. The precipitated RNA is washed to remove impurities, and then resuspended in DEPC water and stored at -80ºC for use in downstream applications. Isolated RNA can be used in RT-PCR, Northern Blot analysis, Dot Blot hybridization, poly(A)+ selection, in vitro translation, RNase protection assay, and molecular cloning.

Selection of Poly(A) RNA using Oligo(dT)

As it was mentioned before, mRNAs comprise only a small percentage of all RNA species in a eukaryotic cell. Enrichment of eukaryotic mRNAs is done by virtue of their poly(A) tail which in most cases is 30-200 nucleotide long. Poly(A)-RNA preparation consists of three steps: (1) hybridization of poly(A)-containing RNAs to oligo-dT molecules connected to a carrier, (2) washing off nucleic acids which do not bind to oligo-dT, (3) elution of poly(A)-RNA from the oligo-dT/carrier combination under low stringency conditions.

Poly(A)-RNA preparation can be done using cellulose-bound oligo(dT), but several carriers for the oligo(dT) molecules have been developed (e.g., magnetic beads used in combination with biotinylated oligo(dT) or oligo(dT)-coupled polystyrene-latex beads).

One should keep in mind that RNA prepared with a standard phenol/chloroform extraction procedure usually also contains traces of gDNA so if the application requires removal of all gDNA from RNA preparation, is recommended using a DNase enzymatic treatment (For detailed information see: Maniatis T, et al. Molecular Cloning: A Laboratory Manual. 2001. Third edition).

DETECTION OF NUCLEIC ACIDS BY ELECTROPHORESIS

This technique relies on the ability of charged macromolecules to move in an electric field with a velocity proportional to their charge and inversely proportional to its size. This would give each DNA or RNA molecule the same velocity as their charge in solution is proportional to their mass. However when moving in a gel matrix, the molecules experience a resistance roughly proportional to their size. Therefore, DNA and RNA molecules separate according to their size during the movement towards the positively charged anode (+) (Table 1).

The separation of large DNA fragments is typically carried out by agarose gel electrophoresis (usually horizontal) while shorter ones on polyacrylamide gel electrophoresis (generally vertical). In both cases, the gel is prepared in different concentrations depending on the size of the DNA fragments that needs to be separated (Figure 2). By modifying the concentration of acrylamide or agarose, higher resolution is achieved over the range of possible sizes of DNA. Denaturing agarose gel electrophoresis is used to check the size and integrity of RNA preparations. RNA can form many different secondary structures, which affect its mobility in an electrical field if it is not maintained in a denatured state. Thus, samples of RNA may be denatured by treatment with formamide and separated by electrophoresis on agarose gels containing formaldehyde.

For staining purposes, Ethidium bromide, GelStar Nucleic Acid Gel Stain, SYBR Safe and SYBR® Green II Gel Stain can be used as they bind both DNA and denatured RNA.

GEL COMPOSITION (%)		DNA RESOLUTION RANGE (kb)
Acrylamide	Agarose	
20		0.006 – 0.1
15		0,025 – 0,15
12		0,04 – 0,2
8		0,06 – 0,4
5		0,08 – 0,5
3,5		1 – 2
	2	0,1 – 2
	1,5	0,2 – 3
	1,2	0,4 – 6
	0,9	0,5 – 7
	0,7	0,8 – 10
	0,6	1 – 20
	0,3	5 – 60

Table 1. **Electrophoresis resolution.**

CELL-BASED DNA CLONING

In 1970, a technique by which the DNA was cut precisely into specific fragments and joined together again in different combinations was developed. Using this approach, a specific DNA fragment was introduced into a vector (i.e., bacteria or virus), in such a way that it would be copied when the cell replicates. This method allows to produce a large number of cells all with identical copies of that piece of DNA. This process was named cloning.

A vector is a "carrier" which allows one to amplify and isolate the DNA fragment of interest. Target DNA fragments lack a functional replication origin, thus they cannot undergo replication within bacterial cells. They need to be linked to a vector molecule. Two types of vectors are most commonly used: *E. coli* plasmid vectors and bacteriophage vectors.

CUTTING AND JOINING DNA MOLECULES

The gene cloning technology was made possible by the discovery of two types of enzymes: restriction endonucleases and DNA ligases. Restriction endonucleases serve to protect bacteria from invading bacteriophages. After recognizing specific short sequence elements in the foreign DNA (4, 6 or 8 pb long), these enzymes cleave the DNA in the vicinity of each such element. The bacterial cell DNA is left uncut because it is protected by DNA methylation. Type II restriction endonucleases recognize a specific DNA sequence or restriction site and cleave the DNA by catalyzing breaks in specific phosphodiester bonds. The cleavage is on both strands of the DNA and results in a double-stranded break. Two types of restriction enzymes exist that differ in the way they cut the target DNA. First, the blunt end cutters are enzymes that cut both strand of the target DNA at the same spot creating blunt ends. Second, the sticky end cutters are enzymes that cut both strand of the target DNA at different spots creating 3'- or 5'-overhangs of 1 to 4 nucleotides known as sticky ends (Figure 3a). Importantly, any blunt end can be joined to any other blunt end regardless of how the blunt end was generated. Sticky ends can be joined to other sticky ends, provided that either the same Type II restriction enzyme was used to generate both sticky ends or that the bases in the overhang are identical and have the correct overhang (Figure 3b).

During in vivo DNA replication, DNA ligase catalyzes formation of 3' → 5' phosphodiester bonds between the short fragments of the discontinuously synthesized DNA strand at a replication fork. In recombinant DNA technology, purified DNA ligase is used to covalently join the ends of restriction fragments in vitro. Ligation is an energy-requiring reaction that occurs in three distinct steps. In the first step, the adenylyl group from ATP is covalently attached to ligase and inorganic phosphate is released. Next, the adenylyl group is transferred from ligase to the 5'phosphate of the DNA in the nick. Lastly, the phosphodiester bond is formed when the 3' OH in the nick attacks the activated 5' phosphate.

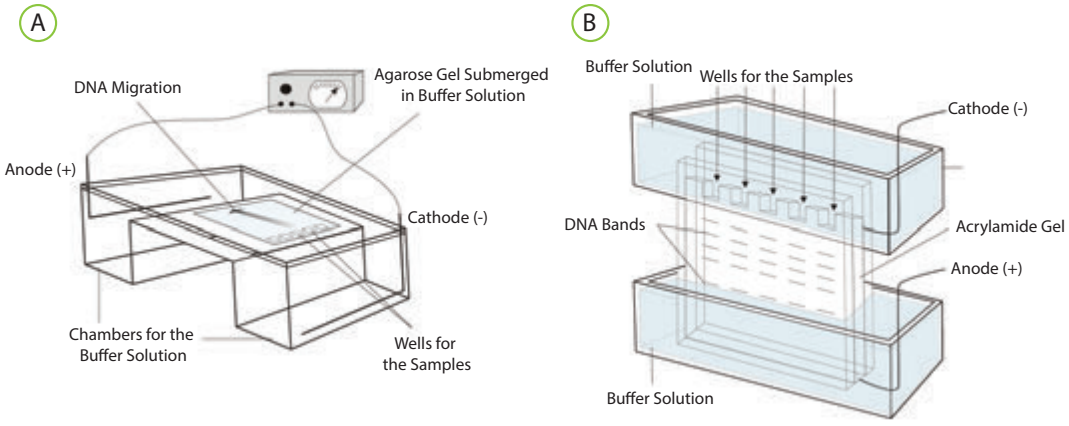


Figure 2. The separation of the DNA fragments is carried out by agarose gel electrophoresis (**A**) or polyacrylamide (**B**). The separation of the RNA fragments is carried out by denaturing agarose gel electrophoresis (**A**).

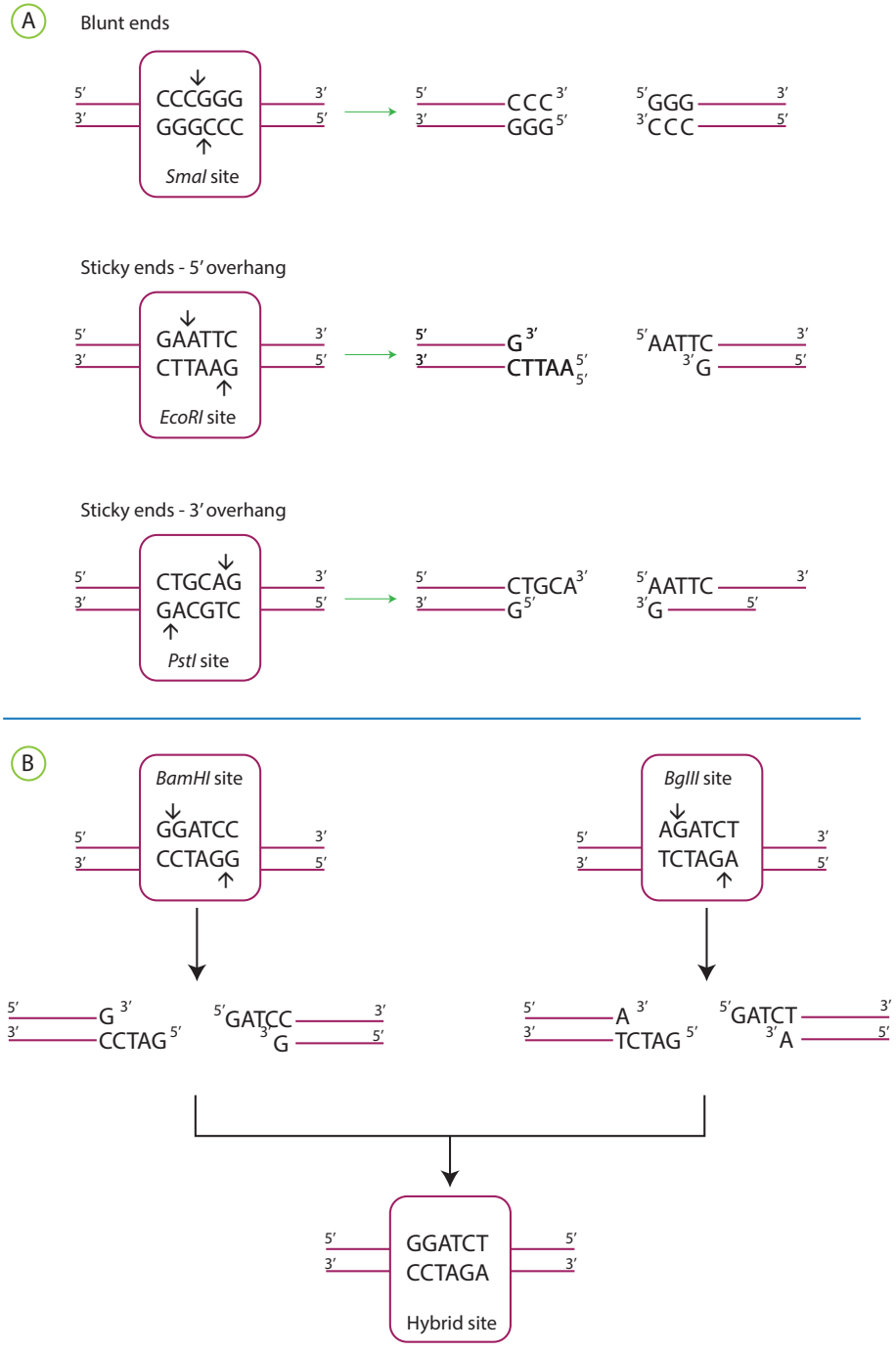


Figure 3. Cleavage of DNA by a Type II restriction enzyme leaves one of three types of ends, depending on the enzyme used. (**A**) The ends generated can be blunt ends, sticky ends with a 5′ overhang, or sticky ends with a 3′ overhang. (**B**) The sticky ends from two molecules cut with two different restriction enzymes can be joined if the overhangs can hybridize.

E.coli plasmid vectors can be constructed with a polylinker- a synthetic multiple-cloning-site sequence that contains one copy of several different restriction sites. When such a vector is treated with a restriction enzyme that recognizes a specific sequence in the polylinker, it is cut at that sequence, generating sticky ends. Next, in the presence of DNA ligase, DNA fragments produced with the same restriction enzyme are inserted into the plasmid. The ratio of DNA fragments to be inserted to cut vectors and other reaction conditions are chosen to maximize the insertion of one plasmid per vector.

TYPE OF VECTORS

Plasmid vectors

Plasmids are circular, double-stranded DNA molecules that are separate from a cell's chromosomal DNA. These extrachromosomal DNAs occur naturally in bacteria, yeast, and some higher eukaryotic cells. In order to be used in DNA cloning, plasmid vectors must have a replication origin, a drug-resistance gene (i,e, ampicillin, kanamycin, tetracycline, chloramphenicol, etc) and a region in which exogenous DNA fragments can be in-

serted (usually a multiple cloning site). A piece of DNA can be inserted into a plasmid if both the circular plasmid and the source of DNA have recognition sites for the same restriction endonuclease. For cloning, both molecules are cut by the restriction endonuclease producing intermediates with sticky and complementary ends. Those two intermediates are recombined by base-pairing and are linked by the action of DNA ligase. As a result, a new plasmid containing the foreign DNA as an insert is obtained (Figure 4).

Bacteriophage vectors

Bacteriophages, or phages as they are commonly known, are viruses that specifically infect bacteria. Their structure is simple, consisting of a DNA (or occasionally RNA) molecule carrying a number of genes, including several for replication of the phage, surrounded by a protective coat or capsid made up of protein molecules. In order to use a phage as a vector it is necessary to digest both target DNA and the phage DNA with restriction enzymes. Once the digestion is performed, the product will have complementary sticky ends, so both molecules can be annealed and ligated. After the resulting recombinant DNA is packaged into phage head, it can be propagated in *E. coli*.

There are two types of bacteriophage infection, lytic or lysogenic (Figure 5). In the lytic cycle, the phage redirects the host metabolisms towards the production of new phages, which are released during the lysis of the cell. In the lysogenic cycle, the genome of the phage typically remains in the host in a dormant stage (prophage) and replicates along with the host, until the lytic cycle is induced. A "lysogenic decision", whether or not to establish a prophage state is made by the temperate phage after infection.

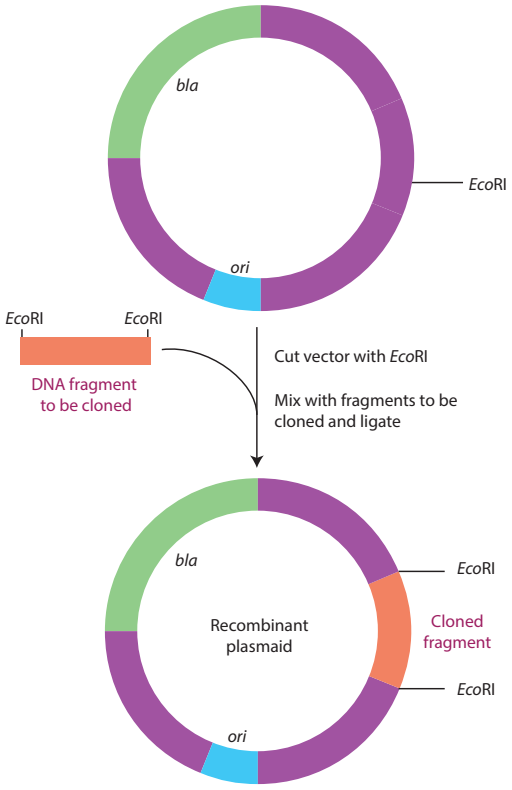


Figure 4. Cloning with a plasmid vector. Fragments of DNA cut with a restriction endonuclease are inserted into a vector that has been cut with a similar enzyme. The DNA fragment and vector are joined by DNA ligase to form recombinant DNA. This process allows the isolation of a large quantity of the DNA fragment for further analysis and manipulation. Abbreviations: beta-lactamase (bla); origin of replication (ori).

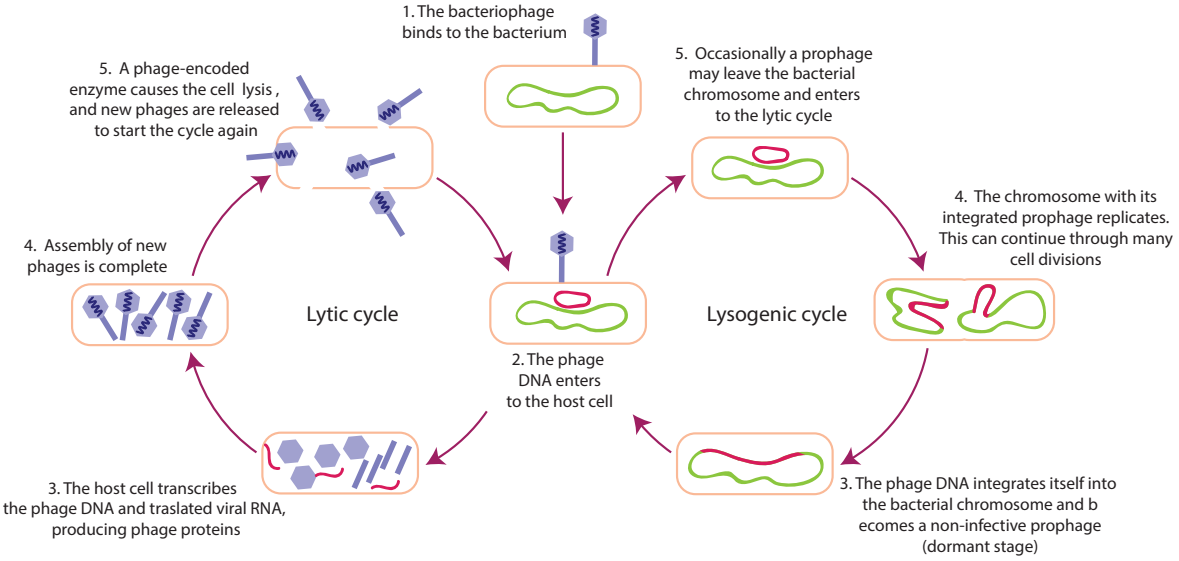


Figure 5. Life cycle of a bacteriophage. Bacteriophages reproduce by a lytic or a lysogenic cycle. During the lytic cycle there is a phage DNA replication followed by synthesis of capsid proteins and a lysis of the bacterial cell after releasing the new phages. Instead, lysogeny is characterized by integration of the phage nucleic acid into the host bacterium's genome. The genetic material of the phage can be transmitted to daughter cells at each subsequent cell division

Lytic cycle of the phages consist on: adsorption, separation of nucleic acids from protein coat, expression and replication of the nucleic acids, virion assembly and release. During the adsorption, the irreversible binding between a phage structure (e.g., tail fibers) and the receptor is accomplished. After adsorption, the cell wall is made penetrable, and the nucleic acid is transported into the cell, whereas the capsid remains outside the cell. Following injection, the genetic material stays in the cytoplasm. In this stage, gene expression, genome replication and morphogenesis occurs, i.e., the formation of the genomes and the capsids (and tails) and the packing of the genomes into the capsids. The phase of the latent period before capsids and genomes are assembled into mature phages is called the eclipse period. The rise period is characterized by the release of mature phages into the environment due to cell lysis and the detection of free phages (virions).

Lysogenic cycle of the phages consist on: adsorption, separation of nucleic acids from protein coat, circularization of the phage chromosome, and repression of the phage genome. Following injection, the nucleic acid sequence is integrated into the host genome and, a phage coded protein, called a repressor, is made which binds to a particular site on the phage DNA, called the operator, and shuts off transcription of most phage genes except the repressor gene. However, when a lysogenic bacterium is exposed to adverse conditions, there is production of proteases which destroy the repressor protein. This in turn leads to the expression of the phage genes, reversal of the integration process and lytic multiplication.

TRANSFORMATION

This is the process by which bacterial cells take up DNA molecules or plasmid cloning vectors. Some species of bacteria such as *Neisseria gonorrhea* naturally take up DNA from their environment, and they are described as being naturally competent for transformation. However, other species such as *E. coli* are not naturally competent, and they need to be treated in a special way to enable them to take up DNA. Two techniques for introducing DNA into bacteria cells are chemical treatment with calcium chloride and electroporation. The first procedure produces a chemical environment that results in DNA attaching to the cell surface; the DNA is then endocytosed by as yet uncharacterized pathways. Meanwhile, electroporation uses an electric field to open up pores in the cell. The DNA presumably diffuses into the cell through the pores.

Once DNA molecules or plasmid cloning vectors have been introduced, a selection of transformed bacteria is carried out. As mentioned before, a plasmid vector must contain a selectable marker whose expression provides a means of identifying cells containing it. The most common marker genes systems are:

- Antibiotic resistance genes: These genes are required to distinguish cells containing the plasmid with the gene of interest from those that did not take up the plasmid. The most commonly used selectable marker genes are ampicillin, tetracycline or chloramphenicol. Those cells that do not have the selectable marker genes will not be able to grow in the presence of the antibiotic targeted by the gene; only those cells that do have the plasmid with the marker gene incorporated will grow.´
- β-Galactosidase gene complementation. β-galactosidase is involved in the breakdown of lactose into its components, glucose and galactose. It is also capable of breaking down the artificial substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to produce a product with a blue color. This enzyme is normally coded by the gene *lacZ,* which resides on the *E. coli* chromosome. Thus, clones with a functional *lacZ* gene can easily be identified because they are blue in color. The family of plasmids to which pUC18 belongs only contains a part of the *lacZ* gene that encodes a fragment of the enzyme called the α-peptide and this partial gene is called *lacZ'*. The multiple cloning site in pUC18 is in the middle of the l*acZ'* gene so cloning into it will result in insertional inactivation of the gene and the resulting clones will be unable to break down X-gal. These recombinant clones will be white, and are easily distinguishable from those containing the recircularized vector, which will be blue (Figure 6).

Finally, recombinant DNA is recovered by growing a culture of cells from a single colony containing the plasmid. Then, the cells are lysed, and the DNA is extracted and purified.

DNA LIBRARIES

A DNA library is a random collection of DNA fragments that ideally contains at least one copy or more of every DNA sequence of a particular organism. Two types of DNA libraries exist, genomic DNA libraries and cDNA libraries.

A genomic DNA library consists of a collection of randomly generated DNA fragments from an organism's genome. This includes regions of the genome that code for proteins, as well as noncoding regions, such as introns, regions between genes, telomeres and centromeres. The first step in creating a genomic library is to break the DNA into manageable size pieces of 5–100 kb, usually by partial restriction endonuclease digest. The random collection of DNA fragments are then purified and ligated into a vector backbone. Bacteriophage λ or cosmid vectors are typically used for genomic libraries. Since a larger insert size can be accommodated by these vectors compared with plasmids, there is a greater chance of cloning a gene sequence with both the coding sequence and the regulatory elements in a single clone.

On the other hand, a cDNA library represents only the DNA that is expressed as mRNA, which is later translated to proteins. cDNA libraries are constructed based on an mRNA population isolated from a specific tissue, cell type,

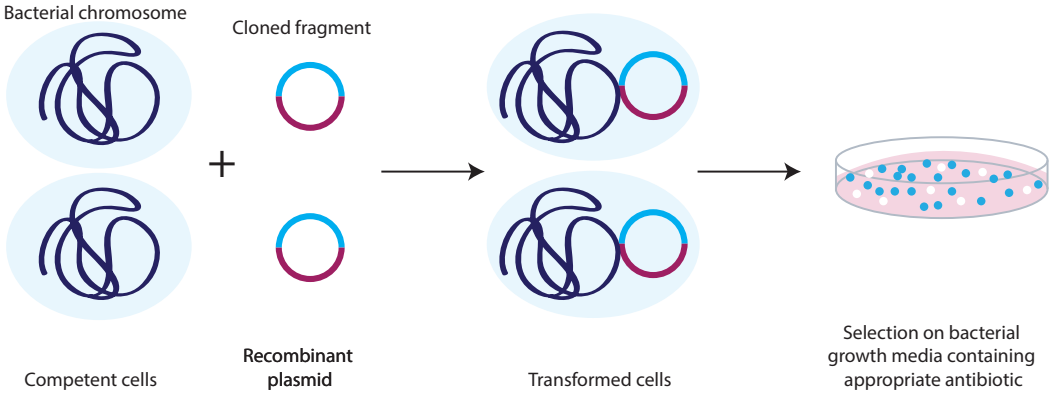


Figure 6. Transformation process.

specific developmental stage or from organisms grown under "stressed" conditions, such as low nutrient availability. The first step in creating a cDNA library consists of poly(A)+ mRNA isolation by specific binding to a complementary single-stranded oligo(dT). The isolated poly(A)+ mRNA can then be converted, using reverse transcriptase, to a double-stranded cDNA. Then, the cDNA has to be digested with a restriction enzyme and cloned into the vector and replicated in bacteria. Bacteriophage λ vectors are commonly used for the construction of cDNA libraries because of the high efficiencies associated with these vectors.

POLYMERASE CHAIN REACTION (PCR)

The principle of the polymerase chain reaction (PCR) technique was first reported in 1983 by Kary Mullis, but only until 1989 the implementation of this technology was made possible with the discovery of the thermostable Taq DNA polymerase enzyme (extracted from the bacterium *Thermophilus aquaticus*). The technique was originally used in the diagnosis of a disease, when a group of researchers identified the gene involved in sickle cell anemia. About the same time the technique was first implemented in forensics and quickly received the greatest honor among scientists, granting its inventor the Nobel Prize in Chemistry in 1993. The technique provided a solution to many of the most fascinating problems of modern biology, including DNA replication.

Initially, PCR was performed manually with incubators at varying temperatures, through which each tube was changed and added the enzyme reaction in each new cycle. On 1985, the thermocycler was invented, allowing automated, fast and precise different temperature cycles with no need to add new enzyme due to the use of thermostable polymerase. This automation step generated a number of different and varied approaches using this technique (Box 1). The purpose of this section is to help use this methodology in the most appropriate manner, in order to optimize resources and correct any problems that may arise in the process.

PRINCIPLE OF THE METHOD

The PCR is a fast, economical and simple approach to generate multiple copies of a segment of DNA from a DNA template. The reaction is performed in vitro by means of an enzyme which generates millions of identical copies based on the template sequence and the knowledge of regions adjacent to the DNA fragment of interest. Based on this information, it is possible to synthesize a pair of oligonucleotides called primers complementary to each DNA strand located in both of the flanking regions. The PCR starts when double stranded DNA is denatured to allow a pair of specific primers to hybridize (anneal) with each of the DNA template contiguous regions. This step is followed by an enzymatic extension reaction in which the Taq DNA polymerase enzyme adds nucleotides complementary to the template strand from the 3 'end of each primer, generating two new molecules of double-stranded DNA. Consequently, each PCR cycle consists of three distinct steps, performed at different temperatures (Figure 7):

1. Denaturation of the double stranded DNA by heating to a temperature between 94 - 98 °C, which creates two single strands of the DNA template.

2. Annealing of the sequence specific primers to the flanking sequence of DNA template between 45 - 68 °C.

3. Primer extension by using Taq DNA polymerase at 72°C, this step must be carried out long enough to produce a DNA product that can even exceed the 1000 bp taking into account the enzyme reaction speed.

During the first cycle of the PCR, the generated product by the extension of the pair of primers will become the new template for the next cycle (Figure 8). Successive cycles will generate an exponential growth in the number of DNA fragments of interest, which will find its completion signal when confronted with the 5' end of each primer. Theoreti-

BOX 1. Polymerase chain reaction (PCR) approaches

- Follow up and diagnosis of disease.
- Microbe and viral low concentration rapid detection.
- HLA typing in transplants.
- Forensic DNA laboratory testing.
- Analysis of Ancient genetic DNA in stored material.
- Nucleic acid probe generation.
- Gene cloning.
- Gene mapping.
- Sequencing.

cally, each cycle can double the DNA molecules generated in the previous cycle, therefore the final amount of DNA can be 2^n, where "n" is the number of cycles. An estimated of 1 ug of DNA can be produced for 25-30 cycles of PCR reaction, starting from 50 ng of genomic DNA, assuming 100% efficiency during the process. After completing the cycles, the PCR product amount is sufficient to be visualized on an electrophoresis gel. The temperature changes during PCR are generally controlled using a thermocycler, consisting of a heating and cooling Peltier block and electronics that allows programming an incubator to regulate various temperatures and times for each cycle of PCR and the length of the process.

CONDITIONS AND COMPONENTS OF A PCR REACTION

Each PCR reaction, either a commercial premade kit or in-lab developed, includes the Taq DNA polymerase enzyme, deoxynucleotide triphosphates (dNTPs) and PCR reaction buffer (usually consisting of a nonionic detergent, KCl and bovine serum albumin). Moreover, the mixture must contain magnesium chloride ($MgCl_2$), cofactor needed for the proper activation and performance of the enzyme and the sequence-specific primers mentioned previously, followed by the appropriate concentration of DNA template. PCR protocols vary considerably according to specific applications; however, the basic considerations for carrying out a typical PCR reaction typically described in Table 2, can be adapted and standardized for the needs that may arise in the laboratory.

STANDARD PARAMETERS OF A PCR REACTION

The primers or oligonucleotides should have a length range between 18 to 30 bases. Oligonucleotide sequences shorter than 18 bases may be repeated several times in a eukaryotic genome and could generate nonspecific PCR products. Furthermore, oligonucleotides longer than 30 bases offer greater difficulty for annealing during the reaction and therefore may be difficult to obtain an amplification product

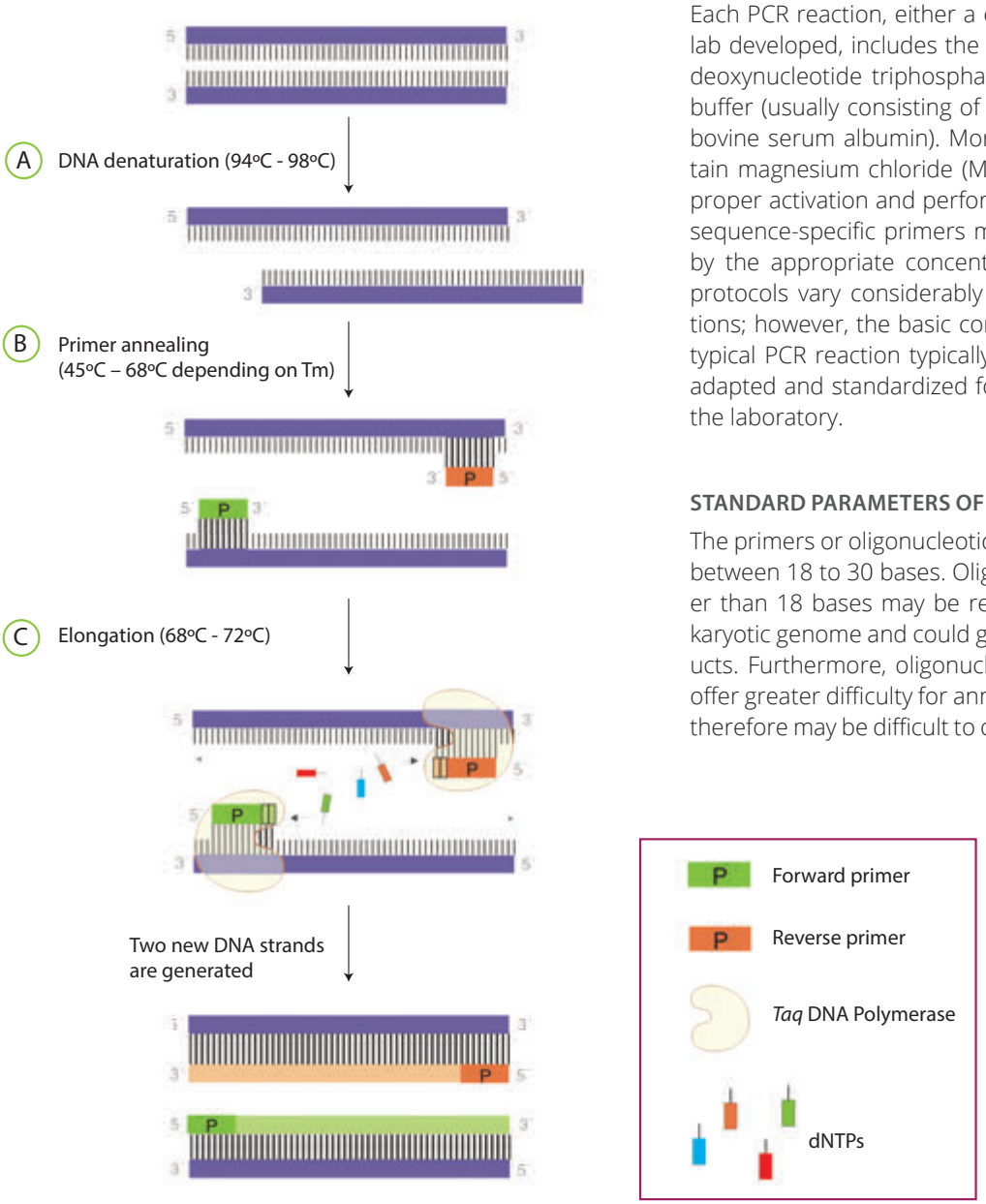
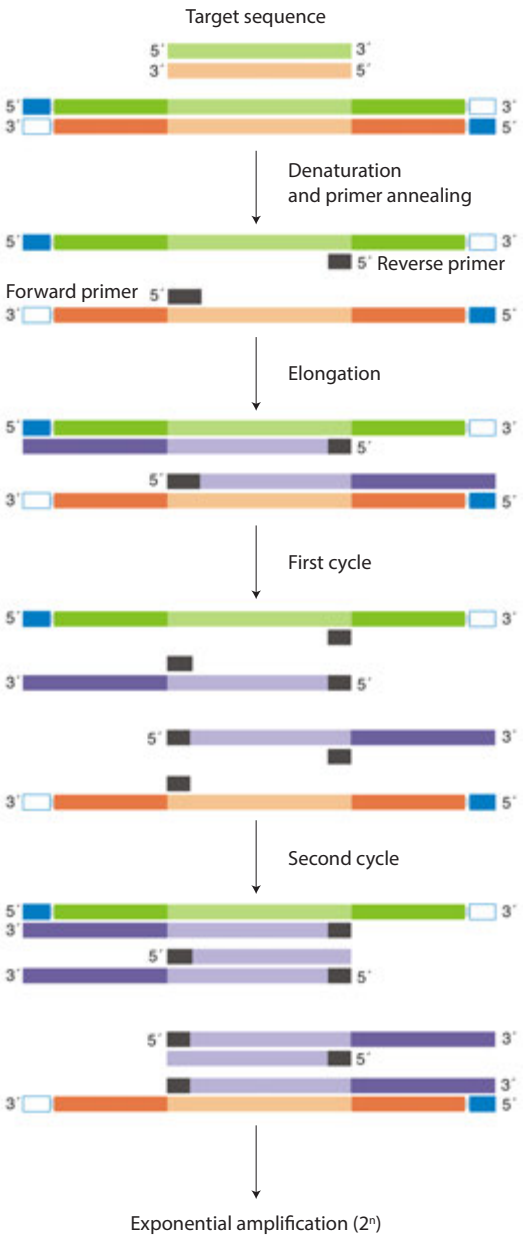


Figure 7. Steps for each of the cycles of a PCR. **A.** Denaturation of the double stranded DNA. **B.** Annealing of the primers to their target DNA sequence. **C.** Extension of the new DNA strands, using Taq DNA polymerase.



during the reaction. Both oligonucleotides (i.e., forward and reverse) must have the same G+C ratio to ensure that the annealing temperature, determined by the ratio of these two bases, is similar for both primers to facilitate DNA amplification. Furthermore, the oligonucleotides should have a minimal complementary capacity to avoid primer dimer in the PCR product. For most PCR reactions a final primer concentration of 0.05 µM to 1 µM is required. This generates an excess of oligonucleotides of 10^7 molecules, with respect to the DNA template, considering a template final concentration of 50 ng in 10 µl of final PCR reaction.

The annealing temperature is based on the average hybridization temperature (Tm) of the primers. This is equivalent to the temperature at which 50% of the oligonucleotides are annealed to their target sequence. For oligonucleotides with less than 20 bases, the approximate Tm can be calculated in degrees Celsius, by using the equation: Tm = 4 (G+ C) + 2 (A + T) where G, C, A and G are the number of bases present in each primer. The Tm is used as the starting point (theoretical) of the annealing temperature, however the optimal specific temperature is generally determined by trial and error, using a thermocycler.

The dNTPs may be used in a concentration of 200 µM, which may contain an initial necessary excess for incorporation in a DNA strand of up to 1 Kb.

An additional key variable in a PCR reaction is the $MgCl_2$ concentration which is required as a cofactor for the Taq DNA polymerase. Excess in the $MgCl_2$ concentration may prevent a complete denaturation of the DNA molecules during each PCR cycle. Moreover, it could also promote nonspecific primer annealing. Low magnesium concentrations can generate alterations during the extension step due to malfunction of the PCR enzyme and therefore do not generate an amplification product. Suitable $MgCl_2$ concentrations are usually in

Figure 8. DNA strand conformation of newly synthetized strands per each PCR cycle.

REAGENT	FINAL CONCENTRATION	VOLUME
Sterile distilled water	Adjust Volume	Adjust volume to 50 µl
PCR Reaction Buffer (10X)	Dilute to 1X	5 µl
Magnesium Chloride (25 mM)	1.5 - 2.0 mM	3 – 5 µl
dNTPs (10 mM)	200 µM	1 µl
Forward Primer (10 µM stock)	0.2 µM (0.05 -1.0 µM)	1 µl
Reverse primer (10 µM stock)	0.2 µM (0.05 -1.0 µM)	1 µl
Taq DNA polymerase	1.25 units	0.25 µl
DNA template	1 ng – 1 µg	----
Final Reaction Volume	----	50 µl

Table 2. Recommended volume and concentrations for master mix reagents used in a standard PCR reaction for amplifying a DNA template. (For detailed information see: http://www.neb.com) .

the range of 1.5-2.0 mM in the final volume of the reaction. The optimal concentration of this cofactor is only established through trial and error testing.

Taq DNA polymerase is the most frequently used thermostable enzyme that extends the DNA starting from the primers at a ratio of 2-4 kb per minute and at optimum temperature of 72°C. Generally, this enzyme is used at a concentration >1 nM or equivalent, which would be >0.1 U per 5 µl of PCR reaction. One disadvantage of the enzyme is the high error rate of the incorporation of bases (one error nucleotide per 100,000 nucleotides extended by reaction cycle). Other commercially available polymerases are the VENT enzyme, isolated from *T. litoralis* or genetically modified forms of the Taq polymerase from *T. acuaticus*, as is the case of the Taq Gold. The latter enzymes have a decrease error rate in nucleotide incorporation during the extension step.

In PCR, incubation temperatures, time and the number of cycles may vary from one protocol to another depending on the approach. A basic and suitable PCR profile for generation of a DNA fragment of about 1000 bp is presented in Table 3 and it is as follows: An initial denaturation cycle for 5 minutes, between 94 to 98°C. The next cycle makes up for the amplification step of the reaction and is comprised of: a denaturation step between 94 and 98°C, no longer than 1 minute, followed by an annealing step between 45 and 68°C which depends on the base composition of the primers and specificity to be achieved in the reaction (i.e., higher temperatures greater specificity). The annealing temperature depends on the oligonucleotide's Tm as mentioned above and the time varies between 30 seconds and one minute. The final temperature for each step of the cycle corresponds to the extension, between 68 and 72°C, which are considered optimal for the polymerase performance. The time for this final step will depend on the size of DNA to be amplified; the Taq DNA polymerase is able to extend up to 4000 bases per minute.

RELEVANT ASPECTS OF THE PCR

Despite the many applications of this technique, its real value lies on the performed quality control, in the knowledge of the method and control of the specific parameters such as: fidelity, specificity, sensitivity and efficiency.

The fidelity is defined as the ability of the enzyme to insert the appropriate nucleotide, relative to the DNA template sequence, and the ability to remove those mistakenly extended in the newly synthesized strand. The fidelity of the reaction

CYCLE	TEMPERATURE	TIME	STEPS
Initial Denaturation	94 - 98°C	2-5min	1
Denaturation	94 - 98°C	1min	30 - 45
Annealing	45 - 68°C (Depending on Tm)	1min	
Elongation	68 - 72°C	1min	
Final Extension	72°C	3-5min	1
Refrigeration	4 - 8°C	Optional	

Table 3. Temperature profile for a PCR product a 1 Kb fragment length.

BOX 2. Avoiding cross-contamination

The PCR laboratory space must have a set of special regulations to ensure the reliability of the amplified DNA. One of the key points to prevent contamination is the existence of separate work areas:

- Pre-PCR area: where the handling of samples and extraction of genomic DNA take place.
- PCR area: this area should be dedicated to store and handle the reagents and materials needed for the preparation of the reaction in order to ensure they will not come in contact with undesired DNA or amplification product. There must be materials separated from the pre-PCR.
- Post-PCR area: This area should be dedicated to store, handle, analyze and verify the performed amplification reaction products. This area might be prone to a higher DNA contamination, so it is necessary to have strict control of the input and output. It is important to carefully manipulate the PCR tubes to avoid splashes and spills that affect the results.
- In addition to the laboratory distribution additional precautions are required:
  - Use of appropriate clothing for a molecular biology laboratory.
  - Exhaustive cleaning bench, preferably with DNA decontaminating solution (0.1 N NaOH, 2% SDS) or 10% bleach.
  - Dilute and distribute reagents in working solutions using sterile laboratory grade water and DNAse and RNAse free sterile tubes.

not only depends on the function of the enzyme but also in the optimization of the conditions. The specificity of the reaction refers to the amplified PCR product similarity of the target sequence to be analyzed. This property depends primarily on the design of suitable oligonucleotides, annealing temperature, the DNA and $MgCl_2$ concentration and the number of cycles of the reaction. Likewise, sensitivity and efficiency refer to the possibility of obtaining a better amplification product, even when the number of molecules of DNA template was very low and mixed with other materials of different genetic origins. In some cases, it is observed that the higher efficiency creates lower specificity, which can be counterproductive to the end result. The goal is always to look for the perfect balance between specificity and sensitivity.

QUALITY CONTROL OF THE PCR

Given the amplification capability of the PCR reaction, it is necessary to maintain strict quality control in each of the assays performed in the laboratory to prevent cross-contamination of the samples, the reagents and the work area that could cause false positives. Quality control seeks to minimize contamination sources and define strategies to help identify them. This control is obtained by designing proper positive and negative controls for each experiment (Box 2).

Positive controls generally correspond to DNA samples containing a high copy number, preferably of known concentration and with results previously confirmed for the target DNA fragment to be amplified. This DNA must have excellent quality and must be stored appropriately in buffered solutions, such as TE buffer (Tris-EDTA), to prevent degradation. Such control will establish the appropriate performance of both the reagents and the protocol for amplification, ruling out any problem generated by the quality of DNA tested.

The negative control corresponds to a reaction containing all components of the master mix, except the target DNA. This control will assess nonspecific amplifications, and will detect the existence of contaminants in the reaction.

Some types of PCR must also contain an internal control for the reaction. This generally corresponds to the inclusion of a pair of primers that amplify housekeeping genes with similar conditions to those of the product of interest; the former to ensure that both the mixture and the temperature profile of the PCR are functioning correctly and also to help rule out false negatives during the analysis of the results.

TROUBLESHOOTING OF PCR REACTIONS

Although the PCR approach is efficient and sensitive, it can sometimes cause problems such as false negatives, false positives or even absence of amplification in the worst case. However, if you follow the recommendations discussed throughout this section and proper standardization of the PCR conditions, it will be easier to solve the problems that may arise. Table 4 summarizes some problems, possible causes, and proposed solutions.

PROBLEM	POSSIBLE CAUSE	POSSIBLE SOLUTION
Absence of amplification	DNA quality	Sample purification to eliminate plausible PCR inhibitors
		Sample re-isolation when degradation problems are present
	DNA concentration	High DNA concentration might inhibit amplification. Low concentration could decrease the likelihood of the oligonucleotides to find the target sequence
	Enzyme	Check enzyme activity in order to confirm if it is inactive or degraded
	No specific oligonucleotides	Check sequence, nucleotide content and length of the oligonucleotides
	Low oligonucleotide concentration	Perform a titration of the oligonucleotides to define the most adequate concentration
	MgCl2 low concentration	Perform a titration to adjust the concentration enough for the enzyme to be able to work.
	Thermal profile conditions	Verify the Tm. Perform a temperature titration to adjust the annealing temperature and extension and time of denaturation
Nonspecific amplification	Concentration and design of the oligonucleotides	Verify length of the oligonucleotides Longer oligonucleotides give better specificity
Not expected amplification product	Oligonucleotide sequence	Verify the localization and complementarity in the target sequence
	Reagent contamination	Verify negative controls.
Oligonucleotide dimer formation	Primer sequence	Verify the complementarity between the pair of oligonucleotides

Table 4. Troubleshooting for the PCR reaction.

REAL TIME PCR

The first description of the real-time PCR technique was by Higuchi et al. in 1993. Higuchi's procedure was performed using ethidium bromide during the polymerase chain reaction (PCR) and using a modified thermal cycler for irradiating UV-rays and a detection chamber. The signal collected was plotted against number of cycles. Final data accounted for the amount of PCR product that was generated during each of the cycles.

Theoretically, the PCR generates copies from a DNA template exponentially; however, due to inhibitors in the reaction, limiting reagents and pyrophosphate molecules build-up, the exponential growth of the molecules is not always observed in practice. Some reactions generate more products than others, therefore quantification is needed. Real time PCR offers the possibility of measuring the PCR products as they progressively accumulate in "real time", facilitating the quantification of PCR product during the exponential phase in order to extrapolate to the initial number of template molecules.

Real time PCR is a method used for quantification of DNA or RNA. In the latter case, RNA is copied by using reverse transcriptase to make complementary DNA strands (Figure 9). Then, for both DNA and RNA the amplification steps are the same.

During real time PCR exponential phase, an initial measurement is performed from the basal fluorescence emission aiming that all samples are comparable and standardized. This signal is calculated on the basis of the amount of fluorescence emitted by the reaction prior to exponential replication and then is plotted at a point where the signal originating from a sample is significantly higher than the fluorescence background signal. Accordingly, the required number of PCR cycles to generate sufficient fluorescence signal exceeds the baseline signal, known as cutoff point (Cp, crosspoint) or threshold cycle (Ct). Cp values are directly proportional to the amount of initial template and are the basis for calculating expression levels or the amount of initial DNA copies.

Currently this approach is thriving and its accessibility, validation, acceptance and expanded application has become widely acknowledged. The surge in the supply of new equipment and reagents capable of carrying out the quantification and detection in real time along with the decrease in costs has resulted in better access for many research groups and diagnostic technology.

REAL TIME PCR PRINCIPLES

The technique relies on the detection and measurement of the PCR products generated during the PCR reaction. Two

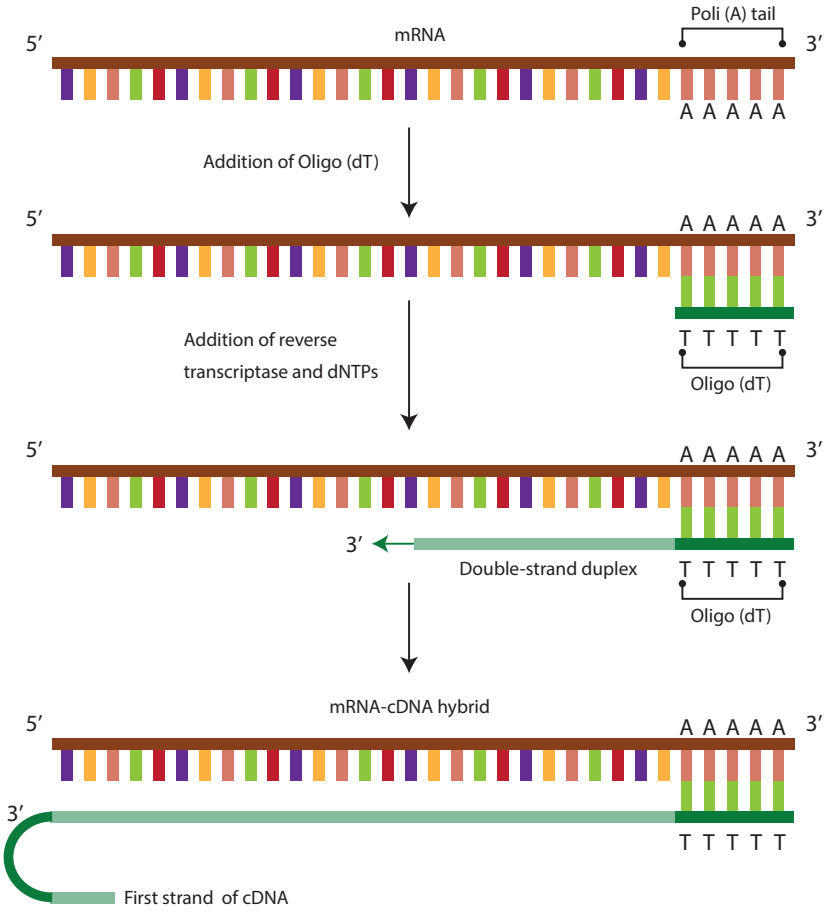


Figure 9. Synthesis of the first strand of cDNA. This process is performed using an RNA-dependent DNA polymerase called reverse transcriptase. As with other polymerases a short double-stranded sequence is needed at the 3' end of the mRNA which acts as a start point for the polymerase. This is provided by the poly(A) tail to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridized (polyT-polyA hybrid). Together with all 4 deoxynucleotide triphosphates, magnesium ions and at neutral pH, the reverse transcriptase synthesizes a complementary DNA on the mRNA template.

major findings led to the discovery of the real time PCR: The first finding was that the Taq polymerase has exonuclease activity from the 5' to 3'; and the second, the production of probes with dual-label oligonucleotides based on the principle of fluorescent resonance energy transfer (FRET).

The hydrolytic or TaqMan probe is a dual-labeled probe marked at the 5' end with a "reporter" fluorochrome (e.g., FAM [6-carboxyfluorescein]) and at the 3' with a "quencher" (e.g., TAMRA [6-carboxytetramethylrhodamine]). While the probe remains intact, the fluorescent emission from the "reporter" is absorbed by the "quencher". In this method, the Taq polymerase cuts the 5′ end and then hybridizes the labeled probe to the DNA template during the extension phase of PCR. By the time the Taq polymerase cleaves the probe, the two markers are separated and the quencher no longer absorbs the emission from the reporter (i.e., no more FRET). Therefore, this allows the reporter to emit its fluorescence which is quantified by the instrument. This process occurs in every cycle and does not interfere with the generation of the PCR product.

Recently, other probe systems have been described including molecular beacons, scorpion probes and hybridization probes. All these probe systems rely on the FRET principle, although not always relying in the nuclease activity of the Taq polymerase. Similarly other chromogens, such as SYBR Green I and ethidium bromide dyes have been used for double-stranded DNA quantification. Nonetheless, these do not apply the same principle of the TaqMan technology or use exonuclease activity of the enzyme.

STANDARDIZATION AND TECHNICAL ANALYSIS

Threshold cycle (Ct)

Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the PCR. The Ct is when the system begins to detect the increase in the fluorescent signal associated with an exponential growth of PCR product during the log-linear phase (Figure 10a). The reporter signal is normalized to the fluorescence of an internal reference dye, to allow for corrections in fluorescent fluctuations caused by changes in concentration or volume, and a Ct value is reported for each sample. This value can be translated into a quantitative result by constructing a standard curve.

Standard curve

The addition of external controls with known DNA concentrations (i.e., standard curve), allows to quantify the initial concentration of the template sequence present in the sample during amplification. This procedure undertakes the acquisition of fluorescence intensification in each cycle; data is plotted on kinetic curves for each of the controls. Consequently, one can control amplification on the initial phases when the concentration of reagents is not yet limited and the effect in the variability and the efficiency is less important. For each sample, the instrument software calculates the number of cycles at which it began to detect an increase in fluorescence relative to the initial signal. The cycle where the increase is detected is Cp or Ct cycle and is inversely proportional to the initial concentration of DNA template present in the sample. Using previously known concentrations of external controls and their corresponding Ct, a standard curve is plotted. With this, it is possible to interpolate the Ct values of each sample and deducing the initial DNA concentration (Figure 10b).

Dissociation curve analysis

Dissociation curve analysis is based on applying a temperature gradient after the PCR to monitor the kinetics of dissociation of the amplified fragments. This application allows to determine the Tm of amplicons and to verify their sequence specificity (Figure 10c). Moreover, it permits the analysis of punctual mutations that can be assessed in different ways. A first approach is the use of complementary probes to the target DNA containing or not the mutation, in this way when the probes hybridize, the probe with the variant will have a greater stability, reflected in a higher Tm. The differences between the average annealing temperatures of the probes should discriminate between wild-type DNA and the mutation. (Figure 10c)

Normalization

RT-PCR-specific errors in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples. This is especially relevant when the samples have been obtained from different individuals, and will result in the misinterpretation of the expression profiles of the target genes.

The accepted method for minimizing these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalized. The ideal internal standard (i.e., housekeeping gene) should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatment. The most widely used constitutive genes are the *β-actin*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal RNA (*rRNA*) genes, although other mRNAs have been used occasionally, for example histone H3 and cyclophilin.

Normalization uses the Ct differences (ΔCt) measured from both the Ct of the target gene and the Ct of the housekeeping gene. The signal difference between two samples is expressed as follows:

$$\Delta Ct \text{ of testing sample} = Ct_{(\text{target gene})} - Ct_{(\text{housekeeping gene})}$$

There are many tools to search validated housekeeping genes:

- BestKeeper selects the least variable gene using the geometric mean from the crude data. It is open access software: http://www.gene-quantification.de/bestkeeper.html

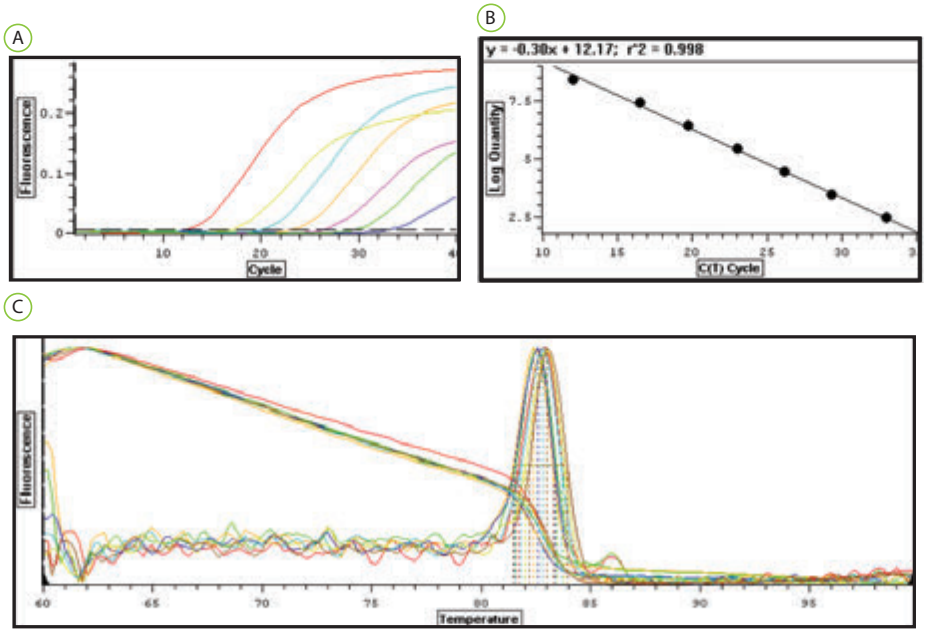


Figure 10. Typical real time PCR results. **A**) Amplification plot illustrating the increase in fluorescent reporter signal with each PCR cycle. **B**) Standard curve derived from a dilution series of a reference RNA. **C**) Dissociation curve for this analysis showing a single, sharp peak, suggesting that only a specific PCR product was generated with this set of primers.

- Gnorm also uses the geometric mean but it uses data converted to copy numbers. It also is open access software: http://medgen.ugent.be/~jvdesomp/genorm/
- Norm-Finder measures the variation level and allows potentially housekeeping genes ranking according to the variation between evaluated groups. This software can be consulted on: http://www.mdl.dk/publicationsnormfinder.htm

DETECTION METHODS USED IN REAL TIME PCR

A wide variety of probes are available for the real time PCR assays. Most of them are useful for the universal real time machines. Below, various probes systems used by this methodology are described.

Hydrolysis or TaqMan probes

For this system, three oligonucleotides are used: a sense/forward primer, an antisense/reverse primer and a dual-labeled probe. These are specific to the target DNA sequence to be analyzed. This method is dependent on the 5'- 3' exonuclease activity of the Taq polymerase.

As mentioned above, the dual-labeled probe consists of a reporter dye at one end and a quencher at the other. While the probe is intact, the fluorescence emission is absorbed by the quencher. The fluorescent reporter marker is covalently bound to the 5', some examples are: FAM (6-carboxyfluorescein), TET (tetrachloro-6-carboxyfluorescein), JOE (2, 7-dimethoxy-4, 5 dichloro-6-carboxyfluorescein), HEX (hexachloro-6-carboxyfluorescein) of VIC. The quencher is attached to the 3' end and may be TAMRA (6-carboxytetramethyl-rhodamine) or DABCYL [4-([4-(Dimethylamino)phenyl]azo)benzoic acid] (Figure 11). An advantage to this type of quenched probes is the reduction of the self-fluorescence.

The proximity of the quencher and the fluorochrome allows FRET, thus no fluorescence signal is originated. During the PCR reaction, if the target sequence is present, the probe specifically anneals and through nuclease activity releases the fluorochrome and the emission of fluorescence is initiated. The intensity of the fluorescence is measured during each cycle of the PCR reaction and is a direct consequence of the amplification process.

Molecular Beacons

Probes of this system exhibit a secondary structure in the form of a loop or hairpin, established from a single-stranded DNA molecule which contains complementary sequences of 4 to 6 nucleotides that comprise the structure (Figure 12). A fluorochrome is attached to one end of the probe and at the opposite end a quencher. The fluorescence is absorbed while the probe maintains its secondary structure due to the proximity of the quencher and the fluorochrome due to FRET. By the time the sequence of the intermediate portion of the DNA probe hybridizes to the template, a conformational change is caused, eliminating the FRET effect and initiating the fluorescence signal which is directly proportional to the amount of template in the sample. The molecular beacon system is suitable for probes used to identify mutations given they can distinguish between sequences that differ by a single nucleotide making them much more specific than other probes of the same length.

Scorpion Probes

The basic elements of this system are: a PCR primer, a blocker sequence for the 5' exonuclease enzyme, a specific probe template sequence and the detection system consisting of the reporter dye and the quencher (Figure 13). The oligonucleotide is part of the probe at its 3' end, the probe

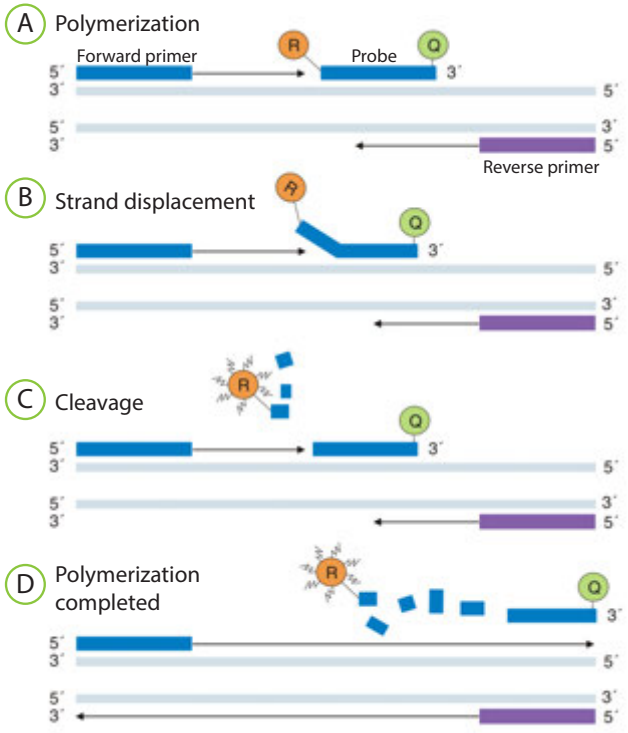


Figure 11. Fluorescence emission from the TaqMan probe system. A) Polymerization: an oligonucleotide probe is constructed containing a reporter fluorescent dye (R) on the 5' end and a quencher dye (Q) on the 3' end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by FRET. B) Strand displacement: if the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended. C) Cleavage: during each extension cycle, the Taq DNA polymerase cleaves the reporter dye from the probe. D) Polymerization completed: once separated from the quencher, the reporter dye emits its characteristic fluorescence. Adapted from: http://www.invitrogen.com

contains a secondary structure formed by complementary sequences (4 to 6 nucleotides) forming a hairpin structure of approximately 20 to 25 nucleotides. The fluorescent reporter is located at the 5' end of the hairpin, while the quencher is located within the system, between the probe and the blocker. The blocking sequence prevents the nuclease activity of Taq polymerase. The probes perform their function after the first primer extension step in the PCR. If the resulting amplicon contains a sequence complementary to the probe, the hairpin opens up and hybridizes to a part of the newly produced amplicon. This causes separation of quencher and fluorophore, starting the emission of fluorescence after disturbing the secondary structure and stopping FRET effect. Scorpion probes generate high specificity, since the fluorescence emission occurs only when the specific target is present in the reaction.

Hybridization Probes

In this system four oligonucleotides are used, two primers and two probes (Figure 14). One probe has a donor fluorochrome on the 3'- end and the another probe has an acceptor fluorochrome on the 5'-end The sequences of the probes are selected such that they hybridize adjacently to each other on the target sequence and bring the donor and acceptor fluorophores in close proximity in order to generate FRET. The acceptor fluorochrome in one of the probes transfers energy allowing the other to fluoresce in a different wavelength. The amount of emitted fluorescence is directly proportional to the amount of template DNA generated during the PCR reaction.

DNA-binding dye

SYBR Green dye is a chromogen that fluoresces when incorporated into double-stranded DNA (Figure 15). When free in solution, this dye shows relatively little fluorescence, but when it binds to double stranded DNA, its fluorescence increases more than 1000-fold. Its advantage lies in that it can be used with any pair of primers. However, its specificity decreases due to the risk of nonspecific amplification products in the PCR. Each real time PCR reaction includes the SYBR green fluorescent dye, Taq DNA polymerase enzyme (usually with integrated uracil DNA glycosylase (UDG) carryover prevention technology), deoxynucleotides including dUTP, UDG, PCR reaction buffer (usually consisting of a nonionic detergent, KCl), $MgCl_2$ as a cofactor, and a reference dye (e.g., ROX). UDG and dUTP prevent the reamplification of carryover PCR products between reactions. dUTP ensures that any amplified DNA will contain uracil, while UDG removes uracil residues from single- or dou-

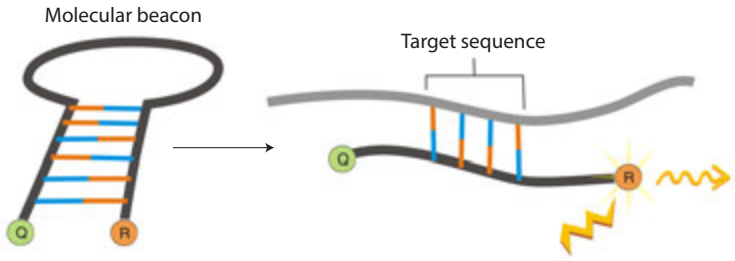


Figure 12. Molecular Beacons are oligonucleotide probes with a stem-and-loop structure. The probe has a reporter fluorescent dye (R) and a quencher dye (Q) at either end but they are designed to adopt a hairpin structure while free in solution, preventing fluorescence emission. By the time the probe hybridizes to the template sequence, the changes in its structure cause the separation of the reporter and quencher enabling fluorescence emission.

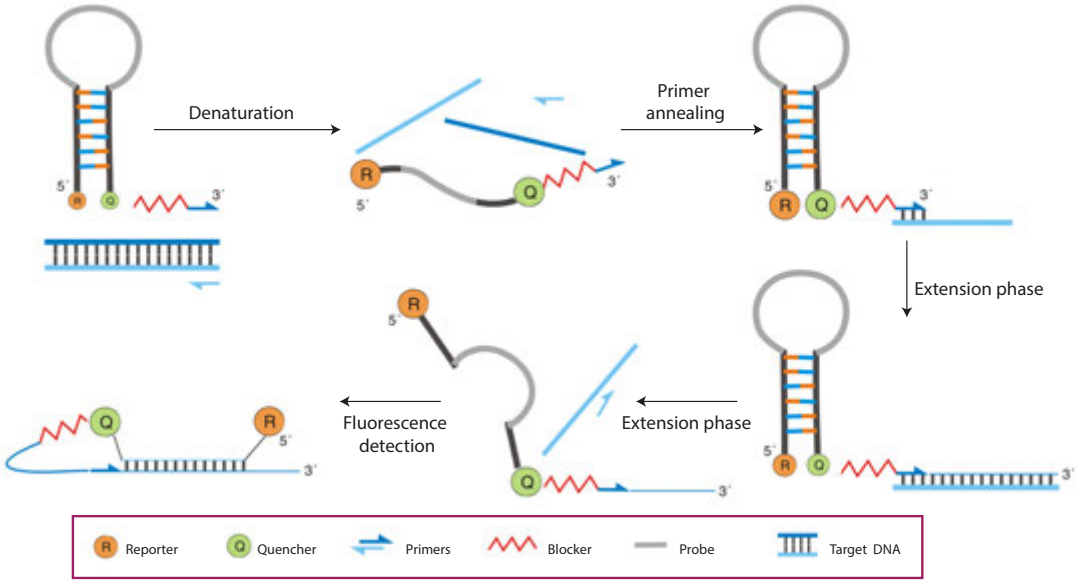


Figure 13. Scorpion probes are single-stranded bi-labeled fluorescent sequence held in a hairpin-loop conformation with a 5' end reporter dye (R) and an internal quencher dye (Q) directly linked to the 5' end of a PCR primer via a blocker. The blocker prevents the polymerase from extending the PCR primer. After extension of the primer by DNA polymerase and its separation from the target DNA by heating, the probe binds to the newly synthesized DNA strand. The distance between the reporter and the quencher increases, allowing fluorescence emission.

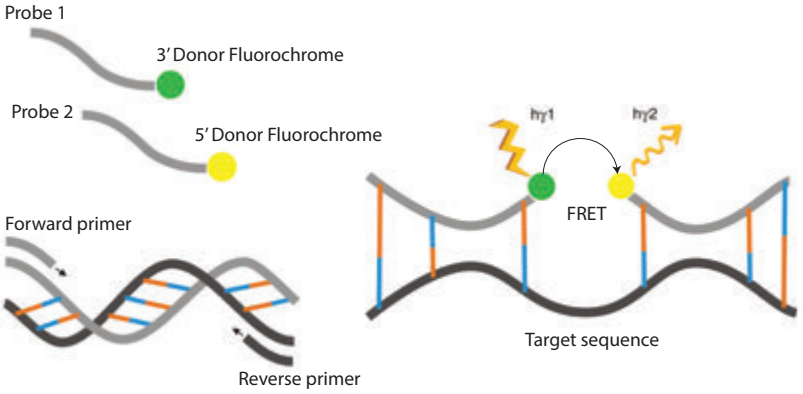


Figure 14. Hybridization probes. This system consists of two oligonucleotides labeled with fluorescent dyes. Interaction of the two dyes can only occur when both are bound to their target. The upstream probe has a donor molecule on the 3'- end and the downstream probe has an acceptor molecule on the 5'-end. During PCR, the two different oligonucleotides hybridize to adjacent regions of the target DNA such that the fluorophores, which are coupled to the oligonucleotides, are in close proximity in the hybrid structure. This allows for transfer of energy from the donor to the acceptor fluorophore, which emits a signal of a different wavelength.

ble-stranded DNA. An UDG incubation step before PCR cycling destroys any contaminating dU-containing product from previous reactions. UDG is then inactivated by the high temperatures during normal PCR cycling, thereby allowing the amplification of genuine target sequences.

COMMON APPLICATIONS FOR REAL-TIME PCR

Relative and absolute quantitation of gene expression

The levels of expressed genes may be measured by absolute or relative quantitative real time PCR. Absolute quantitation measures the actual nucleic acid copy number in a given sample. This requires a sample of known quantity of the gene of interest that can be diluted to generate a standard curve. Meanwhile, relative quantification is based on the expression levels of a target gene versus a housekeeping gene.

Absolute quantification: In this method, a standard curve is first constructed from nucleic acid of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. The dynamic range of the performed calibration curve can be up to nine orders of magnitude from $< 10^1$ to $> 10^{10}$ start molecules, depending on the applied

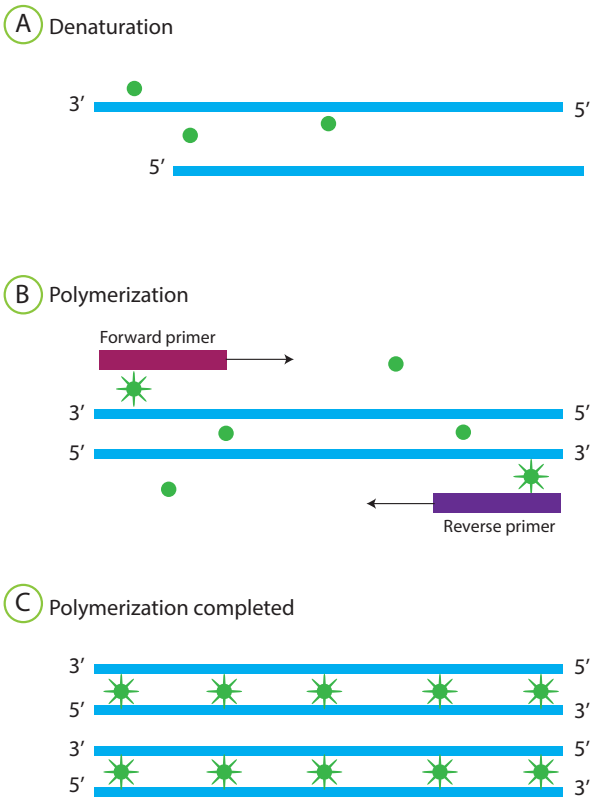


Figure 15. SYBR green dye assay chemistry. A) Denaturation: when DNA is denatured, the SYBR green dye is released and the fluorescence is reduced. B) Polymerization: during the PCR, taq DNA polymerase amplifies the target sequence, which creates the PCR product. C) Polymerization completed: as the PCR progresses, more amplicons are created. Since SYBR Green binds to all double-stranded DNA, the result is an increase in fluorescent intensity proportional to the amount of PCR product produced. Adapted from: http://www.invitrogen.com

standard material. The final result is always reported relatively compared to a defined unit of interest, e.g., copies per defined ng of total RNA, copies per cell, copies per gram of tissue, copies per ml blood, etc.

Relative quantification: This method determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of a housekeeping gene. Calculations are based on comparative Ct method. This involves comparing the Ct values of the samples of interest with a control such as a non-treated sample or RNA from normal tissue. The Ct values of both samples of interest and controls samples are normalized to an appropriate housekeeping gene (Equations 1 and 2).

Eq 1. $\Delta Ct \text{ of testing sample} = Ct_{(\text{target gene})} - Ct_{(\text{housekeeping gene})}$

Eq 2. $\Delta Ct \text{ of control sample} = Ct_{(\text{target gene})} - Ct_{(\text{housekeeping gene})}$

Then, two types of relative quantification models are available and published: without efficiency correction (Equations 3 and 4):

Eq 3. $\text{Relative expression} = 2^{-(\Delta Ct \text{ of testing sample} - \Delta Ct \text{ of control sample})}$

Eq 4. $\text{Relative expression} = 2^{-\Delta\Delta Ct}$

and with kinetic PCR efficiency correction (Equation 5):

Eq 5.

$$\text{Relative expression} = \frac{(E_{\text{target gene}})^{-\Delta Ct \text{ (testing sample - control sample)}}}{(E_{\text{housekeeping gene}})^{-\Delta Ct \text{ (testing sample - control sample)}}}$$

Allelic discrimination

This type of assay is a multiplexed (more than one primer/probe pair per reaction), end-point (data is collected at the end of the PCR process) assay that detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single-nucleic polymorphism (SNP) site in a target template sequence. For each sample in an allelic discrimination assay, a unique pair of fluorescent dye detectors is used, for example, two TaqMan probes that target an SNP site. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2).

The key to success in this type of reactions corresponds to a proper design of probes and primers as shown in Table 5. Another key point is the size of the fragment to be amplified, since the reaction is much more effective when amplicons between 70 and 150 bp are produced. However, larger amplicons can be processed but perhaps further standardization will be needed.

Since the reaction is carried out in the same mixture for both alleles, an increase in the fluorescence emission of one reporter signal will indicate a homozygosity state for that sample, while an increase of fluorescence in both reporters will point to a heterozygosity genotype (Figure 16). The fluorescence emission data produced is interpreted by a computer program, processing the results in a code points representing each one of the samples analyzed, scattered on a plane in which the X-axis and Y-axis correspond to each of the possible alleles of the polymorphic site (Figure 17).

Results generated by this technique are usually analyzed by a computer in three separate analyzes:

1. A multicomponent analysis by determining the contribution of each fluorochrome relative to the total fluorescence detected.

2. The normalization of the fluorescence emission determined against a negative control reaction, and a negative and the positive control for allele 1 and allele 2.

PRIMERS	PROBES
30-80% G+C Content.	
Do not include sequences with three consecutives Gs	
Tm of 58 - 60ºC	Tm of 65 - 67ºC
Both forward and reverse primers should be as close to the probe as possible, without overlapping	Should not contain G at the 5' end
G+C content at the 3' end should be less than 2	Sequence should contain more Cs than Gs

Table 5. Characteristics and selection of probes and primers for allelic discrimination assays.

3. Fluorescence emission calculation for each allele in each reaction

OTHER TECHNIQUES TO ANALYZE DNA OR RNA

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLP)

The protocol usually consists of amplification of the target DNA using PCR, digestion of the DNA into fragments using restriction endonucleases, which cut the double stranded DNA at specific sequences, and separation of the fragments by agarose gel electrophoresis. DNA sequence polymorphisms display different migration profiles from wild-type fragment patterns. Thus a specific profile for each different genotype is produced.

SOUTHERN AND NORTHERN BLOT HYBRIDIZATION

Southern blot hybridization refers to the detection of specific DNA fragments that have been separated by gel electrophoresis. After the electrophoresis the separated DNA fragments are denatured and transferred to a nitrocellulose (or nylon) membrane sheet by blotting. In the blotting the gel is supported on a sponge in a bath of alkali solution, and buffer is sucked through the gel and the sheet by paper towels stacked on top of the nitrocellulose sheet. The buffer denaturates the DNA and transfers the single stranded fragments from the gel to the surface of the sheet, where they adhere firmly. The nitrocellulose sheet containing the bound single-stranded DNA fragments is peeled off the gel and placed in a sealed plastic bag or a box together with buffer containing labeled DNA probe specific for the target DNA sequence. The sheet is exposed to the probe under conditions favoring hybridization. After the hybridization, the sheet is removed from the bag, washed thoroughly to remove unhybridized probes and viewed using autoradiography or ultraviolet light depending on the labels used (radioactive of fluorescent). An adaptation of Southern blotting is Northern blotting, in which RNA molecules are electrophoresed through the gel instead of DNA.

MICROARRAYS

An array is an orderly arrangement of samples where matching of known and unknown DNA samples is done

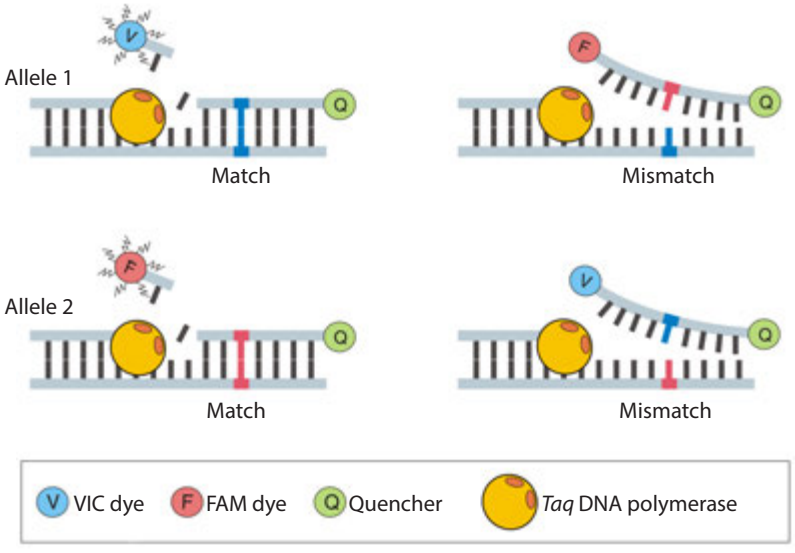


Figure 16. Experimental design of an allelic discrimination assay with fluorochrome labeled probes. The presence of a mismatch between the probe and the target sequence destabilizes the probe during the extension phase, reducing the cleavage efficiency of the probe. The possible results are the following: only VIC signal (homozygous for allele 1); signal for only FAM signal (homozygous for allele 2), the two signals (heterozygous).

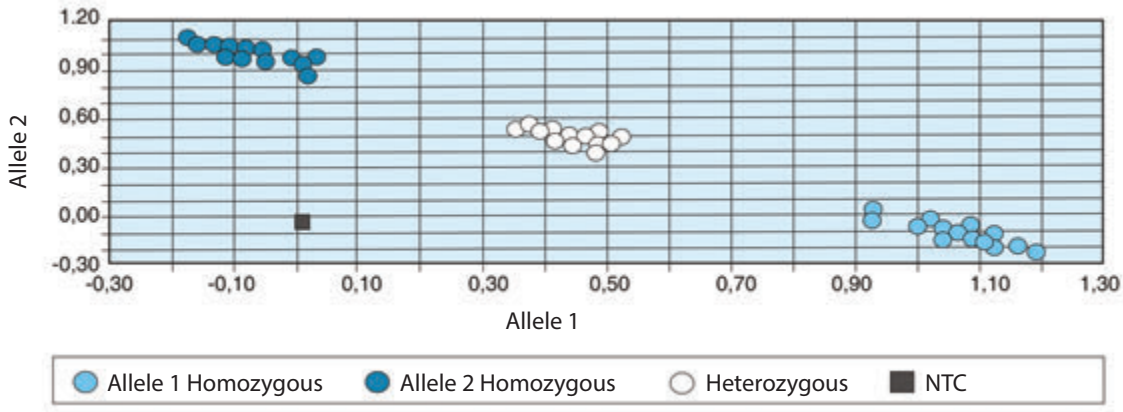


Figure 17. Allelic discrimination results plot. Top left side of the graph show individuals homozygous for allele 1, heterozygous individuals are shown in the center of the graph and in the bottom right individuals homozygous for allele 2. NTC: Non-template Control.

based on base pairing rules. An array experiment makes use of common assay systems such as microplates or standard blotting membranes. The sample spot sizes are typically less than 200 microns in diameter and microarrays usually contain thousands of spots.

Thousands of spotted samples known as probes (with known identity) are immobilized on a solid support (a microscope glass slide, a silicon chip or nylon membrane). The spots can be DNA, cDNA, or oligonucleotides. These are used for determining complementary binding of the unknown sequences thus allowing parallel analysis for gene expression and gene discovery. An experiment with a single DNA chip can provide information on thousands of genes simultaneously. An orderly arrangement of the probes on the support is important as the location of each spot on the array is used for the identification of a gene.

Depending upon the kind of immobilized sample used for constructing the arrays and the information obtained, the Microarray experiments can be categorized in three ways:

- Microarray Expression Analysis: In this experimental set-up, the cDNA derived from the mRNA of known genes is immobilized. Typically two samples are prepared. One sample has cDNA from the normal tissue labeled with a green fluorescent dye and the other has cDNA from the diseased tissue labeled with a red fluorescent dye. Both samples are applied simultaneously on the microarray and allow to hybridize. After washing off the unhybridized material, the array is analyzed. The ratio of fluorescent signal is recorded for each spot. Housekeeping gene spots allow to standardize the results. If the gene is over expressed in the diseased condition, the corresponding spot will have higher ratio of red to green fluorescence. If the gene is under expressed in the diseased condition, the ratio of signals on the corresponding spot will be reversed. This method can be also used for example to analyze different expression patterns in different tissues, different phases of development of the same tissue, different responses of tissues to treatment or different responses of liver tissue to exposure to a drug to study its toxicity.
- Microarray for Mutation Analysis: For this analysis, the researchers use gDNA. The genes might differ from each other by as little as a single nucleotide base (SNP). A DNA microarray is an assembly of DNA specific probes attached to a solid surface. DNA microarrays are generally used to measure the expression levels of genes simultaneously or genotypes over multiple regions of a genome. Each DNA microarray contains probes of a specific DNA sequence. These can be a short section of a gene or other DNA element that are used to hybridize cDNA or genomic DNA sample under high-stringency conditions. Probe and target hybridization is usually detected and quantified by detection of light intensity on the labeled targets to determine relative abundance of nucleic acid sequences for each specific probe (For more specifics and applications of DNA microarrays the reader is directed to chapter 18).

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Recommended Web sites

1. http://www.proligo.com/
2. http://www.qiagen.com/
3. http://www.neb.com/
4. http://www.lifetechnologies.com/

47

A PRIMER ON CURRENT AND COMMON SEQUENCING TECHNOLOGIES

John Castiblanco

INTRODUCTION

Efforts to resolve the sequence of nucleic acids began on the 70s and peaked in 2000 after the completion of the first human genome drafts. Sequencing methods have evolved over time, starting from the Maxam and Gilbert chemical method and continuing to the Sanger enzymatic method giving rise to automated sequencing. All these highly used techniques jump started the development of innovative sequencing strategies (i.e., Next Generation Sequencing - NGS) addressing our anticipated future needs of throughput and cost, and enabling a multitude of current and future applications in genomic research. NGS platforms can generate about five hundred million bases of raw sequence to billions of bases in a single run, presenting both opportunities and challenges for data management, storage, and especially analysis. Most of these novel methods rely on parallel, cyclic interrogation of sequences from spatially separated clonal units. This chapter presents a brief description of the basic concepts of past, present and some of the future sequencing platforms and their technology.

BACKGROUND

Evolution of DNA sequencing methods peaked around year 2000 and is based on the contributions of mainly four researchers. Foremost, Allan Maxam and Walter Gilbert developed in the 70s a chemical method of DNA sequencing, in which terminally labeled with radioactive phosphorus DNA fragments were subjected to base specific chemical cleavage and the reaction products were separated by gel electrophoresis. In an alternative approach in 1977, Frederick Sanger perfected the sequencing method by the use of chain-terminating dideoxynucleotide analogs that caused base-specific termination of primed DNA synthesis. In this method the primer was typically labeled with radioactive phosphorus. Finally Leroy Hood, along with his colleagues, Michael Hunkapiller and Lloyd Smith, in 1986, modified the Sanger method to a higher throughput configuration by using fluorescently labeled dideoxynucleotides. Such approach allowed avoidance of radioactive compounds with limited lifetime and instead use of stable fluorescent probes. In addition the analysis of all nucleic bases could be done by reading just one electrophoresis lane instead of four and the reading process could be automated.

This high-throughput configuration was used in the sequencing of the first human genome, completed in 2003 through the Human Genome Project, a 13-year effort with an estimated cost of $2.7 billion. Thanks to the improvements and automation of the method, in 2008, another human genome was sequenced over a 5-month period for approximately $1.5 million. The completion of the first human genome drafts was just a start of the modern DNA sequencing era which resulted in further inventions and new, advanced strategies of high-throughput DNA sequencing, so called next generation sequencing (NGS).

The development of NGS strategies is addressing our needs of sequencing throughput and cost, in a way which enabled a multitude of current and future applications in genomic research. These advanced methods required development of new bioinformatic tools as an essential prerequisite to accommodate the analysis of huge amounts of data produced in the process. Currently, there are several NGS platforms commercially available, with additional new platforms on the horizon. To accelerate the pace of development of new methods, the US National Human Genome Research Institute (NHGRI) announced in August 2008 funding for a series of projects as part of its Revolutionary Genome Sequencing Technologies program, with a goal of sequencing of a human genome for $1000 dollars or less (Figure 1).

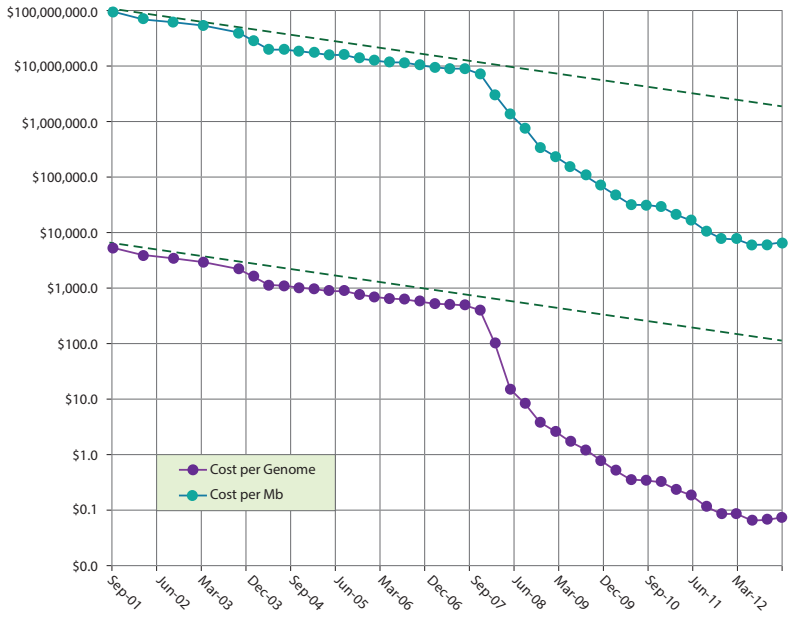

Figure 1. The National Human Genome Research Institute (NHGRI) has tracked the costs associated with DNA sequencing performed at the sequencing centers funded by the Institute. The cost-accounting data are summarized relative to two metrics: (A) "Cost per Megabase of DNA Sequence" - the cost of determining one megabase (Mb; a million bases) of DNA sequence; (B) "Cost per Genome" - the cost of sequencing a human-sized genome. To illustrate the nature of the reductions in DNA sequencing costs, each graph also shows hypothetical data reflecting Moore's Law, which describes a long-term trend in the computer hardware industry that involves the doubling of 'compute power' every two years. Both graphs use a logarithmic scale on the Y-axis. The sudden and profound out-pacing of Moore's Law beginning in January 2008, represents the time when the sequencing centers transitioned from Sanger-based to 'second generation' (or 'next-generation') DNA sequencing technologies. Data obtained from http://www.genome.gov/sequencingcosts/

Besides the advancement of sequencing techniques, this past decade will be remembered as the decade of genome research, owing to the publications of the first composite of human genomes and many draft genomes from other organisms. The speed at which new genomes can now be sequenced has been facilitated by the development of potential NGS technologies and assembly methods. This chapter will describe the current and past sequencing technologies, their history, applications as well as future, emerging applications.

FUNDAMENTALS OF SEQUENCING PLATFORMS

Sanger sequencing is based on the electrophoretic separation of chain-termination products obtained in individual sequencing reactions. When compared with the new sequencing platforms, to some extent, most of them present a similar technological base. Historically, these types of innovations have been given an order depending on the time of discovery or report. Below is the description of the most commonly used platforms.

FIRST GENERATION DNA SEQUENCERS

In 1975, Frederick Sanger introduced the concept of DNA sequencing and published a rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. In 1977, two landmark articles for DNA sequencing were published, Sanger's enzymatic dideoxy DNA sequencing technique based on the chain-terminating dideoxynucleotide analogues and the Allan Maxam and Walter Gilbert's chemical degradation DNA sequencing technique in which terminally labeled DNA fragments were chemically cleaved at specific bases and separated by gel electrophoresis. Thus, these publications were responsible for the introduction of the first automated DNA sequencer which was subsequently commercialized by Applied Biosystems (ABI), the European Molecular Biology Laboratory (EMBL) and Pharmacia-Amersham, later General Electric (GE) Healthcare. The refinement and commercialization of the sequencing method led to its broad dissemination throughout the global research community.

Chain-terminating Maxam and Gilbert Chemical Method

Initially, as the first method of DNA sequencing, this procedure was the most commonly used; however, it was slowly displaced by the enzymatic method (See text below). The Maxam and Gilbert method is based on chemical hydrolysis, applicable to DNA fragments up to 250 nucleotides and consisted of three steps (Figure 2):

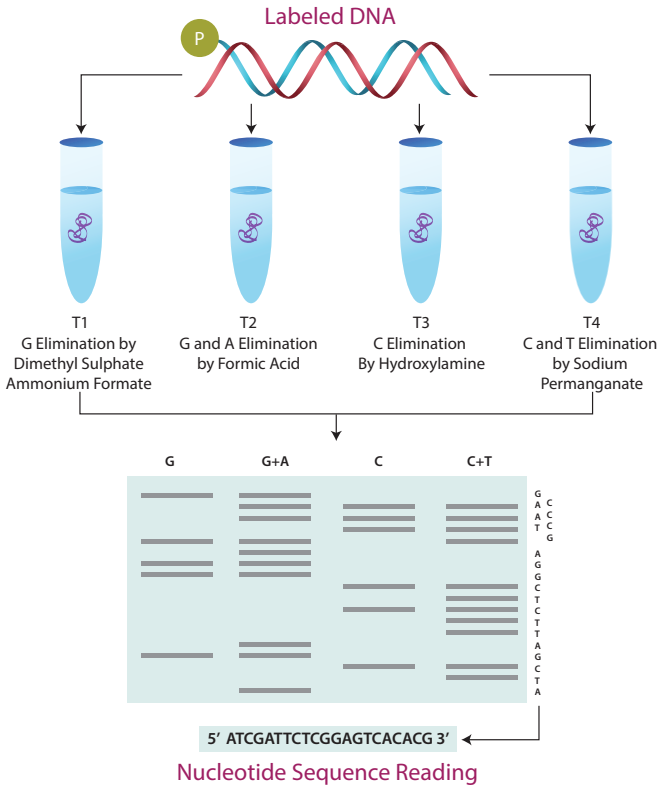

Figure 2. Maxam and Gilbert Chemical Sequencing Method. T=Treatment. Adapted from (2).

Probe Labeling: This process is based on chemical degradation of quite large samples of DNA. It can be performed from a double-stranded DNA or single-stranded genomic DNA isolated from any organism and obtained by enzymatic cleavage or other selective fragmentation process. This step consists on the labeling of one of the ends of the DNA strand. Typically, the 5' end of the strand is labeled with radioactive phosphorus using the polynucleotide kinase enzyme and radioactive ATP. Similarly, the use of fluorochromes on the 3' end of the DNA chain can be applied.

DNA Nucleotide Modification and Fragmentation: This is the most important step and it sought to generate fragments of single-stranded DNA, labeled at one end with variable size. Thus, size strand would indicate the order of the sequence, according to the last nucleotide. This process is carried out by using chemicals that generate specific modifications to each nucleotide and must be carried out in four different tubes. In the case of guanine, dimethyl sulphate and 1% of 50 mM ammonium formate would be added. These compounds first methylate guanine and then remove the base (depurinate) DNA and finally break the DNA strands at all locations where this base is present. For removal of guanine and adenine, 80% of formic acid is used indiscriminately. In the case of cytosine, 4M hydroxylamine is used; for removing cytosines and thymines high concentrations of salts (e.g., potassium permanganate 0.1M) are used. The chemical reactions that are generated in the tubes should be under strict control of time, temperature and reagent concentration.

Separation and analysis of the fragments: This is the final step of the process and it is aimed to establish the sequence of the targeted DNA strand by using high resolution polyacrylamide gels. The final sequence is determined by the migration of the DNA fragments according to size and depending on the base and chemical treatment. As the result of reactions, four mixtures are generated containing DNA fragments terminated with: G, G+A, C, and C+T, and their analysis allow the assignment of all bases constituting DNA strands. Some difficulties with this analysis come from the fact that each reaction is separated in a different well of a polyacrylamide gel and multiple factors including presence of salt, loading of the wells and temperature of the gel influence band migration.

The Enzymatic Sanger Method

The method is built on DNA synthesis based on very small DNA template which is being analyzed. The process relies on the addition of modified nucleotides (i.e., 2', 3'-dideoxynucleotide triphosphates), whose incorporation into a growing DNA chain terminates the DNA extension. The method is performed *in vitro*, by a PCR reaction that extends a single strand of DNA using a specific primer. The primer is marked with a radioactive or fluorescent probe. Typically four extension reactions are carried out separately in parallel, each of which with a different dideoxynucleotide triphosphates (i.e., dideoxy-ATP, dideoxy-TTP dideoxy-GTP dideoxy-CTP) (ddNTPs), structural analogues of the deoxynucleotide triphosphates (dNTP).

The product of the four extension reactions is visualized on a high resolution polyacrylamide gel (Figure 3). One advantage of the Sanger method is that each of the four reactions gave direct information about only one base. The application of this technique was initially very laborious and allowed only to solve short sequences of 100 to 300 bases at the time. The method must avoid the 5'-exonuclease activity that most polymerases possess and involve a high fidelity enzyme. Originally, results would have to be read and interpreted manually, which might introduce errors to the technique. For this enzymatic method, separation of the fragments and the analysis of the sequence follow the same principle used in the chemical method explained above.

Automated Sequencing

The possibility of using different fluorochromes incorporated for each nucleotide in a reaction allowed the Sanger enzymatic method modified by Leroy Hood, to be automated in the same sequencing reaction (Table 1). Taking advantage

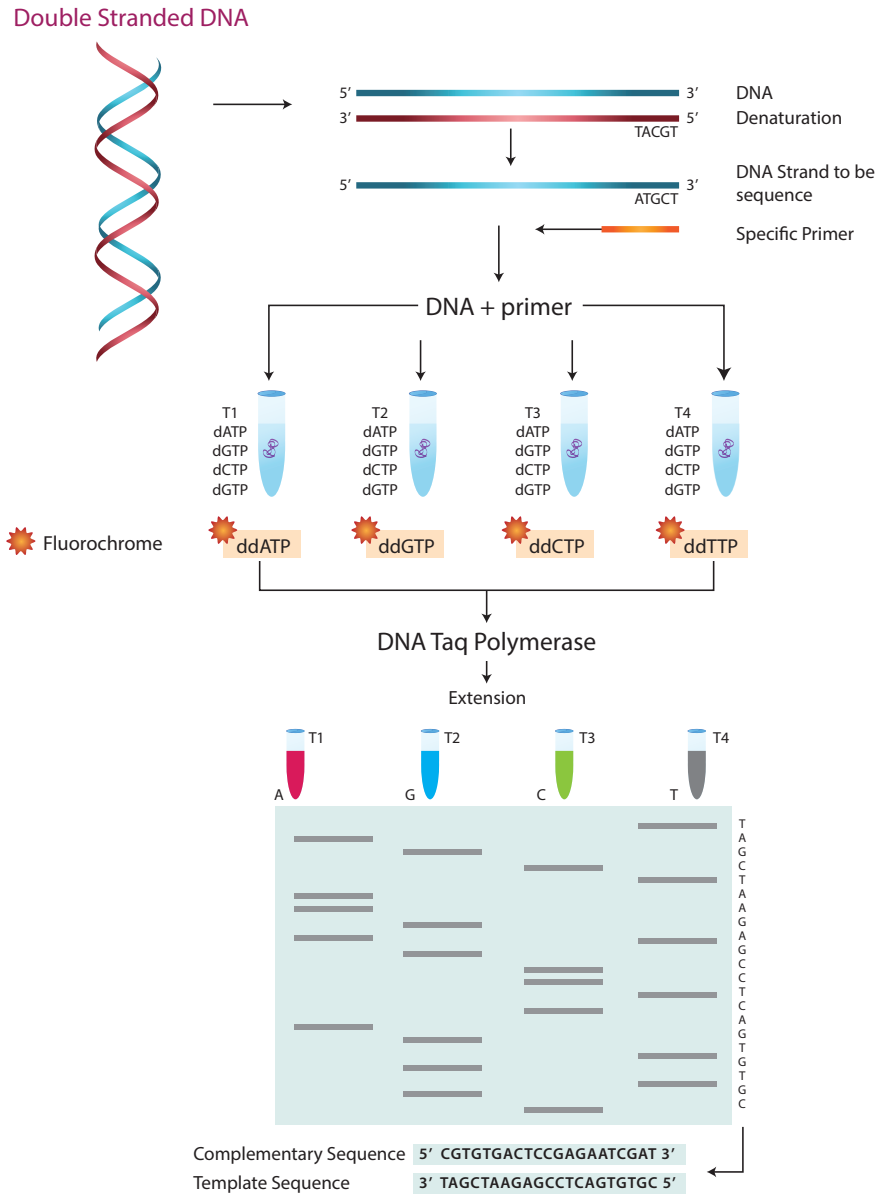

Figure 3. Sanger Enzymatic Sequencing Method. Adapted from (2).

of the fact that fluorescence acquisition can be performed continuously, while gel or capillary electrophoresis is in progress sped up the process and standardized it further. Using this method the DNA molecules of smaller size pass through the gel faster and emit fluorescence at a specific wavelength, that allows determining the nucleotide to which it corresponds. This method greatly increased the size of the DNA fragments that could be sequenced in a single experiment (Figure 4).

In 1996, Applied Biosystems, Inc. (ABI) introduced the first commercial DNA sequencer that used a slab gel electrophoresis unit, known as the ABI Prism 310. Two years later, the considerable labor of pouring slab gels was replaced with automated reloading of 96-well capillaries with a polymer matrix by the ABI Prism 3700 (Figure 4). This automated DNA sequencer was successfully used in the sequencing of the first human genome. At the time, methods used were refinements of the basic 'dideoxy' method introduced by Sanger.

NEXT GENERATION SEQUENCING (NGS) PLATFORMS

The initial birth of the NGS platforms was driven by the technological convergence of pyrosequencing and emulsion PCR. In 1993, Nyren and colleagues described a sequencing approach based on chemiluminescent detection of pyrophosphate released during polymerase mediated deoxynucleoside triphosphate (dNTP) incorporation which led as a foundation for the

ABBREVIATION	NAME	λ_{max} / nm (Absorption)	λ_{max} / nm (Emission)	EMITTED COLOR
FAM	(5 or 6)- Carboxyfluorescein	494	518	Yellow
JOE	(5 or 6)- Carboxy-4',5-dichloro-2'7'-dimethylfluorescein	522	550	Red
TAMRA	(5 or 6)- Carboxytetramethyl-rhodamine	555	580	Puple – Black
ROX	(5 or 6)- Carboxy-X-rhodamine	580	605	Blue - Green

Table 1. **Available fluorochromes for automated sequencing.**

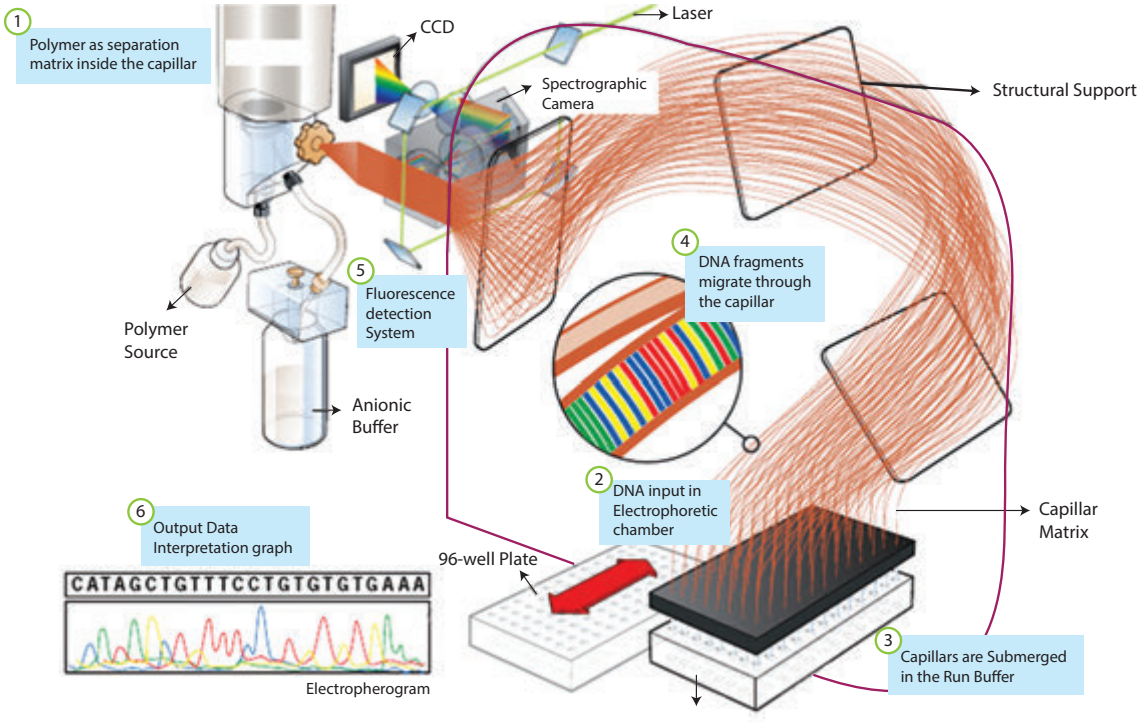

Figure 4. Automatic sequencing scheme using a 96-well capillary electrophoresis which detects the fluorescence emission through a spectrophotometric camera and which wavelength is recorded and translated into an electropherogram. Adapted from (3).

commercial development of pyrosequencing by 1998. In general, the principle of pyrosequencing technique is based on the "sequencing by synthesis". It differs from Sanger sequencing because it depends on the detection of pyrophosphate release during nucleotide incorporation, rather than chain termination with dideoxynucleotides. Around the same time, Tawfik and Griffiths described a single-molecule PCR procedure in microcompartments consisting of water-in-oil emulsions (i.e., emulsion PCR) (Figure 5a).

In 2000, Rothberg founded 454 Life Sciences and developed the first commercially available NGS platform, known as the GS 20, launched in 2005. The GS 20 was successfully validated by combining single-molecule emulsion PCR with pyrosequencing and performing shotgun sequencing on the entire 580.069 bp of *Mycoplasma genitalium* genome at 96% coverage and a 99.96% accuracy in a single run.

In the following years, Roche acquired 454 Life Sciences and introduced the second version of the 454 instrument, the GS FLX titanium. Sharing the same core technology as the GS 20, the GS FLX flow cell is referred to as a "picotiter well" plate, made from a fused fiber-optic bundle (Figure 5a). For sequencing, a library of template DNA is prepared by fragmentation via nebulization or sonication (Figure 6). Then, these fragments several hundred base pairs in length are end-repaired and ligated to adapter oligonucleotides, diluted to single-molecule concentration, denatured, and hybridized to individual beads containing sequences complementary to adapter oligonucleotides. The beads are compartmentalized into water-in-oil microvesicles, where clonal expansion of single DNA molecules bound to the beads occurs during emulsion PCR. After amplification, the emulsion is disrupted, and the beads containing clonally amplified template DNA

are enriched to separate those with successful PCR products from those without any products. The beads are again separated by limiting dilution, deposited into individual picotiter-plate wells, and combined with sequencing enzymes. Loaded into the GS FLX, the picotiter plate functions as a flow cell wherein iterative pyrosequencing is performed by successive flow addition of the four dNTPs. A nucleotide-incorporation event in a well containing clonally amplified template produces pyrophosphate release and picotiter-plate well–localized luminescence, then is transmitted through the fiber-optic plate and recorded on a charge-coupled device camera. With the flow of each dNTP reagent, wells are imaged, analyzed for their signal-to-noise ratio, filtered according to quality criteria, and subsequently algorithmically translated into a linear sequence output (Figure 6).

SECOND NGS PLATFORMS

Second NGS platforms can generate about five hundred million bases of raw sequence (e.g., Roche) to billions of bases in a single run (e.g., Illumina, SOLiD). These novel methods rely on parallel, cyclic interrogation of sequences from spatially separated clonal amplicons (Figure 5). Currently, the three leading second NGS platforms are commercially available (i.e., Roche based on pyrosequencing chemistry, SOLiD based on sequencing by sequential ligation of oligonucleotide probes, and Illumina based on sequencing by reversible dye terminators) (Table 2). Below, a brief description of the basic concepts of each platform and technology is presented, for complete laboratory methods, technical aspects and sample preparation, the reader should consult each platform's website or some of the comprehensive reviews presented in the recommended readings.

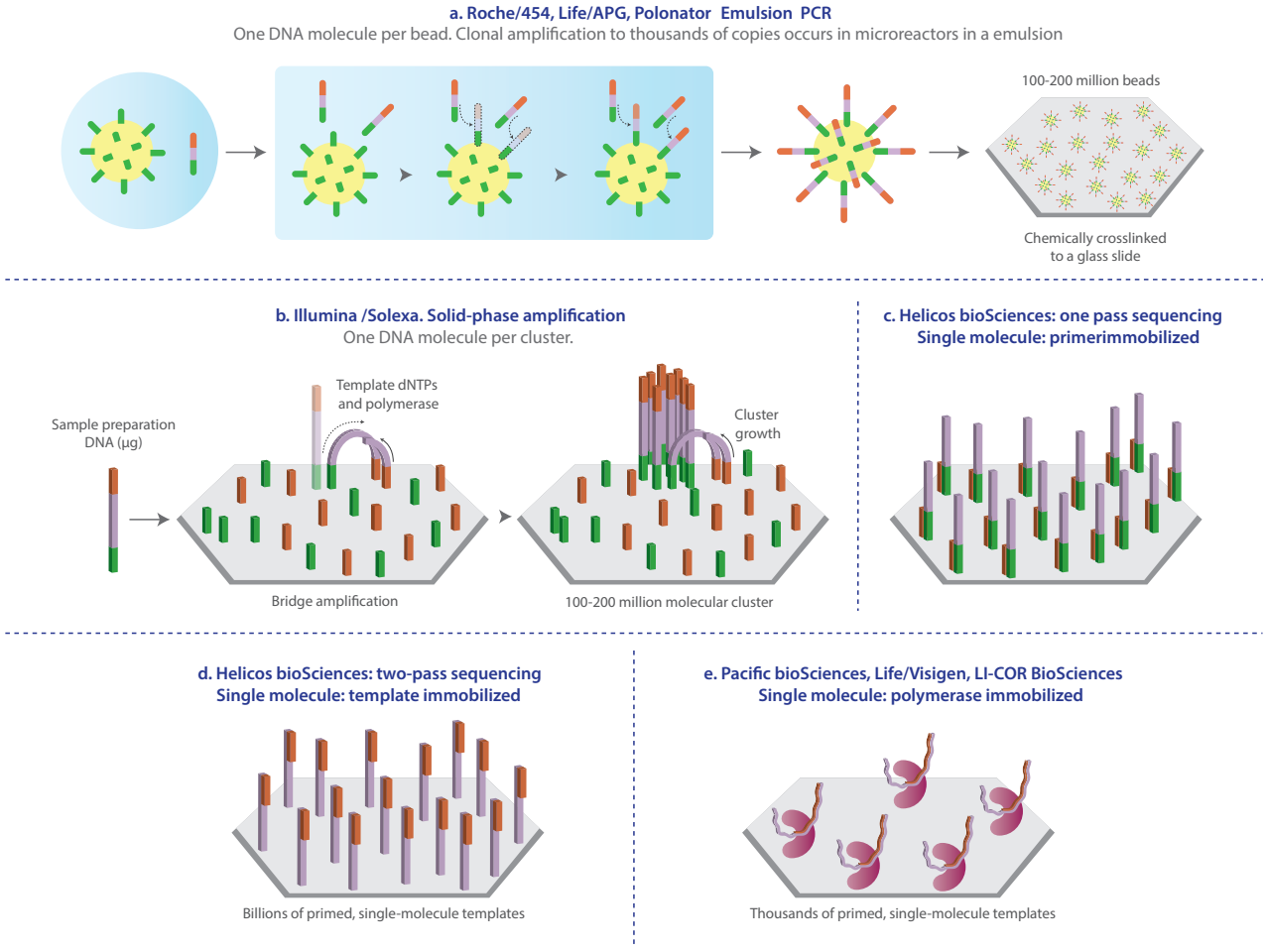

Figure 5. Template immobilization strategies. In emulsion PCR (a), a reaction mixture consisting of an oil–aqueous emulsion is created to encapsulate bead–DNA complexes into single aqueous droplets. PCR amplification is performed within these droplets to create beads containing several thousand copies of the same template sequence. Solid-phase amplification (b) is composed of two basic steps: initial priming and extending of the single-stranded, single-molecule template, and bridge amplification of the immobilized template with immediately adjacent primers to form clusters. Three approaches are shown for immobilizing single-molecule templates to a solid support: immobilization by a primer (c); immobilization by a template (d); and immobilization of a polymerase (e). dNTP, 2'-deoxyribonucleoside triphosphate. Adapted from (5).

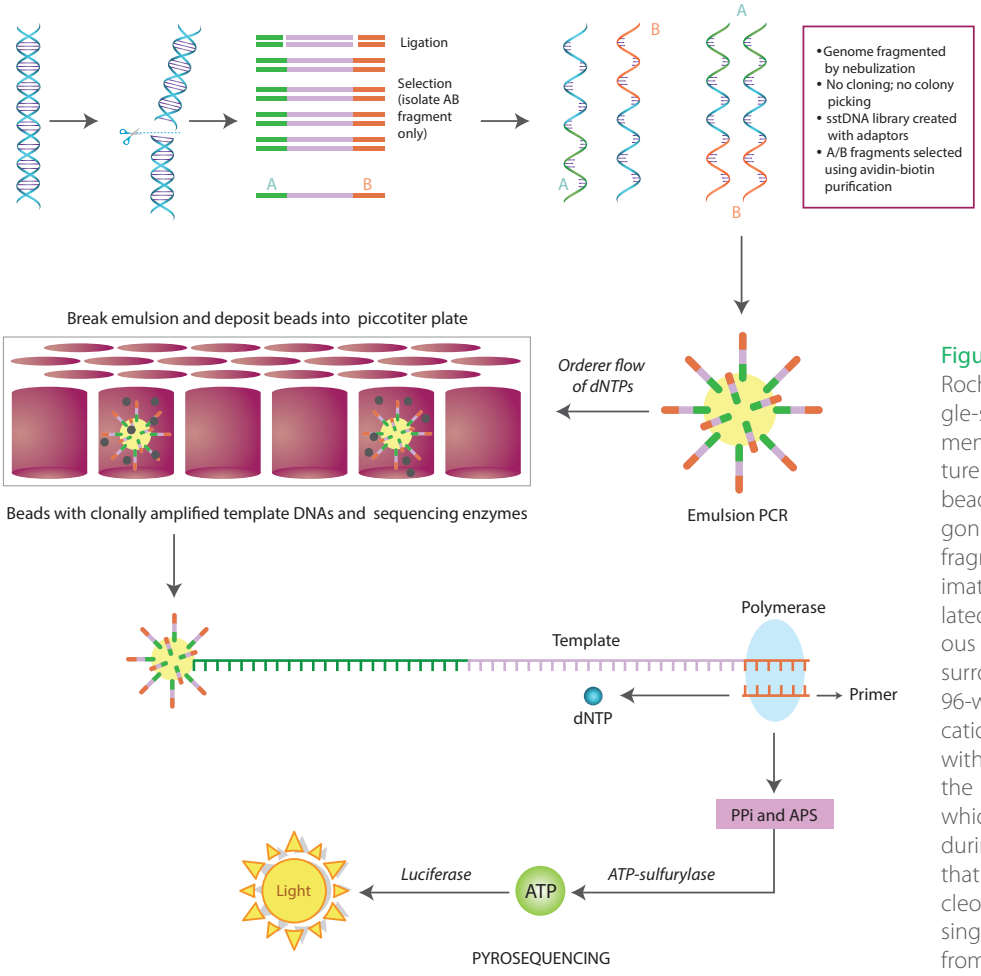

Figure 6. The method used by the Roche/454 sequencer to amplify single-stranded DNA copies from a fragment library on agarose beads. A mixture of DNA fragments with agarose beads containing complementary oligonucleotides to the adapters at the fragment ends are mixed in an approximately 1:1 ratio. The mixture is encapsulated by vigorous vortexing into aqueous micelles that contain PCR reactants surrounded by oil, and pipetted into a 96-well microtiter plate for PCR amplification. The resulting beads are covered with approximately 1 million copies of the original single-stranded fragment, which provides sufficient signal strength during the pyrosequencing reaction that follows to detect and record nucleotide incorporation events. sstDNA, single-stranded template DNA. Adapted from (7).

SOLiD (Supported Oligonucleotide Ligation and Detection) platform

In 2007, Applied Biosystems refined the 454 technology and released the SOLiD (Supported Oligonucleotide Ligation and Detection) platform which is a short-read sequencing technology based on ligation. This approach was developed in Church's laboratory (Harvard Medical School) and its performance was demonstrated by resequencing of the *Escherichia coli* genome.

Sample preparation in the SOLiD is similar to the 454 technology. DNA fragments after sonication are ligated to oligonucleotide adapters, attached to beads in dilution favoring a single DNA molecule per bead, and clonally amplified by emulsion PCR. After cleaning and enrichment, beads are immobilized onto a flow cell surface. Sequencing begins by annealing an oligonucleotide complementary to the adapter at the adapter–template junction (Figure 7). Instead of providing a 3′ hydroxyl group for polymerase-mediated extension, the primer is oriented to provide a 5′ phosphate group for ligation to interrogation probes during the first "ligation sequencing" step. Each interrogation probe is an octamer, which consists of two probe-specific bases followed by six degenerate bases with one of four fluorescent labels linked to the 5′ end (Figure 7).

The two probe-specific bases consist of one of 16 possible two-base combinations. In the first ligation-sequencing step, thermostable ligase and interrogation probes representing the 16 possible two-base combinations are present. The probes compete for annealing to the template sequences immediately adjacent to the primer. After annealing, a ligation step is performed, followed by wash removal of unbound probe. Fluorescence signals are optically collected before cleavage of the ligated probes, and a wash is performed to remove the fluorophore and regenerate the 5′ phosphate group. In the subsequent sequencing steps, interrogation probes are ligated to the 5′ phosphate group of the preceding pentamer. Seven cycles of ligation, referred to as a "round," are performed to extend the first primer. The synthesized strand is then denatured, and a new sequencing primer offset by one base in the adapter sequence is annealed. Five rounds total are performed, each time with a new primer with a successive offset. By this approach, each template nucleotide is sequenced twice. A six-day instrument run generates sequence read lengths of

PLATFORM	AMPLIFICATION	READ LENGTH	THROUGHPUT	SEQUENCE BY SYNTHESIS
Currently Available				
Roche/GS-FLX Titanium	Emulsion PCR	400-600 bp	500 Mbp/run	Pyrosequencing
Illumina/HiSeq 2000, HiScan	Bridge PCR	2 x 100 bp	200 Gbp/run	Reversible terminators
ABI/SOLiD 5500xl	Emulsion PCR	50 -100 bp	>100 Gbp/run	Sequencing-by-ligation (octamers)
Polonator/G.007	Emulsion PCR	26 bp	8-10 Gbp/run	Sequencing-by-ligation (monomers)
Current or future 3rd NGS platforms				
Helicos/Heliscope	No	35 (25-55) bp	21-37 Gbp/run	True single-molecule sequencing (tSMS)
Pacific BioSciences/RS	No	1000 bp	N/A	Single-molecule real time (SMRT)
Ion Torrent/PostLight	No	N/A	N/A	Semiconductor-based pH sequencing
Oxford Nanopore Technologies	No	35 bp	N/A	Nanopores/exonuclease-coupled
Visigen Biotechnologies	No	>100 Kbp	N/A	Base-specific FRET
U.S. Genomics	No	N/A	N/A	Single-molecule mapping
Genovoxx	No	N/A	N/A	Single-molecule sequencing by synthesis
NABsys	No	N/A	N/A	Nanopores
Electronic BioSciences	No	N/A	N/A	Nanopores
BioNanomatrix/nanoAnalyzer	No	400 Kbp	N/A	Nanochannel arrays
GE Global Research	No	N/A	N/A	Closed complex/nanoparticle
IBM	No	N/A	N/A	Nanopores
LingVitae	No	N/A	N/A	Nanopores
Complete Genomics	No	70 bp	N/A	DNA nanoball arrays
base4innovation	No	N/A	N/A	Nanostructure arrays
CrackerBio	No	N/A	N/A	Nanowells
Reveo	No	N/A	N/A	Nano-knife edge
Intelligent BioSystems	No	N/A	N/A	Electronics
LightSpeed Genomics	No	N/A	N/A	Direct-read sequencing by EM
Halcyon Molecular	No	N/A	N/A	Direct-read sequencing by EM
ZS Genetics	No	N/A	N/A	Direct-read sequencing by TEM
Genizon BioSciences/CGA	No	N/A	N/A	Sequencing-by-hybridization

Table 2. Characteristics and principal applications of next-generation sequencing platforms. Adapted from (1).

35 bases. Sequence is inferred by interpreting the ligation results for the 16 possible two-base combination interrogation probes. With the use of offset primers, several bases of the adapter are sequenced. This information provides a sequence reference starting point that is used in conjunction with the color space – coding scheme to algorithmically deconvolute the downstream template sequence.

ILLUMINA GENOME ANALYZER/HiSeq

Shankar Balasubramanian and David Klenerman founded Solexa in 1998 and by 2006, the first "short read" sequencing platform (i.e., the Solexa Genome Analyzer) was commercially launched and later acquired by Illumina. The Genome Analyzer, now the Hiseq and on a smaller scale the MiSeq, uses a flow cell consisting of an optically hollow transparent slide with eight or two individual lanes to which oligonucleotide anchors are bound, respectively (Figure 8).

Sample preparation for Illumina sequencing is started by fragmenting template DNA into lengths of several hundred base pairs followed by end-repair in order to generate 5′-phosphorylated blunt ends. Next, the polymerase activity of the Klenow fragment is used to add a single adenine base to the 3′ end of the blunt phosphorylated DNA fragments, in order to ligate oligonucleotide adapters which have an overhang of a single T base at their 3′ end. Adapter oligonucleotides are complementary to the flow-cell anchors.

Under limiting-dilution conditions, adapter-modified single-stranded template DNA is added to the flow cell and immobilized by hybridization to the anchors. DNA templates are amplified in the flow cell by "bridge" amplification, which relies on captured DNA strands "arching" over and hybridizing to

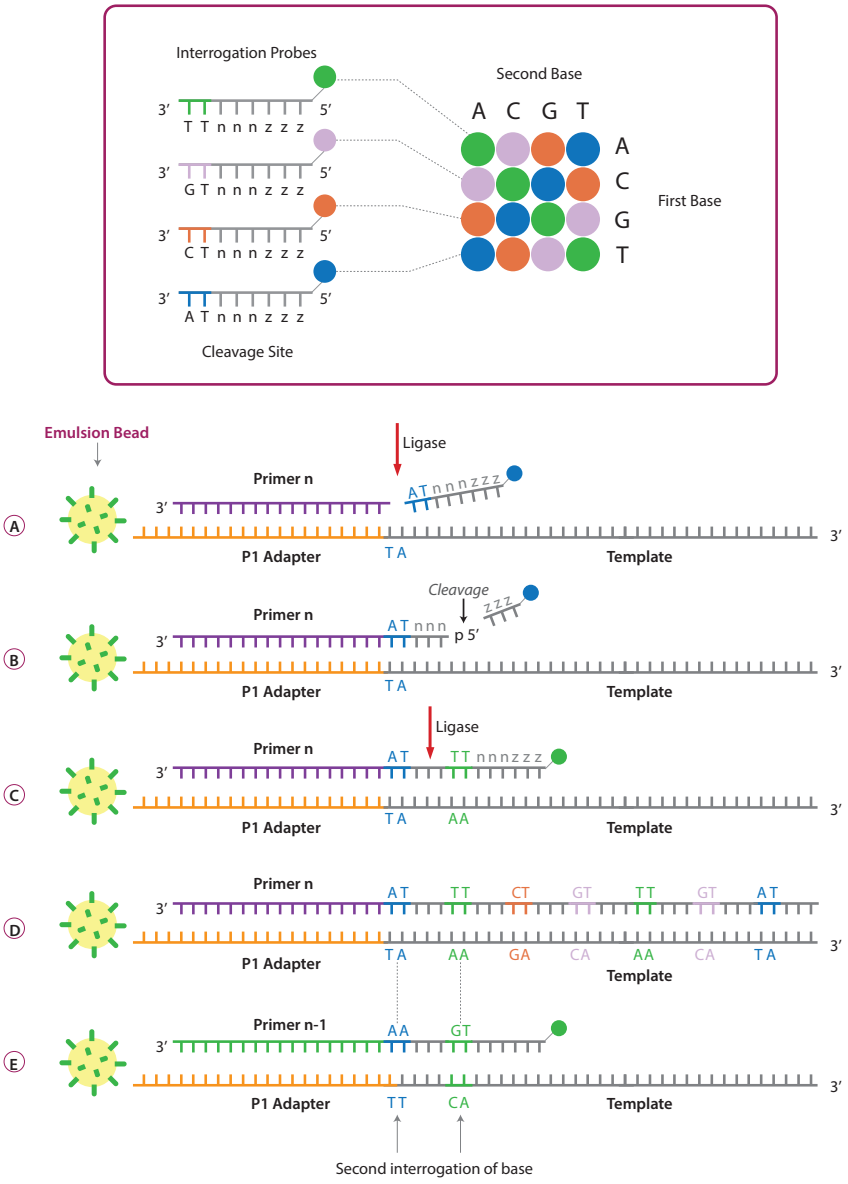

Figure 7. Applied Biosystems SOLiD sequencing by ligation. 1) SOLiD color-space coding. Each interrogation probe is an octamer, which consists of (3′-to-5′ direction) two probe-specific bases followed by 6 degenerate bases (nnnzzz) with one of four fluorescent labels linked to the 5′ end. The two probe-specific bases consist of one of 16 possible 2-base combinations. 2) (A) The P1 adapter and template with annealed primer (n) is interrogated by probes representing the 16 possible two-base combinations. The figure shows the two specific bases complementary to the template are AT. (B) After annealing and ligation of the probe, fluorescence is recorded before cleavage of the last 3′ degenerate probe bases. The 5′ end of the cleaved probe is phosphorylated (not shown) before the second sequencing step. (C) Annealing and ligation of the next probe. (D) Complete extension of primer (n) through the first round consisting of 7 cycles of ligation. (E) The product extended from primer (n) is denatured from the adapter/template, and the second round of sequencing is performed with primer (n - 1). With the use of progressively offset primers, in this example (n - 1), adapter bases are sequenced, and this known sequence is used in conjunction with the color-space coding for determining the template sequence by deconvolution. In this technology, template bases are interrogated twice. Adapted from (7).

an adjacent anchor oligonucleotide. Multiple amplification cycles convert a single-molecule DNA template to a clonally amplified arching "cluster," with each cluster containing approximately 1000 clonal molecules (Figure 8). Approximately 5 x10^7 separate clusters can be generated per flow cell.

For sequencing, clusters are denatured, and a subsequent chemical cleavage reaction and a wash leave only forward strands for single-end sequencing. Sequencing of the forward strands is initiated by hybridizing a primer complementary to the adapter sequences, followed by addition of polymerase and a mixture of four differently colored fluorescent reversible dye terminators. The terminators are incorporated according to sequence complementarity in each strand in a clonal cluster. After incorporation, excess reagents are washed away, the clusters are optically interrogated, and the fluorescence is recorded. With successive chemical steps, the reversible dye terminators are unblocked, the fluorescent labels are cleaved and washed away, and the next sequencing cycle is performed (Figure 8). The newest optical modifications enable the analysis of higher cluster densities. The former is coupled with ongoing improvements in sequencing chemistry and projected read lengths of 150-plus bases. Illumina and other NGS technologies have devised strategies to sequence both ends of template molecules; this is known as paired-end sequencing and provides positional information that facilitates alignment and assembly, especially for short reads.

THIRD GENERATION NGS PLATFORMS

For second generation NGS platforms, sequencing is based on PCR amplification of DNA fragments in order to make a

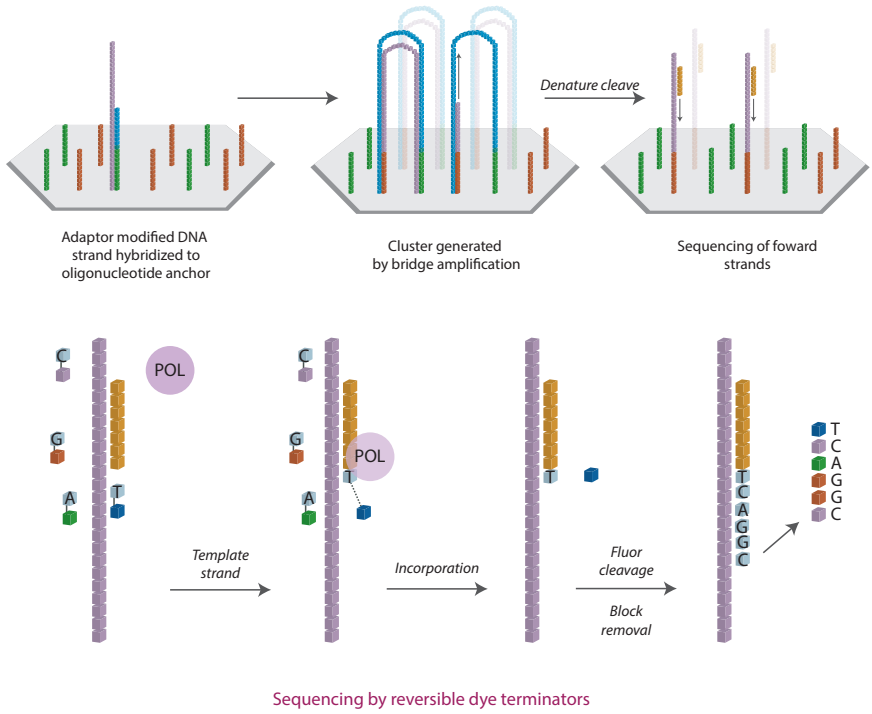

Figure 8. Illumina sequencing. Adapter-modified, single-stranded DNA is added to the flow cell and immobilized by hybridization. Bridge amplification generates clonally amplified clusters. Clusters are denatured and cleaved; sequencing is initiated with addition of primer, polymerase (POL) and 4 reversible dye terminators. Postincorporation fluorescence is recorded. The fluor and block are removed before the next synthesis cycle. Adapted from (7).

luminescent signal strong enough for reliable base detection by CCD cameras. In some instances, this amplification might introduce base sequence errors or favor certain sequences over others by changing the relative frequency and abundance of various DNA fragments that existed before amplification.

As a solution to overcome this plausible amplification bias, together with ultimate miniaturization and the minimal use of biochemicals, sequence could be determined directly from a single DNA molecule (i.e., realtime sequencing by synthesis) without the need for PCR amplification. Current platforms searching to sequence a single DNA molecule are referred to as "Third generation NGS platforms", below are descriptions of some of the new, already available technologies.

HeliScope™ Single Molecule Sequencer

By 2003, the first single-molecule sequencing platform, the HeliScope, was available from Helicos BioSciences (http://www.helicosbio.com) with a company reported sequence output of 1 Gb/day from a single instrument. By avoiding clonal amplification of the template DNA, the method involved simple sample preparation including fragmenting sample DNA and polyadenylation at the 3' end, with the final adenosine fluorescently labeled. Denatured polyadenylated strands are hybridized to poly(dT) oligonucleotides immobilized on a flow cell surface at a capture density of up to $1x10^8$ template strands per square centimeter. After the positional coordinates of the captured strands are recorded by a charge-coupled device camera, the label is cleaved and washed away before sequencing (Figure 9).

A sequencing cycle consists of DNA extension with one of four Cy5-labeled dNTPs added to the flow cell, followed by nucleotide detection and by imaging with the HeliScope sequencer. After label cleavage and washing, the process is repeated with the next Cy5-labeled dNTP. Each sequencing cycle consists of the successive addition of polymerase and each of the four labeled dNTPs, known as quad. The number of sequencing quads performed is approximately 25–30, with read lengths of up to 45–50 bases having been achieved. The sequencer is capable of sequencing up to 28 Gb in a single sequencing run and takes about 8 days. It can generate short reads with a maximal length of 55 bases.

PacBio RS sequencer

Founded in 2004, Pacific Biosciences describes their platform as single molecule real time sequencing by synthesis (SMRT) based on the properties of zero-mode waveguides (ZMWs) (Figure 10). In late 2010, the PacBio RS sequencer was released to a limited set of customers, and by early 2011, the platform was commercially released. To use the instrument requires reagent packs for DNA preparation and sequencing, as well as, small plastic cells called "SMRT Cells". Each cell is slightly less than one centimeter square and contains thousands of ZMWs (Figure 10).

The SMRT principle relies on single-molecule realtime sequencing and uses phospholinked fluorescently labeled dNTPs. Sequencing a DNA fragment is performed by a single DNA polymerase molecule attached to the bottom of each ZMW, so that each DNA polymerase resides at the detection

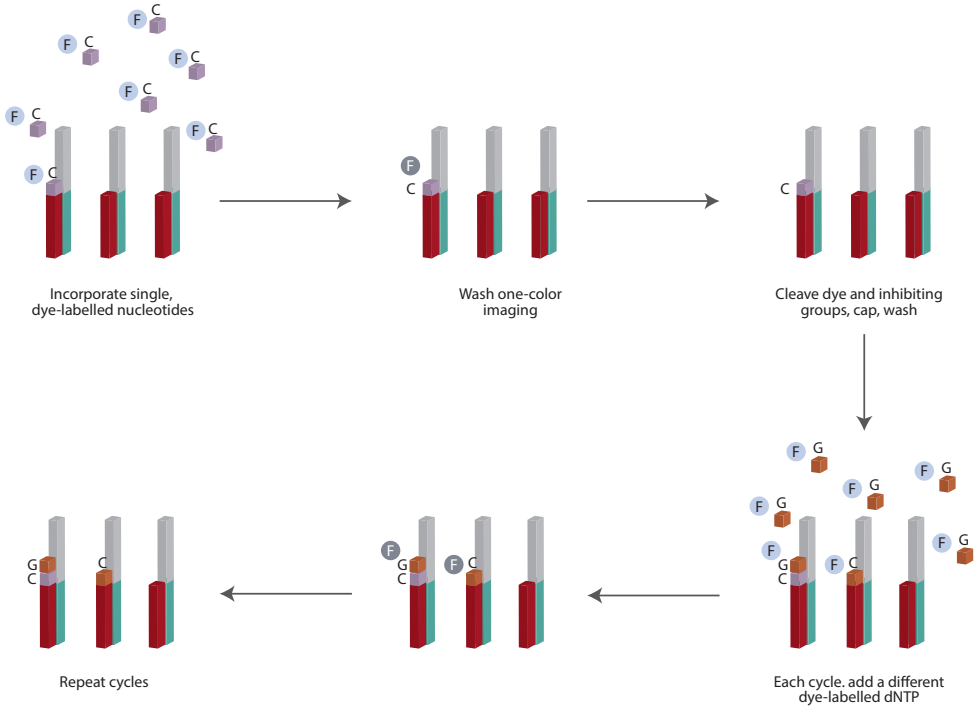

Figure 9. Helicos HeliScope Sequencer Workflow Fragments are captured by poly-T oligomers tethered to an array. At each sequencing cycle, polymerase and single fluorescently labeled nucleotide are added and the array is imaged. The fluorescent tag is then removed and the cycle is repeated. Adapted from (5).

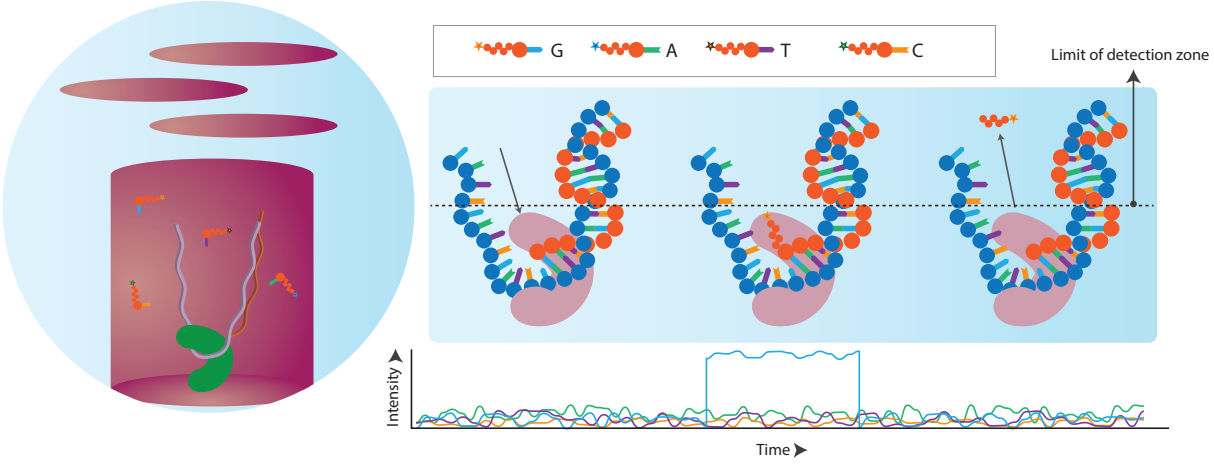

Figure 10. Pacific Bioscience four-colour real-time sequencing method. a) The zero-mode waveguide (ZMW) design reduces the observation volume, therefore reducing the number of stray fluorescently labeled molecules that enter the detection layer for a given period. These ZMW detectors address the dilemma that DNA polymerases perform optimally when fluorescently labeled nucleotides are present in the micromolar concentration range, whereas most single-molecule detection methods perform optimally when fluorescent species are in the pico- to nanomolar concentration range. b) The residence time of phospholinked nucleotides in the active site is governed by the rate of catalysis and is usually on the millisecond scale. This corresponds to a recorded fluorescence pulse, because only the bound, dye-labeled nucleotide occupies the ZMW detection zone on this timescale. The released, dye-labeled pentaphosphate by-product quickly diffuses away, dropping the fluorescence signal to background levels. Translocation of the template marks the interphase period before binding and incorporation of the next incoming phospholinked nucleotide. Adapted from (5).

zone of ZMW (Figure 10). Each well is a nanophotonic chamber in which only the bottom third is visualized, producing a detection volume of approximately 20 zL (zeptoliters, $2x10^{-20}$ L). During the sequencing reaction, the DNA fragments are immobilized to the well bottom and four differently labeled dNTPs are added to be elongated by the DNA polymerase while each base is held within the detection volume for tens of milliseconds, orders of magnitude longer than the amount of time it takes for a nucleotide to diffuse in and out of the detection volume. Laser excitation enables the incorporation events in individual wells to be captured through the optical waveguide, with the fluorescent color detected reflecting the identity of the dNTP incorporated.

For sequencing, Pacific Biosciences uses a modified phi29 (φ29) DNA polymerase that has enhanced kinetic properties for incorporating the system's phospholinked fluorescently labeled dNTPs. In addition, the phi29 is highly processive, with strand-displacement activity. By taking advantage of these properties, Pacific Biosciences has demonstrated sequencing reads exceeding 4000 bases when a circularized single-stranded DNA molecule is used as template. In this configuration, the phi29 polymerase carries out multiple laps of DNA strand displacement synthesis around the circular template. The observed errors, including deletions, insertions, and mismatches can be addressed by developing a consensus sequence read derived from the multiple rounds of template sequencing. A downstream secondary open source bioinformatic analysis tool is available from the "SMRT Portal" on the Pacific Biosciences web page.

Nanopore DNA sequencer

By 2008, Oxford Nanopore Technologies licensed the technology from Harvard University, University of California Santa Cruz (UCSC) and other universities with the objective to develop a solid state nanopore technology to sequence DNA and identify biomarkers, drugs of abuse and a range of other molecules. By 2012, they revealed their first working device. Likewise, other companies are pursuing a combination of nanopore technologies not only for DNA sequencing but for a complete plethora of applications and uses. The technique was developed from studies on translocation of DNA through various artificial nanopores and contrary to most current DNA sequencers, sequencing a DNA molecule with this platform is free of nucleotide labeling and detection.

Oxford Nanopore's proprietary nanopore-based sensing chemistries are operated on an electronics-based platform called the GridION™ system. This enables the scaled-up measurements of multiple nanopores and the sensing, processing and analysis of data in real time. A single instrument, a GridION™ node, operates with a single-use cartridge that contains the necessary reagents to perform an experiment. Recently, the company announced to have come up with a DNA sequencing machine, called the MinION™, the size of a USB memory stick which costs approximately $900 and can sequence simple genomes, but not full human genomes (Figure 11). Considerable funding is being directed toward a variety of nanopore technologies under development as part of the goal of achieving the $1000 genome. However, the technologies are under intense development and scrutiny given its promised applications.

DNA sequencing with a Nanopore instrument relies on converting of electrical signal of nucleotides by passing through a nanopore which is an α-hemolysin pore covalently attached with a cyclodextrin molecule (i.e., the binding site for nucleotides) embedded in reconstituted lipid bilayers. Nanopores are situated in individual array wells, and single DNA molecules are introduced into the wells and progressively digested by an exonuclease. The principle of this technique is based on the modulation of the ionic current through the pore as a DNA molecule traverses it, revealing characteristics and parameters (diameter, length and conformation) of the molecule (Figure 11). During the sequencing process the ionic current that passes through the nanopore is blocked by the nucleotide being cleaved by exonuclease from an interacting cyclodextrin DNA strand. The time period of current block is characteristic for each base and enables the DNA sequence to be determined.

The Ion Torrent sequencing technology

By 2010, the first postLightTM sequencing technology, also known as Ion Torrent, was introduced by its CSO, Jonathan Rothberg. Now part of Life Technologies the Ion Torrent platform creates a direct connection between chemical and digital information enabling fast, simple, massively scalable sequencing by developing the world's first semiconductor-based DNA sequencing technology. The new technology gathers sequence data by directly sensing hydrogen ions produced by template-directed DNA synthesis, offering a route to low cost and scalable sequencing on a massively parallel semiconductor-sensing device or "ion chip". The reactions are performed using all natural nucleotides, and the individual ion-sensitive chips are disposable and relatively inexpensive. The system was validated by sequencing three bacterial genomes and the human genome of Gordon Moore, author of Moore's law.

The massive parallel sequencing of the Ion Personal Genome Machine (PGM™) sequencer works on the basis of "base" principle. This means, if a nucleotide dATP is added to a DNA template and dA is incorporated into a strand of DNA then a hydrogen ion will be released. The charge from that ion will change the pH of the solution and can be detected directly by the ion sensor without scanning, cameras and light. The technological device uses a high-density array of micro-machined wells used to perform this biochemical process in a massively parallel way with each well holding a different, single molecule DNA template. Beneath the wells is an ion sensitive layer and beneath that a proprietary Ion sensor. The PGM™ sequencer sequentially floods the chip with one nucleotide after another. More recently using the same approach semiconductor sequencing technology, the

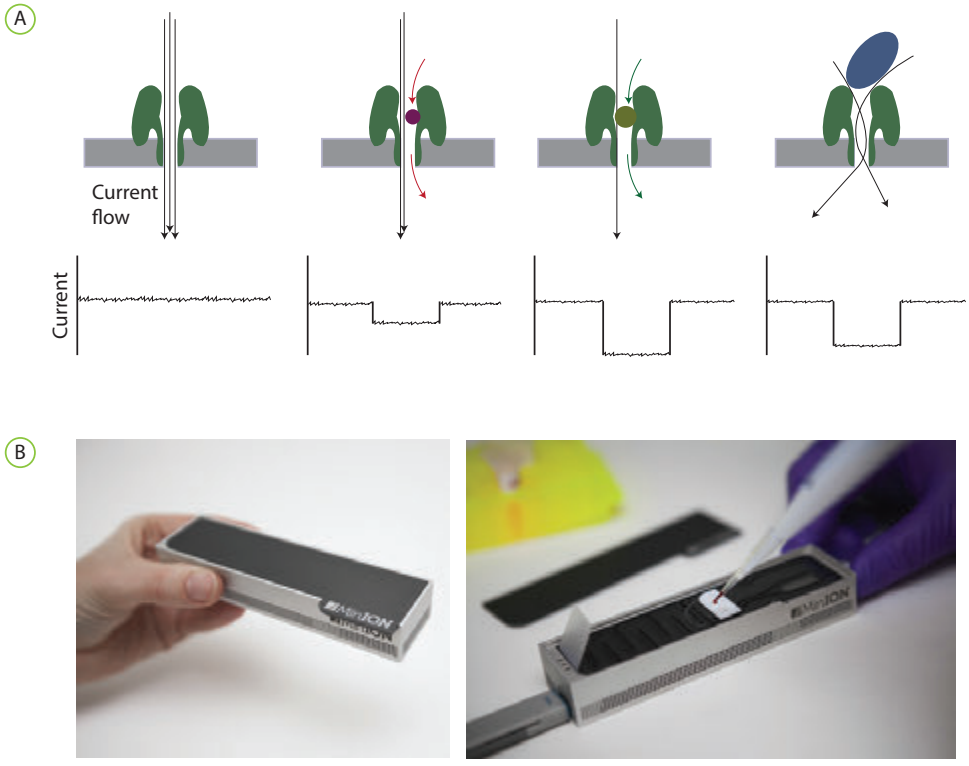

Figure 11. Oxford Nanopore Technology. A) Diagram shows a protein nanopore set in an electrically resistant membrane bilayer. An ionic current is passed through the nanopore by setting a voltage across this membrane. If an analyte passes through the pore or near its aperture, this event creates a characteristic disruption in current. By measuring that current, it is possible to identify the molecule in question. For example, this system can be used to distinguish between the four standard DNA bases G, A, T and C, and also modified bases. It can be used to identify target proteins, small molecules, or to gain rich molecular information, for example to distinguish the enantiomers of ibuprofen or molecular binding dynamics. B) The MinIONTM device is a miniaturized single molecule analysis system, designed for single use and to work through the USB port of a laptop or desktop computer. Images are of an alpha prototype of the MinIONTM device, final device expected to be at least half this size. http://www.nanoporetech.com/technology/the-minion-device-a-miniaturised-sensing-system.

Ion Proton™ sequencer was commercialized with chip densities up to a 1000-fold greater than the PGM™. The Ion Proton™ sequencer is directed towards whole-genome sequencing applications.

Multiplex polony technology

The multiplex polony sequencing is a "distant relative" of the classical polony technology which was mainly developed by Jay Shendure and Rob Mitra in Church's laboratory at Harvard University. They worked out ways to sequence *in situ* polonies using single-base extension which can achieved 5-6 bases reads, some of this principles were used as the foundation of the highly parallel sequencing by synthesis used in 454 Life Sciences method or sequencing-by-ligation method for SOLiD sequencers as well as others. However, several years later, Shendure and Porreca changed almost everything of the original approach in order to make this multiplex sequencing technology work.

In this technique, several hundred sequencing templates are deposited onto thin agarose layers and sequences are determined in parallel. This method presents increase of several orders of magnitude the original number of samples which can be analyzed simultaneously. It has the advantage, in terms of large reduction of the reaction volumes, requiring smaller amounts of reagents and resulting at a lower cost. The designed instrument, known as Polonator G.007, is capable to generate 10 to 35 Gbp per module on a 2.5 day run. The instrument can couple with 200 of these modules to collect 100 diploid genomes at 30X coverage in 5 days, with the remaining 5 days used for repeating any weak runs to assure 98% coverage at $1x10^{-5}$ accuracy. With the significant reduced volume of reagents, the cost per unit volume is lower (i.e., about 10-fold) and the company hopes to meet the goal of $1000 per genome soon.

NGS DATA ANALYSIS

NGS experiments generate unprecedented volumes of data, presenting challenges and opportunities for data management, storage, and specially analysis. NGS data often start as large sets of tiled fluorescence or luminescence

images of the flow cell surface recorded after each iterative sequencing step. This volume of data requires an intensive data-pipeline system for data storage, management, and processing. Data volumes generated during single runs of the 454 GS FLX, Illumina, and SOLiD instruments are approximately 15 GB, 1 TB, and 15 TB, respectively.

The main processing feature of the data pipeline is the computationally intensive conversion of image data into sequence reads, known as base calling. First, individual beads or clusters are identified and localized in an image series. Image parameters such as intensity, background, and noise are then used in a platform-dependent algorithm to generate read sequences and error probabilities. Although many researchers use the base calls generated by the platform-specific data-pipeline software, alternative base-calling programs that use more advanced software and statistical techniques have been developed. The quality values calculated during NGS base calling provide important information for alignment, assembly, and variant analysis. Although the calculation of quality varies between platforms, the calculations are all related to the historically relevant phred score, introduced in 1998 for Sanger sequence data. The phred score quality value (q) uses a mathematical scale to convert the estimated probability of an incorrect call (e) to a log scale ($q=-10.\log_{10}[e]$). Miscall probabilities of 0.1 (10%), 0.01 (1%), and 0.001 (0.1%) yield phred scores of 10, 20, and 30, respectively.

NGS error rates estimated by quality values depend on several factors, including signal-to-noise levels, cross talk from nearby beads or clusters, and dephasing. Substantial effort has been made to understand and improve the accuracy of quality scores and the underlying error sources, including inaccuracies in homopolymer run lengths on the 454 platform and base-substitution error biases with the Illumina format. Study of these error traits has led to examples of software that require no additional base calling and those improved quality-score accuracies improve sequencing accuracy. Quality values are important tools for rejecting low-quality reads, trimming low-quality bases, improving alignment accuracy, and determining consensus-sequence and variant calls.

Alignment and assembly are substantially more difficult for NGS data than for Sanger data, mainly because of the shorter reads lengths in NGS. One limitation of short-read alignment and assembly is the inability to uniquely align large portions of a read set when the read length becomes too short. Similarly, the number of uniquely aligned reads is reduced when aligning to larger, more complex genomes or reference sequences because of their higher probability of repetitive sequences. Unique alignment or assembly is reduced not only by the presence of repeat sequences but also by shared homologies within closely related gene families and pseudogenes. Non-unique read alignment is handled in software by read distribution between multiple alignment positions or leaving alignment gaps. De *novo* assembly will reject these reads, leading to shorter and more numerous assembled contigs. These factors are relevant when choosing an appropriate sequencing platform with its associated read length, particularly for *de novo* assembly.

Error rates for individual NGS reads are higher than for Sanger sequencing. The higher accuracy of Sanger sequencing reflects not only the maturity of the chemistry but also the fact that a Sanger trace peak represents highly redundant, multiple terminated extension reactions. Accuracy in NGS is achieved by sequencing a given region multiple times with each sequence contributing to coverage depth. To assemble, align, and analyze NGS data requires an adequate number of overlapping reads which reflect the depth of coverage per nucleotide. In practice, coverage across a sequenced region is variable, and factors other than the Poisson-like randomness of library preparation that may contribute to this variability include differential ligation of adapters to template sequences and differential amplification during clonal template generation. Studies have shown that coverage of less than 20- (20x) to 30-fold (30x) begin to reduce the accuracy of single-nucleotide polymorphism calls in data on the 454 platform. For the Illumina system, higher coverage depths (50x to 60x) have been used in an effort to improve short-read alignment, assembly, and accuracy, although coverage in the 20-30x range may be sufficient for certain resequencing applications (Figure 12). Coverage gaps can occur when sequences are not aligned because of substantial variance from a reference. Alignment of repetitive sequences in repeat regions of a target sequence can also affect the apparent coverage. Reads that align equally well at multiple sites can be randomly distributed to the sites or in some cases discarded, depending on the alignment software. In de novo–assembly software, reads with ambiguous alignments are typically discarded, yielding multiple aligned read groups, or contigs, with no information regarding relative order.

A large variety of software programs for alignment and assembly have been developed and made available to the research community. Most use the Linux operating system, and a few are available for Windows. Many require a 64-bit operating system and can use >16MB of RAM and multiple central-processing unit cores. The range of data volumes, hardware, software packages, and settings leads to processing times from a few minutes to multiple hours, emphasizing the need for sufficient computational power.

Although a growing set of variations in alignment and assembly algorithms are available, there remains the trade-off between speed and accuracy in which many but not all possible alignments are evaluated, with a balance having to be struck between ideal alignment and computational efficiency. NGS software features vary with the application and in general may include alignment, de novo assembly, alignment viewing, and variant-discovery programs. Currently a wide range of applications for NGS are being implemented and still each new application brings together new challenges in ways to analyze or get the most out of the samples and obtained sequence. Table 3 contains some of the currently used and under development approaches, for more and in deep information of each the reader is directed to the recommended reading documents.

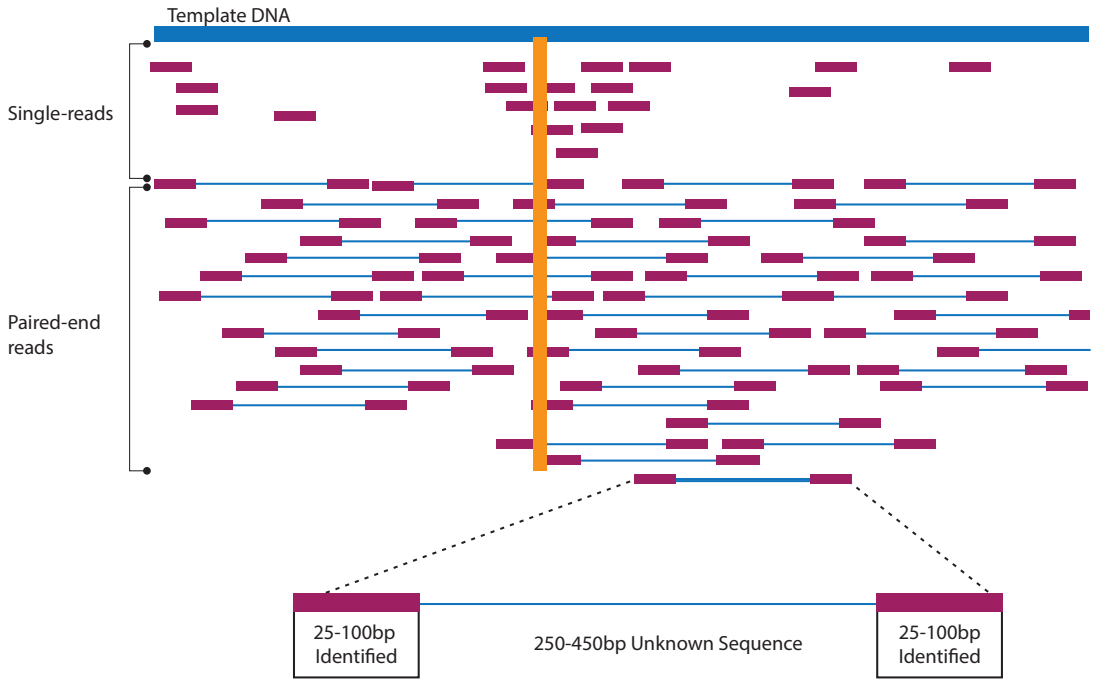

Figure 12. Sequence read types. Short reads are mapped to the template molecule to as A) Single-reads or B) Paired-end reads. Each provides information to reveal sequence polymorphisms in the template, e.g., a SNP (red and yellow). Abundance of reads is used as a quantitative measure of the abundance of the template, and the particular areas of the template covered by reads reveal the internal structure of the template, e.g., the presence of exons and introns.

APPROACH	TYPE OF OBTAINED SEQUENCE INFORMATION
DNA-Seq	Genome sequence
Targeted DNA-Seq	Subset of a genome (for example, an exome)
Methyl-Seq	Sites of DNA methylation, genome-wide
Targeted methyl-Seq	DNA methylation in a subset of the genome
DNase-Seq, Sono-Seq, FAIRE-Seq	Active regulatory chromatin
MAINE-Seq	Histone-bound DNA (nucleosome positioning)
ChIP-Seq	Protein-DNA interactions (using chromatin immunoprecipitation)
RIP-Seq, CLIP-Seq, HITS-CLIP	Protein-RNA interactions
RNA-Seq	RNA (transcriptome)
FRT-Seq	Amplification-free, strand-specific transcriptome sequencing
NET-Seq	Nascent transcription
Hi-C	Three-dimensional genome structure
Chia-PET	Long-range interactions mediated by a protein
Ribo-Seq	ribosome-protected mrNA fragments (that is, active translation)
TRAP	Genetically targeted purification of polysomal mRNAs
PARS	Parallel analysis of RNA structure
Synthetic saturation mutagenesis	Functional consequences of genetic variation
Immuno-Seq	The B-cell and T-cell repertoires
Deep protein mutagenesis	Protein binding activity of synthetic peptide libraries or variants
PhIT-Seq	relative fitness of cells containing disruptive insertions in diverse genes

Table 3. Some of the current applications of NGS. Adapted from Shendure et al 2012. *FAIRE-seq*, formaldehyde-assisted isolation of regulatory elements–sequencing. *MAINE-Seq*, MNase-assisted isolation of nucleosomes-sequencing; *RIP-Seq*, RNA-binding protein immunoprecipitation-sequencing; *CLIP-Seq*, cross-linking immunoprecipitation-sequencing; *HITS-CLIP*, high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation; *FRT-Seq*, on-flowcell reverse transcription–sequencing. *NET-Seq*, native elongating transcript sequencing. *TRAP*, translating ribosome affinity purification. *PhIT-Seq*, phenotypic interrogation via tag sequencing.

Recommended readings

1. Hou R, Yang Z, Li M, Xiao H. Impact of the next-generation sequencing data depth on various biological result inferences. Sci China Life Sci. 2013;56:104-9.
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48

ANALYSIS OF PROTEINS AND ANTIBODIES

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INTRODUCTION

This chapter describes different techniques that profit the specificity of the interaction between antibodies (Ab) to a specific antigen (Ag). Some of the techniques may allow the qualification or quantification of proteins. In addition, base on the properties of the antigen – antibody interaction, it is easy to detect either antigen or antibody in a wide variety of biological samples. These techniques are widely used for both diagnostic and research purposes. Finally, thanks to enzymatic labeling and an antigen-antibody reaction, it is now possible to detect and quantify an analyte of interest; in such methods, generation of a colored or light reaction allows the final quantification by visual or spectrophotometric techniques.

GENERAL ANTIBODY CHARACTERISTICS

POLYCLONAL ANTIBODIES

Polyclonal antibodies are a pool of different antibodies targeting many epitopes within the same antigen. It is said that this kind of antibodies are different because they are produced by diverse B cell clones. Therefore, they have dissimilar affinities and specificities for the antigen. These antibodies are prepared from immunized animals, preferentially in rabbits. However, they can be also produced in goats, cows and pigs. To produce polyclonal antibodies, immunization with the target antigen is made in the animal during 3 to 8 months using multiple injections through this period. Sometimes, an adjuvant (e.g., Freund's complete or incomplete) or immunogenic peptides (e.g., bovine serum albumin-BSA, ovalbumin-OVA) are also added to the antigen for enhancing the immune response. Finally, the polyclonal immunoglobulins are purified from animal serum.

MONOCLONAL ANTIBODIES

In contrast to polyclonal antibodies, monoclonal antibodies are immunoglobulins targeting a single epitope and produced only by a single B-cell clone. Monoclonal antibodies are produced typically by fusing myeloma cells with spleen cells isolated from a mouse or rabbit that have been immunized with the desired antigen. Monoclonal antibodies produced in rabbits, unlike those produced in mice, have more affinity and can recognize multiples epitopes including those with small size. Animals are immunized with the desired antigen for 2 to 4 months and the response to the antigen is tested by measuring antibody titers in their sera. Once the right levels of immunoglobulins are detected, B-cells are isolated from spleen and co-cultured with immortal cell lines, forming fused cells called hybridomas. The hybridomas can be grown indefinitely in especial culture media for production of large quantities of a specific immunoglobulin type. These monoclonal antibodies are finally purified and used in different biological applications.

As mentioned above, polyclonal and monoclonal antibodies have different characteristics, thus it is extremely important to choose the right antibody for the right application (e.g., immunostaining, ELISA, diagnosis, research, etc). (Table 1)

ANTIBODY AFFINITY

Antibody affinity is the strength to which an antibody molecule binds an epitope (antigenic determinant). This affinity can be intrinsic or functional. Intrinsic affinity applies to monovalent interaction (i.e., strength of binding between one antibody combining site and one epitope or antigenic determinant). Functional affinity applies to multivalent interactions (i.e., overall strength of binding between multivalent antigens and antibodies).

MONOCLONAL ANTIBODIES		POLYCLONAL ANTIBODIES	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Consistency	Recognition of single epitope	Better for tissue specimens	Increase cross-reactivity
Less variability		Recognition of multiple epitopes	Production depends of animal's life
Produced constantly by hybridomas		Robustness	

Table 1. **Monoclonal vs. polyclonal antibodies.**

For applications such as immunostaining, it is important to take into account several factors such as antibody affinity and electrostatic charge. For example, it is not recommended to use very long times for formalin-fixation because the electrostatic charge within the tissue can be altered, thus lowering the antibody's affinity.

Another important factor related to antibody affinity is its hydrophobicity because once the immune complex is formed; it will precipitate in the presence of soluble reagents. However, this factor is not of great importance during immunohistochemistry because this approach is based on a immobilized antigen capturing the antibody, instead of its precipitation.

It is important to mention that immune complexes formed by antigens and antibodies can dissociate during washing steps. Therefore, it is important to use buffers with low salt concentrations, high pH and work at low temperatures because they will diminish dissociation of the immune complex. Nevertheless, this problem is overcome using gentle agitation and using high affinity monoclonal or polyclonal antibodies.

Intrinsic affinity is a result of the hypervariable region, which is responsible for the antibody specificity. This specificity is generated by hydrogen bonds, Van der Waals forces and electrostatic interactions. Taking in mind all these factors mentioned above, it is possible to measure an antibody's affinity using the association constant (Ka), which ranges between 10^3 and 10^{10} lt/mole. Therefore, when the Ka is high, we need less antigen concentration to saturate antibody binding sites.

On the other hand, the functional affinity is defined as the time needed to achieve the higher staining intensity. Therefore, if the antibody reaches its intensity in a minimum time compared with others antibodies; it will have more functional affinity than the others.

ELISA

The enzyme linked immunosorbent assay (ELISA) is a sensible, versatile, precise, reproducible, quantitative and qualitative technique for the determination of antigens or antibodies in a biological sample. This immunoassay technique is widely used in diagnostic and biological research. Ever since its description over 30 years ago, it has been modified and adapted for multiple applications. This technique has been sucessfully used in research and diagnostic of infectious diseases, epidemiology, endocrinology and immunology among others. The development of specific antibodies and increasingly adequate reagents have revolutionized the applications and easiness of this technique, in particular, most of the procedures have been streamlined making them shorter, with better reproducibility and sensitivity, allowing a better assessment of antigen-antibody reactions.

ELISA was described by Engall and Perlmann and developed by Weemen and Schuurs in the 70s. The assay consists of a solid phase immunoassay. This technique replaced the Radio Immuno Assay (RIA), which was used in many laboratories before the development of the ELISA. Moreover, it displaced immunofluorescence and agglutination techniques, because of the automation possibilities and the ability to analyze a large number of samples at the same time. Furthermore, the ELISA utilizes the properties of enzymes to expand and develop an antigen-antibody reaction generated in concentrations ranging in pico and/or attomoles.

PRINCIPLE OF THE METHOD

The basic principle of the ELISA technique is the use of an Ag or Ab conjugated to an enzyme which is capable of reacting with its substrate, producing a colored reaction when an immunological interaction between Ag and Ab has ocurred. The color change is monitored visually (qualitatively) or by using spectrophotometric (quantitative) measures in order to determine the relative amount of analyte present in the sample. A key essential step in this type of assays is the separation of the bound labeled enzyme during the reaction and the free or non-specific labeling generated during the test. Similarly, in the case of antibody determination even discrimination and quantification of isotypes depending on the specificity of the used antigen can be achieved.

TYPES OF ELISA

There are many different ways to design an ELISA. The choice depends on the nature of the sample, availability of reagents, as well as the sensitivity, accuracy and precision required for the analysis. In many occasions, it is not necessary to have an accurate measurement of the concentration of a protein in the sample. Under certain circumstances it is sufficient to have a qualitative identification (i.e., presence or absence) of the analyte. Likewise, there are other cases

in which a ranking as high, medium or low (i.e., semicuantitative) may provide enough information resulting in the outcome of a test. In most cases it is necessary to determine exact amounts of the analyte and this amount is obtained with more quantitative stringent assays.

Non-competitive ELISA

This type of ELISA is the most commonly used variety. It consists of a solid phase immunoassay for quantification of the amount of Ag or Ab present in a sample. This type has two basic variants depending of what is bound to the solid phase either antigen-binding or antibody-binding. The non-competitive ELISA in some cases can have a low specificity but high sensitivity.

Coating of the antigen into the solid phase

These are the simplest types of non-competitive assays, also known as indirect ELISA. In this test, the Ag is fixed to the solid phase, which could be either high capacity or low capacity binding, as it is going to be discussed below. Then, the sample (containing Ab) is added to generate Ag-Ab binding. This Ag-Ab interaction will be detected by adding a second specific Ab, enzyme-labeled to identify the class of immunoglobulin that is bound to the Ag (Figure 1). This second Ab is referred to as the detection Ab and is usually bound to biotin, which has high affinity for avidin, then allowing detection of the complex avidin-biotin Ab. This type of test is widely used in microbiological diagnosis and autoantibody testing.

Antibody binding to the solid phase

Known as capture assay, also refers to a sandwich ELISA, which uses two antibodies, one that binds to the solid phase and another antibody that is labeled. The first antibody has the function of "capturing" the specific antigen in the sample. The second antibody also binds specifically to the antigen and is labeled with an enzyme for developing a reaction either symmetrically or asymmetrically (Figure 2a and 2b).

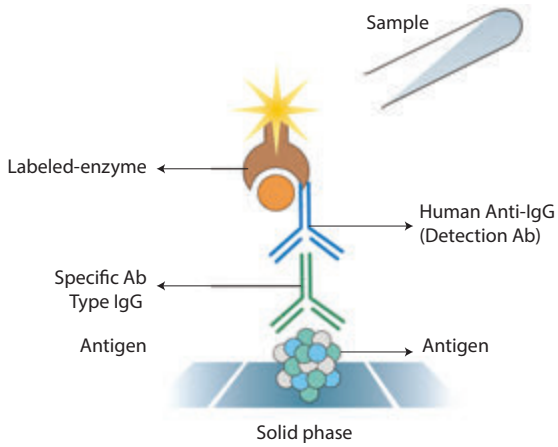


Figure 1. Fixing of an antigen to the solid phase in ELISA. ELISA is based on the detection of an antigen or antibody bound to a solid phase which binds to an antibody (or antigen as appropriate) enzymatically-labeled, which emits color.

Symmetrical assays are those in which the capture antibody binds specifically to the Ag. Then, the complex Ag-Ab is detected by a second addition of the same capture Ab but now enzymatically-labeled. Symmetrical assays use polyclonal antibodies because there are few recognigtion sites for monoclonal Ab available for capturing and at the same time they have to be labeled with biotin and/or another enzyme (Figure 2a) to increase the signal.

Asymmetrical assays are those where the capture and detection Ab are different. These are generated by affinity purified Ab and used in combinations of monoclonal antibodies for capture and polyclonal antibodies as detectors. This type of assay achieves high specificity during capture and maximum detection sensitivity due to the large number of available Ab labeled-enzyme. Since the detector is polyclonal, it offers the advantage of having multiple epitopes

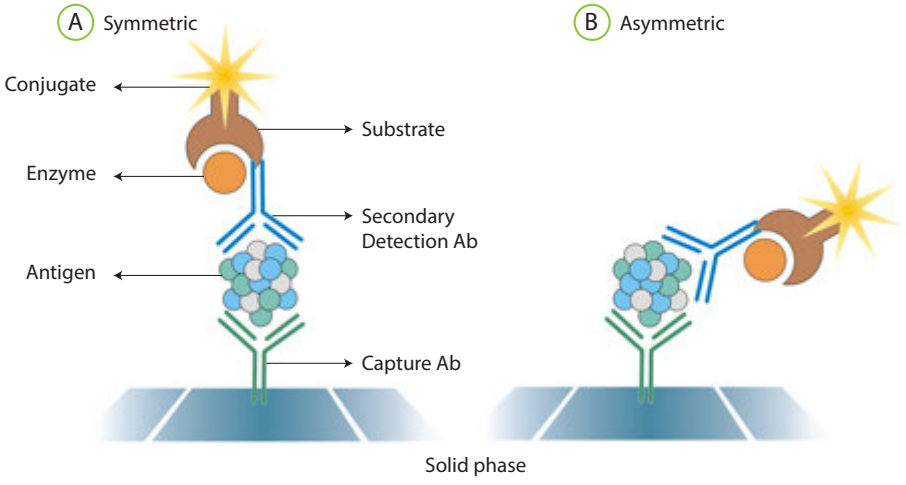


Figure 2. Fixing of an antibody to a solid phase. **A**. Scheme of symmetrical ELISA reaction. **B**. Schematic of an asymmetric reaction.

that can interact with the Ag and provides the ability of a second enzyme labelling (Figure 2b).

Competitive ELISA

The competitive ELISA, unlike the non-competitive, allows an estimate of the amount of Ag or Ab particularly at times when this can not be purified from the medium in which it is located. This type of ELISA provides results of very high specificity but low sensitivity. This ELISA can be used for identification of different antigenic determinants of the same molecule, always taking into account the concentrations of Ag, Ab and its respective competitor, added to the reaction.

Competitive ELISA for Ag Detection

In this method, the Ag can be measured in two ways. i) The Ag bound to the solid phase is the same Ag that is mixed with the sample and the enzyme-labeled specific Ab (Figure 3a). ii) The Ab is fixed to the solid phase and a labeled-Ag is added together with the same Ag enzymatically-labelled present in the sample, which can react with the solid phase-bound Ab (Figure 3b).

Competitive ELISA for Ab Detection

This is the most widely used type of assay. It is used to compare the specificity of antibodies in antigenic structure analysis with monoclonal antibodies. It may be performed in two ways: i) By fixing an Ag to the solid phase and the Ab present in the sample competes with enzyme-labeled Ab for binding to the Ag (Figure 4a). ii) Moreover, an Ab can be fixed to the solid phase, whereas the Ab present in the sample competes with the Ab bound to the solid phase enzyme-labeled Ag in the reaction (Figure 4b).

PROCEDURE, REAGENTS AND MATERIALS

The ELISA technique is performed using either commercial kits or previously tested standardized techniques developed in each laboratory, letting adjust the method to the needs of the analyst. The latter are most widely used in the research areas while the former have more application in clinical diagnosis. For both methods, the requirements are similar and described below.

Sample conditions

Many of these assays are used for diagnosis, for which the most suitable sample is serum, preferably cooled and without presence of hemoglobin, since it can generate background noise during the assays. Similarly, excessive lipids in the sam-

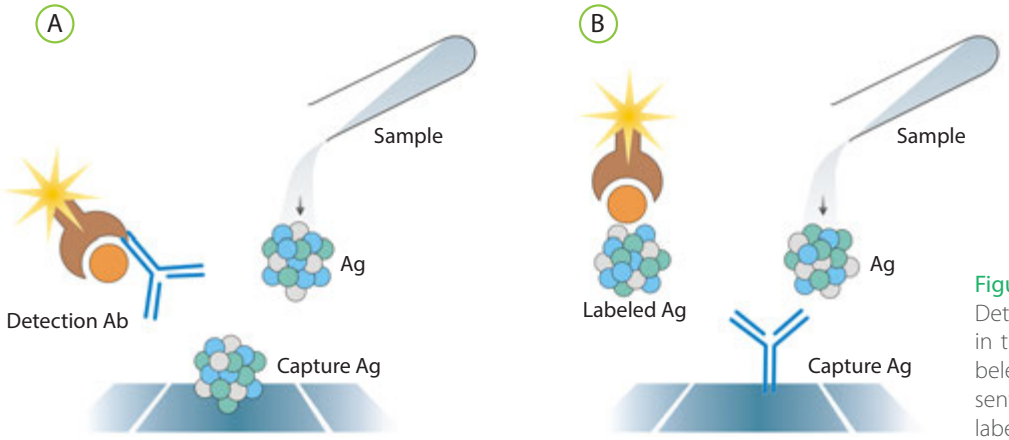


Figure 3. Competitive ELISA for Ag Detection. **A**. The antigen present in the sample competes for the labeled antibody. **B**. The antibody present in the sample competes with a labeled antigen for other antigen.

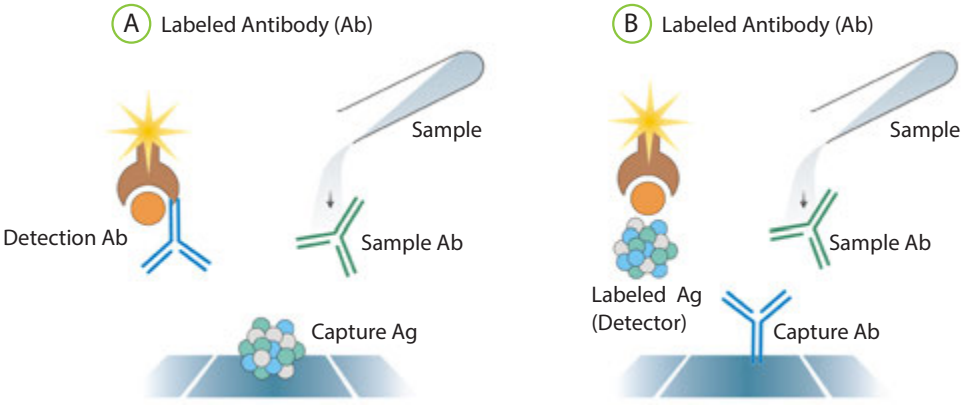


Figure 4. Competitive ELISA for Ab Detection. **A**. Detecting an antibody in a sample by competition with a labeled antibody. **B**. Detection of antibody present in the sample by competition with a labeled antigen.

ple can cause problems. The serum used should be handled under sterile conditions to prevent microbial contamination that could interfere with the result. Other types of samples that can be used are body fluids (i.e., tears, saliva, cerebrospinal fluid, urine) or cell culture supernatants, always taking into account the handling and the conditions required for each sample for the preservation of protein Ag and/or Ab.

Solid phase

The solid phase material is critical to the type of test and the goal of the method, so the solid phase has been divided into two main categories based on the ability to bind proteins, high or low capacity.

High-capacity solid phase

This category encompases agarose particles, cellulose and nitrocellulose papers. This phase can be activated with cyanogen bromide, which allows fixing of a wide range and amount of proteins to the surface. This method is easy to perform and can be inhibited by the addition of salts, such as TRIS. This solid phase with the bound Ab or Ag can be stable for years, and small amounts of protein are sufficient to obtain a good reaction.

Low-capacity solid phase

The polyethylene materials belong to this type of phases, which are available in microplate format, the most used. The major advantage of using this phase is its ability to bind a sufficient and homogeneous amount of protein, reducing the possibility of background noise during measuring of the absorbance. This material is easy to handle and there is a great variety of plates, depending on the needs of the analyst.

Sensitization

After selecting the type of solid phase, the next consideration is to sensitize the chosen solid phase. This consists in fixing the protein (Ag or Ab) to the selected surface. This step can be done directly or indirectly.

Direct sensitization: This is a way to prepare the solid phase passively, by direct adsorption of the protein to the surface. Proteins differ in their capability to adsorb, whereby it becomes necessary to adjust the method according to the needs and characteristics of each test. The efficiency of sensitization depends on two critical factors: i) The integrity of the protein used since it must not be damaged or degraded and ii) The binding strength that is present in the solid phase material. Sensitized proteins can be oriented in a specific direction, for example joining the hydrophobic residues of the protein to the plastic microplates will decrease the amount of Ag determinants that may be exposed.

Indirect Sensitization: This method uses protein intermediates for attaching to a selected surface; this confers an advantage in reproducibility, sensitivity and specificity for the technique. This sensitization can be accomplished through an Ag or an Ab.

Ag Indirect Sensitization: This is one of the best ways to bind Ag to the surface of an ELISA plate by an Ab (Figure 5). This technique has the advantage of increasing the specificity of the assay because the non-specific Ag unbound Ab may not adhere to the desired surface and the stability of the bound Ag to the surface will be much better . Furthermore, the antigenic determinants are less affected and this step and will increase the sensitivity of the assay. This type of sensitization can use both monoclonal and polyclonal antibodies.

Ab Direct Sensitization: This method is the least used, since not many antibodies have the ability to bind to the solid phase and to retain Ag binding sites to capture the Ag of interest, thus the Ab activity is lost in great proportion. However, this technique is recommended in cases where the Ab has difficulties to bind to the solid phase, such as human IgG1 monoclonal.

Types of Ag for Sensitization: This requires special considerations, such as the physicochemical nature of the molecule and its biological role. It is always preferable to use pure Ags, but in many cases the crude extracts are the only source of the material of interest. The sources of Ag can be very diverse, such as cell surface Ags for which it is necessary to fix the entire cell to the solid phase. Another option is the mixture of Ags, when unable to reach purity of the specific molecule, for this type of Ags the use of agarose solid phase or nitrocellulose is recommended. One more way is to perform Ag sensitization with the use of peptides, or protein isolates synthetically manufactured.

Types of Ab for Sensitization: The Ab sensitization can be accomplished in two different ways: i) using polyclonal antibodies that can be obtained by absorption; by antisera with high titres of antibodies; by affinity purified immunoglobulins, or by sensitization with fragments of the F (ab) fraction of antibodies. ii) by monoclonal antibodies which are

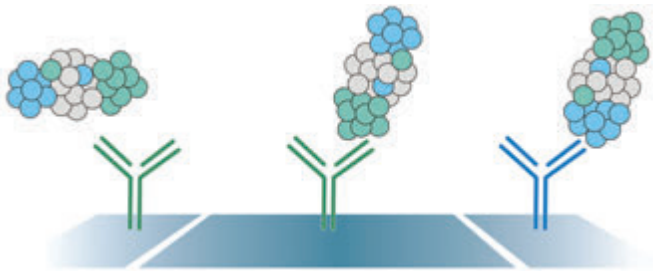


Figure 5. Indirect antigen sensitization. The binding to the antigenic epitope determines the orientation of the capture antigen.

more specific, but more difficult to obtain given that they require the development of a specific immune response, then methods are required for ascites collection after inoculating an animal with the specific Ag for generating a clone of antibody-producing B lymphocytes, or cell culture supernatants. Also, the concentrations obtained by these methods are very low.

Enzyme labeling and detection

The most used enzymes for ELISA are the horseradish peroxidase (HRP), alkaline phosphatase (AP) and β-D-galactosidase (β-GAL). All these enzymes are very efficient, however, it is important to determine the optimal conditions of pH and temperature.

Detection

In the immunoassays one detector is basically a molecule that has specificity for the target analyte in the sample to be detected. The detector may be directly labeled with enzyme, what is known as direct ELISA, or can be labeled indirectly (i.e., indirect ELISA). The detector, in the case of ELISA, may be an Ag or an Ab, but in all cases must specifically bind to the molecule of interest.

Ab as a detector

The detector Ab may be labeled directly, especially if it is a monoclonal Ab, there may be a second enzyme-labeled Ab. Antibodies can also be used as linkers between the detection Ab and the labeled Ab, combining binding sites of the detector Ab to increase sensitivity (Figure 6 a, b and c).

Ag as detector

This mode of detection is not commonly used and in the case of direct ELISA is almost impossible to be functional; however, there are cases where it is necessary. Thus, it is recommended to create a detector complex (indirectly) in which the Ag is specifically linked to an Ab-containing the labeled enzyme or by the use of small chemical groups or biotinylated haptens, although they could be less efficient for Ag-binding (Figure 7a and b).

Substrate

The choice of substrates will depend on the enzyme used, the color ratio required in the sample, the stability of the compound, the toxicity and cost. For HRP, there are a variety of substrates available, this enzyme reduces hydrogen peroxide (H_2O_2) and oxidizes a second substrate. Thus when this enzyme is used, it is always necessary to add H_2O_2. The most used substrates are OPD (o-phenyleneamine) which absorbs at 475 nm; ABTS (5-aminosalicylic acid, 2,2-azino-di (3-ethyl-benstiasolin sulfonate-6) which absorbs at a length of 414 nm and TMB (3,3 '5, 5'-tetramethylbenzidine). Meanwhile for the AP, the most used substrate is NPP (p-nitrophenyl phosphate) which absorbs at 405 nm.

Conjugate Method

This method is widely used and is aimed mostly to join the best enzyme to the antibody or antigen used in the ELISA, promoting the stability of the reaction. The conjugate will be composed by the enzyme, Ag or Ab and a third compound which may be a chemical, protein, or by high affinity reactions like avidin/biotin (Figure 8).

QUALITY CONTROL

There are several critical factors in the quality and reliability of the test, which could be classified as pre-analytical, analytical and post-analytical, as shown in Table 2. To control for these errors is necessary to include positive and negative controls within the assay. A negative control reaction corresponds to a phosphate buffer, which does not include the sample to be analyzed, this will ensure that there is not

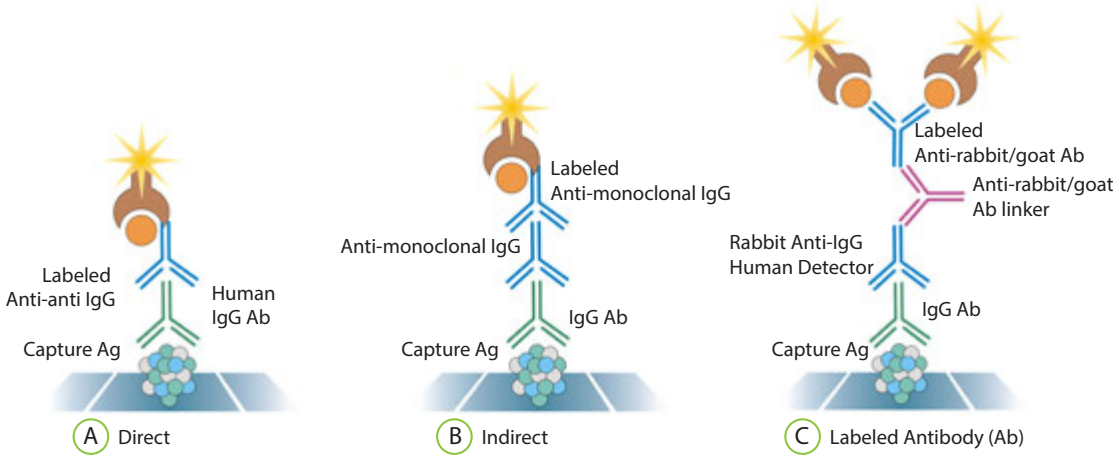


Figure 6. Detection of antibodies. **A.** Direct **B.** Indirect **C.** Complex, wherein antibodies are used as a bridge for increasing the sensitivity and specificity of the test.

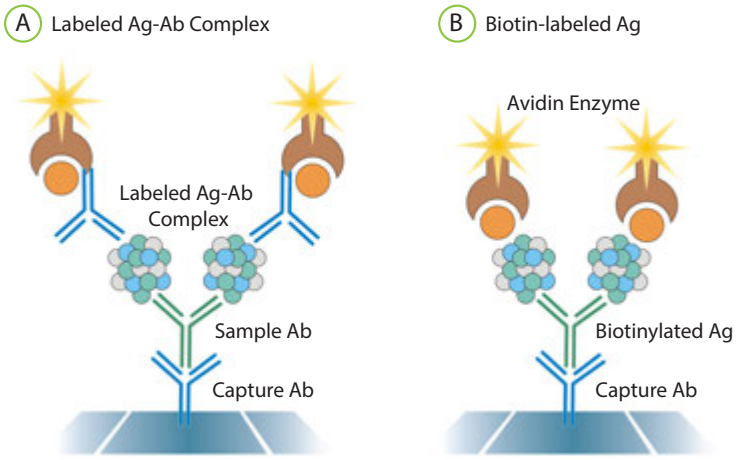


Figure 7. Indirect enzymatic reaction for detection of antigens. **A**. Detection through an labeled Ag-Ab complex. **B**. Detection through a labeled antigen.

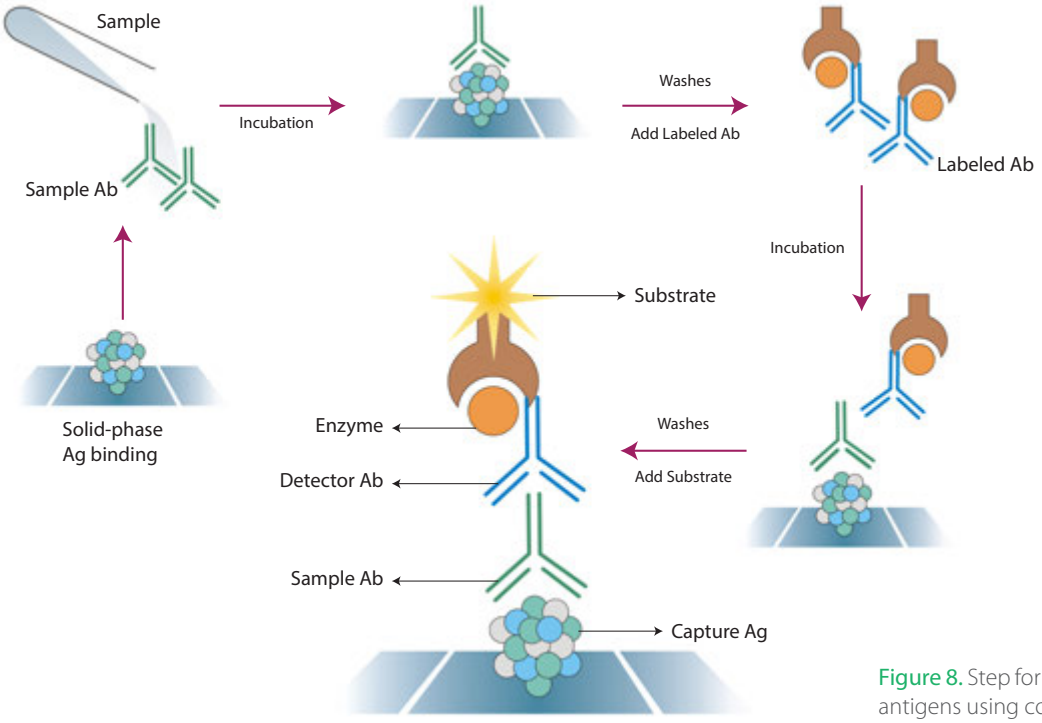


Figure 8. Step for the detection of antibodies or antigens using conjugated method.

an Ag-Ab reaction generated and thus it is used to determine background noise present in the reactions. A strong positive control may correspond to known Ag or Ab concentration. In addition, each of the samples must be analyzed in duplicate or triplicate, if possible. When the ELISA is to be standardized in the laboratory, it is necessary to identify all possible variables and test each one, until they are are reproducible. Similarly, there must be a standard curve to determine the normalization and correlation between the absorbance of the color reaction and the concentration of the sample, as is discussed in the analysis of results below.

RESULTS ANALYSIS

The results obtained in an ELISA may be qualitative, semi-quantitative or quantitative, as mentioned above, depending on the characteristics of the method. Each of the results can be expressed qualitative (Presence/Abscense), semiquantitative (Units) and quantitative (e.g. mg/dL). The crude results are given in optical density (OD). For quantitative results, ELISA data can be interpreted in comparison to a standard curve (i.e., serial dilutions of a known Ag) in order to calculate precisely the concentrations of Ag in samples. These parameters include the cutoff, the reference curve, the lower limit and titers (concentration) of the reaction. Troubleshooting for this technique is listed on Table 3.

PHASE	FACTOR	CAUSE
Pre-analytic	Patient preparation	Intake
		Alcohol
		Tobacco
		Exercise
	Sample collection	Hemolisis
		Hyperlipidemia
		Dilution
Analytic	Sensitization	Lower amount of protein
		Excess of bound protein
		Protein detachment
	Antigen-antibody reaction	Reaction time
		Non-optimal temperature
	Color development	Labelling alteration
		Inspecific developer
		Developer time
		Lack of binding between Ag and Ab
Post-analytic	Unexpected results	Flaws in the analysis
		Reaction saturation
		Linearity loss
		Standard curve alteration
		Errors in the calculations

Table 2. Critical factors in quality control for ELISA.

Cutoff point

The cutoff point is the base line to define which samples are positive for diagnostic ELISA. It is calculated from OD values in negative samples. Cutoff value is equal to the mean optical density (OD) plus 2 or 3 Standard Deviations. Sometimes it is calculated considering a 10% error and the background noise given by the negative control results (No sample).

Standard curve

This is the only parameter that allows calculating quantitative data from an ELISA. It assumes there is a linear relationship between the amount of color generated and the concentration of analyte present in the sample. Samples with known concentration of Ag ("standards") are used for creation of the standard curve and then this data is used for measuring the concentration of unknown samples by interpolation on the graph. The linearity of the curve depends on the concentration of the captured protein, sample dilution and development of the technique on each of its steps. The standard curves are expressed in logarithmic or semilogarithmic coordinates with the concentration on the X-axis against the OD on the Y-axis. The final estimates of the linearity and the correlation between the values of the X-axis with respect to the Y-axis must be calculated by linear regression, using the following equation: Y = Intercept + Slope (X) or Log Y = Intercept + Slope (Log X). An adequate correlation is expressed by a correlation coefficient (r^2) close to 1 (Figure 9).

Lower limit

The lower limit is the OD value corresponding to the background noise detected by either a reaction in which all reagents are present, except a blank sample or by a reaction with a sample to be considered as negative control. A good lower limit is whose lower limit is not zero OD, but which is sufficiently low as not to mask weakly positive reactions in the analyzed samples.

Titers

Titres deal directly with the sample concentration in the reaction and with the cutoff in the assay. The titers will be detected once the lower limit is exceeded in the assay and can be expressed as the concentration of analyte in the sample that can be detected by the assay.

WESTERN BLOTTING

This approach - also known as immunobloting - consists of protein separation (based on molecular weight) and detection using specific antigens recognized by mono- or polyclonal antibodies. This method is based on the transfer of proteins from a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) to an adsorbent membrane. The most commonly used samples analyzed using Western blotting extracts of, cells, microorganisms and environmental specimens. In this section we will go deeper into every step of the protocol: sample preparation, gel electrophoresis, blotting, blocking, detection and analysis.

PROBLEMS	POSSIBLE CAUSES
1. Negative controls with positive reactions	• Reagents or sample contamination • Inappropriate washing • High antibody concentration
2. Sample results out of range	• Samples with low concentration of proteins • Samples with high concentrations of proteins
3. Variations in samples and standards	• Pipetting errors • Not uniformly or adequate plate washing • Not well mixed samples or reagents • Presence of precipitated particles in the sample • Insufficient agitation • Contamination
4. High background	• Solutions or background contamination • Interference of matrix used or non specific binding (Unproper blocking) • Not enough washing steps • Prolonged time of incubation with the conjugate • Reaction not stopped • Substract incubation in presence of light • High incubation temperatures
5. No signal	• Incorrect Antibodies • No addition of substrate solution • Presence of sodium azide
6. Low signal	• Standard improperly stored • Insufficient antibody • Low concentration of target protein • Inappropriate: - Reagents concentrations - Incubation temperature (low) - Incubation time - Agitation - Reagents pH
7. High Signal	• High concentration of target protein • Prolonged times: - plate incubation - detection incubation - Substrate solution incubation
8. Slow color development	• Inappropriate temperature • Weak conjugate • Solutions contamination

Table 3. **Troubleshooting ELISA.**

SAMPLE PREPARATION

Cell lysate is the most common sample used, but tissue homogenate or extracts can also be used. For solid tissues it is necessary to homogenate or sonicate them, while cells are typically lysed using sonication. Detergents, salts and buffers must be added to promote the lysis and to solubilize proteins. Proteases and phosphatase inhibitors are also added to prevent protein degradation and dephosphorylation by endogenous proteases and phosphatases present in the whole cell extract. Sample preparation should be done at low temperatures to avoid protein degradation or denaturing.

To compare samples it is important to know protein concentration, therefore it is necessary to measure it accurately using either spectrophotometer or colorimetric assays, such as bicinchoninic acid assay (BCA). This helps to estimate the volume necessary for loading the samples into the gel wells. The samples are then diluted into a loading buffer which typically contains bromophenol blue as a tracking dye. As with all experiments, positive and negative controls are needed. A positive control should be a known target protein and negative control is typically a null cell line (e.g., β-actin or GAPDH). An example of a sample preparation protocol is presented in Box 1.

GEL ELECTROPHORESIS

After the sample is prepared, it is loaded into wells of a polyacrylamide gel with sodium dodecyl sulphate (SDS-PAGE) for protein separation. The separation of proteins is influenced by their molecular weight, electric charge, and the isoelectric point (pI). For this process two polyacrilamide gels are needed. The first one is called the stacking gel and is acidic (pH 6.8), has a lower concentration of acrylamide and larger pores. These conditions putting all the proteins with a net negative charge, thus aligning them within the gel to further go into the separation gel. The second gel is called separation gel and is basic (pH 8.8), and has a higher concentration

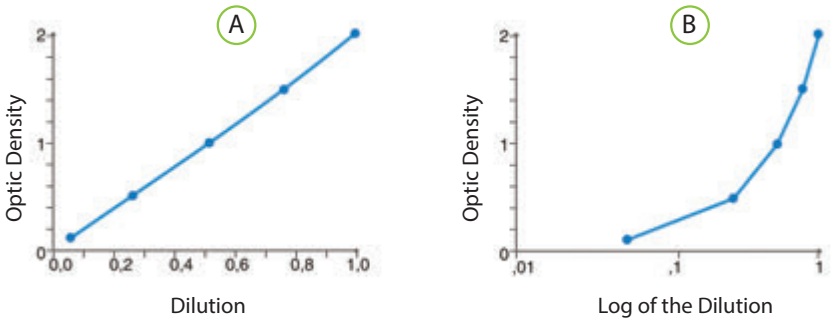


Figure 9. Graphic display of results analysis. A. Linear graph corresponding to the correlation between the concentration of antigen or antibody (dilution) in the sample detected by the OD vs. absorbance of the reaction. B. Curve corresponding to the correlation between the logarithm of the concentration of antigen or antibody (dilution) in sample vs. the logarithm of the OD detected by the absorbance of the reaction.

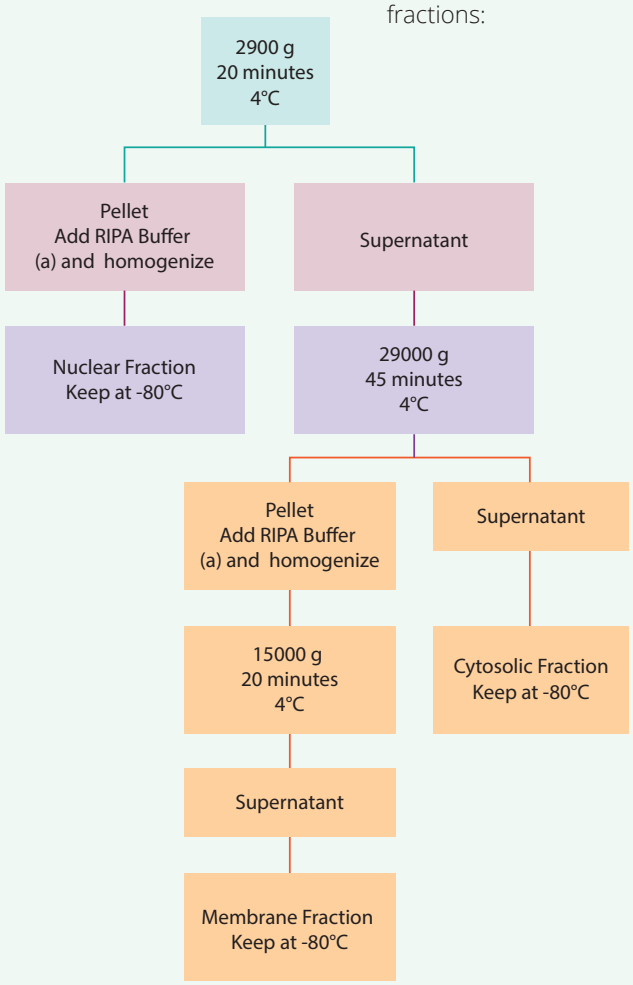
Box 1. Sample preparation. Homogenization and separation of subcellular fractions

A. Solutions:

1. RIPA buffer, containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton 100, 1% Sodium deoxycholate and 0,1% SDS (a solution).
2. Add to RIPA Buffer (b solution):
   - Phenylmethanesulfonylfluoride (PMSF) 1mM
   - Ethylenediaminetetraacetic acid (EDTA) 1mM
   - Dithiothreitol (DTT) 1mM

B. Protocol

1. Weight the tissue, and add RIPA buffer (b solution) according to tissue weight. Approximately, 0.5 L of RIPA buffer per 125 g of tissue.
2. Homogenize the tissue, using a blender in low power by intervals of 30 seconds (avoid bubbles)
3. Follow the next diagram of centrifugation steps in order to separate the different subcellular fractions:



of polyacrylamide; therefore, pores are narrower and proteins are separated by size. Smaller proteins pass through the pores easier and faster than large proteins. Acrylamide concentration will determine gel resolution; therefore a higher concentration makes a better resolution for low molecular weight proteins. Conversely, a lower concentration will be better for high molecular weight proteins. During electrophoresis, a voltage is applied and the proteins which are negatively charged will be pulled down toward the positive electrode (Figure 10). The speed of protein migration depends on their size. It is important to pay attention to the voltage and the temperature of the gel because when a high voltage is used, the gel overheats easily which alters the bands formed by the proteins. It is also possible to separate proteins using 2D- gel electrophoresis. Proteins are separated by their isoelectric point in the first dimension and by their molecular weight in the second one.

BLOTTING

To perform antibody detection, the proteins must be accessible to them. To facilitate that, proteins are transferred from the gel onto a membrane. Binding of protein to membrane is facilitated by hydrophobic and charge interaction. The gel is placed on a membrane. Then the gel and the membrane are placed in an electric field, producing the movement of proteins to the membrane in the same position they had within the gel. To get a good image resolution, it is important to assure a close contact of the gel with the membrane and to put the membrane in the right position: between the positive electrode and the gel. The transfer is preferentially done under wet conditions as that helps for the transfer of big proteins. However, the transfer can also be done in semi-dry conditions. As it was mention at the beginning of this chapter, there are different types of membranes such as nitrocellulose and PVDF. The advantages of the first one is that it retains the proteins because it has high affinity for them; however, this membrane is fragile and it cannot be used for repeat probing. Although, both membranes allow reprobing and storing; nevertheless, PVDF produces much more of background signal. To check if the transfer was uniform, a staining with Coomassie Blue of Ponceau dyes can be performed. To confirm the presence of a specific protein using Western blotting, the membrane is further probed using a reaction with antibodies.

BLOCKING AND WASHING

It is important to highlight the role that blocking has on protein detection. This step prevents the unspecific binding between antibodies and the membrane. This is performed with 3-5% bovine serum albumin (BSA) or non-fat dry milk, which is diluted in Tris-Buffered Saline and Tween 20 (TBST). An advantage of using dry milk is its low-price, but a limitation is that it may interfere with the detection of some molecules. For example, one of the milk components is casein which is a phosphoprotein that may be recognized by some antibodies used during the procedure (e.g., anti-phospholipids). BSA in phosphate buffer saline (PBS) or TBST can be used for incubation of the primary antibody, thus allowing its reuse if it is needed. The importance of blocking step is that it prevents false positive results. After the blocking

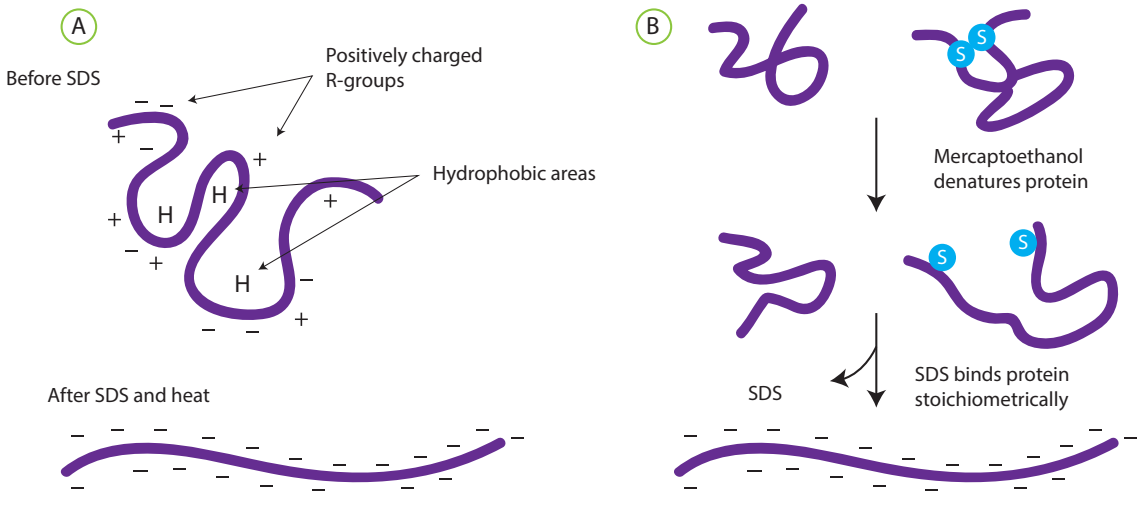


Figure 10. Negatively charge conversion of protein within the sample. **A**. For western blot proteins must be charged negatively in order to be separated within the polyacrilamyde gels. Therefore, the sample buffer and the gels have sodium dodecyl sulfate (SDS) which dissociates hydrophilic bonds and binds stoichiometrically to the positively charged amino acid radicals (R). **B**. In addition, sample must be heated after the procedure to ensure the dissociation and the rupture of hydrogen and non-covalent bonds. However, for stronger bonds other reagents must be used (e.g 2-Mercaptoethanol).

step and the addition of primary antibody, a wash step is necessary to remove unbound antibody. It is important to measure the time of the washing step because a long wash can reduce the signal even leading to false negative results.

DETECTION

After antibody incubation, a second antibody conjugated with a reporter enzyme such as HRPO or alkaline phosphatase is used to detect the protein of interest. To accomplish this goal it is necessary to follow two steps. The first step requires a primary antibody. During this step, an antibody concentration of approximately 0.5-5 µg/mL is used with gentle agitation to the membrane. However for each single case it is important to standardize the optimum concentration of the antibody. The second step involves the secondary antibody which is added after the washing step. This antibody is directed against a specific constant region of the primary antibody and is taken from animals (e.g., anti-mouse, anti-goat, etc). The detection is achieved using an enzyme-conjugated secondary antibody that reacts with a specific substrate producing a colorimetric or light signal which is captured by a light sensitive film. *Colorimetric and light detection* depend on the incubation of the membrane with a solution that responds to the reporter enzyme. For colorimetric detection, the solution will make the dye insoluble and it will precipitate, thus producing the band staining. *Chemiluminescence* requires an incubation of the product with a luminescence solution which will produce light when it exposed to the reporter enzyme. In this case, a light sensitive film is used, which captures the luminescence emission from the nitrocellulose membrane.

Nowadays, there is a one step approach in which the primary antibody contains also the property of protein recognition and, additionally, contains a detection molecule. Such antibody is incubated as it was mentioned before and then protein is detected just after single washing step.

ANALYSIS

It is important to highlight that this tool is semi-quantitative because it does not give us absolute values of concentration or amounts of proteins. The reasons are differences in loading and transfer rates of samples. Sometimes multiple bands are found on the membrane and, it is necessary to compare stained bands with the markers in order to establish the molecular weight of the target protein. Positive controls (e.g., β-actin) should have the same signal intensi-

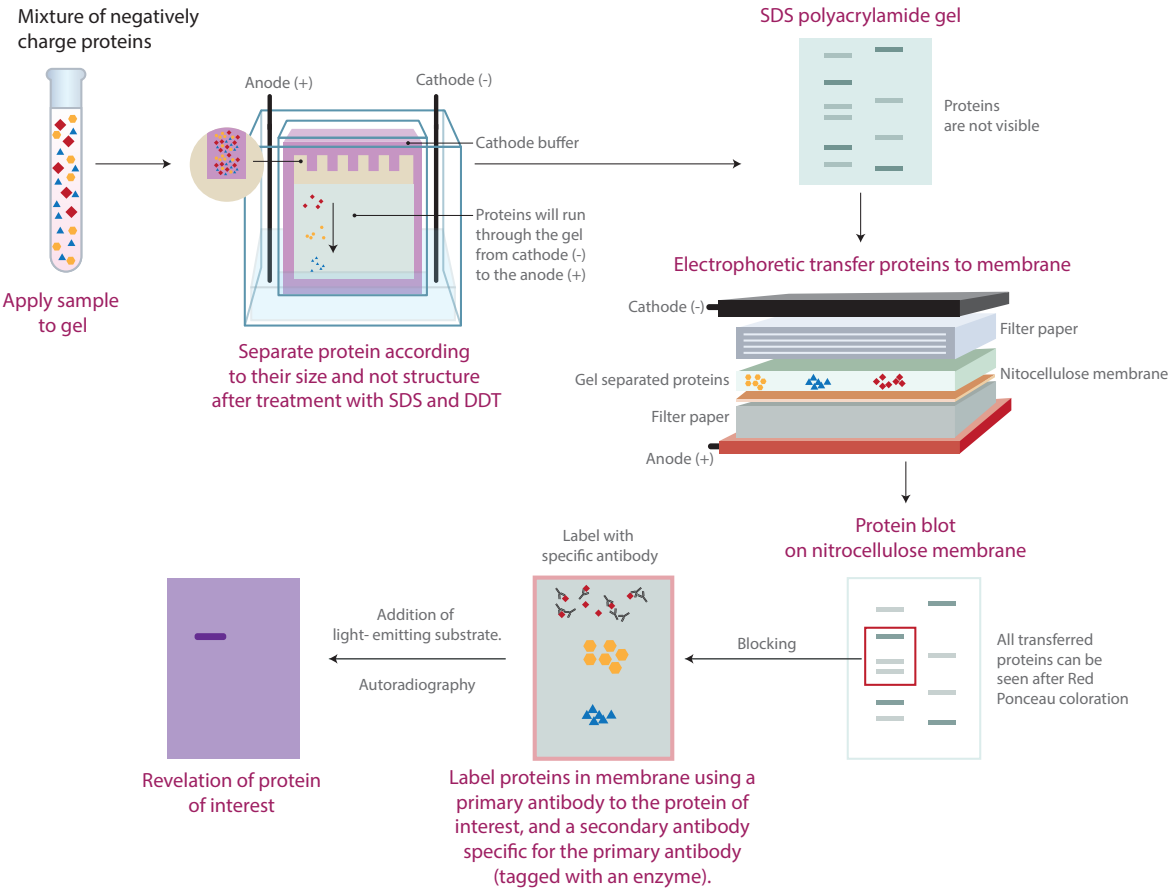


Figure 11. Schematic representation of western blot and immunotransference for immunoblot procedures.

ty in all experiments to validate the results. Protein levels may be analyzed by spectrophotometry or densitometry. For further information about a Western blot protocol and assembly, go to Box 2 and Figure 11. For troubleshooting unexpected results, please look at table 4.

IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMISTRY

Immunofluorescence and immunohistochemistry are methods that use antibodies for detection and localization of proteins and other antigens within biological samples. Tissues and other samples are prepared by a process call "fixation" which is usually done directly on microscopy slides. As a result, cells or tissues retain cellular antigen distribution and preserve its cellular morphology. Before the detection, samples are subjected to a permeabilizing process exposing the antigens which are usually not accessible, thus allowing the antigen detection within the structures. These methods are widely used in autoimmune disease (AD) research and diagnostics. They are especially useful to identify structures to which antibodies are bound within the fixed cells. In addition, these methods allow identification of regions within cells that are important for binding of proteins or specific antigens.

Box 2. Western blot protocol

A. Sample Preparation from cell culture (everything should be done on ice)
1. Wash cells with cold PBS and gently rocking
2. Centrifuge at 1500 rpm for 5 minutes. Discard supernatant
3. Add 180 µL of cold cell lysis buffer (20mM Tris, 100mM NaCl, 1mM EDTA, 0.5% Triton 100X)
4. Add 20 µL of protease inhibitor
5. Incubate for 30 minutes on ice. Centrifuge at 12,000 rpm for 10 minutes at 4°C
6. Store supernatant in a fresh tube on ice or at -20°C or -80°C
7. Measure protein concentration on a spectrophotometer.
8. Add 50 µg of protein extract in each well and add 5 µL of loading buffer
9. Heat sample in a dry plate at 95°C-100°C for 5 minutes
10. Load 15 µL of sample in each well and 6 µL of load marker
11. Run gel for an hour or until dye is at the bot tom of the gel

B. Blotting
1. Cut filter sheets from gel size and a nitrocellulose membrane with same size
2. Wet with transfer buffer (48mM Tris, 39mM glycine, 0.04% SDS, 20% methanol) the sponge and filter paper
3. Take out the gel, remove stacking gel and prepare the "sandwich" without air bubbles
   Sponge → 3 filter papers → electrophoresis gel → 3 filter papers
4. Locate sandwich on a electroblotting equipment in correct orientation and add transfer buffer to cover the sandwich
5. Run for 30 to 60 minutes at 100 V with cooling overnight at 14 V

C. Blocking and washing
1. Block membrane with blocking buffer (5 mL of 5% nonfat skim milk diluted in TBST) for 1 hour
2. Dilute primary antibody in blocking buffer
3. Discard blocking solution
4. Add 5 ml of diluted primary antibody and incubate 30 to 60 minutes on a shaker at room temperature
5. Wash membrane with TBST or TBS for 15 minutes. Repeat thrice.
6. Dilute secondary antibody HRPO- or AP-anti-Ig conjugate in blocking buffer
7. Add diluted secondary antibody and incubate for 30 to 60 minutes with gentle agitation at room temperature
8. Wash membrane with TBST or TBS for 15 minutes. Repeat thrice

D. Detection
1. Place the membrane into the chemiluminescence detection solution for 3 min at RT
2. Drain off the excess of solution with a filter paper
3. Go to dark room and use the red light
4. Take 3 X ray films and put them with the membrane. Expose them for a minute (time may vary according to the user needs)
5. Put the films into revealed solutions and let them dry.

E. Notes:
1. Electrophoretic and transfer buffers are different. The first one is composed by TRIS, Glycine, and SDS. Instead, the second one has TRIS, glycine and methanol.

PROBLEMS	POSSIBLE CAUSES
1. Unusual Bands	• Protease degradation - Use fresh sample and keep it on ice - Unspecific antibody use (e.g., polyclonal antibodies)
2. Blurry Bands	• High voltage • Air bubbles during transfer
3. No Bands	• Antibody: - Improper antibody - Low concentration • Antigen: - Absent antigen - Low concentration • Prolonged washing • Contaminated buffers
4. Weak Bands	• Low antibody or antigen concentration • Antigen masks by nonfat dry milk
5. High Background	• High antibody concentrations • Old buffers • High exposure times, specially on films sensitive to light
6. Irregular spots on the blot	• Improper transfer - Air bubbles • Uneven agitation during incubation • Improper washing • Antibody binding to blocking agents • Secondary antibody aggregation

Table 4. Troubleshooting western blot.

It is important to highlight that these techniques have the same basic steps as the ELISA and Western blot. The steps in common for all these techniques are: fixation of the sample onto slides or plates, blocking in order to avoid unspecific signal, detection by the use of one or two antibodies, and analysis. Therefore, in this section we are not going to explain all these steps. However, when the sample is a tissue, it is important to notice that when using immunofluoresce and immunohistochemistry methods, the sample must be blocked with inactivated serum and not with BSA or dry-fat milk. This is an important step because immune cells may be present within the tissue. Such approach avoids an unspecific response because primary antibodies can bind to antibody receptors present in immune cells' membrane. (e.g. Mast cell).

INMUNOFLUORESCENCE

The detection of antigen-antibody complex in immunofluorescence (IF) is based on the same principles of flow cytometry: as antibodies are dyes with fluorochromes (See chapter 45). However, as it was described before, the cells are fixed on a slide instead of being suspended in a fluid, and the analysis is made by fluorescent microscopy. There are two kinds of immunofluorescence based upon the antibodies that are being used. First, just one antibody is used which binds directly to the target antigen; therefore, this technique is called direct immunofluorescence (DIF). Second, it is called indirect immunofluorescence (IIF) when two antibodies are used and because the antibody allowing the detection binds to a primary antibody that recognizes the antigen on the slide (Figure 12). A general protocol is described in Box 3.

In DIF, fixed cells are stained with antibodies directly linked with a fluorochrome. Therefore, DIF is used to confirm the presence of suspicious antigens in cells. On the other hand, IIF is used for evaluation of antigens co-localized within the sample or the presence of specific antibodies by the use of slides containing known antigens. Noteworthy, this method is widely used for detection of antinuclear antibodies (ANAs), which are common in some AD such as systemic lupus erythematous (SLE). As an example, in SLE for appropriate IIF ANA tests, it is recommended to use tumor cell line (HEp-2) substrate. These cell lines increase the method sensitivity and facilitate the identification of specific antibody patterns when they are compared to differentiated tissue cells; e.g., rodent organ sections which were mostly used in the past.

Analysis

Immunofluorescence is an effective and sensitive tool for protein analysis. Moreover, this approach can be combined with structural and biochemical studies to increase its efficiency. Even though this technique is easy to perform and cost-effective, it is important to pay attention to critical parameters such as fixation, permeabilization, determination of antibody specificity and a careful selection of antibodies and type of tissues to ensure accurate results. If the epitope is hidden, it will not be recognized and bound by the antibody. To overcome this inconvenient, it is necessary to

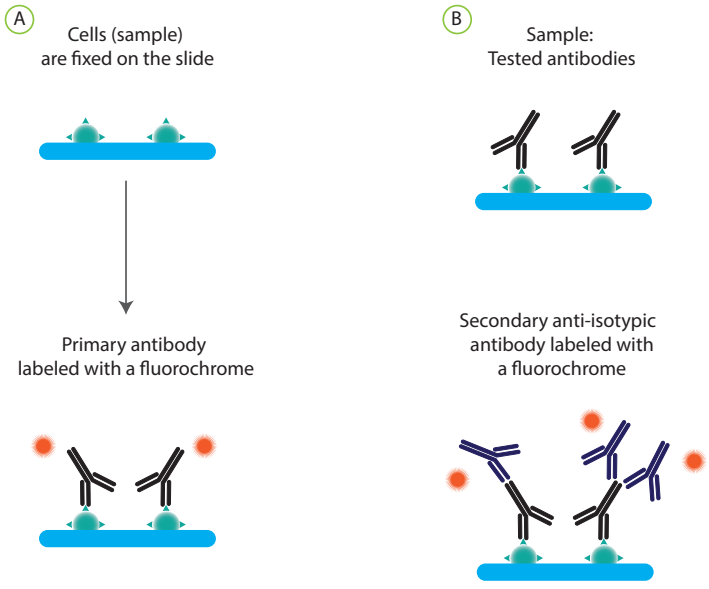


Figure 12. Immunofluoresce. **A**. Direct immunofluorescence, the primary specific antibody is dyed with the fluorochrome. **B**. Indirect immunofluorescence, primary antibody recognizes the antigen on the slide and subsequently, the secondary labeled antibody can recognize the primary antibody. Adapted from (6).

Box 3. Immunofluorescence for auto-antibodies detection

A. Sample and reagents
1. For auto-antibody detection, it has been widely used cell lines. For example, Hep-2 is a human epithelial cell line which is validate for ANAs detection.
2. Positively charged slides are covered well by well with the fixed cells.
3. Positive and negative controls
4. Serum samples
5. Secondary antibody (dye with a fluorochrome)

B. Procedure
1. Serum samples must be fresh or thaw gradually.
2. Homogenize the sample to ensure all the antibodies will be into the solution
3. Centrifuge the samples at high speed for 1 minute in order to precipitate contaminants
4. According to assay, make appropriate dilutions of the samples.
5. Add the sample and the controls to the proper well in the slide. Ensure that the entire well will be cover with the sample.
6. Incubate according with the assay, usually 30 minutes, in a humid environment.
7. Tilt the slide and wash it at least three times for 5 minutes. Be careful to not induce mixing of the samples nor add the buffer directly to the wells.
8. Add the secondary antibody to each well. Ensure that the entire well will be cover with it.
9. Tilt the slide and wash it at least three times for 5 minutes, being careful not to induce mixing of the samples nor add the buffer directly to the wells.
10. Add mounting media and a cover slide.
11. Analyze by using a fluorescent microscopy.
12. Report pattern and dilution in positively wells

C. Notes:
1. In order to have good results it is extremely important avoid dryness of the well during all the procedure, because the tissue or the cells can lose their conformation and their structures.

use during the procedure permeabilizing reagents which expose the epitope. Another important point to highlight is the localization of antigen because there are some antibodies which cannot go into the nucleus; therefore, the epitope will not be achieved and no signal will be produced.

Another critical factor to control in immunofluorescence is antibody specificity. Thus, it is important to include the following controls: 1) A sample incubated only with the secondary antibody to determine non-specific binding sites. 2) Control slide containing cells that either do not express

and/or have high expression of the target protein. These controls ensure that fluorescence is due to antibodies targeting the protein of interest. Finally, it is essential to establish optimal concentrations of the primary and secondary antibody by testing a range of dilutions in order to minimize non -specific binding sites.

For common problems and troubleshooting in immunofluorescence please refer to Table 5.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) uses the same principles of immunofluorescence although with some differences. It allows the analysis of tissue structure and localization of a specific marker within it. The antibody-antigen interaction is visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein or fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy.

This technique can be performed using samples with different previous treatments such as cryopreserved slides or paraffin-embedded tissue slides (Box 4).

Immunohistochemistry allows the detection of more than one antigen simultaneously given the fact that a careful selection of antibodies with different fluorophores can be used. The success of immunostaining depends on a number of factors such as source, specificity, and quality of the primary antibody; and whether it is monoclonal or polyclonal. Antibodies chosen improperly are cause of a cross reaction that leads to erroneous results. Other crucial factors for the results are: specimen conditions, fixation procedure, and the antigen detection strategy. To detect more than one antigen, it is necessary to combine two primary monoclonal antibodies which are produced in two different species, as well as the use of secondary antibodies that must specifically recognize the constant fraction related to the species of primary antibody (Figure 13).

Analysis

Although IHC and immunofluorescence use similar methodology, there are some reasons that make IHC more accessible to researchers: IHC does not require special equipment as IF; allows antigen localization and determination of cell or tissue morphology; staining last for years, and enzymes or substrate used in IHC are light insensitive. Even though, IHC is easier to perform than IF, they both have the same critical factors for performance as: fixation, cautious antibody selection and dilutions, and use of appropriate controls. Negative controls could be slides previously exposed to antibody-specie specific serum (e.g., rabbit's serum if rabbit's antibody is used) or isotype-specific Ig as primary antibody. Controls are also useful for determination of non-specific binding sites to secondary antibody and optimal primary antibody dilution. Positive controls should also be included to validate proper activity of the different reagents. IHC can be done also with fluorochrome labeled antibodies.

Fixation is a key parameter for both IHC and IF, because it may interfere with the antigen-antibody complex formation. Acetone or paraformaldehyde are the most commonly used fixatives, they cause less antigen denaturation, permeabilization and maintain cell or tissue morphology and structure. In contrast, antigens from formalin-fixed samples are more affected than those fixed with acetone. One more point to take into account during the IHC analysis is the previously use of HRPO, because endogenous peroxidases can interfere with the reaction, especially when a tissue has a lot of macrophages or granulocytes. For this reason, some protocols propose to incubate the sample with hydrogen peroxide to diminish peroxidase activity. Results can also be altered if antigens are not used under the right conditions. Thus, it is necessary to optimize antigen-specific conditions such as buffers pH and heating or cooling conditions.

As mentioned before, one of the advantages of IHC compared to IF is the long storage period (years) for samples that could still be read. For this reason, it is important to choose a primary antibody with high sensitivity.

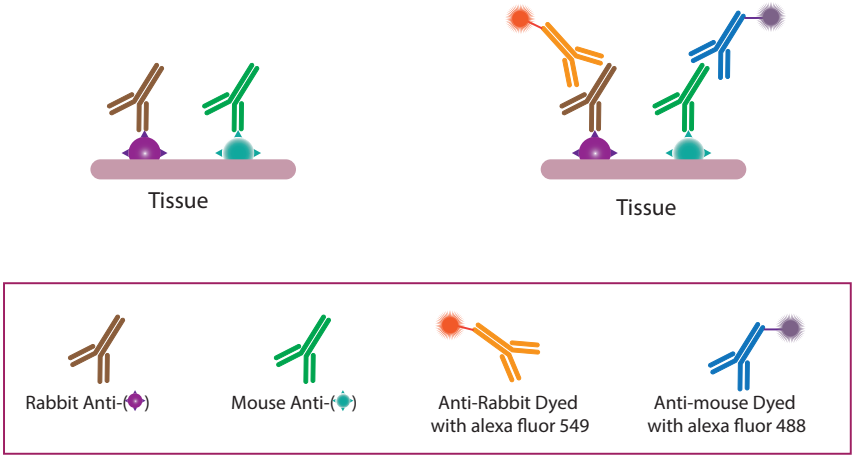


Figure 13. Example of proper selection of antibodies for IHC. Antibodies should be chosen considering the host species production and the dyes.

For common problems and troubleshooting in immunohistochemistry please refer to Table 6.

CONCLUDING REMARKS

As it was stated at the beginning of this chapter, all the techniques mentioned play a critical role for protein analysis. It is important to highlight the recognition of the critical factors of each one of them to avoid problems during the analysis of the results (See tables 3, 4, 5 and 6). Additionally, all techniques depend on antigen-antibody interaction, thus it is necessary to keep in mind the nature of the antigen and the kind of antibody which is going to be used, depending on the chosen technique. For example, epitopes found by blotting are linear, in contrast, structural epitopes can be found with other approaches such as ELISA, immunofluorescence and immunohistochemistry. Similarly, the specific localization of an antigen can be determined by immunofluorescence or immunohistochemistry, but no by western blot or ELISA.

Box 4. Immunohistochemistry

Immunohistochemistry can be done from paraffin slides or frozen tissue sections by two different procedures.

A. Paraffin tissue slides:

1. After tissue is fixed to the slide. Sample must be dewaxed and rehydrate by serial washes with alcohol for 10 minutes each step. Ethanol 100%, 90%, 75%, 50% and finally distillate water.
2. Mask antigens must be exposed in order to make them accessible for antibodies. For this purpose, it is commonly used Sodium citrate 10 mM solution at 80°C for 30 minutes.
3. In order to block unspecific biding incubate with horse serum at room temperature for 2 hours.
4. Tilt the slide and wash it once for 5 minutes being careful not to add the buffer directly to the tissue.
5. According with the protocol incubate the primary antibody in a humid environment for 1 hour at room temperature or overnight at 4°C.
6. Tilt the slide and wash it at least three times for 5 minutes being careful not to add the buffer directly to the tissue.
7. According with its specificity, add the secondary antibody to each well. Ensure the entire well will be cover with the sample.
8. Tilt the slide and wash it at least three times for 5 minutes being careful not to add the buffer directly to the tissue.
9. If it is the case of a colorimetric assay, add the appropriate substrate for the antibodies conjugates.
10. Add mounting media and a cover slide.
11. Analyze by using microscopy.

B. Frozen tissue sections / free – floating stainnig:

1. Choose tissue sections according to the structures will be analyzed, place the tissue in plates with wash solution in continuous agitation.
2. Wash the cryopreservative solution five times for 5 minutes.
3. Mask antigens must be exposed in order to make them accessible for antibodies. For this purpose, it is commonly used Sodium citrate 10 mM solution at 80°C for 30 minutes.
4. Wash five times for 5 minutes
5. In order to block unspecific biding incubate with horse serum at room temperature for 2 hours.
6. Wash once for 5 minutes
7. According with the protocol incubate the primary antibody in a humid environment for overnight at 4°C.
8. Wash five times for 5 minutes
9. According with its specificity, add the secondary antibody to each well. Incubate for 2 hours at room temperature.
10. Wash five times for 5 minutes
11. If it is necessary, add a counter staining as DAPI (for fluorescence). Wash once for 5 minutes
12. If it is the case of a colorimetric assay, add the appropriate substrate for the antibodies conjugates.
13. Wash five times for 5 minutes
14. Place the tissue on a charge slide and let it dry.
15. Add mounting media and a cover slide
16. Analyze by microscopy

C. Notes:

1. Wash buffer can be PBS (phosphate buffer saline) or PB (phosphate buffer)
2. Horse serum or another inactivate mammal serum must be used in order to block unspecific binding.
3. It is better to incubate the primary antibody overnight at 4 °C to insurance assay specificity.
4. In order to have good results it is extremely important avoid that well be dry during the process, because the tissue or the cells can lost their conformation and their structures.

PROBLEMS	POSSIBLE CAUSES
1. Weak or no staining	• Inactive primary or secondary antibody • Improper storage of antibodies • Excesive freezing/thawing cycles (serum samples and antibodies) • Absence or low concentrations of target protein • Low concentration (high dilutions) of antibodies • Inadecuate incubation times • Inadecuate fixation • Epitope modified by fixation procedures (e.g., FFPE) • Unproper unmask antigen • No compatibility between primary and secondary antibody • Alterations on enzyme substrate • Steps omission • Antibodies storage in a lightly place
2. Non specific staining	• High: - Antibody concentration - Incubation times - Temperature • Species used for antibody production is the same for sample analysis • Dry samples
3. High background	• Not enough washing steps • Samples with endogenous enzymes • Insufficient blocking of non specific binding sites • High antibody concentration • High incubation temperatures • Cross-reaction of fluorocrome with the PBS or other buffers • Inadequate fixation • Species used for antibody production is the same for sample analysis • Dry samples

Table 5. Immunohistochemistry troubleshooting.

PROBLEMS	POSSIBLE CAUSES
1. Weak or no staining	• No compatibility between primary and secondary antibody • Low concentration of antibodies (high dilutions) • Improper storage of antibodies • Too much freezing/thawing cycles of antibodies. (Especially secondary Ab) • Improper dilutions • Absence or low concentrations of target protein • Mask antigen • Antibodies storage in a lightly place • Epitope modified by fixation procedures (e.g., FFPE) • Insufficient deparafinization • Buffer contamination • Target protein is located in nucleus and antibody can not go into it
2. Non specific staining	• High antibody concentration • Samples with active endogenous enzymes • Specie used for antibody production is the same for sample analysis • Dry samples
3. High background	• Insufficient blocking of non specific binding sites • High antibody concentration • High incubation temperatures • Secondary antibody damaged • Inadecuate washing • Epitope modified by fixation procedures (e.g., FFPE) • Samples with active endogenous enzymes • Cross-reaction of chromogen with the PBS

Table 6. Immunohistochemistry troubleshooting.

Recommended readings

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49

EPIGENETIC TOOLS IN AUTOIMMUNE DISEASES

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INTRODUCTION

As has been mentioned in the chapter 22, *Epigenetics and Autoimmunity*, epigenetic mechanisms play a key role in regulation of gene expression by gene transcription activation or inhibition. Nowadays, this field is growing and research about epigenetic mechanisms and their regulation has been intensified in the last years. Even more, studies about the improvement of these techniques is increasing to go further into epigenetic-disease relationships and how epigenetic mechanisms could predispose to development of complex diseases (autoimmunity, cancer, etc.). Therefore, epigenetic approaches will open the door to new knowledge about how epigenetic mechanisms interfere in disease progression and what are the differences between healthy individuals and specific-disease patients.

Before explaining in detail every technique, it is important to mention that there is not a gold standard or specific protocol for a particular epigenetic mechanism. Instead, its study depends from a combination of approaches ranging from simple to high-throughput technologies. It is noteworthy to point out that epigenetic mechanisms are tissue-specific; therefore, it is essential to highlight the relevance of performing all epigenetic studies in a tissue-specific manner regarding to the disease we want to address. In this chapter, we are going to give an overview of the widely used epigenetic tools used for analyzing the methylation status and histone modification in a genome-wide or a loci-specific manner.

METHYLATION ANALYSIS

It is important to remember that approximately 5% of cytosines are within CpG islands and about 80% of them are methylated. The significance of this methylation state lies on its localization within genome and its influence in aberrant gene expression, thus promoting the development of complex diseases. Methylation tools allow the study of DNA methylation profiles within whole genome (methylome), or within a specific CpG, gene or loci. Currently, there are vast numbers of tools for methylation analysis; however, because of space concerns, we cannot go into details about each one of them. Hence, this section will emphasize the most widely used methylation analysis techniques which are based on restriction endonucleases (Figure 1) and bisulphite conversion (Figure 2).

WHOLE METHYLOME ANALYSIS

Techniques for whole methylome analysis help to quantify methylcytosine concentrations by high-performance separation approaches. These procedures are based on the enzymatic hydrolysis of the entire DNA to obtain the complete cytosine composition of whole genome. This process can be achieved by *high-performance liquid chromatography (HPLC),* which is a quantitative tool based on DNA hydrolysis to deoxyribonucleo*tides*, and then to deoxyribonucleo*sides*. This hydrolysis is made by using enzymes such as deoxyribonuclease I and nuclease P1, as well as treatment with alkaline phosphatase. Products from this process are then separated by HPLC. Regarding methylation, cytosine and 5-methylcytosine (5-mC) are identified and quantified by comparing the relative absorbance of 5-mC with the cytosine absorbance at 254nm. Some limitations of this technique are: high-quality and quantity of DNA are required (approx. 2.5 µg) in addition to special and sophisticated equipment; precipitation of elution buffers in the chromatography column; also, it does not provide information about genomic specific-positions of

aberrant methylation. For all these reasons, this technique is not recommended for high-throughput analyses. To bypass this drawback, other techniques have been used to perform whole methylome analysis such as *the methyl-accepting assay* (or M. SssI acceptance assay) and the luminometric methylation assay (LUMA).

The methyl-accepting assay uses the bacterial enzyme SssI DNA methyltransferase to methylate cytosine at CpG sites. For this purpose, DNA is incubated with the radio-labeled enzyme S-adenosylmethionine (SAM), which will donate methyl groups to unmethylated cytosines. As a result, all non-methylated CpGs in the sample will become radioactively methylated. The product of this reaction is then immobilized on a nitrocellulose membrane, and the radioactivity is measured using a beta-counter. Thus, low methylation of starting material correlates with high reactivity and vice versa. The limitations of this technique are enzyme instability, excessive radioactivity and difficulties during DNA homogenization; hence, it is important to start with identical DNA quantities if two samples are to be compared.

Taking into account all limitations mentioned above, *LUMA* could be a better option for whole methylome analysis because it has low requirements, it is not time-consuming and it is appropriate for high-throughput analysis. To perform luminometric methylation assay (*LUMA*), DNA sample is divided into two samples which will be then digested by three different restriction enzymes: one insensitive to DNA methylation added to both samples, other sensitive to methylation added to the first sample, and the last enzyme is an isoschizomer of the second enzyme (insensitive to CpG methylation) added to the second sample. After digestion, products are analyzed by pyrosequencing (Figure 3A). As we have mentioned before, this assay also needs high DNA quality because it is based on extension reaction.

The techniques mentioned above are the most widely used to perform whole methylome analysis. However, there are other techniques which also accomplish this aim such as: *high-performance capillary electrophoresis (HPCE), chloroacetaldehyde assay and bisulfate-based PCR methods* which are commonly used to assess methylation patterns of repetitive elements.

CpG LOCI ANALYSIS

Nowadays, there are a lot of techniques or approaches to perform this kind of analyses and the majority of them involve an initial amplification of the target sequence. The problem with this approach is that DNA polymerases do not distinguish between 5-mC and cytosine. For this reason, restriction enzymes and chemical modification have been implemented to overcome this problem. These procedures consist on base modification through methylation-sensitive restriction endonucleases (MSREs) or bisulphite conversion to allow the differentiation between cytosines and 5-mC. Moreover, to accomplish the aim of the study sometimes it is necessary to use these techniques together with other approaches such as: PCR (see chapter 46), hybridization, pyrosequencing and arrays. It is important to highlight the variety of techniques used for specific or individual methylome analysis; therefore, this section will be divided *into individual CpG loci analysis and genome-wide analysis*. As it was mentioned at the beginning of this section, it will only cover the most widely used approaches. Before going deeply into these tools we will explain restriction enzymes and bisulphite modification assays.

Methylation-Sensitive Restriction Endonucleases (MSREs)

As previously described, this approach is used together with other techniques such as PCR and southern blot hybridization, depending of the quantity of initial DNA (approx. 1 µg and 10 µg, respectively). MSREs assays are based on recognition of specific target sequences and DNA modifications. Therefore, these assays use isoschizomers with different sensitivities to methylated cytosines within the CpG target sequence. The most widely used restriction enzymes for this technique are *HpaII* and *MspI* (Table 1). In this method, DNA is divided into two samples: the first one is digested with a methylation-sensitive enzyme (it does not cut methylated sites, e.g. *HpaII*); the second sample is digested with a methylation un-sensitive enzyme (it will cut independently of methylation pattern, e.g. *MspI*). As a result, DNA methylation can be confirmed when there is no evidence of cleavage in the sample incubated with the methylation sensitive enzyme (Figure 1). This approach has some limitations such as incomplete digestion (false positive results), *a priori* knowledge of the sequence, and intrinsic resistance to some target sites. Also, all CpGs loci located outside the recognition sites will not be analyzed; for this reason, the sensitivity of this technique depends on the restriction site used and its distribution within the DNA sequence. On the other hand, the advantages are: ease of performance and robustness.

Bisulphite modification

This approach has become the gold standard for methylation analyses and it is based on the sodium bisulphite conversion of DNA. It requires between 1 ng to 5 µg of genomic DNA to run the protocol and it can be used for qualitative and quantitative analyses. Similarly as MSREs approach, this technique uses PCR, MSREs, sequencing and arrays. The bisulphite conversion converts un-methylated cytosines into uracils, which are used for differentiation between methylated and un-methylated DNA. DNA is first completely denatured by alkali treatment and then it is incubated with bisulphite for 4-18 hours at 60 – 95°C (time and temperature vary from protocol to protocol). As a consequence, sulphonated cytosines undergo hydrolytic deamination and they are converted into uracil sulphonates. The products desulphonate under alkali treatment and become uracils. During PCR, the uracils behave as thymines. Hence, as we mentioned before, un-methylated cytosines will become uracils, while methylat-

Sensitive to methylation	Insensitive to methylation	Restriction Site
HpaII	*MspI*	C CGG GGC C
SmaI	*XmaI*	CC CGGG GGGC CC
AgeI	*CspAI*	AC CGGT TGGC CA
Bsp199I	*SfuI*	TT CGAA AAGC TT
Kpn2i	*AcdII*	TC CGGA AGGC CT
Cfr10I	*BssAI*	RC CGGY YGGC CR
AvaI	*BmeT110I*	CY CGRG GRGC YC

Table 1. Isoschizomer restriction enzymes for methylation analysis.

ed ones will remain as cytosines (Figure 2). The advantages of this approach are that methylation patterns of any CpG from the genome can be determined; it can be done on paraffin-embedded tissue (FFPE) or poorly purified DNA; and the integrity of genomic DNA will remain conserved. On the other hand, the limitations of this method are: partial denaturation which will produce artifacts; incomplete desulphonation by pH changes causing alterations on polymerase activity; and partial conversion of non-CpG sites (false positives results).

INDIVIDUAL CpG LOCI ANALYSIS

Methylation Specific PCR (MSP)

MSP is the most widely used technique for this type of analysis because it is a qualitative approach to analyze CpG site methylation. This is based on the discrimination between methylated and un-methylated DNA after bisulphite treatment, hence two PCR reactions must be performed to obtain the results. The first one is done with methylation specific primers and the second one with specific primers for un-methylated DNA (Figure 2A). It is noteworthy to mention the importance of primers design because bisulphite modified DNA will not have complementary strands. Therefore, we have to consider the following key points for designing specific primers of methylated and un-methylated DNA: annealing temperature between 55°C and 65°C for each strand; PCR products between 80-175 base pairs (bp); primers should have at least two CpG, and they must contain non-CpG cytosines. The advantages of this tool are that it

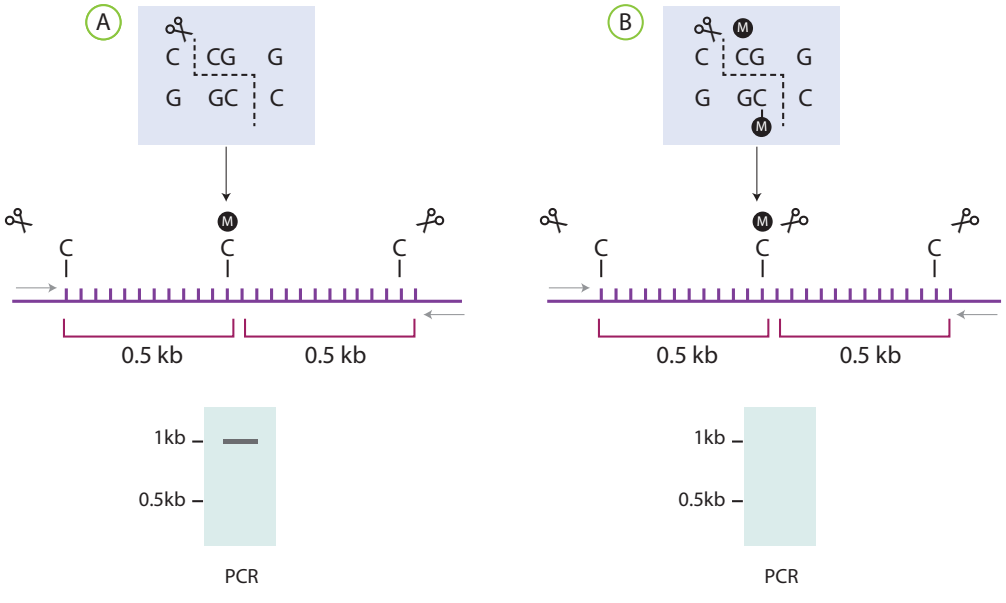


Figure 1. Methylation-sensitive restriction endonucleases. **A.** Restriction sites for *HpaII* and result from PCR analysis for methylated and un-methylated CpG sites using *HpaII*. **B.** Restriction sites for *MspI* and result from PCR analysis for methylated and un-methylated CpG sites using *MspI*. Black circles indicate methylated cytosines. Arrows are PCR primers.

does not require special equipment, it has great sensibility so it can be used with low DNA quantities and FFPE tissue (*in situ MSP*), and it is a fast and economic approach for diagnostics. Despite its advantages, it is important to remember that bisulphite conversion sometimes is incomplete, thus this tool needs appropriate controls such as methylated DNA modified by bisulphite, un-methylated DNA also modified by bisulphite and non-modified genomic DNA. Another limitation of this tool is that it is a qualitative approach; hence, results will be analyzed just as positive or negatives. Therefore, hemi-methylation patterns within a gene will be reported as methylated or un-methylated, since this type of patterns cannot be distinguished. Considering these disadvantages, it is necessary to verify the results with others techniques and also interpret them carefully, especially if the results are for clinical purposes.

Combined Bisulphite Restriction Analysis (COBRA)

This tool is a semi-quantitative approach to analyze methylation patterns within a short DNA sequence. Therefore, this technique uses bisulphite-modified DNA which will be amplified using two primers flanking the endonuclease recognition sequence. Once the DNA is amplified by PCR, a restriction endonuclease recognizing the CpG of interest, but no other cytosine residues (e.g. *BstUI*) will be used to digest the PCR product. As a consequence, cytosines from the initial methylated DNA will remain as cytosines when bisulphite modification is made, thus allowing the digestion by the restriction endonuclease. On the other hand, cytosines from initially un-methylated DNA will be converted to uracil and will not be recognized by the endonuclease, so digestion will not take place (Figure 2B). Then, digestion products are quantified first by electrophoresis and then by southern blotting. An advantage of this approach is that bisulphite already specifies where methylated sites within CpG sites are, thus we can use restriction enzymes to look for methyl-CpGs. Limitations of this tool are the availability of endonucleases, incomplete digestion and incomplete conversion.

Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE)

This tool is also based on bisulphite-converted DNA, but it does not require endonuclease activity as COBRA; thus every cytosine residue could be analyzed. This approach uses a primer extension reaction to determine the cytosines and thymines in the bisulphite-modified DNA. A PCR with specific primers is performed for amplification of the modified DNA, and its products are then purified by electrophoresis. This product is denatured and other internal primers will anneal flanking the CpG of interest. Also, radioactive deoxycytidine triphosphate (dCTP) or deoxythymidine triphosphate (dTTP) nucleosides will be added together with the DNA polymerase. Hence, methylation will be quantified by the inclusion of these radioactive molecules through the use of a phospho imaging equipment. As a consequence, if the radioactivity signal is higher with the dCTP, it will mean that DNA was methylated. In contrast, if the radioactivity signal is higher with the dTTP, it will be associated with un-methylated DNA (Figure 2C). Nowadays this approach can be done by fluorescence-based tools or pyrosequencing, to avoid the use of radioactive materials which are hazardous and require special equipment. This technique can be used in the clinical setting because of its specificity and sensitivity, and because it requires only small amounts of DNA. Its limitations include the PCR bias and the difficulty to design primers without CpG dinucleotides when there are CpG-rich regions within the sequence of interest.

MULTIPLE CONSECUTIVE CpG LOCI ANALYSIS

Bisulphite Pyrosequencing (PS)

PS is a gold standard for multiple consecutive CpG loci analysis and it is based on the addition of nucleotides into the DNA, which will be converted into light signal. In this method, DNA polymerase adds a complementary deoxyribonucleotide triphosphates (dNTPs) to the primers hybridized to the template, and this incorporation released a pyrophosphate (PPi). As a result, the PPi is converted quantitatively into ATP by the ATP sulfurylase in the presence of adenosine 5'-phosphosulfate.The ATP acts as a fuel for the luciferase, which then converts luciferin to oxyluciferin generating a light signal. This signal is proportional to ATP quantities released and the number of base repeats within the DNA sequence. Unincorporated nucleotides are degraded by the apyrase enzyme, and the next nucleotides in the sequence are added (Figure 2D). In the case of methylated DNA, dCTP and dTTP will be added successively when they arrive at a methylated cytosine position. As a consequence, the signal ratio will correlate with the quantity of methylation within CpG site. The advantages of this technique are that it can be used for high-throughput analysis and it also allows the fast quantification of DNA methylation status in any sequence within the genome. However, disadvantages of this tool are: problems with specific primers design because of the low temperatures used during PS reaction, and the low sequencing distance per run (100 – 150 bp), thus limiting the number of CpG regions.

MethyLight

A useful tool for multiple and consecutive CpG analyses is the *MethyLight*, which is a technique based on bisulphite modification and the TaqMan protocol for RT-PCR. It is a quantitative approach and - as same as MSP technique - it uses specific primers designed to amplify the un-methylated and methylated DNA (Figure 2E). The RT-PCR uses fluorescent probes that are specific to every sequence fragment which will be amplified by specific primers (for more information about Taqman Probes, go to Chapter 46). Serial dilutions from un-methylated and methylated control DNA are needed in each assay to create the standard curves. An advantage of this tool is the detection of hypermethylated low frequency alleles (highly sensitive), which can be used as biomarkers for

clinical purposes. For example, this tool allows comparison between very low methylation levels in normal tissue samples and hypermethylated cancerous tissue.

Other tools

Besides the techniques mentioned so far, there are other tools that may be helpful for multiple consecutive CpG loci analysis such as ***MSP*** (previously mentioned), cloning, MassArray and Maxam-Gilbert sequencing. These three tools will be briefly explained.

Cloning is a tool based on bisulphite-converted DNA and PCR for DNA amplification using specific primers. After PCR, products are cloned into a vector and transferred into bacteria. Then, plasmid DNA is extracted and sequenced (Figure 2F). This tool is time-consuming, but it provides methylation maps of a DNA sequence and allows determination of allele-specific methylation patterns. A limitation is the great quantity of clones that has to be sequenced to establish the amount of methylation within a specific region. For more information about Cloning go to Chapter 46.

Another tool is the ***MassArray*** which uses linker primers specific for bisulphite-modified DNA. The antisense primer has a T7-promoter sequence in its linker sequence, thus T7-RNA polymerase is used for transcribing the DNA strand. Therefore, un-methylated CpG loci converted to TpG by bisulphite will result as CpA in the RNA, while methylated CpG will remain the same (Figure 2G). This assay also uses RNase T1 enzyme which cleaves RNA after guanosine residues, hence it will cleave methylated cytosine residues and the resulting fragments could be analyzed by MALDI-TOF (Matrix-assisted laser desorption/ionization-time of flight mass spectrometry). This method requires an *a priori* knowledge of the DNA sequence. Some limitations of this approach are the failure to analyze small CpG fragments and the need of expensive equipment. However, the main advantage is the high resolution analysis of large sequences.

The last tool used for this kind of analysis is ***the Maxam-Gilbert sequencing*** which is based on chemical reactions to cleave DNA fragment after a specific nucleotide. This approach uses hydrazine and piperidine to induce cleavage at cytosine or thymidine residues. The product of this reaction will be amplified, radio-labeled and separated by electrophoresis. Therefore, when there is a 5-mC, the hydrazine will fail to cleave the fragment as it does with cytosine. As a result, bands from 5-mC will not appear on the autoradiogram. To perform this technique, it is also necessary to know *a priori* the DNA sequence and another important disadvantage is the use of hazardous chemicals.

MULTIPLE CpG LOCI ANALYSIS (MULTIPLEX)

This type of analysis can be done by methylation-sensitive restriction enzyme and various PCR (*MSRE-PCR*) or by *methylation-sensitive arbitrarily primed PCR (AP-PCR).* The first tool is based on the wide digestion of genomic DNA samples with methylation-sensitive enzymes. The products of the digestion will be then amplified by multiple PCR using primers flanking CpG loci. The advantage of this approach is the low quantities of starting DNA required to perform the technique. However, this tool has the same disadvantages that has been discussed before (go to *MSRE analysis*). The other analytical tool is the ***AP-PCR*** which is based on PCR, and it is used to find methylation changes and isolate specific DNA fragments associated with these changes. To perform this analysis, DNA sample is divided into three samples. The first one is used as a positive control and it is digested by an endonuclease not cutting at *CG* sites (e.g., RsaI). The second and third samples are digested by methylation sensitive and insensitive enzyme (e.g., *HpaII* and *MspI*, respectively). The digestion products are then amplified by AP-PCR with the addition of nucleotides and one or several primers. Therefore, primers anneal to many target sequences to produce a specific primers fingerprint. The PCR products can be separated by electrophoresis and the bands isolated from the gel gives further information about selected sequence (Figure 3B). The advantage of this tool is that it can detect small methylation changes even in samples as small as 200 ng of genomic DNA. Limitation of this tool (the same as the approaches mentioned above) is that it does not provide information about association between methylation changes and gene expression.

GENOME-WIDE ANALYSIS

Immunoprecipitation (IP)

This approach is commonly used to isolate methylated DNA by monoclonal antibodies with specificity for 5-mC (anti-5mC), or to isolate DNA fragments with methylated CpG dinucleotides by methyl binding domain proteins (MBD). This tool is analogous to IP used for chromatin analyses (explained further in *histone modification analyses*). It uses genomic DNA, which is digested with restriction enzymes or sonicated, and then divided into two samples. The first sample is used as a control or "input" which is not treated, and the second one is used for IP. DNA is denatured and incubated with a primary antibody such as anti-5mC or anti-MBD. Then, these complexes are captured by a secondary antibody, and the unspecific bound DNA is removed by several washing steps. The specific bound complexes are precipitated and purified to elute methylated DNA from the protein complex. This methylated fragment could be compared with the input using PCR, arrays or sequencing (Figure 4). Keeping this approach in mind we will further discuss different IP methylation analyses.

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP uses anti-5mC antibody to isolate with high specificity methylcytosine from a DNA sequence. The procedure is similar to immunoprecipitation (Figure 4A). It is important to highlight that this tool needs specific conditions to enhance its efficiency. Therefore, it is important to optimize the sonication to obtain fragments between 300 and 1000 base

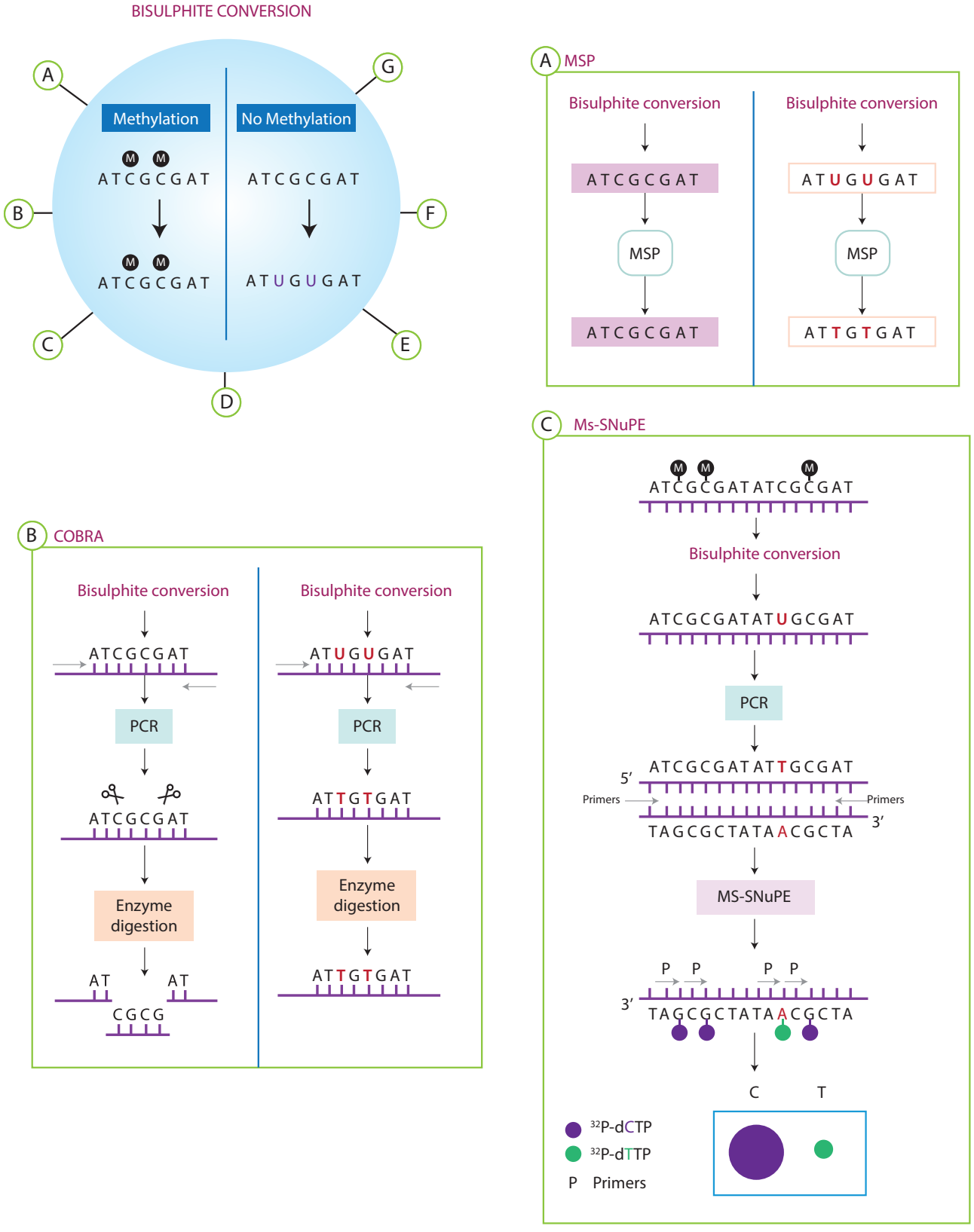
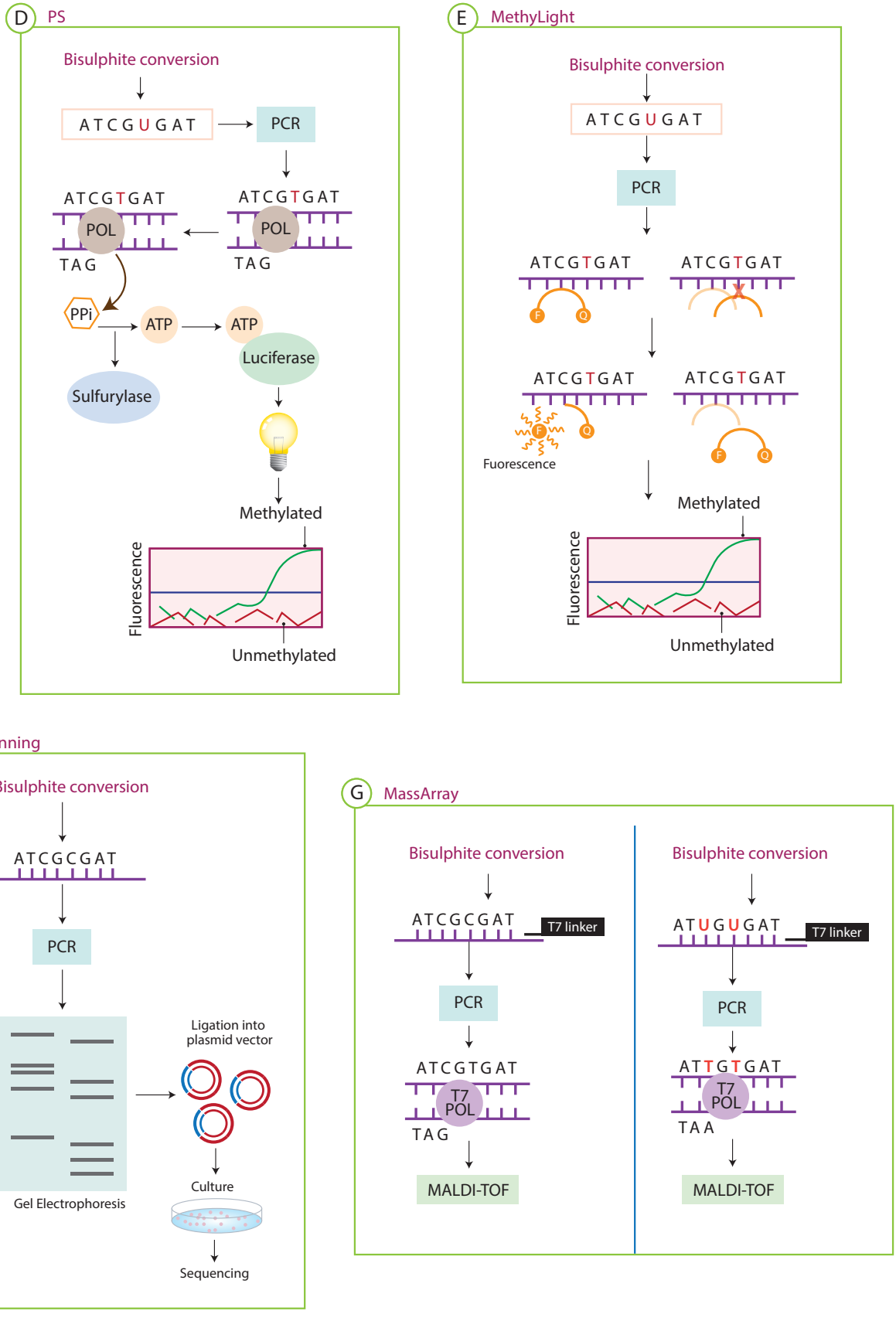


Figure 2. Methylation analysis tools based on bisulphite conversion. **A.** Methyl Specific PCR (MSP), **B.** Combined Bisulphite Restriction Analysis (COBRA), **C.** Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE), **D.** Bisulphite Pyrosequencing (PS), **E.** MethyLight, **F.** Cloning, **G.** MassArray.


D PS
Bisulphite conversion
ATCGUGAT
PCR
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Luciferase
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E MethyLight
Bisulphite conversion
ATCGUGAT
PCR
ATCGTGAT
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F Clonning
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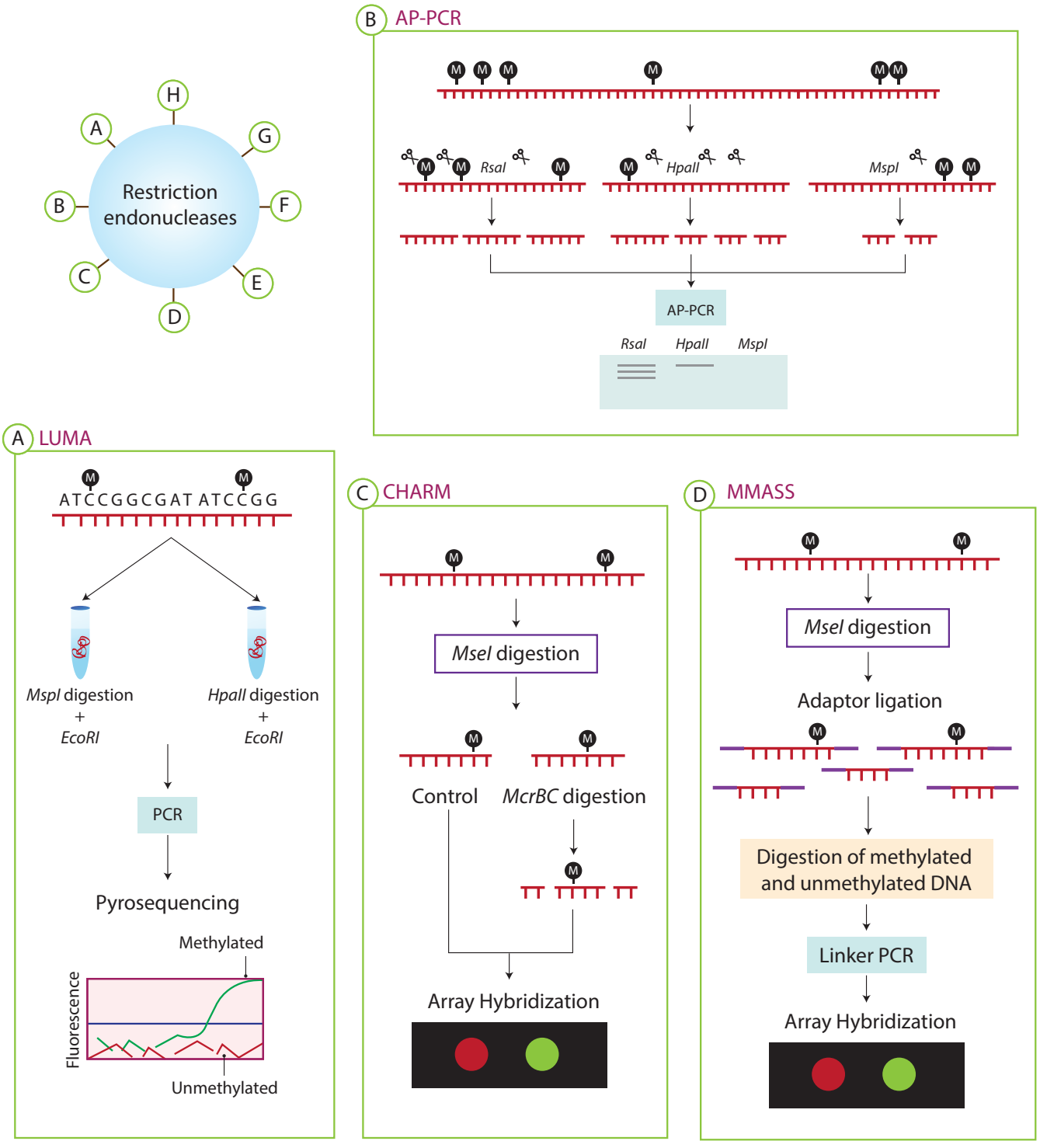
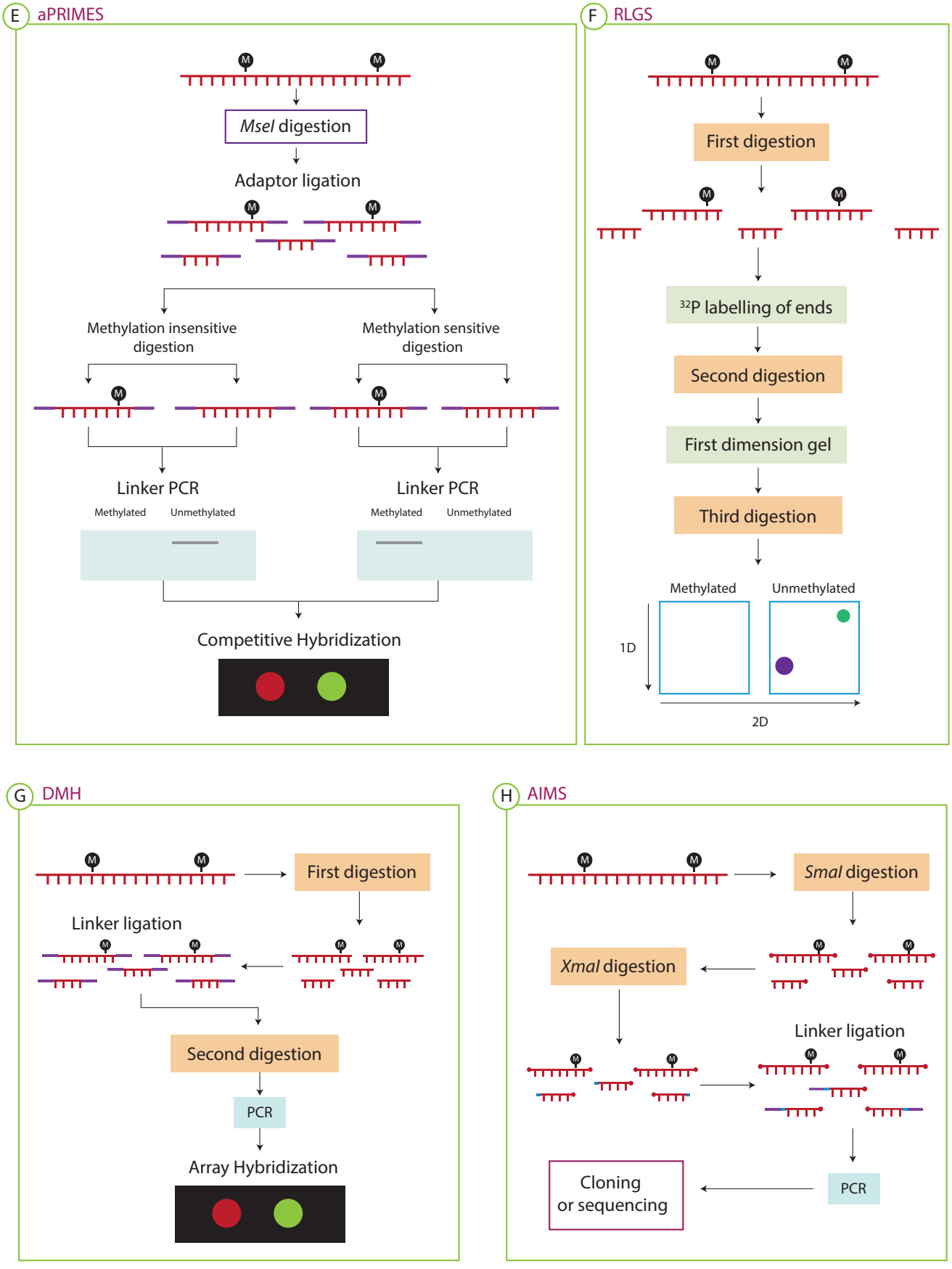


Figure 3. Methylation analysis tools based on restriction endonucleases digestion. **A.** Luminometric Methylation Assay (LUMA), **B.** Methylation-sensitive Arbitrarily Primed PCR (AP-PCR), **C.** Comprehensive High-throughput Arrays for Relative Methylation (CHARM), **D.** Microarray-based Methylation Assessment of Single Samples (MMASS), **E.** Array-based Profiling of Reference-Independent Methylation Status (aPRIMES), **F.** Restriction Landmark Genomic Scanning (RLGS), **G.** Differential Methylation Hybridization (DMH), **H.** Amplification of Intermethylated Sites (AIMS).


E aPRIMES
MseI digestion
Adaptor ligation
Methylation insensitive digestion
Methylation sensitive digestion
Linker PCR
Methylated
Unmethylated
Linker PCR
Methylated
Unmethylated
Competitive Hybridization
F RLGS
First digestion
32P labelling of ends
Second digestion
First dimension gel
Third digestion
Methylated
Unmethylated
1D
2D
G DMH
First digestion
Linker ligation
Second digestion
PCR
Array Hybridization
H AIMS
SmaI digestion
XmaI digestion
Linker ligation
PCR
Cloning or sequencing

pairs. Also, complete denaturation is needed because anti-5mC has more affinity for single DNA strands, so re-annealing will decrease its affinity. Additionally, this tool can be used in FFPE tissue and samples with low quantity of DNA. Currently, there is a modification of this technique named ***methyl-binding PCR (MB-PCR)***, which uses anti-5mC antibody immobilized onto the PCR tube surface to capture methylated fragments and to amplify them within the same tube (Figure 4B). The products from this IP can be analyzed and quantified by microarray or next generation sequencing, thus helping the construction of human methylome maps. A disadvantage of this approach is its inability to identify methylation changes in a specific nucleotide because DNA fragments will have hundreds of them. Furthermore, it is necessary to perform accurate sonication, complete denaturation and a careful antibodies' standardization to avoid possible biases.

Methyl-CpG immunoprecipitation (MCIp)

This approach is similar to MeDIP. Its major difference is the protein used for capturing methylated DNA fragments. This protein is composed of a recombinant methyl-CpG binding antibody, which it is formed by the fusion of a MBD (methyl-CpG- binding domain) with the human IgG1 Fc portion. Therefore, protein complexes have a greater CpG sensitivity than anti-5mC. This protocol uses MBD-Fc protein complex linked to sepharose beads to capture methylated DNA fragments. Next, denatured DNA is incubated with the complex and centrifugated to eliminate unbound DNA (Figure 4C). After this, the product is washed with increasing NaCl concentrations to elute fragments holding the methylated CpGs. The advantages of this tool are its usefulness in locus-specific or genome-wide analysis, and the separation of DNA methylated fragments into portions of increased methylation density during their elution with the salt gradient. This feature allows quantification of DNA methylation density for hypo- or hyper-methylated samples. A limitation of this tool is that the affinity of the protein complex for 5-mC increases as methylation density is higher.

Methylated-CpG island recovery assay (MIRA)

This tool is similar to MCIp; however, it uses methyl-CpG binding domain protein 3-like 1 (MBD3L1: enhances transcriptional repression of methylated DNA by MBD2) fused with MBD2 to form a MBD complex. It is important to highlight that MBD2 has an increased binding affinity for methylated CpGs. The protocol for this technique is the same as was mentioned before (Figure 4D). However, in contrast to previously approaches, MIRA uses a glutathione affinity matrix which can be hybridized on microarrays and it is compatible with ChIP. Also, it has higher specificity than other tools, because its affinity to methylated DNA reduces the possibility of false positive results. This tool has the same limitation as MCIp; additionally, MBD complex binds preferentially to densely methylated CpGs and it requires more than 2 methylated CpG sites for binding.

MICROARRAYS

This tool uses bisulphite-modified DNA as template in a PCR reaction. After amplification, the product is hybridized on glass slides containing oligonucleotide probes, which can distinguished between methylated and un-methylated cytosines from a specific CpG site. The hybridization signals can be detected by fluorescence (typically using Cy3 and Cy5 dyes). Therefore, each probe is designed to hybridize the sequence surrounding a CpG region. This approach can detect as little as 3% of methylated sites and approximately 20% of the differences in methylation patterns between samples. The advantages of microarrays are: multiple genes could be analyzed at the same time and on the same array, it provides absolute methylation values, and is not a time consuming technique. The limitation of this tool is the fact that if CpG loci are not present in the array, methylation patterns from these specific sites will not be determined. Therefore, the array will show us only methylation status from CpG island, and it will exclude methylation patterns from informative sites such as CpG shores or gene bodies. Nowadays, there are a lot of commercial microarray platforms available, as well as custom-made arrays depending of what researchers want to focus on.

For this type of analysis, ***SNP-chips (MSNP)*** also have been used, which contain specific SNPs oligonucleotide microarrays covering the whole genome. This type of microarrays has high resolution and has been employed in methylation analysis. Another approach for this kind of methylation study is the ***comprehensive high-throughput arrays for relative methylation (CHARM),*** which uses a designed array and statistical algorithms for analysis. In CHARM method, once the sample has been prepared (e.g. with methylation dependent restriction enzyme such as McrBC), it is hybridized into a microarray designed with the maximum number of CpG loci (Figure 3C). The product of this microarray is then analyzed with a genome-wide algorithm, which takes into account information from patterns of neighboring sequences. The advantage of this approach is that it allows a wide analysis of samples with high density, specificity and sensitivity. There are others arrays-based techniques such as ***microarray-based methylation assessment of single samples (MMASS)*** and ***array-based profiling of reference-independent methylation status (aPRIMES).*** These approaches combine restriction enzyme digestion with microarrays, and they can be also used for individual CpG loci analysis (Figures 3D and 3E).

WIDE METHYLATION ANALYSIS COMPARING TWO SAMPLES

The first approach is named ***Restriction Landmark Genomic Scanning (RLGS)*** and it is used to recognize differentially methylated DNA from two different samples. The technique uses methylation sensitive restriction endonucleases to create a four base fragment. Then, this product is extended by a polymerase using radio-labeled nucleotides. After this step, the DNA segment is further fragmented with a *non methylation sensitive* restriction enzyme and next, DNA is separated by electrophoresis in first dimension. Finally, a

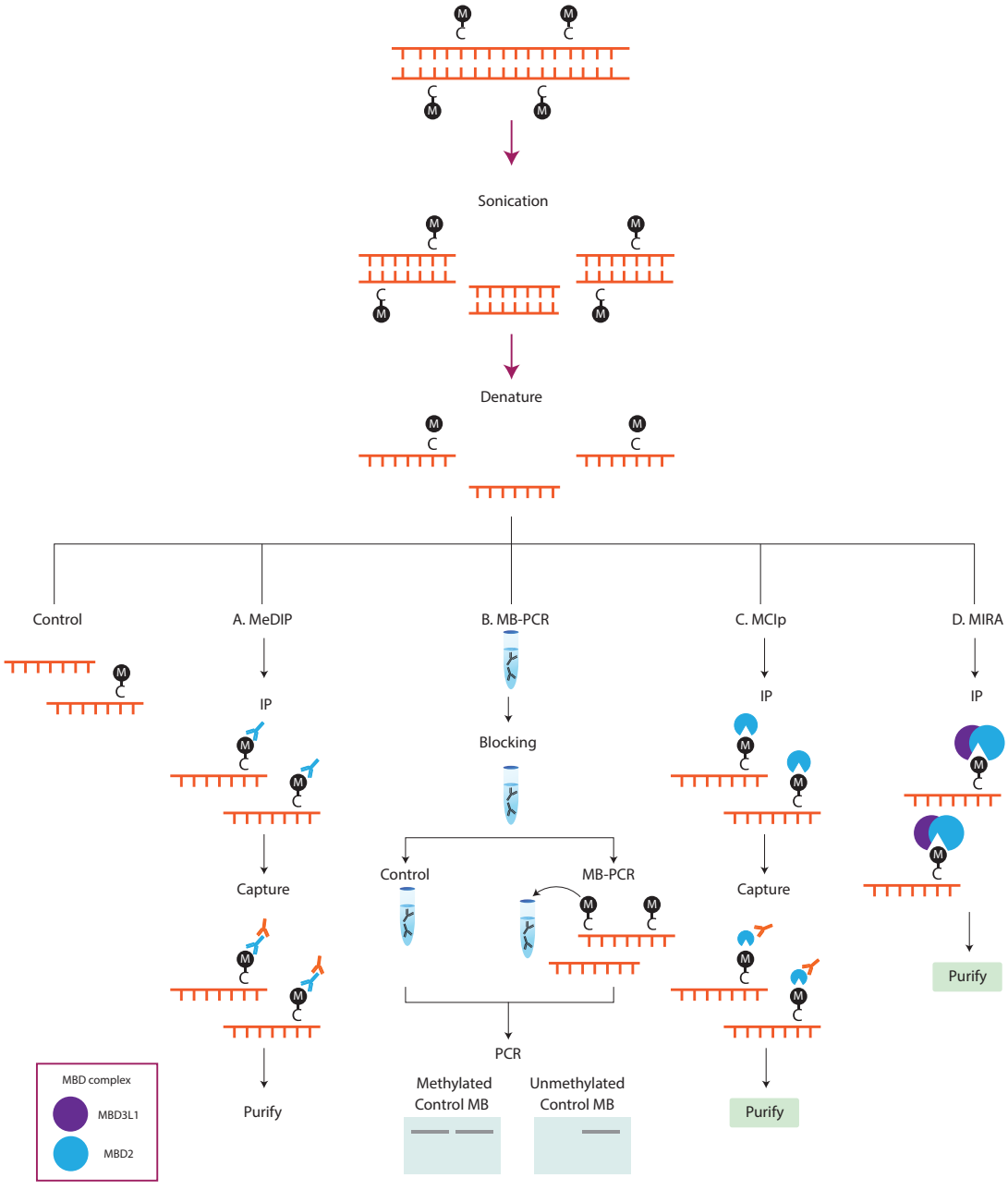


Figure 4. Methylation analysis tools based on immunoprecipitation. **A.** Methylated DNA Immunoprecipitation (MeDIP), **B.** Methyl-Binding PCR (MB-PCR), **C.** Methyl-CpG Immunoprecipitation (MCIp), **D.** Methylated-CpG Island Recovery Assay (MIRA).

gel digestion is performed with a third *non methylation sensitive* enzyme. The product is separated by 2D-electrophoresis, and finally, it is compared by autoradiography (Figure 3F). Therefore, two identical RLGS have to be done to compare the methylated sites in both samples. Its limitations are: long time required, need for large quantities of DNA (5 µg), difficulty of interpretation. RLGS does not give information about location of CpG sites in promoter regions.

The second approach often used for this type of analysis is ***differential methylation hybridization (DMH)***. It also uses restriction enzymes to digest DNA sample, but it needs endonucleases with no recognition for CG dinucleotides within its recognition site (e.g. *MseI*). After digestion, linker ligation is done on DNA and; again, DNA is digested by a *methylation sensitive* enzyme (e.g. *HpaII*). Therefore, un-methylated fragments will be cleaved, while methylated ones will remain intact, and they will be then amplified by PCR. The PCR products from each sample can be subsequently compared using microarrays (Figure 3G). Finally, the last approach named ***amplification of intermethylated sites (AIMS)*** uses

SmaI digestion to remove un-methylated DNA, and then remaining DNA is digested by an enzyme producing sticky ends (e.g. *XmaI*). Next, linker ligation is performed followed by PCR amplification. As a result, PCR products can be separated by electrophoresis or used for cloning or sequencing to identify differentially methylated genes (Figure 3H).

For a summary of all techniques mentioned during this section and their requirements about initial DNA concentrations, please go to Figure 5.

HISTONE MODIFICATION ANALYSIS

Genomic DNA from eukaryotes is found in combination with histones, non-histone proteins and RNA. All these elements together constitute the chromatin, which has a highly dynamic folding and organization within the cellular nucleus. Nucleosome is the initial folding level and responsible for chromatin packaging. This unit is formed by four histone octamers (two copies of H2A, H2B, H3 and H4). Around them, there is compacted DNA of approximately 147 bp. As

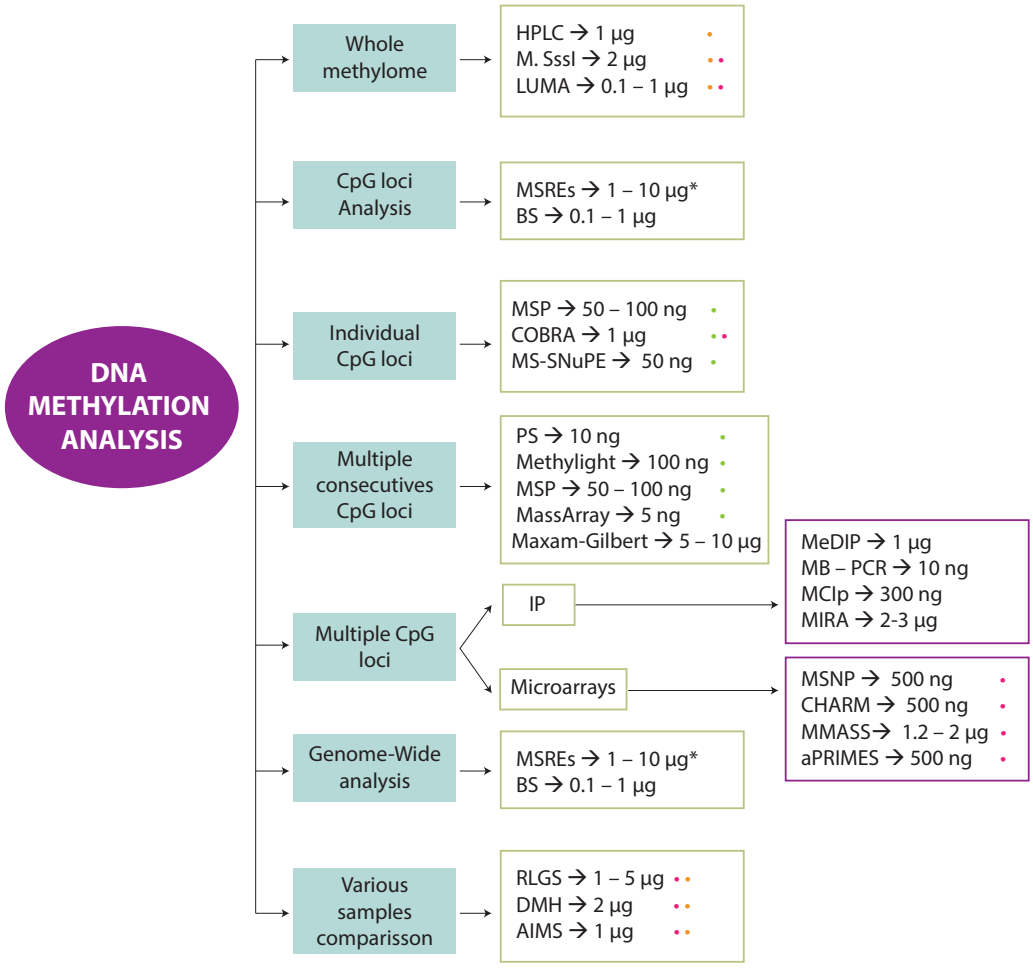


Figure 5. Summary of tools for methylation analysis.

* MREs DNA concentration varies depending on if it is used for southern blot (10 µg) or for PCR (1 µg)

• These tools can be also used for Genome-Wide methylation analysis

• These tools use restriction enzymes for methylation analysis

• These tools use bisulphite modified DNA for methylation analysis

Abbreviations: *HPLC*: high-performance liquid chromatography, M. *SssI*: methyl-acception assay, *LUMA*: luminometric methylation assay, *MSREs*: methylation-sensitive restriction endonucleases, *BS*: bisulphite modification, *MSP*: methylation specific PCR, *COBRA*: combined bisulphite restriction analysis, *MS-SNuPE*: methylation-sensitive single nucleotide primer extension, PS: bisulphite pyrosequencing, *MSRE-PCR*: methylation-sensitive restriction enzyme and various PCR, *AP-PCR*: methylation-sensitive arbitrarily primed PCR, *MeDIP*: Methylated DNA immunoprecipitation, *MB-PCR*: methyl-binding *PCR, MCIp*: methyl-CpG immunoprecipitation, *MIRA*: methylated-CpG island recovery assay, *MSNP*: SNP-chips, *CHARM*: comprehensive high-throughput arrays for relative methylation, *MMASS*: microarray-based methylation assessment of single samples, *aPRIMES*: array-based profiling of reference-independent methylation status, *RLGS*: Restriction Landmark Genomic Scanning, *DMH*: differential methylation hybridization, *AIMS*: amplification of intermethylated sites.

a result, compacting levels increase and they start the formation of nucleosome columns, which interact simultaneously to produce helixes and chromatin loops. Interestingly, these loops are organized on merged layers, thus producing visible chromosomes during cell metaphase because of their tridimensional structure. Moreover, chromatin organization allows differentiation between highly condensed chromatin which is transcriptionally silent (heterochromatin) from less condense one which is transcriptionally active (euchromatin). DNA packaging determines the accessibility of transcription factors to certain genomic regions. Hence, they perform regulatory functions during gene transcription, repair, recombination and DNA replication.

As was mentioned on Chapter 22, some epigenetic modifications may affect structurally the chromatin order, thus altering their chemical properties and certain DNA bases. All these changes are called *Histone Posttranscriptional Modifications*. They are reversible and take place on the amino-terminal tail of histones. These modifications include arginine and lysine acetylation and methylation, serine phosphorylation, and lysine ubiquitination, among others. It is important to mention that all these changes are performed by enzymes such as histone acetyl-transferases (HATs), histone methyl-transferases (HMTs), histone deacetylases (HDACs) and histone demethylases (HDMs).

Historically, classic experimental tools have been used to study histone modification such as: protein hydrolysis, using radio-labeled precursor molecules, mass spectrophotometry, Western Blot and immunofluorescence using specific antibodies recognizing H4 acetylation, H3 methylation, and H2A phosphorylation. The main disadvantages of these methods are their level of difficulty, their cost, and the fact that sometimes they can produce cross-reactions. Nowadays there are many protocols based on molecular biology which are designed to study more in deep chromatin and its interactions. Thus, in this section the most commonly used approaches for studying histone modifications will be discussed.

ELECTROPHORETIC METHODS

Eastern Blotting

This approach is one of the most commonly used methods in biochemistry because it allows post-traslational modification (PTM) analyses such as carbohydrates epitopes, lipids and proteins glyco-conjugated, etc. Moreover, this technique is considered a modified Western Blot (go to Chapter 48 for further information about Western Blot) and similarly to this approach, it includes tissue preparations from whole tissue or cell cultures. When solid tissues are studied, first they are broken down using a blender, a homogenizer or by sonication. Important differences between these methods are detergents, salts and buffers employed to enhance cells lyses and proteins' dissolution. The sample's proteins are then separated by electrophoresis using a polyacrylamide gel and buffers loaded with sodium dodecyl sulfate (SDS) to maintain polypeptides in a denatured state. Next, samples are loaded into gel wells, and proteins migrate at different speeds depending on their size during electrophoresis. In order to make proteins accessible to antibody detection, they are moved from gel onto a nitrocellulose membrane, which contains a modified antibody against the protein of interest linked to a reporter enzyme. Hence, a signal is produced when protein-antibody complex is exposed to an appropriate substrate. Even though this method detects protein modifications, it may present cross-reactions and give false positive results because of its lack of specificity during protein attachment. These limitations complicate the identification of posttranslational modifications within a sample.

HPCE (High-Performance Capillary Electrophoresis)

This method combines electrophoresis used for protein separation with an electrolyte-filled capillary. Therefore, this approach is used for isolation of solutes regarding their ability to migrate within a capillary with an electric gradient. This process allows highly efficient separations, a precise quantification, and the possibility to perform it in an automated manner. Nevertheless, the histone analysis will be limited to small cell populations. As consequence, adjustments have been made using chemical modification within the capillary surface, thus avoiding the interaction between histone cations and the silica surface which prevents false positive results.

FREE SPACE NUCLEOSOME DISTRIBUTION AND POSITIONING IDENTIFICATION ANALYSIS

There are several experimental methods used for analysis of nucleosome distribution along the genome, and to determine if they are randomly or orderly distributed. These methods have some common steps between them including chromatin breaking by sonication or nuclease digestion using deoxyribunoclease (DNase) and micrococcal nuclease (MNase), chromatin immunoprecipitation (ChIP), microarrays and high-throughput sequencing.

Micrococcal Nuclease Digestion (MNase-SEQ)

Micrococcal nuclease is an endonuclease used for isolation of nucleosomal DNA. Once the double strand is cleaved, this enzyme will digest the exposed extremes and will not stop until it finds an obstruction caused by nucleosome or DNA-binding proteins. Depending on enzyme concentrations or exposure time, isolated DNA will show a "scale" effect on the electrophoresis gel. Therefore, each level will represent the presence of a nucleosome. However, depending on every scale size, it will suggest different types of nucleosomes. For example, approximately 150 bp scale belongs to mononucleosomal DNA, 300 bp to dinucleosomal DNA, and 450 bp to trinucleosomal DNA (Figure 6). After electrophoresis, DNA is isolated from the agarose gel and then, it can be processed by hybridization methods or

microarrays. Consequently, the results can be confirmed by high quality sequencing to determine DNA sequences and its localization within the nucleosome.

DNase I Digestion (DNase-SEQ)

In comparison with the micrococcal method, *DNase I* digestion allows identification of free DNA nucleosomes. Studies performed decades ago and some recent findings have demonstrated that regions with transcriptionally active genes are more susceptible to *DNase I* digestion (DNA hypersensitive sites – DHSs) than other genomic regions. Typically, euchromatin is completely digested with a small amount of *DNase I*. Subsequently, the product is purified and digested by a second restriction enzyme recognizing the sites flanking the DHS. Finally, nucleosome analysis is carried out by Southern Blot using specific probes, therefore to perform this approach, knowledge of the target DNA sequence is required (Figure 7).

Additionally, the study of DHSs in a genome-wide manner is now possible thanks to the microarray development and advances in molecular biology tools. Previous studies used techniques such as DNase-Chip and DNase-array. DNase-Chip uses *DNase I* chromatin digestion to create DNA ends that are then ligated to biotinylated linkers. These biotinylated fragments are then captured by streptavidin, labeled with a fluorochrome, and hybridized to high-density oligonucleotides (microarrays). DNase-array depends on two *DNase I* cleavages, which release the regions containing DHSs. After cleavage, there is a fragment-size selection in which smaller fragments are isolated to ensure DHS sites. Then, these fragments are labeled with fluorochromes and hybridized on microarrays.

It is important to highlight that both approaches allow the recognition of hypersensitive *DNase I* sites and the active or inactive chromatin domains. However, both techniques are laborious, and they are used for studies with small cellular populations. Furthermore, it is recommended to confirm the results by either direct or high-throughput sequencing.

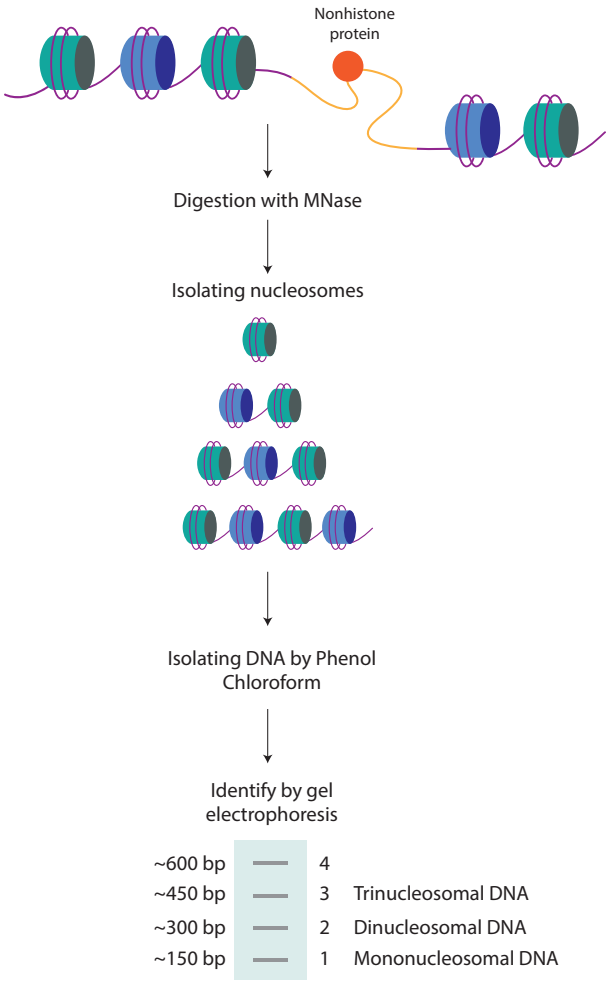


Figure 6. MNase-Seq Micrococcal Nuclease Digestion. Adapted from (10).

FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements)

This tool was developed to identify all nucleosome-depleted genomic regions using few reagents, thus decreasing the labor time during the process. The first step of this approach involves cell cross-linking by fixing with formaldehyde. Then, chromatin is isolated and fragmented by sonication. Subsequently, DNA is isolated using phenol-chloroform-isoamyl alcohol (PCI). This last process exposes free protein-chromatin fragments, which will be found in the aqueous phase of the reaction tube. Finally, fragments can be analyzed by hybridization with high-density oligonucleotides (FAIRE-chip) or by sequencing (FAIRE-seq) (Figure 8). On the other hand, all bound protein fragments within the organic phase will be retained.

SONO-SEQ

This tool is also based on cell cross-linking by fixing with formaldehyde and chromatin sonication. However, it uses reverse cross-linking with a subsequent DNA isolation by phenol-chloroform-isoamyl alcohol. Once DNA is isolated, next step is fragment selection depending on their size (between 100-350 bp), which are then analyzed by sequencing. In contrast with FAIRE, this approach includes the fragment size selection step (Figure 9). The idea behind this selection is that fragments with open chromatin regions are more easily to exclude than closed ones. Therefore, size selection step is important because larger fragments will represent the chromatin that is harder to fragment. This guarantees to obtain only active chromatin pieces and the identification of bigger size-fragments, which represent closed chromatin non-accessible for transcription factors.

CHROMATIN STRUCTURE ANALYSIS BY THE PRESENCE OF HISTONE MODIFICATIONS

Histone modification distribution and its variants, as well as chromatin components within specific DNA sequences, can

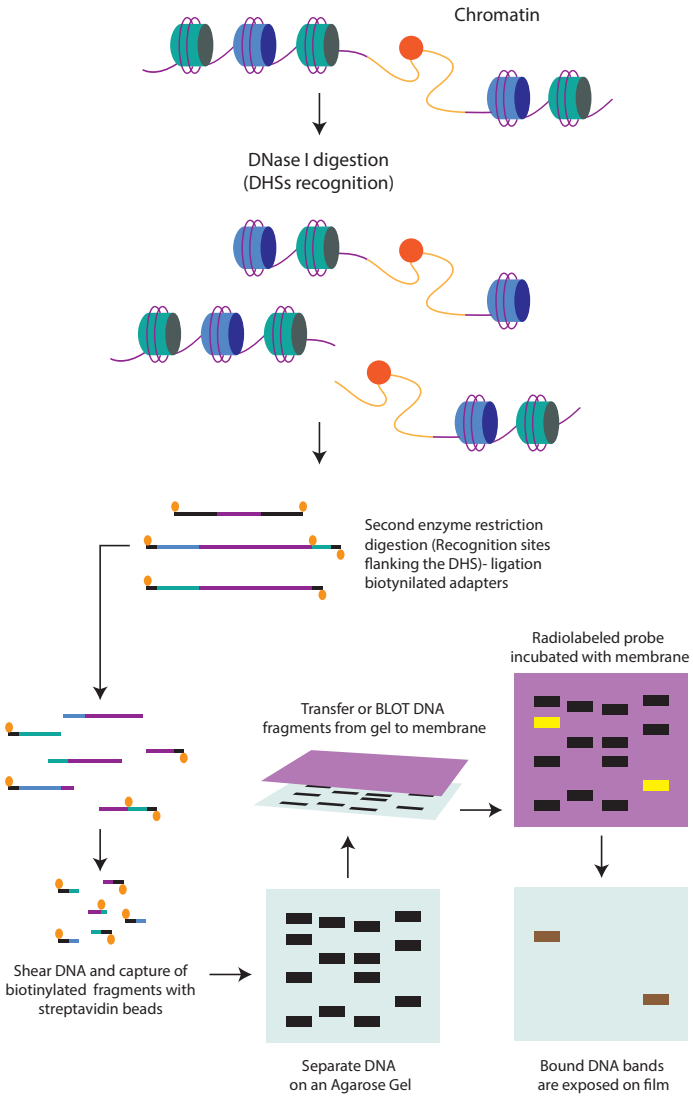


Figure 7. DNaseI Digestion

provide valuable information about chromatin functions and proteins interactions. For this purpose, the most widely used tools are:

ChIP (Chromatin Immunoprecipitation)

ChIP is a biochemical method commonly used to determine protein localization within the genome, or histone modifications such as phosphorylation, methylation, ubiquitination, etc. This approach is based on the use of antibodies against target proteins, which can be diluted or within the chromatin. First, DNA from cells is cross-linked by fixing with formaldehyde to secure protein-protein and protein-DNA interactions. Then, immunoprecipitation of protein-DNA complexes is carried out by using specific antibodies. These antibodies recognize modified histone epitopes from sonicated chromatin. Subsequently, immunoprecipitated fragments are amplified by PCR to determine if they are enriched or not depending on each antibody (Figure 10).

ChIP approach, together with the sequencing (ChIP-seq), is a technique useful for identification of transcription factors binding sites. Both methods allow the generation of genome-wide histone modification maps. Moreover, they allow comparative analyses between histones' dynamic. The drawback is that these analyses do not give us information about nucleosome positioning.

MagPIE (Magnetic Protein Immobilization on Enhancer DNA)

This is a simple, rapid and multiparametric assay which uses flow cytometry immunofluorescence to study interactions between transcription factors, chromatin structure and DNA. For this purpose, genomic regions of interest are amplified by PCR using sequence-specific primers linked to MagPIE primer sequences, which are predicted to have low TF-binding affinity. Subsequently, DNA is further amplified using a MagPIE biotinylated reverse primer and a MagPIE

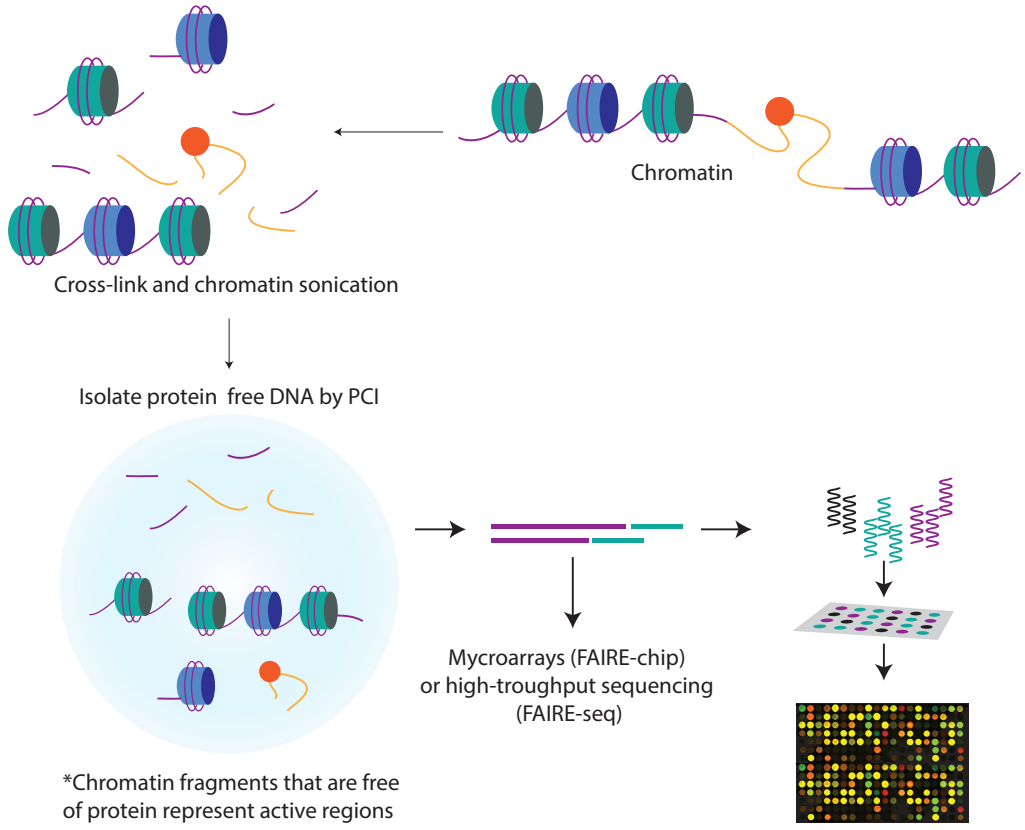


Figure 8. Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE)

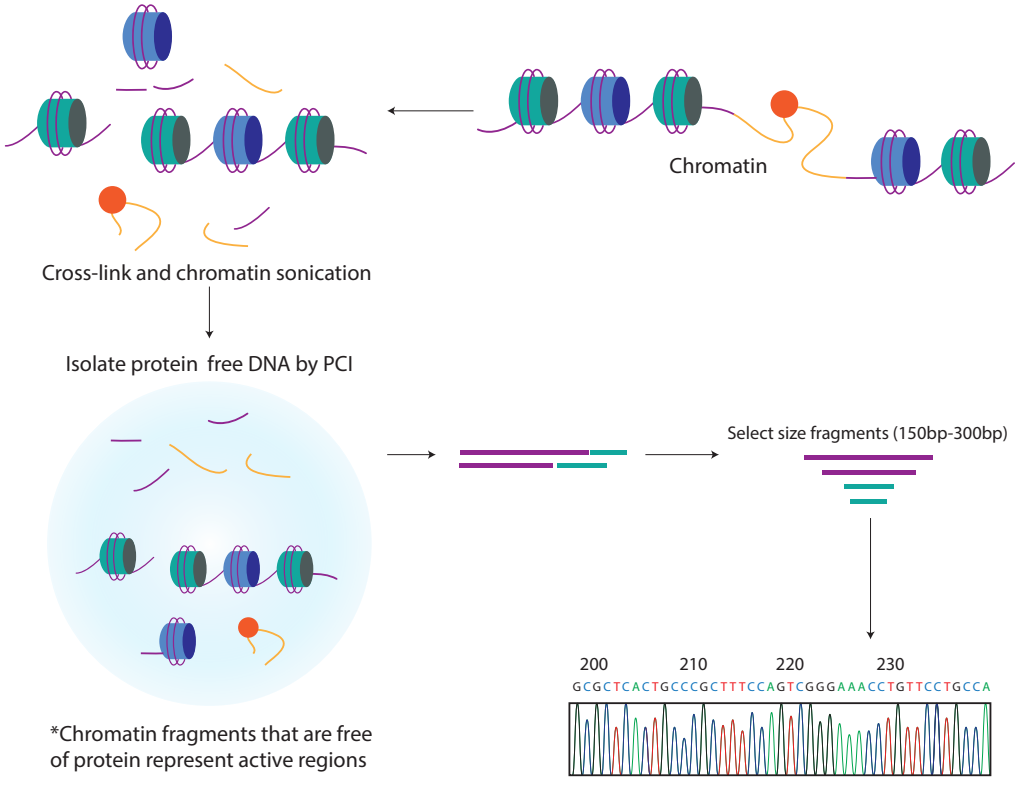


Figure 9. Sono-Seq

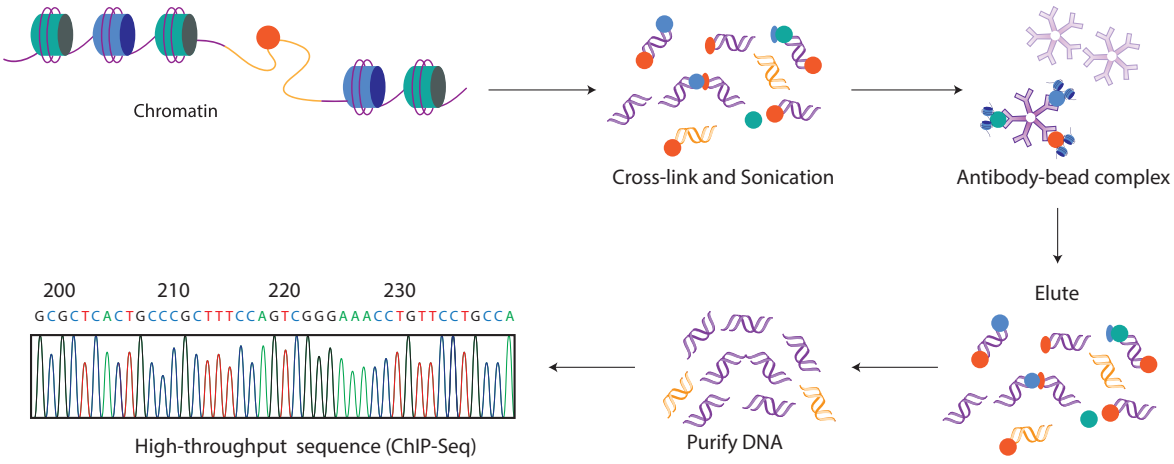


Figure 10. Chromatin Immunoprecipitation (ChIP).

forward primer tagged with a fluorophore. Next, biotinylated DNA is captured by streptavidin-coated magnetic beads. This immobilized DNA is incubated with nuclear protein to lysate the nucleus and then, sequence-specific nuclear factors are pull down in the presence of an alternating copolymer poly (dI:dC) DNA during a 10-min binding reaction at 37°C. Noteworthy, this polymer is used as DNA substrate for DNA methytransfease activity evaluation, because its structure similar to double-stranded RNA is considered a synthetic analog of double-stranded RNA. Finally, transcription factors and other DNA-bound proteins are immunostained with fluorescent-tagged antibodies and its fluorescence intensity is then analyzed by flow cytometry. It is important to highlight that MagPIE allows analysis not only of transcription factors binding sites, but also of epigenetic modifications because nuclear lysate allows DNA to be modified by methylation and be packaged into chromatin. Therefore, chromatin modification could be seen through flow cytometry using immunostained antibodies.

LONG-RANGE CHROMATIN INTERACTIONS DETECTION

Different studies regarding histones and molecular interactions within the genome, have suggested that distal genomic regions within the linear strand of DNA can physically interact with each other. This is achieved by formation of chromatin loops; hence, they regulate gene transcription by getting closer to the distal DNA regions, enhancers and gene proximal promoters. Therefore, the locations and numbers of such loops involved during transcription are been investigated by 3D studies. These studies help furthering the knowledge on chromatin dynamics and the interacting regions within chromatin. In this section, we will describe the most commonly used techniques for studying distal genomic regions within chromatin.

3C (Chromosome conformation capture)

This method was the first established to identify global chromatin interactions. This approach includes cells treatment with formaldehyde to fix (cross-linked) *in vivo* protein - protein and protein-DNA interactions. This is followed by chromatin digestion with restriction enzymes that recognize and cut 6 bp sites, thus generating ligation fragments which are in very close proximity (intramolecular ligation). Then, the cross-linking is reversed and the frequency of ligation events between genomic regions of interest is determined by quantitative polymerase chain reaction (qPCR) using specific primers (Figure 11). Noteworthy, when ligation fragments of interest are unknown, it is important to design primers flanking the promoter and all the restriction sites. In contrast, when ligation fragments are known, primers should be designed only for the interacting target region. It is important to take into consideration for primer design that 3C allows detection of interactions among regions localized in a distance between 100 bp and 1000 bp. Moreover, to detect ligation products between two fragments, PCR primers must be designed in sense or antisense direction. Additionally, a variation of the original 3C method substitutes the restriction enzyme digestion with a sonication step to produce chromatin fragmentation. Therefore, sonication will generate smaller fragments which can be presented with higher affinity and molecular specificity than larger fragments.

4C (Circular chromosome conformation capture and chromosome-conformation capture-on-ChIP)

This approach allows the impartial detection of all genomic regions interacting within a region of interest. Nowadays, there are two variants of this method. In the first one, the binding of the particular region of interest with the fragments is performed in a circular manner. This process

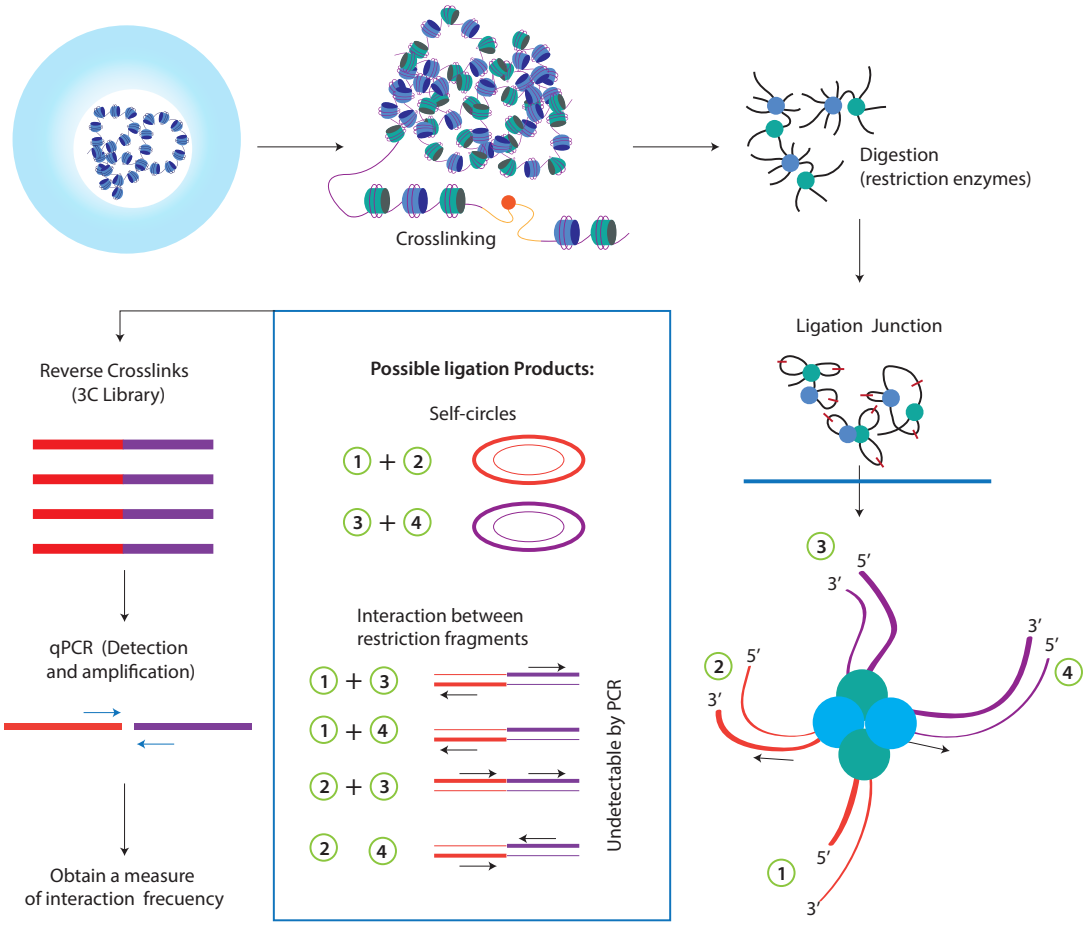


Figure 11. 3C (Chromosome conformation capture). Adapted from Dostie *et al.* (10).

occurs after the restriction enzyme recognizes and cuts 6 bp sites (e.g., *EcoRI*, *HindIII*) as it is in the 3C step. Next, the circular junction produces the binding between the extremes of one fragment with the ends of the particular region of interest. Therefore, the method 4C is represented by the circular chromosomal conformation. After chromosomal binding, cross-linking is reversed and amplification of region of interest is done by PCR using specific primers (Figure 12A). Finally, the interacting regions and the target element are identified by microarrays and high-throughput sequencing.
In the second variant, a cleavage of 6 bp is done with restriction enzymes to digest the chromatin cross-linked. Because the products of this digestion are lineal fragments, only an end of each one of them can bind to a particular sequence of interest. After this step, the cross-linking is reversed and the fragments forming the linear pieces are cleaved with a 4 bp cutter restriction enzyme. This product will then induce an auto-ligation thus producing circular sections, which will be analyzed similarly to the circular variants form during 3C (Figure 12B).

5C (Chromosome conformation capture carbon copy)

This assay is a high-throughput version of 3C. It contains the same 3C steps, but with a main difference in the last step used for identification of the interacting fragments. Specifically, multiple PCR primer pairs are used, and each one of them contains different genomic sequences of interest. The primer mix is combined with a 3C genomic library to allow the annealing of only a specific number of primer pairs which enlarge the ligation junctions of the 3C library fragments. This mix contains two types of 5C primers: 5C forward and 5C reverse primers. These are designed in such a way that forward and reverse primers anneal across ligation products present in the 3C library. 5C primers that annealed next to each other are then ligated by Taq ligase allowing the generation of 5C library. The library is amplified with universal PCR primers - multiplex ligation-dependent probe amplification (MLPA) - annealing the tails of the 5C primers. Only the ligated primer pairs, reflecting a portion of the 3C library containing the interacting fragments are amplified (Figure 13). These PCR products are then identified by microarrays or high-throughput sequencing.

6C (Combined chromosome conformation capture ChIP cloning)

This method is based on the 3C procedure; but, it also includes a ChIP step after the intermolecular ligation step.

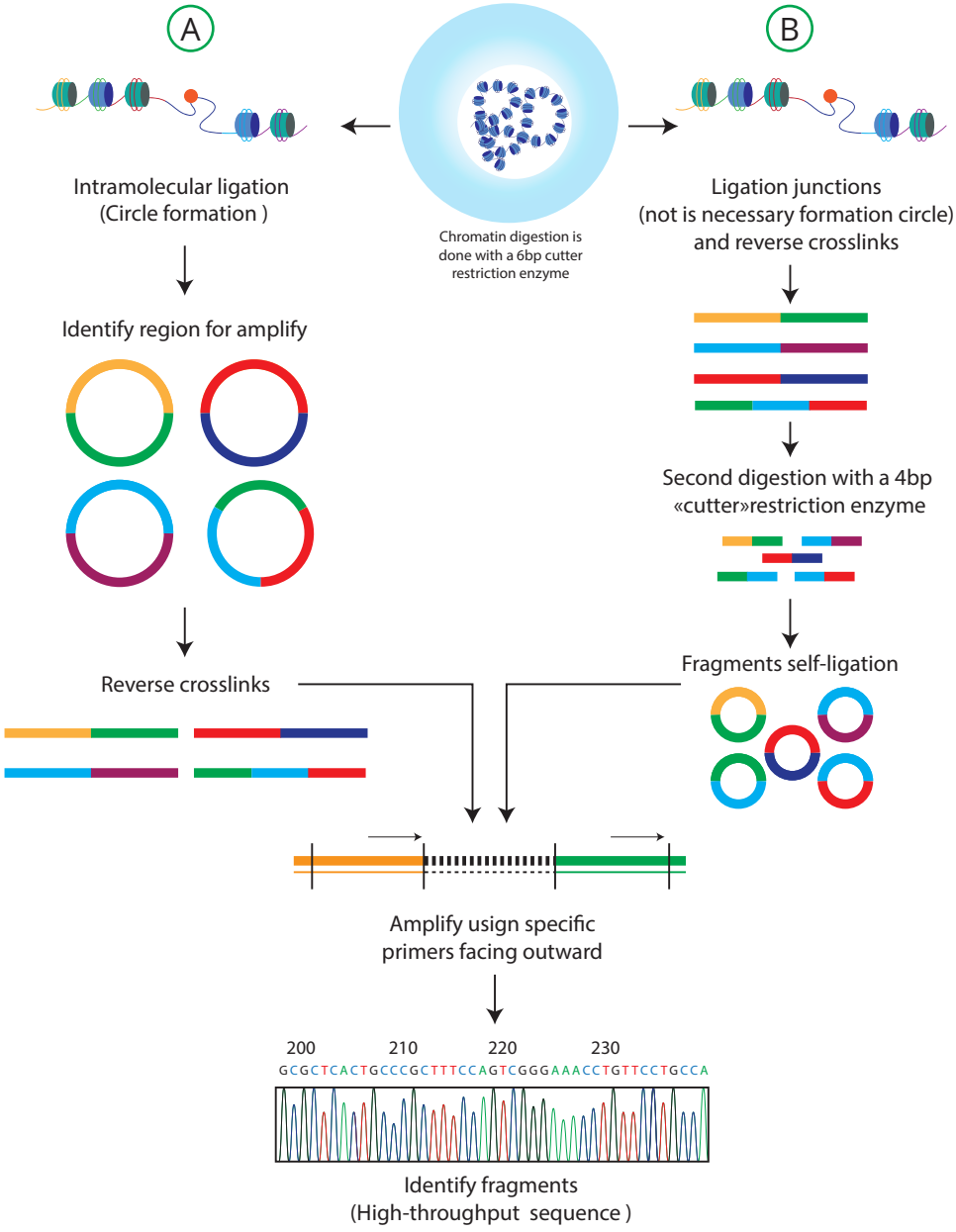


Figure 12. 4C (Circular chromosome conformation capture). A. 4C-1, B. 4C-2. Adapted from Dostie *et al.* (10).

This process is followed by the ChIP DNA isolation, cloning into a vector, and subsequent transformation into bacteria. The clones are then cleaved into multiple fragments after digestion with the same restriction enzyme used to build the 3C model. Later, these fragments are sequenced from both ends to identify their interacting partners. This method has the advantage of the annealing specificity. However, this approach is time-consuming, and does not allow genome-wide studies because it requires restriction enzymes and individual cloning.

The methods mentioned above allow an initial approximation to identify specific sites for chromatin interaction, but they require the design of large scale, high-density oligonucleotides to assess each interaction site within the sequence. Because of this fact, they are not recommended for metagenomic analyses. Therefore, new techniques in molecular biology have been developed to identify global chromatin interactions such as "global looping interactions", which was designed for yeast and human research.

Global 3C Interactions (HI-C)

This assay principally requires cellular cross-linking by fixing with formaldehyde as well as chromatin digestion with restriction enzymes. The digestion process produces an overhang in each 5´end of DNA fragments which are filled with complementary nucleotides, and one of these nucleotides is

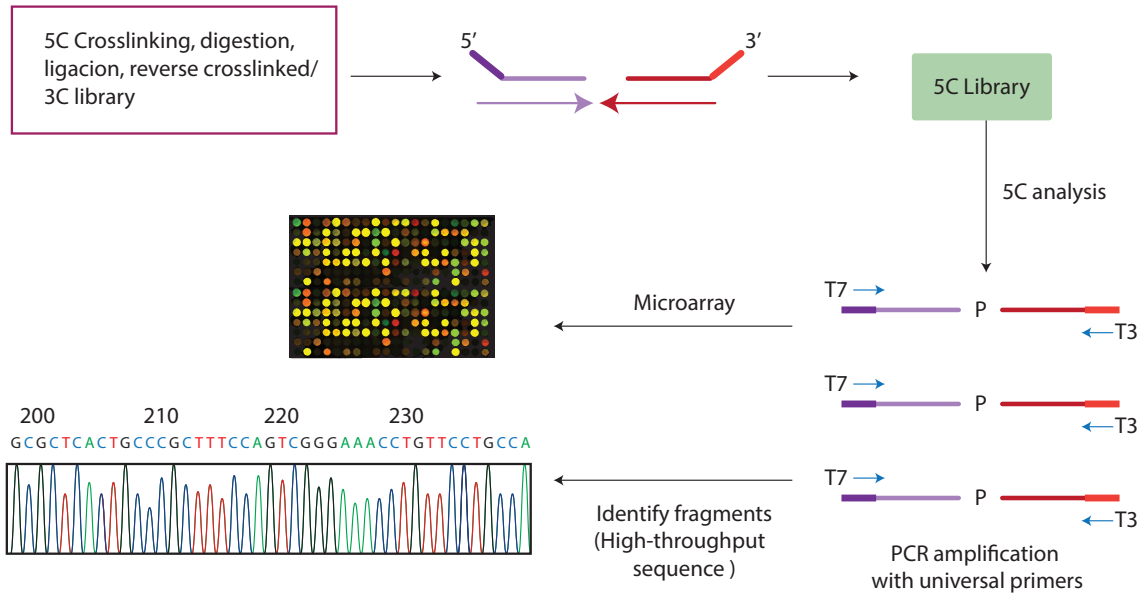


Figure 13. 5C (Chromosome conformation capture carbon copy). Adapted from Dostie *et al.* (10).

biotinylated. After this step, chromatin dilution is done and ligation is performed to favor intramolecular binding events in a circular manner. Subsequently, cross-linking is reversed and fragments with biotin at their ligation junctions are amplified and then isolated with streptavidin. Finally, they are identified by high-throughput sequencing (Figure 14). Nowadays, application of Hi-C in humans shows that open and closed regions of chromatin are located at different parts of the nucleus.

CHIA-PET (Chromatin Interaction Analysis by Paired-End Tag Sequencing)

The ChIA-PET method, like Hi-C, allows the detection of long-distance chromatin interactions in an unbiased fashion. The main steps of this technique include cellular cross-linking and chromatin sonication. After the steps mentioned for Hi-C, ChIP is carried out to enrich the fragments bound by a particular protein of interest. The fragment ends are then ligated to biotinylated linkers, which contain *Mme1* restriction sites. At the same time, under dilution conditions, biotin process allows the binding of different chromatin fragments by its complementarity. Because chromatin is folded during this step, next step is to reverse cross-linking and then, a digestion with the *Mme1* restriction enzyme is performed. It is important to highlight that this restriction enzyme cuts 20 bp downstream of its restriction site. After digestion, isolating fragments containing the ligation junctions are captured by streptavidin, and are then identified by paired-end sequencing.

The target fragment of interest will be formed by the binding of each middle portion from previously digested *Mme1* biotinylated fragments. Therefore, when the biotinylated tags are mapped taking into account the reference genome, they will be represented by a sequence with a length of less than 3,000 bp between each other. It is important to mention that ligation events between different fragments within chromatin may be represented by biotinylated tags located in regions from the same or different chromosome, thus reflecting long-distance interactions. An advantage of this approach is that it detects protein binding sites, which could be found easily by performing a standard ChIP experiment.

CONCLUDING REMARKS

As has been shown along this chapter, there are many options for epigenetic analysis. The choice of analysis tools depends on the goal of the study and what questions researchers want to analyze. It is important to highlight that epigenetic analyses are not made exclusively using "gold standard" techniques; but by a combination of approaches ranging from simple to high-throughput technologies. Another important point to take into account when researchers want to study epigenetic mechanisms is that all of them are tissue-specific. Therefore, it is essential to know *a priori* what is the target tissue in the study and how it was stored. The last point is important because depending on the tissue storage (e.g., FFPE or frozen), DNA concentrations may vary. For example, is well known that low DNA concentrations are extracted from FFPE tissues, while higher concentrations are extracted from frozen tissue. Therefore, it is important to choose the right technique which will give the best results when using low or high DNA concentrations. Also, researchers have to take into consideration, what kind of study they

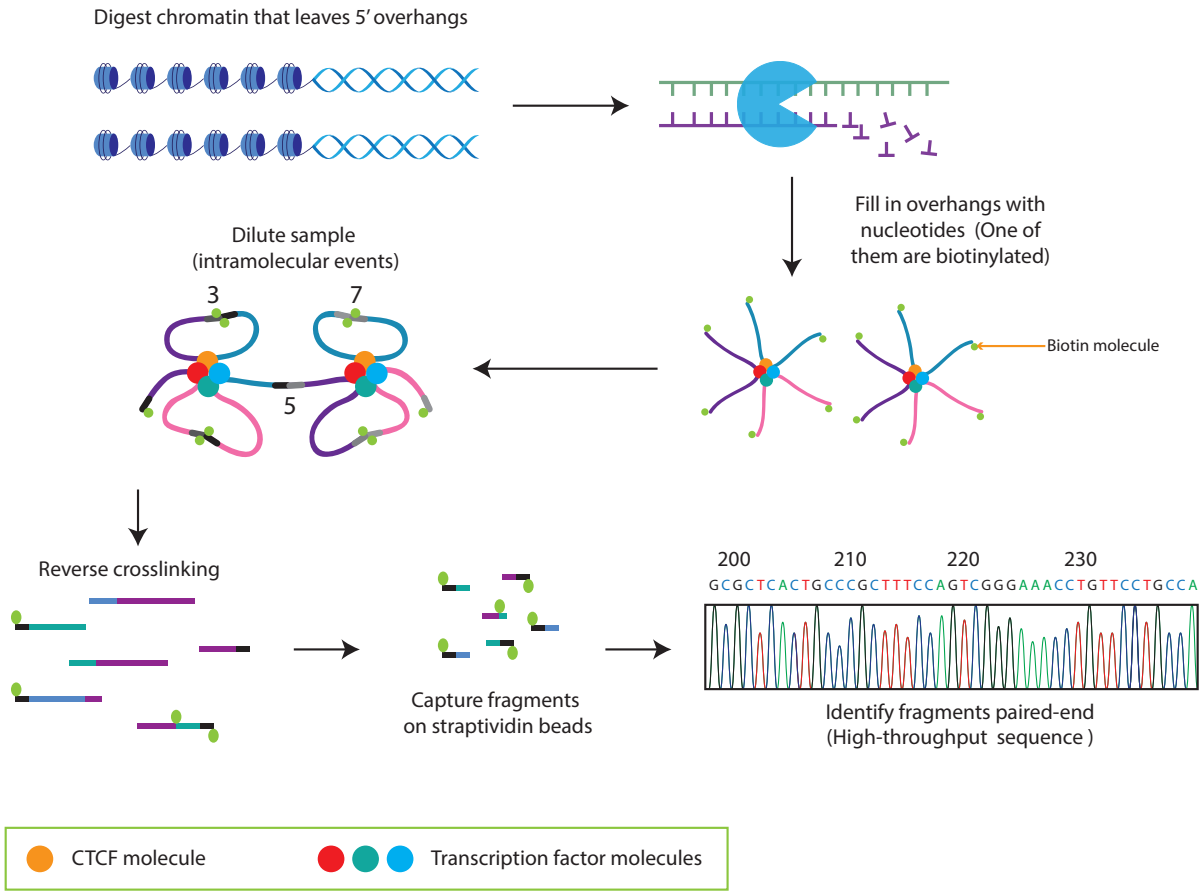


Figure 14. Global 3C Interactions (HI-C). Adapted from Dostie *et al.* (10).

want to perform (genome-wide vs. site specific) because depending on their hypothesis, researchers can choose the better option to accomplished their goal of study, and use the method that is available in their laboratories. Finally, considering how fast epigenetic studies have been growing through recent years, we can expect that in the near future there will be more high-throughput techniques which would be even more sensitive and specific to study DNA methylation and Histone modifications.

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INDEX

A

Abatacept, 414-416, 464, 469, 684,
Acetyl choline receptor, 118, 332,
Acetylation, 20, 29, 129, 172, 187, 346, 355, 360
Acute aseptic meningitis, 335
Adalimumab, 414, 464, 469, 649
Addison´s disease, 239, 240, 264, 588
Adrenocorticotropic hormone (ACTH), 201
Adultered rapeseed oil, 330
Adult-onset Still's disease, 648
Agarose, 757
Age at onset, 238-239, 381, 485, 486, 498, 573, 635, 636, 659
Age, 260
Age-related macular degeneration, 68
Aging, 185-195
Aging–associated B cell (ABC), 192
AIRE (Autoimmune regulator), 25-27, 523
Alemtuzumab, 706, 712
Allelic discrimination, 772
Allelic exclusion, 86, 103
Allergy, 69
Allogeneic transplantation, 705
Alopecia areata, 264, 551,
Alternative activated macrophages (AAM), 309
Alveolar epithelial cells, 33, 38
Alveolar macrophage, 330
Alzheimer's disease, 700, 702
Amoebas, 247
Amplification of Intermethylated Sites (AIMS), 820, 823, 824
Amyloidosis, 401, 403, 476, 478
Anakinra, 414, 650
Anaphylatoxins, 59-61
Anatomical barriers, 31
ANCA-associated vasculitis, 466, 469, 621-623, 632-634, 638, 686, 702, 703
Ancestral 8.1 haplotype, 278
Ancestry, 238, 241, 242
Androgens, 201
Anion-exchange, 756
ANKRD55, 290
Ankylosing spondylitis, 239, 243, 261, 264, 271, 277, 344, 404, 647
Annelids, 248
Annexin, 228, 445, 456, 457, 459, 600
Anoikis, 220
Antagonistic pleiotropy, 242
Antibiotic resistance, 761
Antibodies, 77, 97, 98, 116-118
Anti-α fodrin antibodies, 489,Ç
Anti-apolipoprotein A-1 antibodies, 657, 659
Anti-β2 glycoprotein-1 antibodies (β2GPI), 345-347, 443, 446, 455, 457
Anti-cardiolipin antibodies, 329, 335, 443, 446, 447, 455-458
Anti-CD3 antibodies, 519, 525, 528-531
Anti-centromere antibodies, 487, 488, 489, 491, 577, 668, 669
Anti-citrullinated peptide antibodies (ACPA), 273, 326, 345, 382, 383, 386, 388, 392, , 396, 398, 485-491, 658, 692, 693
Anti-deaminated gliadin peptides antibodies, 588-590
Anti-double-stranded DNA antibodies (anti-dsDNA), 53, 54, 107, 121, 329, 331, 334, 337, 359, 362, 429, 431, 433, 434, 436, 437, 460, 462, 490
Anti-endomysium antibodies, 585, 589, 590
Anti-GAD autoantibodies, 524, 525, 527, 528, 530
Anti-glomerular basement membrane antibodies, 622-624
Anti-gp210 antibodies, 574, 576, 577
Anti-histone antibodies, 333
Anti-IA2 autoantibodies, 524, 525, 527
Anti-idiotype antibodies, 116
Anti-insulin autoantibodies, 105, 524, 525, 527, 528
Anti-islet antibodies, 525, 527, 528
Anti-Ku antibodies, 489, 490
Anti-La/SSB antibodies, 479-491
Anti-malondialdehyde-modified LDL antibodies, 659
Anti-mitochondrial antibodies, 489, 490, 573, 576
Anti-modified citrullinated vimentin antibodies (anti-MCV), 393, 659
Anti-neutrophil cytoplasmic antibodies (ANCA), 490, 564, 624
Anti-nuclear antibodies (ANA), 279, 281, 328-333, 428, 431, 435, 436, 485, 486, 489, 490, 543, 564, 565, 574-577
Anti-nucleosome, 431, 433
Anti-phospholipid antibodies (aPL), 307, 443, 447, 448, 455-459, 490, 655, 669
Anti-phosphorylcholine antibodies, 659
Anti-Ro/SSA antibodies, 431, 462, 489-491
Anti-Sm antibodies, 431, 436, 437
Antiprothrombin antibodies 457
Anti- thyroglobulin antibodies (TgAb), 538-554
Anti-thyroperoxidase antibodies (TPOAb), 538-554
Anti-tissue transglutaminase, 585, 588-590
Anti-TSHR antibodies (TRAb), 538-547, 549, 550

Antibody affinity, 793
Antibody analysis, 793-810
Anticoagulant, 743
Anticonvulsants, 333
Antigen, 47, 77-80, 82, 84-92, 97-101, 103-105, 107, 108, 114-121, 250, 251, 252, 292,307, 348, 361
Antigen presentation, 173, 176-181
Antigen presenting cells (APCs), 79, 82, 97, 105, 116, 117,127, 169, 176-178, 560, 733
Antimalarials, 660, 664, 666
Antimicrobial peptides (AMPS), 35
Antiphospholipid syndrome, 69, 239, 240, 303, 344, 345, 655, 665, 666, 713
  Epidemiology, 443, 444
  Obstetrics consideration, 450
  Pathophysiology, 444, 445
  Clinical manifestations, 445
  Pregnancy, 455-459
  Treatment, 448-451
Anti-thyroid drugs, 544, 550
Anti-TNF, 403, 414-416, 433, 464, 469
AP-1, 4, 25, 50, 80, 81, 89
APC, 332
Apoptosis, 22, 48, 54, 66, 78, 82, 85, 87, 89, 100, 101, 103, 105-107, 115, 116, 118, 126, 219-225, 278, 295, 324, 328, 358, 359, 361, 499, 733
APRIL (A proliferation inducing ligand), 86, 89, 92, 101, 106, 108, 135, 157, 501
Argonaut protein family (Ago proteins), 721
Array-based Profiling of Reference-Independent Methylation Status (aPRIMES), 820, 822, 824
Artificial immune networks (AINE), 373-377
Asolute quantitation, gene expression, 771
Aspirin, 448, 449, 458, 467
Association studies, 257, 260, 265, 291, 285, 286
Atacicept, 108
ATF-2, 88
ATG16L1, 290, 295
ATG5, 290, 295
Atherosclerosis, 483, 653-655
ATM, 190, 195
ATR, 190
ATXN2, 288
Atypical hemolytic uremic syndrome, 68
AUF1, 19
AU-rich element (ARE), 14, 19
Autoimmune ecology, 238, 241, 242, 321, 323, 336
Autoimmune gastritis, 239
Autoimmune hepatitis, 239, 329, 333, 336, 481, 485, 488, 490, 563-570, 575, 577
Autoimmune hepatitis, type I, 564-570
  Clinical manifestations, 564
  Diagnostic criteria, 566
  Immunopathogenesis, 568
  Laboratory findings, 564
  Recurrence, 566, 567
Autoimmune hepatitis, type II, 565
Autoimmune lymphoproliferative syndrome (ALPS), 116
Autoimmune mucocutaneous blistering disease, 702
Autoimmune-polyendocrinopathycandidiasis-ectodermal dystrophy (APECED), 10
Autoimmune tautology, 237-245, 479, 550, 653
Autoimmune thyroid disease (AITD), 382-403, 480, 481, 485, 486, 488, 489, 497, 519, 524, 537-544, 547, 550-554, 576, 659, 660
Autologous transplantation, 705, 706
Automated Sequencing, 779
Autonomic nervous system, 201-208
Autophagy, 225
Azathioprine, 437, 463, 464, 466, 468, 488, 505, 507, 528, 530, 564 566, 578, 628, 629, 632, 634-636, 649, 662, 664
Azurocidin, 228

B

B7, 82, 89, 116, 176
B220, 86, 98
B cell, 20, 26, 28, 77-93, 97-108, 113-119, 190-194, 361, 699, 703, 705, 709, 723
B cell-activating factor (BAFF), 86, 89, 92, 100, 101, 105-107, 500-502
B cell-attracting chemokine (BCA), 100
B cell follicles, 84, 100
B cell helper neutrophils (Nbh), 92
B cell linker protein (BLNK), 88
B cell maturation factor (BCMA), 89, 106, 108
B cell receptor (BCR), 20, 51, 77, 85-90, 92, 97, 100, 103-107, 115, 118, 192, 193
B cell tolerance, 103, 105, 106, 115
B lymphocyte stimulator (BLyS), 105
Bacillus Calmette-Guerin (BCG), 51
Bacteria, 761
Bacteriophage λ, 761, 762
Bacteriophage/Phage, 760
BANK1, 26-27, 288, 293
Basophils, 36, 92
Bcl-2 protein family, 221, 230
BCL-6, 84
Behçet's disease, 467, 623, 635, 636, 648
Belimumab, 108, 508
Beta2 (β2)-microglobulin, 120, 169, 174, 175, 177, 181, 483, 489
Beta cell (β cell), 120, 121
Beta-adrenergic receptor, 205
Beta-globin, 4
Beta-haemolytic streptococci, 118
Bim, 191
Biodiversity, 247
Biologic agent, 699, 703
Biological pleiotropy, 242
Biological therapies, 705
Biomarkers, 722, 732,
Biosensors, 728, 735
Biostatistics, 257, 265
Bisulphite modification, 814-816, 824
Bisulphite Pyrosequencing (PS), 816, 818, 824
BLK, 288, 293
Blood drawing, 743

Blood sample, 743
Bone marrow, 77, 78, 85-87, 90-92, 97, 99, 101-104, 706, 709
Borrelia spp, 539
BP1, 86
Breastfeeding, 458
Briobacept, 93, 108
British Isles Lupus Assessment Group, 664
Bromocriptine, 205
Bullous pemphigoid, 602-604
Butterfly rash, 432
Bystander activation, 304, 306, 307

C

C reactive protein, 32, 295, 396-398, 404-406, 654, 657
C1 inhibitor, 62, 66, 68, 70, 71
C1q receptor for collagen region, 64
C1q, 57-68, 118, 228-231
C1q-binding protein, 65
C1r, 58-60, 68
C2 receptor inhibitor, 64, 65
C2, 296
C2a, 58-60
C2b, 58-60
C3, 434, 462
C3a, 57-68
C3a-desArg, 57-61
C3aR, 57, 61, 64, 65, 69
C3b, 57-68, 447
C3c, 59, 61
C3d, 57-68, 89, 118
C4, 334, 362
C4a, 57-68, 428
C4b 57-68, 428
C4-binding protein, 62, 63
C5a, 62, 63, 226, 447
C5a-desArg, 58-61
C5aR, 64, 65
C5b, 58-61
C5L2, 64, 65
C6, 58-61
C7, 58-61
C8, 58-61
C9, 58-61
Calcineurin, 80
Calmodulin, 80
Calnexin, 177-179
Calreticulin, 177-179, 274, 275
Cancer, 19, 24, 69, 683-686, 721
Capillaroscopy, 611, 612
Capping, 6, 7, 9, 10, 12-14, 29
Carboxyl terminal domain (CTD), 6-8, 10, 11
Carboxypeptidase-N, 61-63
Cardiovascular disease, 653-682
Cartilage, 381, 387, 391, 393, 394, 395, 398-400, 407, 409-411
Caspase-3, 221, 330
Caspase-8, 116, 221
Caspase-9, 330
CAT, 4
Catalase, 228
Catastrophic antiphospholipid syndrome (CAPS), 443, 444, 447-449
Cathelicidin, 35
Cathepsin G, 228
CBLB, 288
CBP (CREB-binding protein), 172
CD1, 26, 79, 90-92, 108, 180-182
CD3, 78-80
CD4, 77, 78, 80, 82, 84, 85, 100, 101, 104, 115, 117, 119, 120, 176, 177, 459-461, 709, 723, 726, 727, 734
CD5, 20, 90, 91, 100, 187, 189, 358
CD6, 289
CD8, 21, 77, 78, 80, 85, 115, 117, 119, 120, 121, 176, 177, 187-194, 361, 709, 711
CD9, 90
CD10, 86, 100
CD11a, 187
CD19, 86, 88-91, 100, 104, 108, 189, 192
CD20, 90, 100, 108
CD21, 90, 100, 108, 118
CD22, 89, 90, 100, 107
CD23, 90, 100, 108
CD24, 86, 91, 100, 108
CD25, 78, 82, 84, 85, 117, 189, 191, 361, 537, 538, 568, 709
CD27, 81, 90, 91, 101, 188, 190, 192, 359
CD28, 19, 21, 81, 82, 89, 116, 118, 119, 188-192, 194,195
CD34, 709, 711
CD38, 90, 91, 100, 101
CD40, 52, 89, 91, 100, 101, 107, 108, 384, 388, 537, 538, 575, 576, 665
CD40L, 81, 82, 84, 89, 92, 101, 288, 555
CD43, 86
CD44, 26, 78, 190, 288
CD44, 190
CD45, 27, 80, 89, 90
CD45RA, 189, 195
CD45RO, 188, 189, 567
CD54, 52
CD56, 189
CD57, 189
CD58, 289
CD69, 288
CD70, 90, 191, 359, 361
CD72, 27
CD79, 87, 100
CD80, 82, 85, 90, 116, 120, 288
CD83, 52
CD85, 188, 190
CD86, 52, 53, 85, 90, 116, 120, 192
CD94, 190, 191
CD95, 189
CD96, 190
CD99, 18
CD122, 82
CD132, 82

CD137, 27
CD158, 191
CD161, 83
CD226, 288, 293, 523
CD247, 78, 88
CDKN2A, 187
cDNA libraries, 761
cDNA, 761, 762, 767
Celiac disease, 239, 260, 277, 351, 404, 483, 519-521, 551, 583-592
Cell aging. See aging
Cell analysis, 741-753
Cell count, 752
Cell culture, 741-753
Cell death, 24, 25, 219-230
Cell lymphotoxicity, 52
Cell lysis, 755
Cell sample, culture, 742
Cell separation, 744-746
Cell storage, 746
Cell Thawing, 746, 747
Cell viability, 752, 753
Central tolerance, 103, 104
Centrifugal elutriation, 745
Centrifugation, density gradient, 744
Centrifugation, differential, 744
Centrifugation, isopynic, 745
Centrifugation, zonal, 745
Centroblasts, 90, 100, 101
Centrocytes, 90, 100, 101
Cerebellar transitory ataxia, 335
Certolizumab, 734
c-fos, 81
CGRP (Calcitonin-gene-related peptide), 204
Chagas' cardiomyopathy, 344
Chain-terminating Maxam, 778
Chemokine-receptor, 137, 138, 161-163
Chemokines, 32, 43, 77, 83, 84, 87, 89, 90, 92, 100, 101, 160-163, 249
Chemotherapy, 706, 707, 711
Chia-PET, 791, 832
Chimerism, 261
ChIP-Seq, 791
Chlamydia pneumoniae, 577
Cholestasis, 573, 577
Chromatin Immunoprecipitation (ChIP), 827, 829
Chromatin Interaction Analysis by Paired-End Tag Sequencing (CHIA-PET), 832
Chromatin remodeling, 5, 7, 8, 15, 18-21, 102, 187
Chromatin, 3, 4, 5, 7-9, 15, 18-22, 102
Chromosome, 3, 15, 21-24, 98, 114, 169, 170
Chron's disease, 360
Churg-strauss syndrome, 466, 467, 621-623
Cigarette smoking, 325-328
CIITA (Class II transactivator), 21, 171, 172
Circadian rhythm, 201, 203, 210-212
cis sequences, 6, 15, 18
c-jun, 81
Class switch recombination (CSR), 87, 100, 103
Class switching, 722
Clearance deficiency, 306, 308, 312
Clearance, 66, 221-225, 295
CLIP (Class II-Associated Invariant Peptide), 178, 179
CLIP-Seq, 791
Clonal anergy, 116
Clonal deletion, 115
Clonal expansion, 82
Clonal ignorance, 116
Clonal selection algorithm (CLONALG), 375
Cloning, 758-762, 817, 819
Clostridium tetani, 308
CLRs, 310
Coagulation system, 32, 67, 68
Cogan syndrome, 626
Collagen type II, 17, 29
Collagen-induced arthritis, 52, 107, 710, 723, 732
Collectins, 32, 40, 41
Combined Bisulphite Restriction Analysis (COBRA), 816, 818, 824
Commensal bacteria, 310-313
Common disease/common variant model, 263, 264, 266
Common genetic factors, 238, 242
Common lymphoid progenitors (CLP), 37, 77
Complement system, 32, 57-71, 118, 173, 249-251
  Alternative pathway, 59-63
  Classical pathway, 58-53
  Lectin pathway, 59-63
Complement factor H-like protein 1, 62, 63, 66
Complement factor H-related, 65, 66, 68
Complement receptor of the immunoglobulin family, 62, 65
Complement receptors, 64-66
Complement regulatory factors, 62
Complementarity-determining regions (CDR), 79
Complotype, 272, 276
Comprehensive High-throughput Arrays for Relative Methylation (CHARM), 820, 822, 824
Concordance rate, 242, 243, 260
Concordance, 271
Congenital heart block, 484
Congenital rubella, 524
Constant region (C), 87, 98, 113
Cornification, 220
Corticosteroids, 502, 505, 507, 508, 588, 596, 645, 713
Corticotropin-releasing hormone (CRH), 201, 210-212, 540
Cortisol, 201, 210-212, 540
Coxsackie virus, 539, 544
CpG Island, 186, 354, 355, 358, 813-815
CpG loci analysis, 814, 815
CpG-oligodeoxynucle¬otides (ODN), 52, 54
CpGs, 19, 51-54
CR2, 27
CRE, 4, 15
CREB (Cyclic-AMP-Responsive-Element-Binding Protein), 171, 172
CREST syndrome, 577
Cross-reaction, 86, 118, 188
Cryoglobuilinemic vasculitis, 630-632

Cryoglobulinemia, 483, 490, 493
Cryoglobulins, 477, 484, 489, 493, 624, 630
CTLA-4 (CD152), 12, 26, 28, 82, 84, 85, 116, 118, 120, 188, 192, 279, 226, 286, 292, 384, 414, 469, 523, 524, 537, 551, 573, 574, 587
C-type lectin receptors (CLRS), 39-41
Cushing syndrome, 520
Cyclophosphamide, 437, 451, 463, 468, 488, 505, 617, 628-637, 649, 662, 686, 706, 707, 712, 713
Cyclosporine, 437, 466, 506, 508, 528, 566, 578, 636, 649, 650
Cytokine dysregulation, 118
Cytokine signaling suppressor proteins (SOCS/CISH), 140
Cytokine, 79, 81-86, 89, 91-93, 97, 100-102, 105-108, 116-121, 133-164, 237, 241, 248-252, 358
Cytokine-receptor, 134-160
Cytolysins, 85
Cytomegalovirus (CMV), 187, 190, 194, 238, 241, 303, 444, 476, 480, 499, 629, 654, 707, 710, 728
Cytopenia, 701, 702, 708, 713
Cytoskeleton, 25, 42, 88, 157, 220, 585, 598,

D

Damage-associated molecular patterns (DAMPS), 39-42, 59, 224
Danger theory and dendritic cell algorithms (DCA), 375
DAP12, 191
DAS28, 326, 327, 401, 405, 406, 453, 659
Decay accelerating factor, 62, 63, 68
Decay constitutive element (CDE), 19
Dectin, 40
Deep protein mutagenesis, 791
Deep vein thrombosis, 443
Defensins, 31, 35, 84, 161, 228, 296
Dehydroepiandrosterone (DHEA), 202, 204
Dementia, 483, 484,
Dendritic cells, 32, 35, 37, 48, 51-54, 65, 77, 78, 80, 84-87, 89-92, 97, 100, 115, 118, 121,124, 126, 142, 144, 147, 157, 169, 181, 189, 193, 208, 221, 225-230, 241, 274, 309, 311, 324, 373, 387, 433, 498, 500-503, 525, 532, 563, 569, 599, 626, 700, 722,
Dermatomyositis, 332, 708, 713
Dermatomyositis, pregnancy, 465
Descriptive epidemiology, 257
Desmocollins, 598-602
Devic' diseases, 68
Diacylglycerol (DAG), 80, 81, 88 153
Differential Methylation Hybridization (DMH), 820, 823, 824
Diffuse systemic sclerosis, 611
Discoid rash, 436
Disease modifying anti-rheumatic drugs (DMARDs), 403, 409, 412-417, 467, 560, 711, 732
Distal elements, 4
Distal renal acidosis, 483
DNA damage response, 188
DNA isolation, 744, 755
DNA libraries, 761
DNA ligase, 758, 760
DNA methylation, 18-20, 103, 186, 187, 351, 353, 354, 356-359, 361, 362, 758, 759
DNA methyltransferase (DNMT), 187, 353, 359, 814
DNA Organic extraction, 756
DNA repair kinases, 190
DNA-binding dye, 770
DNA-PKCs, 190
DNase I Digestion (DNase-SEQ), 826, 827
DNase I sensitive sites, 5
DNA-Seq, 791
DNase-Seq, 791
dNTPs, 763, 782
Downstream the promoter element (DPE), 4, 7, 8
D-penicillamine, 330
Drosha, 720, 723, 724
Drosophila melanogaster, 248
dsRNA, 40, 41, 53, 248, 720, 722
Duffy antigen receptor for chemokines (DARC), 249

E

Early arthritis clinics, 691
Early B cell factor (EBF), 97, 102
Eastern Blotting, 825
Echinoderms, 248
Eco-Doppler, 730
Ecology, 241, 311, 321, 322, 480, 499,
Ecosystem, 314, 321, 322,
Eculizumab, 71
Elastase, 34, 35, 37, 61, 226, 228
Electrophoresis, 757, 758
Electroporation, 761
ELISA, 794-801
  Competitive, 796
  Conjugate, 798
  Cutoff, 800
  Enzyme , 798
  Limits, 800
  Non-competitive, 795
  Sample, 794
  Sensitization, 794
  Solid phase, 794
  Standar curve, 800, 802
  Substrate, 798
  Titers, 800
Elongation complex (TEC), 8, 9
Elongation, 6-10, 12-14, 18, 20
Encephalitis, 335
Endogenous retrovirus, 241, 281, 304, 347,
Endosome, 50, 52, 153, 178-181, 248, 541
Endothelial cells, 35, 38, 39, 43, 58, 63, 65, 67, 68, 79, 80, 83, 89, 127, 142, 143, 160, 219, 221, 249, 294, 388-390, 393, 444, 445, 447 449, 500, 563, 564, 584, 611, 612, 623, 630, 632, 634, 654, 655, 665,
Endothelial dysfunction, 326, 553, 653, 654, 657, 658, 659, 660, 662, 666,
End-stage renal disease, 328, 664
Entosis, 220
Environmental agents, 241, 322, 323, 524,
Environmental exposures, 187, 260, 322, 323, 325, 336,
Eosinophilic granulomatosis with polyangiitis, 239, 621, 623, 633,

Eosinophils, 35, 36, 57, 61, 65, 83, 92, 142, 143, 156, 162, 164, 225, 312, 578, 603-605, 632, 633, 700,
Epidemiology, 257, 265, 271, 272, 285, 322-325, 357, 381, 427, 443, 475, 519, 543, 544, 549, 573, 583, 646,
Epidermolysis bullosa acquisita, 432, 598, 604,
Epigenetic mechanisms, 5, 15, 18-20, 103, 129, 146, 186, 352, 353, 365, 428, 539, 813, 832
Epigenetics, 19, 186, 187, 241, 315, 324, 351, 352, 357, 362, 384, 428, 539, 576, 728, 813
Episcleritis, 402, 403, 435, 637, 643, 645, 648
Epistasis, 242, 243, 257
Epithelial cells, 18, 31-33, 35, 38, 39, 57, 63, 78, 83, 92, 100, 123-129, 142-144, 147, 150, 154, 155, 162, 164, 169, 225, 249, 389, 390, 433, 486, 490, 498-504, 573, 575, 586
Epithelitis, 123-129, 475, 481, 482, 498, 667
Epithelium, 31-33, 38, 83, 90, 115, 123-129, 147, 150, 155, 160, 433, 475, 491, 498, 500, 553, 573, 575, 577, 578, 584-587, 598, 602, 648, 701, 734
Epitope spreading, 105, 118, 306, 307, 334, 343, 345, 359, 386, 429, 526, 604,
Epitope, 33, 67, 77, 115, 118, 120, 121, 127, 128, 226, 271, 273-275, 277, 304-308, 326, 334, 343-348, 359, 373, 382, 383, 386, 387, 405, 428, 429, 431, 444, 499, 526, 551, 564, 584, 600, 602, 603, 604, 605, 654-658, 700, 710, 793, 794, 795, 797, 806-810, 825, 827
Epratuzumab, 93, 107, 108
Epstein-Barr virus (EBV), 137, 194, 238, 241, 281, 193, 303-305, 329, 345,382, 386, 444, 476, 480, 498, 499, 548, 654, 742,
ERAP1 (ER-Associ¬ated Aminopeptidase 1), 180
Erosion, 185-188, 191, 194, 195, 327, 358, 387, 391-414, 553, 599, 601-605, 633, 692, 693
ERp57, 177-181
Erythropoietin, 134, 136, 139, 141, 207
Escherichia coli, 228, 303, 376, 531, 575, 783,
ESR1, 187
ESR2, 187
Estradiol, 204, 206, 210, 431, 460
Estrogens, 201, 204, 207, 334-336, 382, 385, 437, 448, 449, 459, 460, 463, 464, 484, 499, 538
Etanercept 139, 334, 414, 469, 477, 650,
Ethidium bromide, 219, 757, 767, 768
ETS1, 288, 293, 361, 587
Euchromatin, 4, 20, 187, 355, 356, 825, 826
EULAR Sjögren's syndrome disease activity index, 494
EULAR Sjögren's syndrome patient reported index, 494
Exon, 3, 4, 10-12, 15, 16, 24-27
Experimental allergic encephalomyelitis (EAE), 54, 105, 117, 120, 274, 709
Extra-articular manifestations (EAM), 381, 385, 391, 393, 395, 398, 401, 551-553, 655, 658-660,
Extra-glandular manifestations, 475, 480-491, 498, 503, 505, 508

F

Factor Ba, 60
Factor Bb, 60
Factor D, 8, 57, 59, 60, 62, 67
Factor H, 57, 59, 62-69, 224
Factor I, 62, 63, 64, 67, 68
FAIRE-Seq, 791, 826, 828
Familial aggregation, 238, 259, 260, 279, 381, 384, 479, 497, 520, 52
Familial autoimmune disease, 238-240, 264, 404
Familial autoimmunity, 238-240, 259, 262, 264, 314, 382, 384, 404, 477, 480, 538, 551, 657, 658
Familial determinants, 257, 259
Fas, 82, 116, 120, 159, 221, 222, 223, 225, 498, 500, 525, 526, 538, 543, 565, 577, 586, 601,
FasL, 82, 85, 116, 120, 135, 157, 221, 223, 294, 498, 500, 526, 565, 569, 586,
Fc receptor, 118, 142, 699, 700
FCGR2A, 290
FCγR, 250, 251, 296,
Fetal loss, 431, 444, 450, 457-460, 467,
Fetal microchimerism, 385, 538-540
FGFR1, 26-28
Fibrin, 17, 28, 33, 60, 68, 226
Fibrinogen, 17, 33, 60,
Fibroblast growth factor (FGF), 26, 28, 83, 84
Fibroblast-like synoviocytes (FLS), 384-394, 494
Fibronectin, 27, 33, 37, 48, 49, 139, 163
Ficolin, 31, 34, 59, 60, 62, 67, 69
Ficoll-Hypaque, 745
Fillagrin, 28, 29
First-degree relative, 259, 260, 277, 404, 489, 521, 527, 528, 551, 573, 583
Flow cytometry, 747-752
FLT3LG (Fms-like tyrosine kinase 3 ligand), 135, 149-151,
Fluorescent resonance energy transfer (FRET), 768
Fluorochrome, 747-750, 768-773, 779, 781, 806-808, 826,
FMLP, 61, 189, 190,
Follicular B Cells (FoB), 86, 87, 90, 92, 100
Follicular dendritic cells (FDCs), 65, 82, 87, 100, 503
Follicular helpers T cells (Tfh), 82, 84, 87, 91, 92, 100, 142, 143, 162
Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE), 791, 826, 828
FOXO, 25, 26, 88, 102, 221
FOXP3, 16, 25, 26, 28, 84, 85, 117, 129, 187, 191, 310, 311, 312, 359, 363, 525, 537, 538, 568, 569, 700, 709,
Fractal, 3, 22, 23
FRT-Seq, 791
FT3, 544, 546-549
FT4, 544, 546-549
Functional genetic studies, 242, 257
Fyn, 80, 81, 88

G

G protein-coupled receptor (GPCR), 23, 61, 101
Gap junctions, 85, 598
GAPDH, 768
Gastroesophageal reflux, 432, 465, 545, 616
GATA-3, 25, 26, 83, 102
GCP-2, 53
GelStar Nucleic Acid Gel Stain, 757
Gender, 201, 209, 237, 238, 241, 258, 261, 322, 326, 331, 333, 381, 484, 485, 629, 653, 656-658, 685, 693,

Gene expression, 3, 53, 81, 86, 88, 117, 129, 137, 146, 150, 152, 164, 170, 172, 185, 186, 187, 190, 202, 107, 210, 211, 249, 287, 314, 333, 351, 353, 355-361, 385, 428, 497, 502, 509, 538, 741, 761, 771, 774, 813, 817
Gene regulation, 3, 5, 19, 81, 314, 351,
Gene therapy, 531
Genetic drift, 272
Genetic epidemiology, 257, 258
Genetic linkage analysis, 257, 265
Genetic susceptibility, 119-121, 203
Genome-wide association studies (GWAS), 5, 260, 263, 264, 266, 274, 285, 286, 287, 382, 428, 497
Genomic DNA, g(DNA), 761-765, 774, 779, 814, 817, 824, 755
Genotype, 237, 242, 259, 262, 265, 272, 273, 286, 291, 351, 524, 574, 589, 772, 773
Germinal center (GC), 64, 83, 84, 86, 87, 91, 100-102, 192
Gestational diabetes mellitus, 520
Giant cell arteritis, 621-623, 625-627
Gilbert Chemical Method, 777, 778
Global 3C Interactions (HI-C), 831, 833
Glomerulonephritis, 54, 68, 123, 328, 403, 434, 479, 481, 483, 509, 624, 629, 631-633, 661
Glucocorticoids, 413, 415, 416, 432, 435-438, 520, 544, 578, 628, 629, 632, 634, 635, 659, 664
Glucose-regulated protein, 18
Gluten challenge, 589, 590
Gluten-free diet, 587-592
Glycemic target, 532
Glycosaminoglycans (GAG), 209, 328, 389, 456, 543, 549, 563
GM-CSF, 35, 52, 189, 226, 249, 388-390, 394, 389, 390,
Gnathostomes, 248, 251
Goblet cells, 83
Goiter, 543-546, 549
Golimumab, 650
GPIIb/IIIa inhibitors, 450
GPR183, 90
Graft-versus-host disease, 477, 705-709
Granulin/Epithelin (GRN), 135, 157-159
Granulocyte-monocyte colony stimulating factor (GM-CSF), 35, 52, 84
Granulocytes, 23, 32, 35, 36
Granzymes, 37, 38, 84, 85, 226, 565
Graves' disease, 202, 328, 326, 328, 335, 348, 486, 521, 523, 537-544, 549, 550
Graves' ophthalmopathy, 549, 550
Growth factors, 19, 81, 102, 133, 706
Growth hormone, 1309, 201-212
Guanidinium isothiocyanate, 757
Guillain-Barre syndrome, 334, 335, 344, 701

H

Halogenated hydrocarbons, 575
Hardy-Weinberg equilibrium, 272
Hashimoto thyroiditis, 265, 486, 488, 537-547
Health assessment questionnaire (HAQ), 401,405,406, 553
Heat shock proteins, 627, 654
Heavy chains (H), 97, 98
Heavy metals, 322
Helicobacter pylori, 499
HeliScope, 784, 786, 787
HELLP syndrome, 448, 461
HeL-N1, 19
Hematopoietic stem cell transplantation, 705-707, 711-713
Hematopoietic stem cells (HSC), 705-709
Hemolytic uremic syndrome, 68, 335, 448
Hemophagocytic sindrome, 699, 702
Henoch-schölein purpura, 621-623, 634, 635
Heparin, 449, 458, 631, 667
Hepatic failure, 564, 568
Hepatitis A, 435
Hepatitis B, 435
Hepatitis C virus, 16, 149, 281, 303, 306, 444, 477, 480, 499
Hereditary angioedema, 68
Heritability, 242, 243, 258, 289,
Herpes Simplex Virus, 654
Heterochromatin, 4, 20, 187
Heterochromatin, 825
Heterozygosity, 34, 272, 450, 520, 772
Hi-C, 791, 831-833
HIC2, 290
High-Performance Capillary Electrophoresis (HPCE), 814, 825
High-performance liquid chromatography (HPLC), 813, 824
HiSeq, 784
Histamin, 36, 41, 61, 83
Histones, 15, 18-20, 35, 126, 129, 226, 228, 230, 355, 356, 359, 360, 363, 428, 433, 710, 824-829
Histone acetyltransferases (HATs), 20, 353, 355, 356, 825
Histone deacetylases (HDACs), 20, 187, 355, 356, 360, 825
Histone demethylases (HDMs), 356, 825
Histone methyl-transferases (HMTs), 187, 356, 825
Histone modification analysis, 824
Histone modification, 18, 103, 129, 186, 187, 351, 353, 355, 356, 358-360, 363
HITS-CLIP, 791
HMGB1, 29, 66, 157, 229, 230,
Homeostasis, 24, 32, 37, 38, 57, 101,108
Homeostatic cytokine receptors, 188
Homocysteine, 449, 654, 656, 657, 662, 663
Homozygosity, 772
Hormone replacement therapy, 334-336, 662, 663
Hormones, 24, 133, 135, 201-212,261, 334, 335, 346, 431, 460, 464
Human FcγRIIa (CD32), 51, 89
Human genome, 3, 134, 160, 257
Human Leucocyte Antigen (HLA), 22, 26-28, 78, 79, 91, 120, 127, 169-182, 260, 265, 271-282, 289, 190, 194, 195, 209, 326, 330, 332, 344-348, 360, 523, 524, 527, 529, 537, 538, 543, 547, 548, 567, 578, 573
Cross-presentation, 120, 127, 180
Genetic organization, 169
HLA class I, 91, 78, 79, 169, 170-180
HLA class II, 78, 79, 90, 170-179
HLA-DM, 170-172, 178, 179

HLA-G, 26, 28
Inheritance, 174
Nomenclature, 173, 174
Non-HLA genes, 285-296
Origin and evolution, 172
Presentation of non-protein antigens, 180-182
Regulation of gene expression, 170-172
Humoral immunity, 77, 97, 574, 655,
HuR, 19
Hürthle cells, 544
Hybridization Probes, 770, 771
Hybridization temperature (Tm), 764
Hydralazine, 252, 334
Hydroxycloroquine, 415, 505
Hygiene hypothesis, 303, 309, 524,
Hypergammaglobulinemia, 107, 482, 487, 489, 491, 493, 496, 501, 508, 566, 568
Hypermethylation, 353, 355, 358, 359, 363
Hyperprolactinemia, 202, 460, 662
Hypersensitivity vasculitis, 621, 622, 635
Hypertension, 401, 435, 443, 461, 483
Hyperthyroidism, 537-549, 551, 553, 554, 537, 539, 543-549, 551, 553, 554
Hypoglycemia, 152, 205, 529, 531
Hypomethylation, 333, 346, 352, 357-359, 361, 362, 384, 724
Hyponatremia, 445
Hypothalamic-pituitary-adrenal axis, 201, 211, 251
Hypothalamic-pituitary-gonadal axis, 201
Hypothalamic-pitutary-thyroid axis, 201
Hypothyroidism, 432, 477, 482, 505, 521, 539-549, 553, 554

I

-Ak, 52
IC3b, 59-62
ICAM-1, 80, 90, 176, 177
ICOS (inducible T cell co-stimulator), 82-84, 90, 118, 119, 723, 724
ICOSLG, 288
Idiopathic inflammatory myositis, 264
Idiopathic thrombocytopenic purpura, 239
Idiopatic immune thrombocytopenia, 701, 702
Idiotype Cross-Reaction, 118
IFIH1, 290, 291
IFN-α (Interferon alpha), 32, 43, 82, 92, 135, 136, 140, 144, 149, 189, 497, 500, 501, 508, 538-540, 650
IFN-β (Interferon beta), 50, 82, 135, 136, 140, 144, 149
IFN-γ (Interferon gamma), 32, 35-38, 41, 43, 52, 53, 82, 85, 91, 102, 135, 136, 140, 142, 146, 149, 171, 177, 191, 209, 294
IFN-κ (Interferon kappa), 144
IFN-λ (Interferon lambda), 135, 140, 146
IFN-ω (Interferon omega), 135, 140, 144
IgA deficiency, 583, 588-590, 701
IKAROS, 97
IKZF1, 288, 523
IKZF3, 290
IL-1, 32, 43, 47, 48, 53, 83, 102, 117, 140, 141, 152-154, 189, 190,
IL-1R, 48, 152-156
IL-2, 80-85, 89, 90, 102, 116, 117, 119, 134-136, 141-143, 189, 192, 289
IL-2RA, 156, 294
IL-3, 134-136, 140, 141, 145
IL-4, 28, 37, 39, 83, 84, 102, 118, 134, 136, 140-142, 144, 189-191, 194
IL-5, 36, 38, 83, 134, 140, 145
IL-6, 19, 43, 53, 54, 83, 84, 89, 92, 101, 102, 117, 135, 136, 138, 140, 143, 147, 148, 152, 189, 190, 193, 194, 249, 352, 358, 361
IL-6R, 26
IL-7, 27, 77, 85, 97, 101, 102, 134, 140-143, 190
IL-8, 35, 38, 83, 142, 160, 189, 190, 194, 249
IL-9, 83, 134, 140-143
IL-10, 19, 32, 43, 53, 83-85, 89-91, 102, 107, 108, 117-119, 135-137, 139, 140, 143, 146, 149, 293, 358
IL-11, 83, 135-136, 140, 143, 147
IL-12, 32, 43, 52-54, 82, 84, 135, 136, 139, 140, 143, 147, 148
IL-12A, 289, 497, 573, 574, 587
IL-12B, 289
IL-12R, 573, 574
IL-13, 83, 84, 134,136, 140-142, 144, 293, 309
IL-14, 135
IL-15, 32, 43, 82, 134, 136, 140-143, 190, 388, 38, 569, 585-587
IL-16, 91, 135, 156
IL-17, 38, 39, 53, 83, 84, 91, 101, 102, 117, 135-137, 163, 164, 230, 289
IL-17R, 12
IL-18, 43, 82, 135, 152-155, 223, 229, 230, 249
IL-18R1, 117
IL-19, 135, 140, 143
IL-20, 135, 140, 143
IL-21, 82-84, 89, 92, 134, 140-143, 190, 191, 294, 312
IL-22, 83, 84, 135, 140, 143, 191, 289, 312
IL-23, 53, 83, 117, 135, 136,140, 141, 143, 147
IL-23R, 289, 293, 312, 332
IL-24, 135, 140, 143
IL-25, 83, 135, 142, 163
IL-26, 135, 140, 143, 289
IL-27, 135, 136, 140, 143, 147, 289
IL-28, 135, 140, 146
IL-29, 135, 140, 146
IL-31, 135, 140, 143, 147
IL-32, 26, 135, 136,
IL-33, 83, 135, 136, 152, 155
IL-34, 135, 149, 150,
IL-35, 85, 135, 136, 140, 143, 147
IL-36, 135, 152, 154, 155
IL-37, 135, 152, 155
IL-38, 135, 152, 155
Illumina, 782, 784, 786, 790
Immune complex, 51-54, 57, 63-70, 100, 104, 107, 118, 121, 250, 251, 278, 295, 305, 324
Immune repair, 711
Immuno aging. See Immunosenescence
Immunoablative therapy, 710
Immunodeficiency linked to chromosome X, 85
Immunodeficiency state, 699
Immunodeficiency Virus (HIV), 149, 156, 163, 194
Immunofluorescence, 805-810
Immunofluorescence, analysis , 806

Immunofluorescence, protocol, 806
Immunogenetic mechanism, 258
Immunoglobulin, 190, 192
Immunoglobulin A (IgA), 87, 90-92, 98, 189, 192
Immunoglobulin D (IgD), 87, 90, 91, 97-101, 103, 107, 192
Immunoglobulin E (IgE), 83, 87, 98
Immunoglobulin G (IgG), 87, 90-92, 98, 104, 107, 118, 121, 189, 192
Immunoglobulin M (IgM), 86, 87, 90- 92, 97-100, 103, 105, 107, 108, 116
Immunoglobulin superfamily, 251
Immunohistochemistry, 805-810
Immunohistochemistry, analysis, 808
Immunohistochemistry, protocol, 809
Immunologic tolerance, 115
Immunological synapse, 80, 85
Immunoprecipitation (IP), 817, 824
Immunoreceptor tyrosine-based activation motif (ITAM), 79, 80, 88
Immunosenescence, 187-199
Immuno-Seq, 791
Immunostimulatory, 51, 54
Immunosuppressive, 102, 117, 426, 427, 437, 437, 459, 461, 463-465, 504, 508, 528, 605, 612, 636, 702, 705-708, 711, 713, 734
Individual CpG loci analysis, 815, 816
Indolamine deoxygenase (IDO), 85
Infection, 186, 187, 188, 192-195, 242, 303-316
Infectious agents, 303, 323
Infectious diseases, 64, 185, 194, 195
Inflammasome, 41, 42
Inflammation, 41-43, 48, 53, 66, 80, 83, 97, 117, 120, 147, 154, 155, 194, 202-204, 352, 358-360, 362, 386. 394, 400, 411, 435, 445, 465, 494, 502, 504, 547, 563, 578, 591, 599, 606, 623, 627, 637, 643, 645, 648, 649, 663, 711, 724, 732, 735
Inflammatory bowel disease, 16, 26, 239, 264, 286, 310, 333, 551, 646, 648
Infliximab, 249, 334, 415, 464, 469, 649, 650, 734
Influenza, 334, 335
Inheritance, 257, 261, 285
Inhibitor of nuclear factor κB (IκB)-kinase complex, 49, 81
Initiation complex (IC), 6-8, 12-14, 170
Initiator element, 3, 4
Innate immune response, 31-43, 47, 295
Innate lymphoid cells, 32, 37
Inositol triphosphate (IP3), 80, 81, 88
Insulin analogues, 529
Insulin pump, 529
Insulin resistance, 402, 520. 529, 556, 558
Insulin, 519, 524-529, 656, 661, 662
Insuline-like growth factor receptor (IGF-1R), 187
Insulin-like growth factor (IGF-1), 187, 201-212
Insulitis, 105, 519, 525, 527, 528
Integrins, 90
Interferonopathy, 501
Internal ribosome entry sites (IRES), 12, 16
International Autoimmune Hepatitis Group score, 564, 565
Interstitial lung disease, 431, 435, 497, 509
Intracrine, 133
Intraepithelial lymphocytes, 32, 162, 585-587
Intrahepatic lymphocytes, 564
Intravenous immunoglobulin, 458, 463, 604, 629, 699-703
  Acute renal failure, 702, 703
  Adjunct therapy, 704
  Adverse event, 703
  High-dose IVIG protocol, 702
  Infusion rate, 702, 703
  Low-dose IVIG protocol, 702
  Premedication, 703
  Prophylaxis, 703
  Purification methods, 702
  Subcutaneus administration, 703
  Sucrose, 703
Introns, 3, 4, 10, 11
Invariant Chain (Ii), 178, 179
Ion Torrent, 788
IRAK (Interleukin (IL)-1R-associated kinase), 49
IRAK1, 290, 721, 723
IRAP (Endosomal insulin responsive aminopeptidase), 180
IRF (Interferon regulatory factors)
  IRF1, 25
  IRF2, 24, 25
  IRF3, 25, 50
  IRF4, 25
  IRF5, 26, 27
  IRF7, 26, 50
  IRF8, 26, 97
IRGM, 290
Ischemia-reperfusion injury, 67
ISR receptor substrate-1, 187
ITAMs (Tyrosine-based activation motifs), 79, 80, 88
ITGAL, 187
ITGAM, 290
ITGAX, 290

J

JAK (Janus activated kinase), 140-151, 290
JAK-STAT pathway, 190
Joint, 119, 257, 327, 330, 360, 361
Juvenile idiopathic arthritis, 29, 204, 209, 646, 648, 706, 708, 709, 710, 713

K

Kaleidoscope of autoimmunity, 237
Kawasaki disease, 28, 71, 243, 286, 622 623, 628, 629, 648, 701
Keratinocytes, 31, 35, 39, 84
Keratoconjunctivitis sicca, 435, 481, 486, 506, 578
Ketoacidosis, 526, 527
KIAA1542, 290
KIF5A, 281, 288
KIR, 38, 139, 188, 190, 191, 251
Kit-ligand, 77
Klrf1, 190
KLRG1, 188
Knodell's histological activity index, 564
Koch's postulates, 305, 306
Kupffer cells, 564
Kynurinines, 85

L

Lactoferrin, 228
Lactose, 761
lacZ, 761
Lamina propria, 91
LAT, 78, 80, 81
Latent-autoimmune diabetes in adult (LADA), 520, 528
LCK, 78, 80, 81
LCR (locus controlling region), 4, 21
Leflunomide, 409, 414-416, 437, 465, 468
Lepromatous leprosy, 119
Leptin, 139, 205
Leucine-rich regions (LRR), 47
Leukapheresis, 706
Leukocyte adhesion deficiencies, 68
Levothyroxine, 546-548, 553
LFA-1, 80, 90, 177, 187
Lichen planus, 432, 578
Lifestyle, 242, 315, 323
Light chains (L), 97, 98, 103, 104, 113, 114
LILRA2, 27, 28
Linkage analysis, 257, 258, 265
Linkage disequilibrium, 265, 272
Lipid rafts, 49, 137
Lipids, 85, 103, 181
Lipopolysaccharides (LPS), 37, 38, 40, 41, 48, 49, 53, 124, 201, 226
Liver biopsy, 446, 564, 565, 577
LL37, 228-230
Loci, 80, 86, 97, 113, 114, 170, 173, 174
Long-lived cells, 91
Low density lipoprotein cholesterol, 325, 654
Low molecular weight heparin, 703
LRRK2, 291
L-selectin, 79, 80, 84
Ludwig histological classification, 578
Luminometric methylation assay (LUMA), 814, 820
Lupus anticoagulant (LAC), 431, 443, 447, 457, 663, 666, 667, 668,
Lupus flare, 208, 460
Lupus nephritis, 427, 431, 433, 434,459, 461, 724, 733
Luteinizing hormone, 206, 207
Lyme arthritis, 344, 356
Lyme disease vaccine, 335
Lymph nodes, 38, 64, 79, 80, 90, 107, 125
Lymphadenopathy, 52, 358, 484, 495
Lymphocytes, 51, 52, 54, 65, 77-92, 97-108, 113-121, 225, 358-362, 709, 710, 732
Lymphoid organs, 78, 79, 86, 90, 100-102, 116, 125
Lymphoid-2 progenitor (LCA-2), 97
Lymphokines, 119, 134
Lymphoma, 436, 465, 475-486, 489, 493, 494
Lymphopenia, 335, 484, 496, 529,
Lymphotoxin-α, 89, 100, 389
Lysogenic cycle, 760, 761
Lytic cycle, 760
Lyzosomes, 77

M

Macrophages, 18, 31, 32, 35-37, 41, 43, 47, 52, 55, 57, 59, 61, 63, 65-67, 82 89, 90, 92, 101, 119, 121, 125-127, 134, 136, 142, 147, 149, 150, 152, 154, 157, 160, 162, 163, 169, 179, 181, 188, 189, 190, 205, 207, 221, 225, 226, 241, 249, 252, 278, 294, 295, 307, 309-312, 325-327, 330, 332, 361, 373, 386-391, 394, 433, 436, 494, 501-503, 525, 526, 530, 564, 573, 585, 598, 606, 523, 626, 630, 650, 654, 662, 700, 713, 721, 722, 732-734, 745, 808
Magnesium chloride (MgCl2), 763, 764, 766
Magnetic particles, 752
Magnetic Protein Immobilization on Enhancer DNA (MagPIE), 827
MAINE-Seq, 791
Major Histocompatibility Complex (MHC), 114-116, 120, 121, 169-182
MHC class I, 38, 52, 120
MHC class II, 21, 37, 52, 120
MHC class III, 170
Malar rash, 432, 436
Malaria, 139, 249, 250, 467
Mammals, 248
Mannose-binding lectin (MBL), 31, 59, 60, 68
MAPK phosphatase-1 (MKP-1), 19
Marginal zone B Cells (MZB), 86, 90, 92, 101, 107
MassArray, 817, 819, 824
Mast cells, 31, 35-37, 41, 58, 83, 142, 147, 160, 225, 241, 312,
Matricrine, 133, 134
Maturation by affinity, 87
Mature B cells, 90, 91, 100-104
Mature peripheral B Cells, 90, 101
Maxam-Gilbert sequencing, 817, 824
MBL-associated serin protease 1 (MASP), 34, 59-63
MCP-1 (Monocyte chemoattractant protein 1), 37, 83, 361, 259
MCP-2 (Monocyte chemoattractant protein 2), 53
Medullary thymic epithelial cells (mTEC), 78
Megaloblastic anemia, 521
Melanocytes, 121, 125
Membrane attack complex (MAC), 58-61
Membrane co-factor protein, 62
Membrane-bound immunoglobulin (mIg), 85-87, 91
Membranoproliferative glomerulonephritis, type II, 68
Memory B cells, 84, 86, 87, 89-91, 97, 101, 190, 193
Memory T cells, 188, 195
Mendelian inheritance, 257, 260
Mesenchymatous cells, 83
Metagenomics, 313-315
Metalloproteinases (MMPs), 89, 133, 138, 360, 361, 428, 498, 585
Metametabolomic, 314, 315
Metaplasia, 83
Metaproteomic, 314
Metatranscriptomic, 314
Metazoans, 247, 249
Methotrexate, 393, 415, 416, 437, 468, 507, 528, 578, 615, 634, 649, 650, 660, 735
Methyl binding domain (MBD), 354, 358, 817
Methyl-accepting assay (M. SssI acceptance assay), 814

Methylated DNA Immunoprecipitation (MeDIP), 817, 822, 824
Methylated-CpG island recovery assay (MIRA), 822, 823, 824
Methylation analysis, 813
Methylation Specific PCR (MSP), 815, 817, 818, 824
Methylation-sensitive Arbitrarily Primed PCR (AP-PCR), 817, 820, 824
Methylation-Sensitive Restriction Endonucleases (MSREs), 814, 824
Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE), 816, 818, 824
methyl-CpG- binding domain (MBD), 822
Methyl-CpG immunoprecipitation (MCIp), 822, 823, 824
MethyLight, 816, 819, 824
Methylprednisolone, 437, 463, 509, 627, 632, 635, 712, 713
Methyl-Seq, 791
MHC. See Major Histocompatibility Complex (MHC)
Microarray, 822, 773, 774
Microarray-based Methylation Assessment of Single Samples (MMASS), 820, 822, 824
Microbiota, 310-313, 587
Micrococcal Nuclease Digestion (MNase-SEQ), 825, 826
Microglia, 65, 67, 150, 154, 252, 361
Microsatellites, 263
Microscopic polyangiitis, 466, 623, 633, 686
MIIC (MHC class II compartment), 178
Mikulicz's disease, 475
Minor salivary gland biopsy, 478, 492
miRNA (MicroRNA), 5, 6, 14, 15, 19, 22-24, 186, 187, 351, 356, 360-365, 385, 428, 719, 721-724
miR-1, 187
miR-140, 187
miR-145, 187
miR181a, 187, 188, 190
miR-34a, 187
miR-449, 187
Mitochondria, 17, 25, 77, 85, 118, 221, 227
Mitogen-activated protein (MAP) kinase, 14, 19, 25, 49, 81, 146, 150, 153, 158, 188, 211
Mitotic catastrophe, 220
Mixed connective tissue disease, 238
MMEL1, 290
Molecular Beacons, 768, 770
Molecular mimicry, 118, 188, 304, 306-309, 334, 344-348
Monoclonal antibody, 71, 107, 108, 160, 163, 211,
Monoclonal antibody, 793
Monocytes, 32, 35, 37, 52, 65, 89, 92, 142, 147, 225, 228
Monogenic disorders, 257
Monozygotic twins, 120, 242, 260, 351
Mosaic of autoimmunity, 19, 328
mRNA (Messenger RNA), 3-6, 8-23, 719, 722-724, 735
mRNA, 757, 761
MUC19, 291
Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), 79
Multifactor complex (MFC), 13, 14
Multifractal, 3, 21, 22
Multiple autoimmune syndrome, 237, 240, 264, 279, 479
Multiple consecutive CpG loci analysis, 816, 817, 824
Multiple CpG loci analysis (Multiplex), 817, 824
Multiple myeloma, 92, 156
Multiple sclerosis, 5, 12, 16, 26, 27, 29, 54, 69, 92, 93, 104, 108, 120, 125, 192, 228, 239, 260, 262, 264, 274, 286, 288-291, 304, 328, 344-348, 351, 359-361, 363, 364, 382, 551, 645, 706-712
Multiplex polony, 789
Myasthenia gravis, 18, 28, 71, 125, 238, 239, 260, 264, 332, 335, 344, 404, 551, 588
Mycobacterium bovis, 51
Mycobacterium tuberculosis, 182, 228, 347, 348
MyD88, 40, 49, 50, 54, 153
MyD88-dependent pathway, 49
MyD88-independent pathway, 49, 50
Myelin basic protein (MBP), 27, 28, 192, 274
Myelin oli¬godendrocyte glycoprotein (MOG), 26-29, 104
Myeloid cells, 65, 77, 92, 121, 223
Myeloid dendritic cells, 52
Myeloperoxidase, 35, 226, 228, 230
Myocardial infarction, 546
Myopathy, 482, 545
Myosin, 156, 228
Myositis, 226

N

Naïve B cells, 102, 190, 192,
Naïve T cells, 78, 79, 83, 119, 187, 188, 191, 195
Nanomedicine, 727, 735
Nanoparticles, 727, 729-736
Nanopore, 784, 788, 789
Natural killer cells (NK), 31, 32, 35, 38, 43, 65, 77, 85, 97, 127, 157, 188, 226, 241, 389, 390, 503, 525, 530, 542, 564, 573, 586, 627, 709
Natural selection, 247, 249, 272
Necroptosis, 137, 158, 159, 220, 225, 230
Necrosis, 36, 66-71, 157-159, 219-221, 225, 229
Negative selection algorithm (NSA), 374-376
Negative selection, 78, 85, 115, 116, 128, 129, 208, 212, 272
Neisseria gonorrhoeae, 303, 307, 344, 456
Nematodes, 248
Neonatal lupus erythematosus, 462, 463
Nerve growth factor (NGF), 135, 157, 159
Netosis, 36, 220, 225, 226,228-230, 360, 363, 428
NET-Seq, 791
Neurodegenerative diseases, 67, 143, 225
Neuroendocrine hormones, 251
Neuroendocrine system, 201, 202, 210, 212
Neuroimmunoendocrine network, 251
Neuron-glial relationship, 252
Neuropathic pain, 252
Neuropeptide Y (NPY), 204, 209
Neuropeptides, 133, 201, 204, 205, 209
Neuropsychiatric systemic lupus erythematosus, 427, 435, 436
Neutrophils, 31, 35, 36, 42, 43, 57-59, 61, 65, 66, 70, 89, 92, 136, 141-143, 146, 147, 160, 162, 164, 188, 189, 225, 226, 228-230, 241, 249, 250, 325, 388-391, 393, 428, 578, 603, 605, 623, 624, 629, 632, 635, 700
Next Generation Sequencing (NGS), 777, 780-789
NFAT, 25, 80, 81 88, 188

NFY (Nuclear Transcription Factor Y), 171, 172
NF-κB (Nuclear Factor κB), 16, 25, 40, 49, 50, 80, 81, 88, 159, 171, 188, 193, 294, 295, 358, 359-363, 384, 389, 390
N-glycan linkages, 118
Niches, 77, 85
Nicotine, 325, 327
Nitric oxide, 26, 28, 35, 204, 241, 274, 303, 326, 329, 389, 391, 444, 498, 569, 615, 617
NLRs (Nod-like receptors), 32, 39-42, 124, 153, 324, 391
NOD mice, 105, 120, 121, 525, 528, 529
NOD2, 41, 290, 295
Nodules, 327, 397, 398, 400-402, 488, 578, 624, 629, 633, 635, 645, 692, 693
Nonsteroidal anti-inflammatory drugs (NSAIDs), 403, 404, 413, 414, 435, 437, 438, 463, 464, 505, 508, 544, 549, 615, 657, 732
Noradrenaline, 203, 204
Northern blot, 757, 773
Notch, 77, 85, 100, 133, 135, 138, 598
Nucleosome, 19-21, 52, 103, 125, 126, 157, 187, 226, 333, 353, 355, 356, 359, 360, 363, 434, 498, 710, 791, 824, 825, 827
NY-ESO-1 antigen, 348

O

Obstructive lung disease, 481, 482, 547
Ocrelizumab, 93, 108
Ocular staining score, 478, 491, 492
Ofatumumab, 93, 108
Oligo(dT), 757, 762
Oligomenorrhea, 545
Oncostatin M (OSM), 135
Operational taxonomic units (OTUS), 314
Optic neuritis, 483, 496, 636
Oral contraceptives, 334, 449, 464
Oral ulcers, 436, 487, 635, 636
Organic solvents, 238, 242, 243, 328-330, 353, 386
Osteoarthritis, 53, 69, 160, 204, 205, 211, 384, 395, 495, 692
Osteoblasts, 149, 154, 388-390, 394, 709
Osteoclast, 57, 147, 149, 154, 274, 275, 387, 389, 390, 394, 395
Osteopenia, 387, 407, 408, 409, 468, 576, 577
Osteoporosis, 203, 402, 404, 416, 577, 588, 590, 592, 664, 692
OTUD3, 290
Outcome Measures in Rheumatoid Arthritis Clinical Trials (OMERACT), 407, 408, 410-413, 625
Overlapping syndromes, 238
OX-40, 82, 84, 158,
Oxidative DNA damage, 186, 313,
Oxidative Stress, 186, 190, 220, 313, 735

P

pp16, 187, 360, 363, 577
p21, 187, 260, 363, 577
p53, 146, 157, 187, 220, 221, 393, 504
p230, 18
P bodies, 356, 357, 721-723
P selectin glycol-protein ligand 1 (PSGL-1), 90
PacBi0 RS, 786
Pancreatic beta cell, 120, 121, 519, 520, 524, 525
Pancreatitis, 228, 432, 435, 476, 481, 483, 520, 629, 634, 637
Pancytopenia, 436
Panniculitis, 432
Pannus, , 387, 393, 394, 401, 411, 413
Paracrine, 35, 82, 133, 134, 156, 157, 390, 504, 563
Parasite, 31, 36, 59, 66, 119, 126, 139, 226, 249, 250, 296, 308-310, 312, 321, 343, 347, 531
PARK7, 288
Parotidomegaly, 476, 481, 482, 491
Paroxysmal nocturnal haemoglobinuria, 68
PARS, 791
Parthanatos, 220
Pathogen-associated molecular patterns (PAMPs), 38, 39, 41, 42, 47, 48, 53, 59, 66, 141, 147, 159, 295, 308, 387, 388
Patient-reported outcomes (PROs), 405, 406
Pattern recognition receptors (PRRS), 31, 33, 35, 38-42, 47-49, 52, 248, 334
PAX5 (paired box gene 5), 97, 102
PCAF (p300/CBP-associated factor), 171, 172
PCR (Polymerase chain reaction), 756, 757, 762-773, 780, 783, 785, 814-824, 827-830
PD-1 (Programmed death-1), 81, 82, 120, 121
Pemphigus, 123, 239, 597-602,702
Peptidomimetics, T cell epitope, 345, 346
Peptidyl arginine deaminase (PAD), 226, 363
Perforins, 38, 82, 85, 227, 526, 586
Periarterial lymphatic sheath (PALS), 100
Peripheral blood mononuclear cells (PBMCs), 21, 54, 66, 155, 194, 195, 210, 358, 361, 362, 384, 724, 745, 751, 747
Peripheral lymphoid tissues, 99
Peripheral node addressin (PNAd), 79
Peripheral tissue antigens (PTAs), 19
Peripheral tolerance, 116, 120, 498
Pernicious anemia, 125, 239, 240, 264, 403, 404, 521, 551, 605,
Peyer's patches, 79, 86, 90
Pharmacogenomics, 243
Phenol/chloroform, 756, 757
Phenotype, 10, 16, 19, 20, 37, 67, 77-79, 82-85, 90, 98, 102, 114, 123, 129, 188, 191, 192, 194, 195, 208, 220, 223, 225, 237, 238, 239, 241-243, 249, 258-267, 274, 286, 289, 311, 322, 323, 330, 348, 351, 353, 361, 376, 382, 391, 392, 445, 449, 479, 480, 489, 529, 527, 538 566, 568-570, 591, 605, 653, 664, 666, , 732, 733
PhIT-Seq, 791
Phosphatidylinositol biphosphate (PIP2), 80, 81
Phospholipase Cγ (PLCγ), 80, 81, 88
Photosensitivity, 431, 432, 436, 479, 487
Phylogeny, 139, 248, 315, 724
PI3K, 24, 88, 145, 146, 150, 151, 157, 159, 190, 193, 221, 389, 390
PIP4K2C, 288
PIR-B, 89
Piwi asociated RNAs (piRNA), 719, 722
PLA2G2E, 290
Plasmid, 348, 758-762, 817, 819
Plasmocytes, 77, 90, 309, 575
Plasmocytoid dendritic cells (pDC), 51, 69, 101, 126, 127, 144 150, 193, 228, 229, 230

Platelets, 35, 36, 39, 89, 162, 169, 250, 307, 388, 389, 390, 405, 430, 436, 444, 445-450, 456, 457, 461, 462, 495, 496, 611, 654, 658, 666, 701, 706
Platyhelminths, 248
Pleiotropy, 242, 243, 257, 266
Poly(A), 757, 762, 767
Polyarteritis nodosa, 239, 264, 335, 622, 623, 629, 638
Polyautoimmunity, 237, 238, 261, 262, 282, 403-405, 479, 485, 486, 488-490, 537, 538, 550-554, 574, 576, 578, 659, 660, 663
Polyclonal antibody, 793
Polydipsia, 530
Polyglandular autoimmune syndrome type II, 240
Polymorphism, 5, 26-28, 34, 40, 41, 67, 104, 128, 156, 173, 206, 249, 250, 251, 260, 263, 264, 265, 272, 274, 288-295, 315, 351, 357, 360, 363, 383, 428, 497, 499, 520, 523, 538, 573, 587, 601,, 657, 658,
Polymyositis/Dermatomyositis, 238, 239, 332
Polyneuropathies, 483
Polynucleotide A tail (poly A),10, 16, 18, 24
Polyphagia, 526
Polyuria, 526
Positive co-stimulatory signals, 81
Positive selection, 78, 115, 128
Positive transcription elongation factor b (P-TEFb), 6-10
Postpartum thyroiditis, 538, 543, 544, 547, 548
Postvaccinal antibodies, 335
pRb, 187
PRDM1, 290, 295
Prednisone, 437, 438, 466
Preeclampsia, 68, 71, 458, 459, 463
Pregnancy loss, 445, 450, 455-469
Pregnancy, 70, 85, 147, 203, 206-209, 261, 431, 445, 447, 455-469
Pre-initiation complexes (PIC), 6- 8, 10, 12, 13, 16
Pre-mRNA, 10, 16, 26-28
Primary biliary cirrhosis, 238, 239, 260, 264, 328, 327, 360, 573-582
Clinical features, 576, 578
Natural history, 578
Prognosis, 578
Primary prevention, 528
Primer, 762
PRKCQ, 288, 294
Procainamide hydroxylamine (PAHA), 333, 358
Procainamide, 333, 358
Progesterone, 201-212, 459, 540
Prolactin, 134, 139, 140,141
Promoter clearance, 6, 7, 8
Promoter, 3-10, 15, 16, 18, 19-22, 24
Prophage, 760, 761
Proptosis, 549
Propylthiouracyl, 550
Protectin, 63, 64, 67 68, 70, 71
Protein A associated with the TLR4 (PRAT4A), 18
Protein analysis, 793-810
Protein kinase C (PKC), 81
Protein sample, 801, 802
Protein tyrosine kinases (PTKs), 88, 89
Proteinase K, 755
Proximal elements, 4
Pseudogene, 24
Pso27, 29
Psoriasis, 84, 239, 259, 264, 606, 607
Psoriatic arthritis, 646, 647
Psychomotor retardation, 545
P-TEFb (Positive transcription elongation factor b), 6-10, 171, 172
PTEN, 24
PTPN2, 288
PTPN22, 26, 28, 104, 126, 279, 281, 286, 288, 292, 382-386, 428, 522, 523, 537, 538, 551, 553
PU.1, 97
Pyroptosis, 137, 152, 220, 225

Q

qPCR (Quantitative polymerase chain reaction), 767-773, 829, 830
Queen-anne´s sign, 543
Quencher, 768-773

R

Rabies vaccine, 335
Race, 252, 331, 381, 605, 634, 663
Radiation, 251, 252, 323, 325, 410, 412, 477, 478, 530, 538, 539, 547, 550, 591, 603, 606, 625, 706, 709, 712
Radio-labeled enzyme S-adenosylmethionine (SAM), 814
RAME model, 264
RANK, RANKL, 135, 136, 157-159, 203, 274, 275, 388, 389, 390, 394, 395, 577
RANTES, 37, 163, 164, 249, 361, 500, 721
RAPID3, 4, 5 (The Routine Assessment of Patient Index Data 3, 4, and 5), 405, 406
Raynaud's phenomenon, 237, 330, 403, 465, 481, 483, 485, 487, 489, 491, 502, 577, 578, 611-613, 668
Reactive arthritis, 397, 647
Reactive nitrogen intermediates (RNI), 34
Reactive oxygen species (ROS), 190, 195, 204, 329
Real time PCR (RT-PCR), 767-772
Recombination activating gene 1 (RAG1), 77-79, 85
Recombination activating gene 2 (RAG2), 77-79, 85, 104
Recombination signal sequences, 85
Recurrence risk ratio, 258, 259
Regulators of G protein signaling (RGS), 101
Regulatory B Cells (Bregs), 91, 107, 108
Reiter's syndrome, 646
REL, 290
Relapsing polychondritis, 239, 251
Relative quantification, gene expression, 771, 772
Relative risk,264, 272, 273, 276, 331
Repetitive elements, 353,
Replication, 3, 21
Reporter, 768
Restriction endonuclease, 758, 759, 815
AcdII, 815
AgeI, 815
AvaI, 815
BmeT110I, 815

Bsp199I, 815
BssAI, 815
BstUI, 816
Cfr10I, 815
CspAI, 815
HpaII, 814, 815
Kpn2i, 815
MspI, 814, 815
SfuI, 815
SmaI , 815
Restriction fragment length polymorphisms (RFLP), 773
Restriction Landmark Genomic Scanning (RLGS), 820, 822, 823, 726
Retrovirus, 304, 346
RFX (Heterotrimeric X-box-binding factor regu¬latory factor X), 171, 172
RFX-associated ankyrin-containing protein (RFXANK), 171, 172
RFX-associated protein (RFXAP), 171, 172
Rheumatic fever, 118, 343, 344
Rheumatoid arthritis (RA), 5, 14, 16-18, 26t-29, 53, 70, 84, 89, 92, 98, 104, 105, 119, 192, 194, 195, 226, 238, 239, 242, 248-251, 259-261, 264, 273, 274, 316, 326, 381-426
Articular involvement, 398-401
Classification, 395-397
Epidemiology, 381
Epigenetics, 384
Etiology, 382-387
genetics, 382-384
Magnetic resonance imaging studies (RAMRIS), 412, 413
Monitoring patients, 404-405
Pathogenesis, 387-395
Pregnancy, 464, 465
Risk factors, 383-387
Treatment, 412-416
Rheumatoid factor, 52, 107, 121, 190, 194, 205, 237, 304, 326, 382, 388, 391, 398, 432, 478, 480, 484-488, 491, 577, 631, 657, 658, 691-693
Ribo-Seq, 791
Ribosomes, 77
RIP-Seq, 791
Rituximab, 93, 108, 414, 437, 450, 464, 468, 469, 488, 508, 650, 703
RLRs, 32, 40, 41
RNA, 755, 756
RNA interference (RNAi), 20, 719
RNA isolation, 757
RNA-induced silencing complex (RISC), 721
RNAP II holoenzyme, 6-11, 15-17, 20, 21
RNase, 757
RNA-Seq, 791
ROR alpha, 26
ROR gamma, 26
RORc, 83
rRNA, 757, 768
RT-PCR, 816
RT-PCR, dissociation curve, 768
RT-PCR, normalization, 768
RUNX3, 289
RXR, 26

S

Salivary gland ultrasonography, 493
Salivary glands (SGs), 107, 304, 362
Salting-out, 756
Sanger, 778-780
Sarcoidosis, 391, 397, 476, 478, 645, 646, 648, 650
Schirmer test, 490-493
Scintigraphy, 492
Scleritis, 435, 643, 645
Scorpion Probes, 769, 771
SDS, 755, 801-803
Secondary prevention, 528
Sedimentation equilibrium, 745
Sedimentation rate, 745
Sedimentation, 744
Selectins, 90
SELENA-SLEDAI, 437
Semaphorin, 204
Senescence, 185, 187-189, 191
Sequencing , 778-791
Serine 2 specific phosphatase (FCP1), 6, 7, 9, 10
Serine 5-selective phosphatase (SCP1), 7
Serositis, 436-438
Serotonin, 83
Sex hormone-binding protein (SHBG), 206-212
SH2B3, 281, 286, 288, 292
Shared epitope, 273-275, 383, 384, 385 , 386, 405
Shared subphenotypes, 237, 238
Sharp-Van der Heijde (S-VdH) Index, 407-409
Shelterin complex, 185, 186, 195
Short-lived cells, 91
SIAE, 291
Sialic acid-binding immunoglobulin-like lectin (Siglec), 90
Sialoendoscopy, 492, 505
Sialometry, 492
Signaling pathways, 3, 14, 16, 20, 24, 25
Signalosome, 88
Silica, 382, 386, 530,
Silicosis, 391
Simplified Disease Activity Index (SDAI), 405, 406
Single-nucleotide polymorphism, 5, 26, 28, 249, 263, 291
SIRT1, 187
Sjögren's syndrome, 18, 69, 70, 89, 102, 107, 123, 127, 226, 238-240, 249, 260, 264, 276, 304, 359, 362, 363, 365, 382, 465, 475-509, 553, 575, 576, 667,684, 723, 730
Skin, 32, 403, 432, 481, 545, 597-607
SLC15A4, 290
Small nuclear ribonucleoproteins, 118
SmD (1-2), 28, 29
Smoking, 238, 241, 252, 323, 325-328, 385, 386, 392, 401
snRNA, 6, 11, 22, 23
SnRNPs, 51
Socio-economic status (SES), 387, 660
SOCS3, 190
Sodium/iodine symporter (NIS), 540, 541
SOLiD, 782, 783, 784, 790

Somatic hypermutation, 87, 100, 103, 114, 115
Somatic pain, 252
Sono-Seq, 791
SONO-SEQ, 826, 828
Sorting, 748, 750
Southern blot, 773
Sp1, 4
Sphingosine 1-phosphate receptor 1 (S1P1), 100, 101
Spleen, 86, 87, 90-92, 99-101, 107
Splicing, 6, 7, 10-12, 15, 16, 18, 22, 27, 28
SPRED2, 288
Spurious pleiotropy, 242
SRE, 4, 15, 16
STAT (Signal Transducers and activators of transcription), 140-152
STAT1, 25, 293, 361
STAT2, 289
STAT3, 25, 190, 289, 293, 358, 361, 362,
STAT4, 25, 82, 290, 293, 382, 384, 428, 497, 574, 578
STAT5, 190
STAT6, 25, 83
Statins, 660, 665
Stem cell factor (SCF)/KitL, 135, 139, 149, 151, 152
Stem cells, 97
Stress, 58, 251, 322, 323, 402, 444, 537, 540, 569
Stroma of the thymus, 77, 85
Studies of adoption-migration, 257
Subacute thyroiditis, 538, 543, 544, 548
Subcellular fraction, 802
Subclinical hypothyroidism, 486, 546, 553, 554
Subphenotype, 237, 238, 241-243, 264, 382, 479, 480, 485-487, 490, 491, 496, 509
Substance P, 204
Sulfasalazine, 414-416, 468, 660
Sulfonamide, 333
Superantigens, 306, 307
Suppressors of cytokine signaling (SOCS), 102, 140
Surrogate light chain (SLC), 86
Swan-neck finger defomity, 399, 407
Swollen joint count (SJC), 404-406
SYBR Safe, 757
SYBR® Green, 757, 766, 770, 771
Synovial fibroblasts (SF), 53, 358, 404-406,
Synthetic saturation mutagenesis, 791
Synthetic theory, 247
Systemic lupus erythematosus (SLE), 5, 17, 18, 20, 25-29, 53, 54, 68-70, 84, 89, 92, 105, 107, 108, 121, 194, 195, 225-230, 238-240, 242, 243, 248-252, 259, 260, 262, 264, 271, 275, 276, 303, 327-334, 405, 427-438, 444, 456, 458-465, 483, 485-490, 521, 538, 543, 551, 553, 643, 661-665, 684, 699, 709-711, 723, 730
  Classification, 436
  Clinical manifestations, 431-436
  Collaborating clinics (SLICC), 437, 664
  Disease activity index, 437, 664
  Epidemiology, 427
  Epigenetics, 428-430
  Genetics, 428-430
  Laboratory findings, 436
  Physiopathology, 428-431
  Pregnancy, 459-464
  Treatment, 437
  Vasculitis, 466
Systemic sclerosis, 207, 226, 465, 480, 487-490, 611-617, 667, 684, 707-712
Systemic sclerosis, pregnancy, 465
Systemic vasculitis, 621-638, 713

T

T cell receptor (TCR), 77, 78, 80, 81, 88, 92, 105, 113-115, 117-120, 188, 190, 191, 195, 346, 709
T cell zone, 84, 100
T cells, 12, 21, 26, 52, 53, 77-92, 97, 101, 102, 104, 105, 107, 113-120, 188-192, 239, 271, 292, 294, 358-361, 705, 709, 713, 723, 722,
Tacrolimus, 437, 649, 650
TAGAP, 288
Takayasu arteritis, 466, 621-623, 627-629
TAP1 and TAP2 (Transporter associated with antigen processing 1 and 2), 178-180
Tapasin, 177-179
Taq DNA Polymerase, 762, 763
TaqMan probe, 768, 769, 771
TATA box (sequence TATAAA in the promoter), 4, 7, 15
T-bet, 26, 82, 83
TBP (TATA binding protein), 7
TCR signaling, 190, 195
Tear break-up time, 490-492
Telomere shortening, 21, 22
Telomeres, 185, 186, 188, 189, 194, 195
telRNA, 6
Tender joint count (TJC), 404-406
Teplizumab, 528
TERC, 186
Terminal deoxynucleotidyl trans¬ferase (TdT), 98, 99
Termination (transcription or translation), 6-11, 13, 15, 20
Ternary complex (TC), 13, 14
TERT enzyme, 186
Testosterone, 201-212, 545
Tetanus toxoid, 307-309, 335, 347
Tetrachlorodibenzo-P-dioxi, 325, 326
TFIIH, 6-9
TGF-α (Transforming growth factor alpha), 32, 43, 102
TGF-β (Transforming growth factor beta), 32, 83-85, 89, 117, 118, 309-312
Th1, 52, 54, 82, 83, 85, 102, 118, 119, 189, 191, 194, 201, 261, 293, 294, 309, 312, 346, 358, 361
Th17, 82, 83, 85, 117, 119, 191, 192, 194, 294, 310, 361, 460, 501, 502, 525, 526, 568, 573, 576
Th2, 54, 82, 83, 85, 102, 118, 119, 142, 164, 189, 191, 194, 293, 294, 310, 460, 501, 525, 575, 654, 711
Th22, 82, 84
Th9, 82, 83
The Framingham Heart Study, 653, 662
Threshold cycle (Ct), 767
Thrombocytopenia, 345, 347, 436, 446, 496, 664
Thrombopoietin, 134, 139, 141

Thrombosis, 443- 451, 457, 458
Thymic cortex, 77, 78
Thymic progenitors (ETP), 77
Thymocytes, 77, 78
Thymus, 77, 78, 85, 92, 102, 103, 113, 115, 116, 189
Thyrocyte, 538-541
Thyroglobulin, 538-542, 547
Thyroid lymphoma, 543
Thyroid ultrasound, 544, 546, 549
Thyroidectomy, 544, 450
Thyroperoxidase, 541, 542, 544
Thyroxine, 541, 544-548
TIA-1, 14
TIAR, 14
Tight junction, 584, 598
TIRAP, 49, 50
Tissue-restricted antigens (TRAs), 19
TMEM39A, 290
TNFAIP3, 290, 291, 523
TNFR2 (Tumor necrosis factor receptor 2), 26
TNFRSF (Tumor Necrosis Factor Receptor Superfamily Members), 101, 158, 159, 188, 286, 290, 294
TNFSF (Tumor Necrosis Factor Superfamily Members), 135, 136, 157-160, 288, 290, 293
TNFα (Tumor necrosis factor alpha), 14, 19, 53, 82-85, 89, 102, 120, 170, 189, 190, 193, 195, 249, 384, 388, 460-462, 630, 719, 721, 723, 725, 735
TNIP1, 290
TNPO3, 290
Tocilizumab, 93, 211, 414-416, 469, 661
Toll receptors and Interleukin 1 domains (TIR), 47, 49, 54
Toll-like Receptors (TLR), 32, 40, 47-54, 90, 107, 248, 294, 295, 310, 311, 324, 332, 334, 360, 387, 388, 391
TLR1, 48, 49
TLR2, 48, 49, 53
TLR3, 48-51, 53, 248
TLR4, 18, 48-50, 53, 252
TLR5, 48
TLR6, 48, 49
TLR7, 18, 48, 51, 53, 54, 118, 248, 290
TLR8, 48, 51, 290
TLR9, 18, 48, 50-54, 118, 248
TLR10, 48
TLR11, 48
Tonsillar crypts, 90
Tonsils, 86, 90
Toxoplasmosis, 643, 646
TRAF (TNF Receptor-Associated Factor), 49, 290, 358, 721
TRAM (TICAM2), 49
Trans element, 4, 12, 15, 16, 24
Trans Golgi network (TGN), 18
Transcription factors IID and IIB (TFIID and TFIIB), 171, 172
Transcriptional factories, 21
Transcriptional machinery, 8, 15, 21
Transcytosis, 584
Transformation, 761
Transforming growth factor (TGF)-β-activated kinase (TAKI), 49
Transgenic mice, 106, 107
Transglutaminase, 29
Transitional B Cells, 90, 99, 100, 104, 107
Translation, 3, 4, 6, 10, 12, 15, 16, 22
Translational medicine, 243
Transverse myelitis, 481, 483, 484, 496
TRAP, 791
T-regulatory cells (Treg), 82-85, 91, 117, 119, 189, 191, 194, 388-390, 460, 501-503, 526, 538, 563, 568-570, 577, 586, 587, 699, 709-711, 723
TREX1, 291
TRIF (TICAM1), 49, 50
Triiodothyrosine (T3), 539, 541, 544, 546-549
Tristhetraproline , 19,
TRIzol, 757
tRNA, 3, 5, 6, 12, 13, 14, 16
tRNA, 757
TSH, 539-552
TSHR, 540-543
Tuberculosis, 249
TYK2, 290
Type 1 Diabetes Mellitus (T1D), 5, 12, 16, 25, 26, 29, 68, 104, 105, 120, 121, 195, 239, 240, 259-262, 264, 271, 276-281, 334, 359, 360, 362, 404, 519-532, 547, 551, 605, 613, 713, 730, 732, 734
Type 2 diabetes mellitus, 520, 528, 531, 654
Type-I genes, 6
Type-II genes, 6
Type-III genes, 6

U

U1 small nuclear ribonucleoprotein (U1-68K), 17
U1 small nuclear ribonucleoprotein (U1-70K), 29
UBE2L3, 290
Ulcerative colitis, 28, 264
Ultrasound, 409, 410
Ultraviolet light, 430, 773
UNC93B1, 18, 53
Urochordates, 248
UTR, 3, 4, 12, 14, 16, 24, 27
Uveitis, 209, 210, 347, 635, 636, 643-651

V

Vaccination, 529
Vaccines, 53, 438
DNA, 438
Hepatitis B, 338, 391, 477, 623,
Variable number nucleotide repeats (VNTR), 523
Variable region (V), 79, 87, 98-100, 102, 113, 115
Vascular cell adhesion molecules, 654
Vascular permeability, 83
Vasculitis, 69, 71, 206, 209, 226, 228, 239, 328, 333, 365, 401-403, 432, 435, 466, 469, 479, 481, 482, 486-490, 495, 502, 621-642
Vasculitis associated with systemic disease, 623, 637
Vasoactive intestinal peptide, 209
Vector, 759-762
Very Low-Density Lipoprotein, 181, 664
Vesicular traffic, 18

Vimentin, 17, 29
Viral infection, 186, 187, 192-195
Virions, 761
Vitamin C, 386
Vitamin D, 382, 385, 386, 468, 483, 484, 504, 524
Vitiligo, 71, 118, 125, 225, 238-240, 260, 264, 286, 287, 404, 521, 551, 588, 598, 599, 605, 606
Vitronectin, 63, 64
Vogt-Koyanagi-Harada syndrome, 646, 648

W

Warfarin, 448-451, 458, 459, 463, 631
Wegener's granulomatosis, 264, 293, 339, 404, 466, 621-623, 632
Western blotting, 800-806
  Analysis, 804
  Blocking, 803
  Detection, 804
  Protocol 707, 805
Whole methylome analysis, 813, 814

X

X-chromosome inactivation, 261, 351
Xeroderma, 543
Xerophthalmia, 431, 476,477, 479, 481, 490, 492, 504, 506
Xerostomia, 475-477, 479, 481, 482, 490, 492, 503-506, 554
X-gal, 761
X-linked genes, 262
X-PID gene, 523

Y

Yellow fever, 335
Yersinia spp, 539

Z

ZAP70, 78, 80, 81, 88, 188
Zero-mode waveguide (ZMW), 787
Zonulin, 584

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"This book attempts to organize the knowledge collected by those who have extensively studied autoimmune diseases (ADs) into a comprehensive overview of how the immune system, governed by its own complex yet limited pathways, turns against self targeting distinct tissues in different conditions according to a balance determined by the genetic predisposition of the host, its gender, environmental circumstances and other co-factors that modify the outcomes of a similar pathological process (...).

This book strikes a great balance at the experimental and translational intersection in presenting cutting edge views of ADs in the broader context of autoimmunology".

Francesco M. Marincola, MD, FACS.
[From the Preface]

The CREA Collection of "Universidad del Rosario" offers to the medical community and related areas, texts on translational autoimmunity, by which new and relevant biomedical discoveries are applied to autoimmune diseases, in their prediction, diagnosis improvement, treatment, prognosis, and prevention.

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