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Influenza Virus-Induced Type I Interferon Leads to Polyclonal B-Cell Activation but Does Not Break Down B-Cell Tolerance[▽]

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The link between infection and autoimmunity is not yet well understood. This study was designed to evaluate if an acute viral infection known to induce type I interferon production, like influenza, can by itself be responsible for the breakdown of immune tolerance and for autoimmunity. We first tested the effects of influenza virus on B cells in vitro. We then infected different transgenic mice expressing human rheumatoid factors (RF) in the absence or in the constitutive presence of the autoantigen (human immunoglobulin G [IgG]) and young lupus-prone mice [(NZB × NZW)F_1] with influenza virus and looked for B-cell activation. In vitro, the virus induces B-cell activation through type I interferon production by non-B cells but does not directly stimulate purified B cells. In vivo, both RF and non-RF B cells were activated in an autoantigen-independent manner. This activation was abortive since IgM and IgM-RF production levels were not increased in infected mice compared to uninfected controls, whether or not anti-influenza virus human IgG was detected and even after viral rechallenge. As in RF transgenic mice, acute viral infection of (NZB × NZW)F_1 mice induced only an abortive activation of B cells and no increase in autoantibody production compared to uninfected animals. Taken together, these experiments show that virus-induced acute type I interferon production is not able by itself to break down B-cell tolerance in both normal and autoimmune genetic backgrounds.

The development of autoimmune diseases depends on both genetic and environmental factors. Among the latter, infections have been shown to play an important part either in triggering or in exacerbating autoimmunity or, in contrast, in preventing it (4). The mechanisms underlying this relationship are mostly unknown. However, two processes have been put forward to explain such an association. The first, molecular mimicry, is antigen dependent and relies on the activation of self-reactive lymphocytes by microbial components structurally similar to self-antigen. The second, usually named "bystander activation," covers all antigen-independent events triggered by a pathogen and possibly leading to activation of autoreactive cells. Indeed, infections are often associated with inflammation and the release of cytokines that contribute to enhanced antigen presentation by antigen-presenting cells, release of sequestered self-antigens, and epitope spreading (11, 37).

Therefore, to understand the mechanisms of B-cell tolerance breakdown, we have established four transgenic (tg) mouse lines, expressing low (Smi)- or intermediate (Hul)-affinity human rheumatoid factors (RF) in the absence or in the constitutive presence of the autoantigen (knock-in for human immunoglobulin G [IgG], Smi × cIgG and Hul × cIgG). Using these models, we have shown that, although RF B cells are immunologically ignorant of their autoantigen (20, 21, 32), chronic infection with *Borrelia burgdorferi* is able to induce autoantibody production. This RF production relies on (i) a direct polyclonal activation of B cells by the bacteria that is T cell independent and (ii) a B-cell-receptor (BCR)-dependent activation of RF B cells that needs T-cell help and that we defined as RF B-cell tolerance breakdown. It is mediated by immune complexes that cross-link the BCR and Toll-like receptors (TLR) on the B-cell surface (33).

Here, we used these RF tg lines, as well as the lupus-prone mouse line (NZB × NZW)F_1, to consider the effects of an acute infection with influenza virus on autoreactive B-cell tolerance. This viral infection is of particular interest because it induces alpha interferon (IFN-α) production (13), which could be of importance in the breakdown of autoreactive B-cell tolerance and in autoimmunity (6, 28, 35).

First, we show that the virus is unable to directly activate purified B cells in vitro and that polyclonal B-cell activation is type I IFN induced. Second, experimental infection with influenza virus of the different tg lines, as well as of the (NZB × NZW)F_1 mice, induces only an abortive activation of both autoreactive and nonautoreactive B cells that does not lead to autoantibody production during the course of the infection.

MATERIALS AND METHODS

Mice. All mouse lines were housed and crossed in our institute's animal facility, in isolator cages. Influenza virus-instillated mice and the noninfected controls were housed in the Molecular and Cellular Biology Institute's animal facility. C57BL/6 and (NZB × NZW)F_1 mice were bought from Harlan (Gannat, France). Type I IFN receptor (IFNR)-deficient mice (type I IFNR KO) on a C57BL/6 background were purchased from the CDTA Institute (Orleans, France). All animal experiments were done in accordance with institutional and national regulations.

The generation of Smi, Smi × cIgG, Hul, and Hul × cIgG mice has been described previously (20, 21, 32). Briefly, RF tg mice are generated as single-chain tg mice (heavy or light) on a C57BL/6 background. Single-chain tg mice are crossed with cIgG knock-in mice generated on a mixed background (129/OLA,

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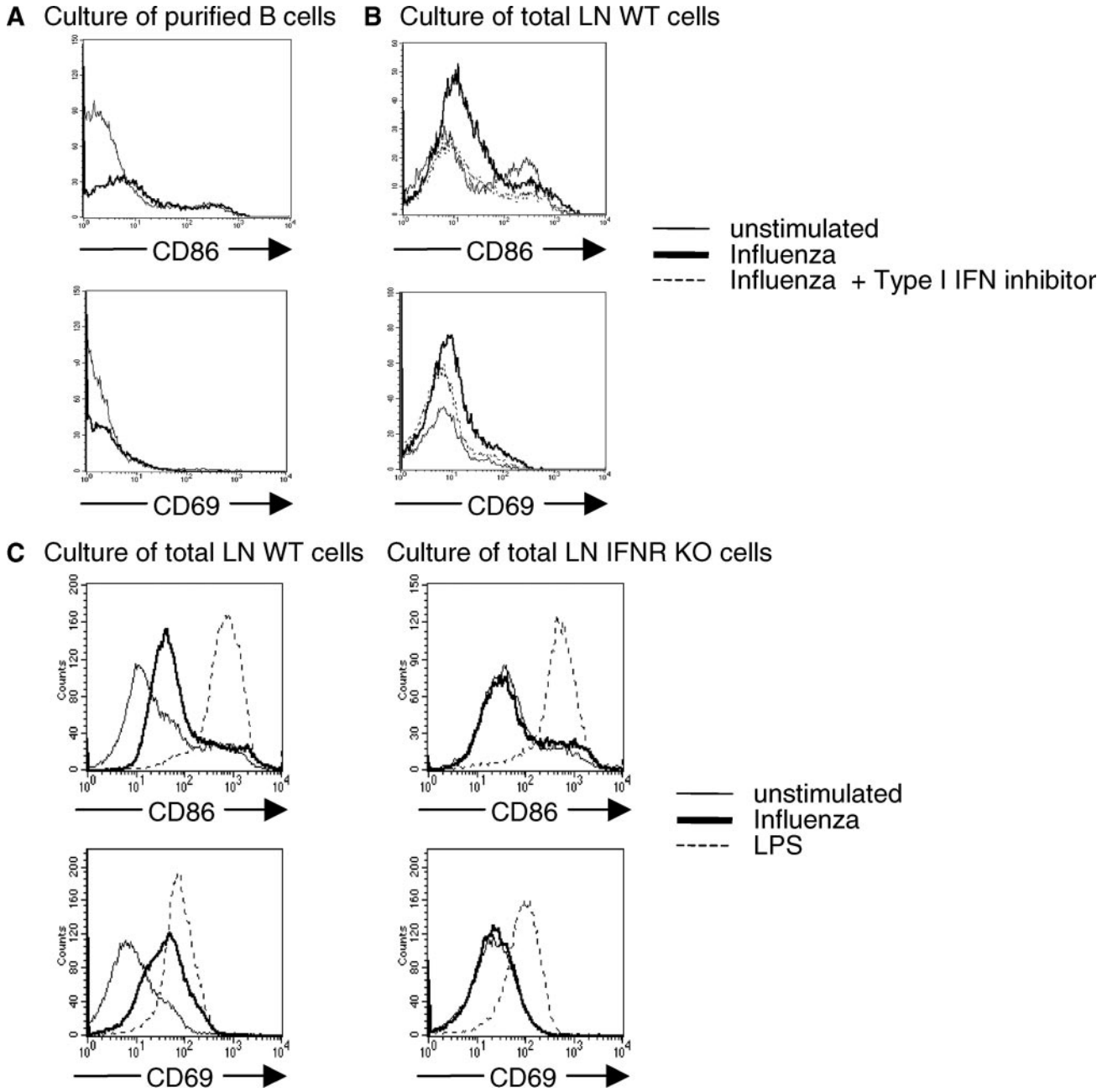

FIG. 1. Influenza virus is not able to stimulate B cells directly but acts through type I IFN production. (A) Stimulation of purified LN B cells (sorted negatively with magnetically activated cell sorting anti-CD43 magnetic beads) with influenza virus (thick line) and stimulation with virus-free allantoic fluid (thin line) were compared according to CD86 and CD69 expression. (B) Expression of activation markers CD86 and CD69 on LN B cells (gated as IgM^+ cells) cultured with total cells from mediastinal and mandibular LN and influenza virus in the presence (dashed line) or the absence (thick line) of B18R (type I IFN inhibitor). (C) Expression of activation markers CD86 and CD69 on LN B cells (gated as IgM^+ cells) cultured with total cells from mediastinal and mandibular LN with influenza virus (thick line), virus-free allantoic fluid (thin line), and LPS (dashed line). Wild-type LN B cells (left) are compared to type I IFNR KO cells (right). The expression of CD86 and CD69 with medium only is identical to the expression with virus-free allantoic fluid, for wild-type and for IFNR KO mice (not shown). WT, wild type.

CB20, mainly C57BL/6, and N6 backcrosses with C57BL/6 mice) and then intercrossed to obtain Smi, Smi × cIgG, Hul, and Hul × cIgG mice on the same genetic background. For experiments, tg mice were always compared to their littermate controls. The cIgG knock-in line was kindly provided by K. Rajewsky (Boston, MA) and expressed chimeric IgG with the human γ-chain C1 region.

Screening of the tg mice was performed as previously described (21). Smi and Hul tg mice were identified after tail DNA PCR typing, and cIgG knock-in mice were selected by enzyme-linked immunosorbent assay (ELISA) (human IgG dosage described in reference 39).

Influenza virus infection. The influenza virus strain A/NT/60/68 (H3N2, influenza virus type A/Northern Territory Hong Kong/strain number 60/1968) was provided by Martine Valette (Faculté de médecine, RTH Laennec, Lyon, France). Virus was grown in the allantoic cavity of 10-day-old embryonated hen's eggs. Virus preparations were quantitated by hemagglutinin (HA) titration.

RF tg mice were challenged intranasally under light anesthesia with influenza virus strain A/NT/60/68 (H3N2; HA titer, 1,260) using a dose (30 μl) predetermined in a group of test mice in order to obtain 20% weight loss. Animals were infected between 8 and 12 weeks of age, except for (NZB × NZW)F_1 mice, which were infected at a predisease state, 17 weeks of age. Uninfected mice were instillated with virus-free allantoic fluid. Animals were sacrificed 5 or 15 days after infection.

Some of the mice were rechallenged with the virus-containing allantoic fluid after the first infection (day 21 [D21]) and sacrificed 4 days later (D25).

Flow cytometry. Preparation of mandibular and mediastinal lymph nodes (LN) and of spleen cell suspensions and their staining for flow cytometry have already been described previously (21). The analysis of the B-cell phenotype was performed by double or triple staining with anti-B220–fluorescein isothiocyanate (FITC), anti-IgM–Cy5 or anti-IgM–phycoerythrin (PE), anti-IgD–biotin, anti-CD19–biotin, anti-CD21–FITC, and anti-CD23–PE monoclonal antibodies (MAbs) (Pharmingen). RF B cells were identified by staining with anti-IgMa–FITC and 17109-biotin (17109 recognizes the tg RF V_κ chain idiotype and was provided by D. A. Carson, San Diego, CA). The T cells were monitored using double staining with anti-CD4–PE and anti-CD8–FITC (Pharmingen). Analysis of the expression of activation markers CD86 and CD69 was performed using anti-CD86–PE and anti-CD69–PE antibodies (Pharmingen). Biotin-conjugated antibodies were revealed using streptavidin-allophycocyanin or streptavidin-PE (Pharmingen). Nonviable cells were excluded and identified by incorporation of propidium iodide (PI; 10 μg/ml; Sigma). The analysis was performed with a FACSCalibur using the CellQuest software package (BD Biosciences).

ELISA. Mice were bled by retro-orbital puncture under anesthesia. Sera were centrifuged (12 min, 10,000 rpm) and stored at −20°C until ELISA.

For murine and human anti-influenza virus IgG dosage, A/NT/60/68 virus and allantoic fluid (at a 1/200 dilution) were adsorbed to 96-well microtiter plates (Falcon, Oxnard, CA) in 0.05 M carbonate-bicarbonate buffer, pH 9.6, at 37°C overnight. The plates were blocked with 0.4% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) at 37°C for 1 h. Following washing with PBS-T, serum diluted in PBS-T containing 0.4% BSA was added and incubated for 1 h at 37°C. Goat anti-mouse IgG or goat anti-human IgG antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) were used as secondary antibodies. The final reaction was revealed by adding H_2O_2 in the presence of 3,3′,5,5′-tetramethylbenzidine. After 15 min at 37°C, the reaction was stopped by addition of 1 M HCl, and the absorbance values were measured at 450 nm. Data were expressed as optical density (OD) values (450 nm) minus background for a 1/500 serum dilution.

ELISAs for serum IgM and IgM RF were done as described previously (21, 33). Binding of total IgM was determined by adding a peroxidase-coupled anti-mouse IgM antibody (Jackson Immunoresearch).

For RF dosage, plates were coated with anti-IgMa (Smi and Smi × cIgG mice) or the MAb H1.10 (Hul and Hul × cIgG mice), and serum RF concentrations were revealed by adding biotin-labeled 17109 antibody and then streptavidin-peroxidase. The H1.10 MAb recognizes the tg Hul RF V_H chain idiotype and was provided by D. A. Carson (San Diego, CA).

For anti-double-stranded DNA (anti-dsDNA) antibodies, calf thymus DNA was absorbed at 100 ng/ml (Sigma); single-stranded DNA (ssDNA) was removed by digestion with S1 nuclease (100 IU/ml; Amersham), and bound antibodies were revealed with an anti-mouse IgM (or IgG)-peroxidase antibody (Jackson Immunoresearch). For ssDNA dosage, plates were coated with ssDNA (Sigma) and total IgM was evaluated using a peroxidase-coupled anti-mouse IgM or IgG antibody (Jackson Immunoresearch).

To measure the reactivity of mouse sera with nucleosomes, ELISA microtiter plates (Falcon, Oxnard, CA) were coated overnight at 37°C with 1 μg/ml nucleosome (as expressed in DNA concentration)-PBS, pH 7.4. The subsequent steps of the respective tests were performed as previously described (25) using mouse sera diluted 1:200 in PBS-T and goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:20,000 in PBS-T. The cutoff points of each assay were determined with the sera from 10 nonimmunized BALB/c mice. Chromatin was prepared from the mouse L1210 lymphocytic leukemia cell line as described previously (22), except that it was not run through a sucrose gradient. The isolated chromatin consisted mainly in mononucleosomes as characterized by 1.5% agarose gel electrophoresis. In parallel, the content in histones was checked by 18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

B-cell proliferation assays. Spleens or LN were removed from C57BL/6 mice or type I IFNR KO mice and teased apart. Cells were washed and suspended in RPMI 1640 with L-glutamine (BioWhittaker) supplemented with gentamicin (40 mg/ml; BioWhittaker), 2-mercaptoethanol (Sigma), and 10% heat-inactivated fetal calf serum (Dutscher). Cells (10^6) were incubated in a 96-well BD Falcon plate (final volume of 200 μl) with one of the following reagents: 10 μg/ml of lipopolysaccharide (LPS) from *Salmonella enterica* serovar Typhi (Sigma), 5% heat-inactivated (30 min at 56°C) influenza virus-containing allantoic fluid, or virus-free allantoic fluid.

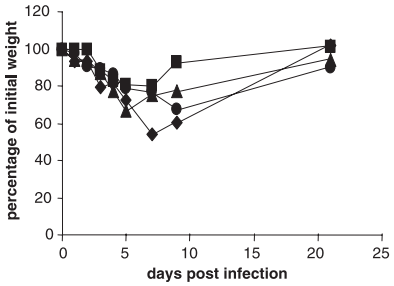

FIG. 2. Mice infected with influenza virus show weight loss. Shown is weight loss in four different individual tg Hul × cIgG mice infected with influenza virus. Each symbol represents one mouse followed between the day of infection and the day of sacrifice.

After 48 h of culture at 37°C, the phenotype of the cells was determined by flow cytometry analysis.

For proliferation assays, cells were labeled with carboxy fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) before addition of virus-free allantoic fluid, LPS, or influenza virus. Suspensions of 2×10^7 cells/ml in 0.1% PBS-BSA were incubated with CFSE at a final concentration of 2 mM for 10 min at 37°C. Cells were then washed and suspended in the culture medium.

For CD86 and CD69 expression, cells were labeled after 48 h of culture as previously described (33).

Cell purification. Spleen or draining LN were harvested from 8- to 12-week-old mice.

Cells were isolated with a magnetic cell separation depletion protocol (Miltenyi Biotec). Single-cell suspensions were depleted of non-B cells with anti-CD43 magnetic beads (Miltenyi Biotec). Purity of B cells was confirmed by staining with anti-B220–FITC antibody (more than 98% purity).

Inhibition of type I IFN effects. During in vitro B-cell proliferation assays, type I IFN was blocked using B18R, a purified soluble form of type I IFNR. B18R (1,000 ng/ml; eBioscience) was added to the cell culture at the same time as LPS, virus-free allantoic fluid, and influenza virus were added.

Anti-CD4 treatment. Nondepleting mouse anti-CD4 antibody (YTS177.9.6.1) in ascites form diluted in PBS (50 μl ascites plus 50 μl PBS per mouse per injection) was administered intraperitoneally 2 days before influenza virus infection and then twice a week until sacrifice (12, 33). Control animals were injected with PBS.

RESULTS

Given our previous results with *B. burgdorferi* infection, and knowing that B cells express TLR3 and TLR7, we first wanted to check that influenza virus was able to directly stimulate B cells.

In vitro, influenza virus activation of LN B cells is mediated by type I IFN. In vivo, influenza virus is known to induce an acute localized infection of the upper respiratory tract and to activate B cells in the draining LN. Thus, we stimulated C57BL/6 total LN cells or purified LN B cells originating from the upper respiratory tract draining LN (mandibular and mediastinal) with either influenza virus (2 HA U/ml) or virus-free allantoic fluid for 48 h. Cell proliferation was evaluated using CFSE staining (not shown), and activation was monitored by surface expression of CD86 and CD69. Influenza virus activated B cells when they were cultured among unpurified total LN cells (Fig. 1B), but it did not stimulate purified LN B cells (Fig. 1A).

We therefore wondered if type I IFN could account for the

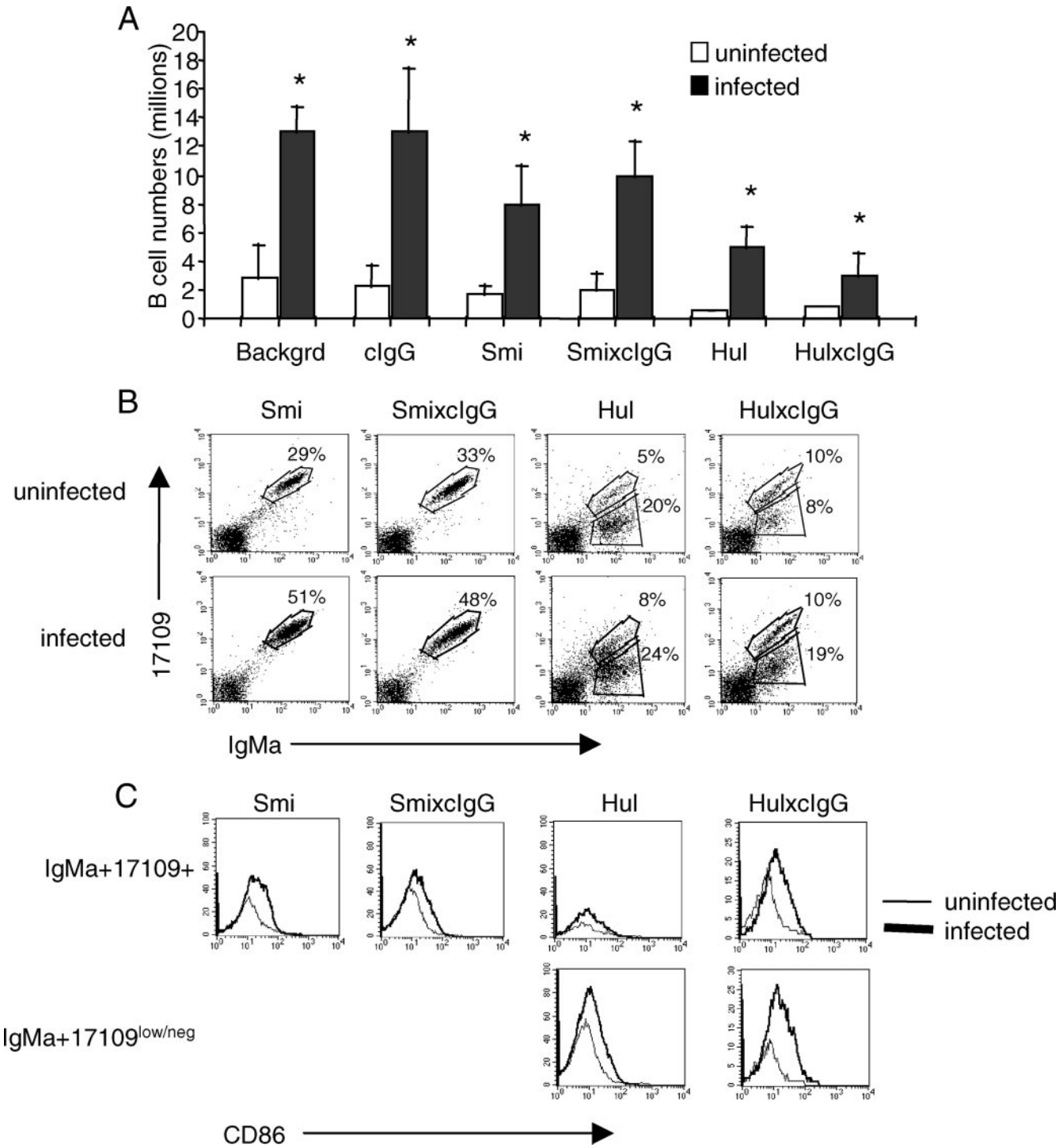

FIG. 3. Influenza virus infection induces a polyclonal expansion and an activation of B cells that are independent of the autoantigen. (A) Absolute LN B220/IgM$^+$ cell numbers in millions in infected (black bars) and uninfected (open bars) mice 5 days after infection. Each value ± standard deviation represents the mean from four to six mice depending on the mouse line. Statistical difference is designated by an asterisk ($P < 0.05$, Mann-Whitney test). "Backgrd" stands for non-tg littermates (C57BL/B6). (B) Flow cytometry analysis of infected and uninfected mouse LN cells 5 days after infection. Viable lymphocytes were gated on forward scatter and side scatter parameters. IgMa$^+$/17109^{hi} staining reflects RF B cells, and IgMa$^+$/17109$^{low/neg}$ staining reflects non-RF B cells (present only in Hul and Hul × cIgG mice). (C) Surface expression of CD86 on gated IgMa$^+$/17109^{hi} (RF) cells and on gated IgMa$^+$/17109$^{low/neg}$ (non-RF) cells (for Hul and Hul × cIgG mice only) in infected mice (thick line) was compared to that in uninfected controls (thin line).

different in vitro effects of influenza virus on purified and nonpurified B cells. We thus performed two types of experiments: (i) we cultured C57BL/6 LN cells with the virus for 48 h either in the absence or in the presence of type I IFN inhibitor B18R (purified recombinant soluble form of type I IFNR). B-cell activation induced by influenza virus was almost completely abolished in the presence of B18R. Expression of activation markers CD86 and CD69 (Fig. 1B) in the presence of B18R returned to levels found in unstimulated cells. (ii) We also stimulated LN cells from type I IFNR KO mice with influenza virus and showed that the virus was not able to activate these cells compared to wild-type LN cells (Fig. 1C).

Thus, these results confirm previous work from Coro et al. (13) showing that influenza virus infection induces the production of type I IFN (IFN-α and IFN-β) which directly activates B cells in vitro.

In vivo, infection with influenza virus of the RF tg mice. Smi, Hul, Smi × cIgG, and Hul × cIgG mice were infected by intranasal instillation of influenza virus A/NT/60/68 (30 μl, HA titer of 1,260) between 8 and 12 weeks of age. Uninfected controls were instillated with virus-free allantoic fluid. Animals were sacrificed for analysis 5 or 15 days after infection. Virus replication occurs quickly after infection in the respiratory tract tissues and draining LN. Viremia then declines due to the formation of immune complexes containing influenza virus antigens and specific IgA or IgG and to the cytolytic activity of CD8 T cells (34, 38). Influenza virus infection induced weight loss in infected animals (approximately 10 to 40% of total weight in tg mice, as shown in the case of the Hul × cIgG tg line in Fig. 2), and initial weight was restored 3 weeks after infection. Mice developed a murine influenza virus-specific IgG response at day 15 postinfection (not shown). But, knock-in mice, both Smi × cIgG and Hul × cIgG, also produce anti-influenza virus cIgG (OD, 0.0 before infection and from 0.2 to over 1.0 15 days after infection; see Materials and Methods). Thus, this experimental infection induces a specific immune response and in vitro type I IFN production, allowing us to directly address the questions of the role of virus-induced type I IFN production in autoreactive B cells and the possible synergy between virus-containing immune complexes and type I IFN on RF B cells.

Influenza virus infection leads to polyclonal LN B-cell expansion and activation. Briefly, four RF tg mouse lines were studied with their appropriate controls. Two of them expressed polyreactive low-affinity RF Smi in the constitutive presence (Smi × cIgG) or in the absence (Smi) of the autoantigen. In those lines, most B cells are RF B cells as they express both heavy and light chains of the RF transgene and are identified as $IgMa^{+}/17.109^{+}$ cells. The other two lines, on the other hand, express monoreactive intermediate-affinity RF (Hul and Hul × cIgG mice). Only 10% of spleen and LN B cells are RF B cells ($IgMa^{+}/17.109^{+}$ cells). Most of the other B cells express the heavy chain of the RF transgene but are $17.109^{low/-}$ (non-RF B cells). We previously showed that those non-RF B cells express in fact endogenous light chains and are therefore unable to bind human Ig. These non-RF B cells are used as internal controls (32).

Five days postinfection, LN B-cell numbers in infected animals increased from three- to fourfold compared to uninfected controls. This increase occurred in all mouse lines whether the autoantigen (human IgG) was present (Smi × cIgG and Hul × cIgG) or not (Smi and Hul) and whatever the affinity of the RF was (Fig. 3A). In the Hul × cIgG tg line, polyclonal B-cell expansion was shown by comparing RF ($IgMa/17109^{hi}$) and non-RF ($IgMa/17109^{low/neg}$) B cells, which were equally expanded (Fig. 3B).

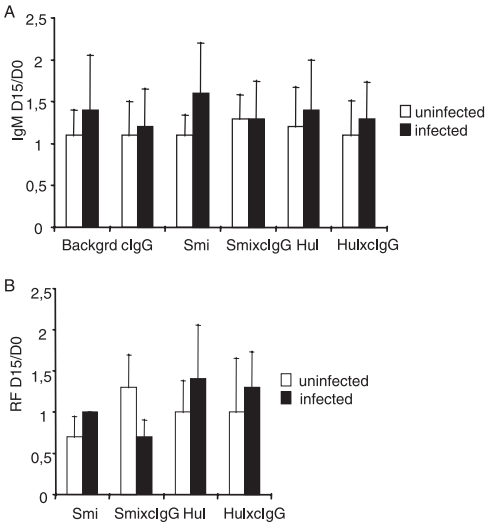

FIG. 4. Influenza virus infection induces no increase in IgM or IgM RF in infected mice compared to uninfected controls. (A) IgM levels were measured by ELISA. Values represent the mean relative increase of the total IgM production from D0 to D15 in infected mice (black bars) and noninfected mice (white bars). Numbers of tested animals are the same as in Fig. 3A. (B) Ratios of serum RF levels between D15 and D0 in infected RF tg mice compared to controls. Numbers of tested animals are the same as in Fig. 3A.

Infection also induced a mild activation of RF and non-RF B cells, as shown by the increase of expression of CD86 on both those cell types (Fig. 3C) in infected animals compared to uninfected controls.

Infection did not induce IgM or IgM RF production, even after viral rechallenge. In contrast to what we observed during *B. burgdorferi* infection, IgM and IgM-RF production levels were not increased in Smi and Hul infected mice compared to uninfected controls between D0 and D15 postinfection (Fig. 4A and B). Also, they were not affected by the constitutive presence of the autoantigen, and in particular, we did not observe any increase of IgM-RF in Smi × cIgG and Hul × cIgG mice. In other words, both the low-affinity and intermediate-affinity RF B cells were no more induced to produce RF in the presence of their autoantigen when stimulated by virus-induced type I IFN.

In 1996, Fazekas et al. (17) showed that viral infection with another strain of influenza virus (A/PR/8/34, H1N1 strain) is able to induce the production of murine RF of different isotypes in BALB/c mice provided that mice are rechallenged at least once with the virus. Thus, in order to allow for better formation of immune complexes between influenza virus and anti-influenza virus human IgG (produced by the knock-in tg mice), we rechallenged the intermediate-affinity Hul × cIgG tg mice with the virus 21 days after the first infection and monitored the specific human IgG anti-influenza virus response as well as the RF production.

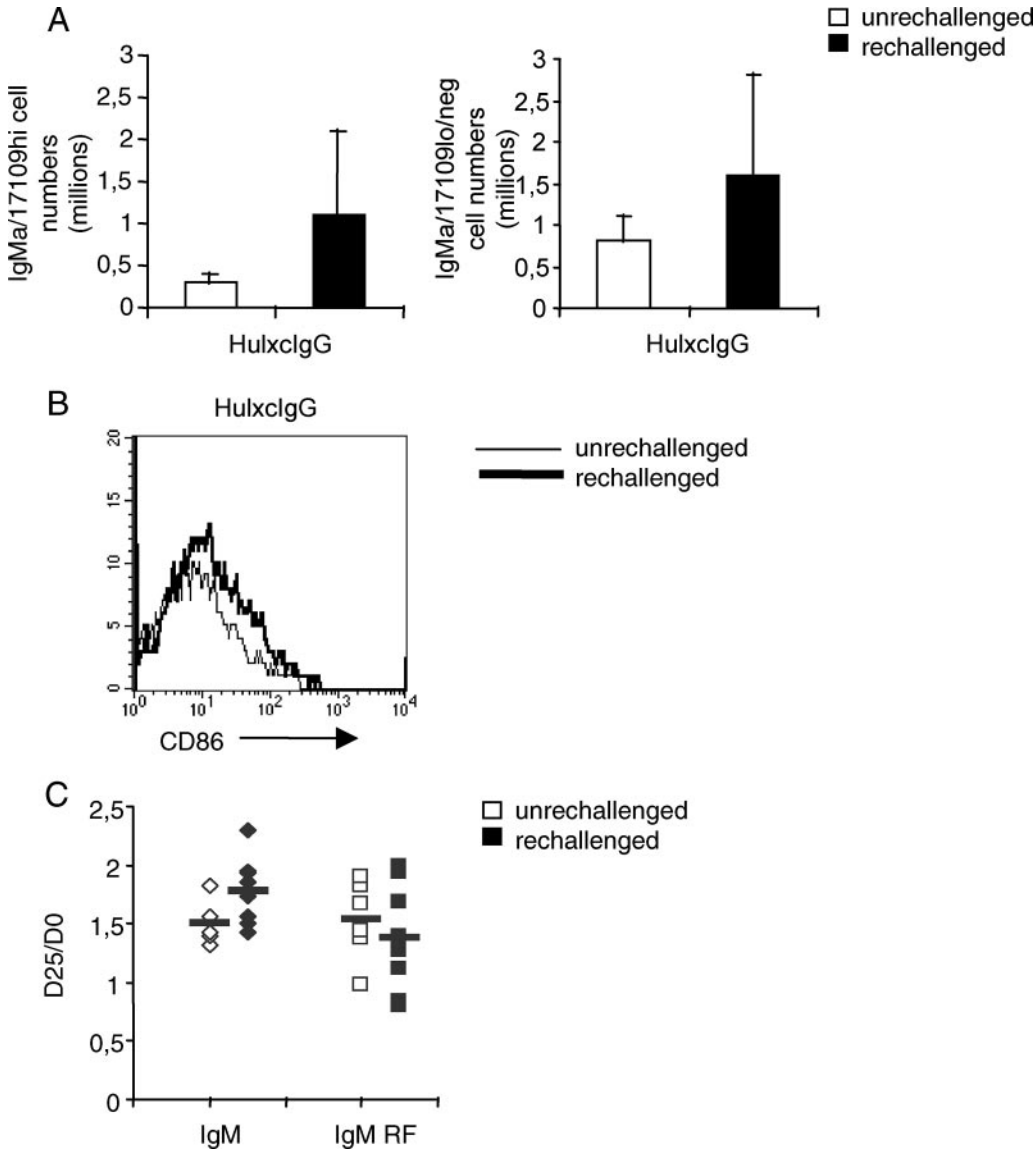

FIG. 5. Influenza virus rechallenge does not lead to RF production. (A) RF ($IgMa^+/17109^{hi}$) and non-RF ($IgMa^+/17109^{low/neg}$) cells are still expanded after viral rechallenge. Absolute RF and non-RF B-cell numbers were determined on D25 after primary infection (4 days after viral rechallenge) in six Hul × cIgG unrechallenged mice and in eight Hul × cIgG rechallenged mice. (B) CD86 expression on RF ($IgMa^+/17109^{hi}$) cells in infected mice (thick line) compared to that in uninfected mice (thin line) 4 days after viral rechallenge. (C) IgM and IgM RF ratios between D25 after infection (4 days after viral rechallenge) and day of infection in infected rechallenged (black symbols) and unrechallenged (open symbols) mice. Each point represents a mouse. IgM and IgM RF levels are not significantly different from those in uninfected control mice ($P < 0.05$, Mann-Whitney test).

Hul × cIgG mice were infected (D0), reinfected with the same dose of virus 21 days later (D21), and finally sacrificed 4 days after the last virus instillation (D25). Serum IgM and IgM-RF were monitored on D5, D15, and D25. Rechallenged mice were compared to mice that were infected once but not reinfected. After virus rechallenge, LN B-cell numbers were increased (two to three times the numbers found in unrechallenged animals). RF and non-RF B cells were equally expanded (Fig. 5A). RF B cells were slightly activated although not to the same extent as RF B cells from mice infected only once (Fig. 5B). In addition, RF production was not different in rechallenged mice compared to unrechallenged controls (Fig. 5C) despite the production of anti-influenza virus human IgG (OD, 0.0 before infection and from 0.2 to over 1.0 on D25 postinfection).

Thus, in our model of influenza virus experimental infection, RF B cells proliferate and are activated but do not produce RF during the course of a polyclonal activation, which is totally independent of the autoreactive nature of the BCR, indicating the lack of synergy between the B-cell effect of type I IFN and the IgG-containing immune complexes binding on RF B cells.

The nonspecific B-cell response to influenza virus does not rely on T-cell help. In the *B. burgdorferi* infection model, T cells are important for the breakdown of RF B-cell tolerance. We

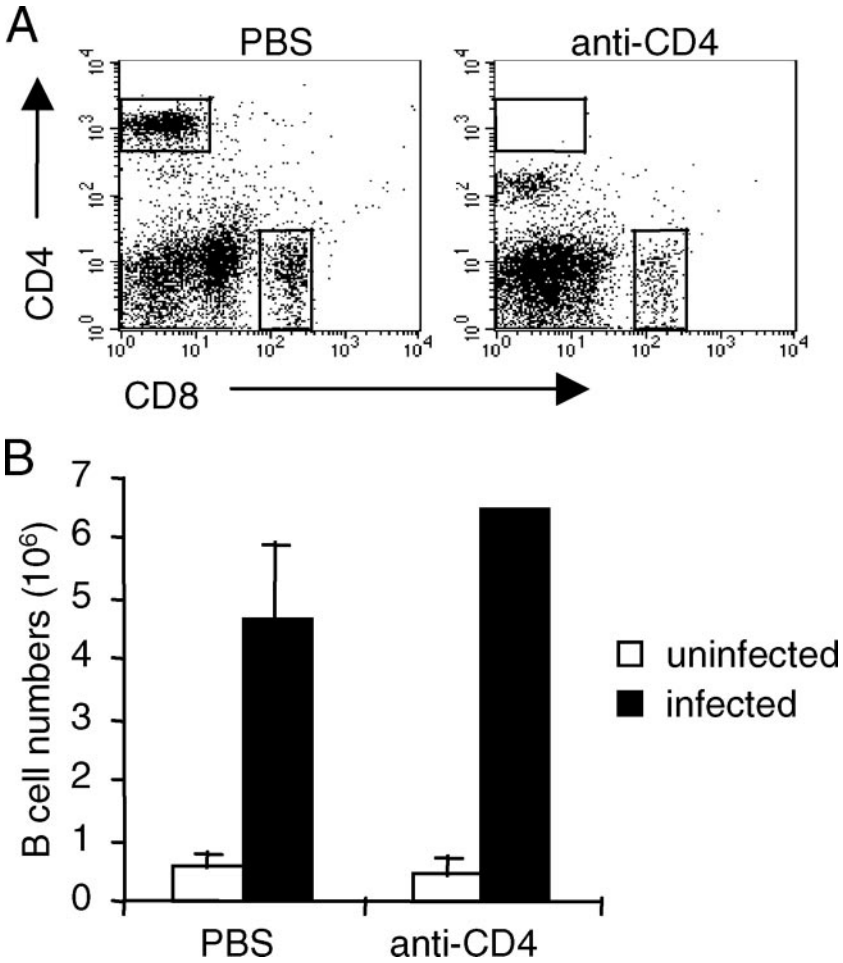

FIG. 6. Polyclonal activation of B cells by influenza virus is independent of the presence of CD4 T cells. (A) Control of CD4 T-cell blockade with the nondepleting anti-CD4 MAb YTS177 2 weeks after infection. (B) Absolute LN B220/IgM$^+$ cell numbers in millions in infected (black bars) and uninfected (open bars) mice, treated or not with anti-CD4 MAb, at 5 days after infection. Each value ± standard deviation represents the mean from four uninfected and untreated mice, five infected and untreated mice, two uninfected and treated mice, and two infected and treated mice. B-cell numbers in anti-CD4-treated and untreated mice are not statistically different ($P < 0.05$, Mann-Whitney test).

therefore wondered what part those cells would play during the infection of RF tg mice with influenza virus. To answer this question, we treated tg mice with a nondepleting anti-CD4 MAb under conditions demonstrated to block CD4 T cells for 1 week (12, 33). Animals were sacrificed 5 days postinfection, and LN B cells were analyzed by flow cytometry. Expansion of B cells was not affected by CD4 T-cell blockade (Fig. 6). IgM and IgM RF were still not increased in the sera of infected mice compared to uninfected controls (not shown).

B-cell response to influenza virus infection is not affected by lupus-prone genetic background. In order to know if the autoimmune genetic background could modify the effect of influenza virus-induced type I IFN production on autoreactive B cells, we used the same experimental infection with influenza virus in (NZB × NZW)F_1 mice at a predisease state (17 weeks of age, 4 months). Seven days postinfection, LN B-cell numbers in infected animals increased from two- to threefold compared to uninfected controls (Fig. 7A). Expression of activation markers CD86 and CD69 was only mildly increased in infected animals compared to uninfected controls (Fig. 7B). Influenza virus infection did not induce any increase in IgM or IgG anti-dsDNA, anti-ssDNA, or antinucleosome autoantibody production (Fig. 7C, D, and E).

DISCUSSION

Our results show that an acute viral infection of RF tg mice and lupus-prone mice, leading to an indirect polyclonal B-cell activation mediated by type I IFN, induces an abortive activation of autoreactive B cells that does not result in autoantibody production even after viral rechallenge. This polyclonal activation does not depend either on the presence of the autoantigen or on T-cell help.

We previously demonstrated that *B. burgdorferi* infection is able to overcome RF B-cell tolerance in the same tg mouse models, i.e., it induces proliferation and activation of RF B cells associated with BCR-dependent RF production. The effects of *B. burgdorferi* infection on RF B-cell tolerance depend on both a nonspecific B-cell activation, most likely based on TLR recognition, and an antigen-dependent signal mediated

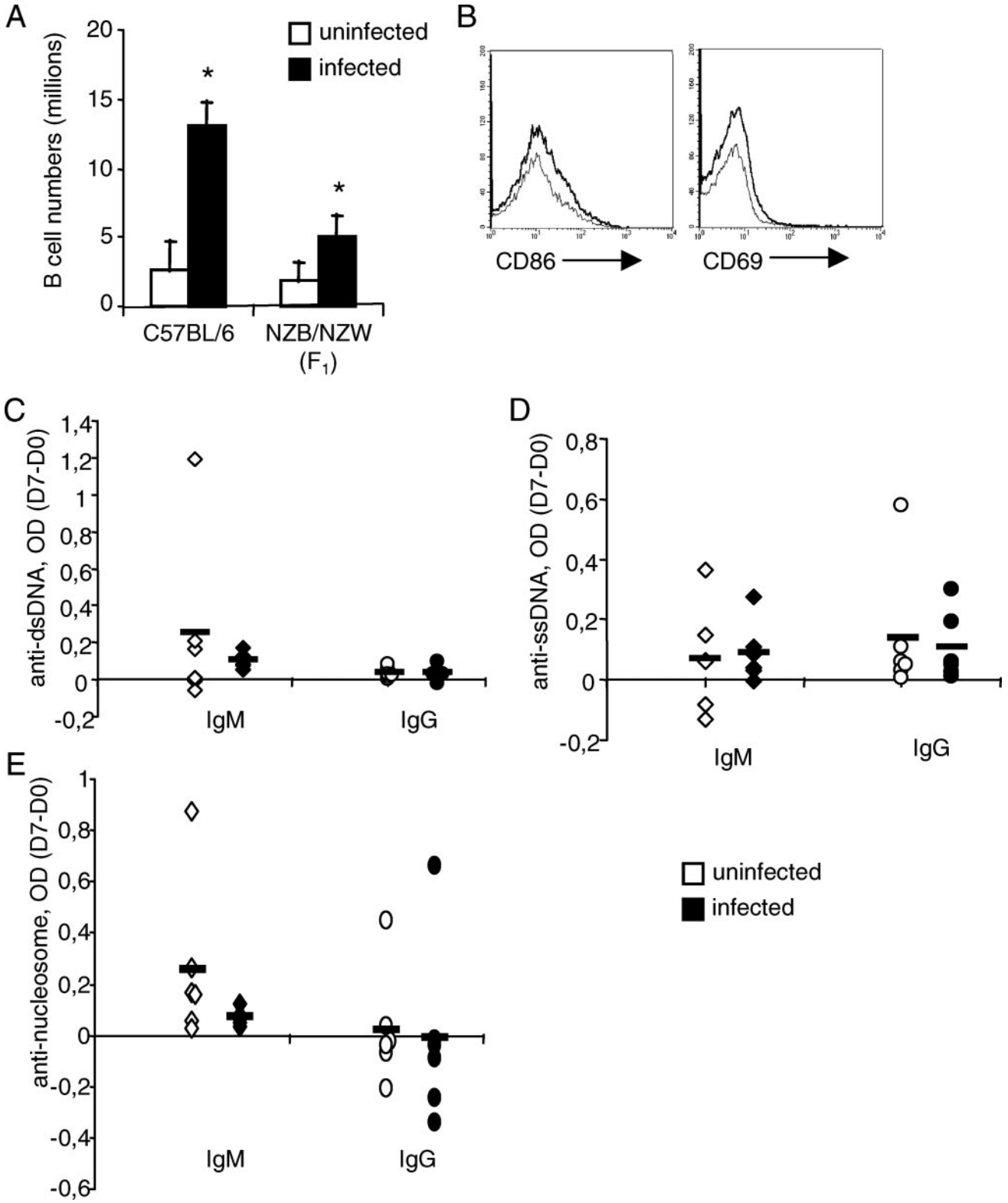

FIG. 7. B-cell response to influenza virus infection is not affected on a lupus-prone genetic background. (A) Absolute LN B220/IgM$^+$ cell numbers in millions in infected (black bars) and uninfected (open bars) mice 7 days after infection. Each value ± standard deviation represents the mean from six mice. Statistical difference is designated by an asterisk ($P < 0.05$, Mann-Whitney test). (B) Surface expression of CD86 on B cells (gated as IgM$^+$ cells) in infected mice (thick line) was compared to that in uninfected controls (thin line). (C) Anti-dsDNA IgM and IgG levels in infected (black symbols) and in uninfected (white symbols) mice 7 days after infection. Each point represents the OD increase (D7 to D0) for one mouse. Evolution of anti-dsDNA IgM or IgG was not statistically different between infected and uninfected mice (Mann-Whitney test). (D) Anti-ssDNA IgM and IgG levels in infected (black symbols) and in uninfected (white symbols) mice 7 days after infection. Each point represents the OD value for one mouse. Evolution of anti-dsDNA IgM or IgG was not statistically different between infected and uninfected mice (Mann-Whitney test). (E) Antinucleosome IgM and IgG levels in infected (black symbols) and in uninfected (open symbols) mice 7 days after infection. Each point represents the OD value for one mouse. Evolution of anti-dsDNA IgM or IgG was not statistically different between infected and uninfected mice (Mann-Whitney test).

by immune complexes containing *B. burgdorferi* antigens and anti-*B. burgdorferi* IgG. These complexes cross-link BCR and TLR on the surface of B cells, which leads to sustained RF production (33). During influenza virus infection of RF mice, direct activation of B cells by the virus is impossible. Nevertheless, B cells express TLR3 (2, 8) and TLR7 (36), which recognize dsRNA (1) and ssRNA (23), respectively, and should therefore be able to detect influenza virus. These receptors are expressed at the endosomal membrane and are thus not present on the cell surface (18, 24). This could explain why B cells on their own do not react to the presence of the virus and why the cross-link between TLR and the BCR that occurs at the surface of B cells during *B. burgdorferi* infection cannot take place. On the other hand, in 2006, Coro et al. showed that influenza virus induces production of type I IFN by respiratory tract epithelial cells, which is able to directly stimulate B cells through type I IFNR (13). We confirmed this result in vitro and showed that stimulation induced by influenza virus of total LN cells leads to a B-cell activation that is reversed by addition of type I IFN inhibitor, suggesting that a non-B-cell population produces type I IFN in the draining LN. Thus, influenza virus first activates epithelial cells or other cells present in the LN, like plasmacytoid dendritic cells (7, 15), which are high type I IFN producers (3, 10, 30), and then activates B cells. Taken together, these results are most striking because IFN-α has often been associated with several autoimmune diseases, and particularly systemic lupus erythematosus (SLE). Patients with severe SLE, for instance, have elevated levels of serum IFN-α during active disease; in addition peripheral blood cells of SLE patients show an IFN-α signature gene expression (5, 9). Moreover, lupus-prone NZB mice deficient for type I IFNR have significantly reduced disease development (35). However, under our experimental viral infection, influenza virus-induced type I production is not able to overcome B-cell tolerance both in nonautoimmune and in autoimmune disease-prone mice.

Polyclonal B-cell activation on its own is thought to be able to induce autoimmunity. As demonstrated by Hunziker et al., chronic murine infection with lymphocytic choriomeningitis virus (LCMV) leads to polyclonal hypergammaglobulinemia. This phenomenon is mediated by CD4 T cells, which are activated by interactions with B cells presenting LCMV antigens processed in a nonspecific manner. Thus, in this chronic model of infection, non-LCMV-specific B cells receive T-cell help and differentiate to produce polyclonal antibodies and autoantibodies (19). The authors demonstrate that these antibodies are of the IgG class and come from an induced switching of the IgM produced in the natural repertoire. The mechanisms underlying nonspecific B-cell activation during infections are numerous; they include direct activation by B-cell mitogens, cytokines released from activated T cells which may substitute nonspecifically for helper T cells, and even T-cell help provided through cognate interaction but independent of BCR specificity (14, 19, 27, 29, 31). However, in contrast to the LCMV infectious model, influenza virus induces an acute infection that is localized almost solely to the upper respiratory tract and draining LN. Moreover, T cells are not involved in the polyclonal activation of B cells that we observed during the course of infection. Therefore, several conditions known to induce autoantibodies during chronic infection with LCMV are not achieved here. This could explain why influenza virus infection induces only an abortive polyclonal activation of B cells that does not lead to a breakdown of RF B-cell tolerance shown by antigen-dependent sustained RF production.

In humans, infectious diseases with a wide variety of pathogens, such as subacute bacterial endocarditis, tuberculosis, or type C viral hepatitis, are frequently associated with high production of RF (16). At variance with our results, RF production can be induced by several rechallenges with influenza virus in BALB/c mice (17). We can suggest that, as for most pathogens, the susceptibility to influenza virus infection depends on the mouse line that is infected and on the strain of virus used for infection. In our model, both were different from the model of Fazekas et al. On the other hand, as hypothesized by Posnett and Edinger (26), the virus needs two distinct pathways to induce RF production. One is due to chronic exposure of B cells to repetitive epitopes on the viral surface that maintains polyclonal B-cell activation through a T-cell-independent mechanism. The other occurs when the virus epitopes are presented in immune complexes and therefore induce a T-cell-dependent and antigen-specific RF production (26). In our model, we suppose that this antigen-specific event is missing, probably due to the incapacity of influenza virus to directly activate B cells. RF are therefore not produced.

Thus, we show that infectious conditions necessary to overcome B-cell tolerance leading to the production of potentially harmful autoantibodies are quite limited and that an acute and localized viral infection inducing type I IFN production does not provide these conditions.

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