

# Chronic fatigue syndrome is associated with diminished intracellular perforin

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## Summary

Chronic fatigue syndrome (CFS) is an illness characterized by unexplained and prolonged fatigue that is often accompanied by abnormalities of immune, endocrine and cognitive functions. Diminished natural killer cell cytotoxicity (NKCC) is a frequently reported finding. However, the molecular basis of this defect of *in vitro* cytotoxicity has not been described. Perforin is a protein found within intracellular granules of NK and cytotoxic T cells and is a key factor in the lytic processes mediated by these cells. Quantitative fluorescence flow cytometry was used to the intracellular perforin content in CFS subjects and healthy controls. A significant reduction in the NK cell associated perforin levels in samples from CFS patients, compared to healthy controls, was observed. There was also an indication of a reduced perforin level within the cytotoxic T cells of CFS subjects, providing the first evidence, to our knowledge, to suggest a T cell associated cytotoxic deficit in CFS. Because perforin is important in immune surveillance and homeostasis of the immune system, its deficiency may prove to be an important factor in the pathogenesis of CFS and its analysis may prove useful as a biomarker in the study of CFS.

**Keywords:** chronic fatigue syndrome, cytoplasmic granules, flow cytometry, killer cells – natural, perforin

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## Introduction

Chronic fatigue syndrome (CFS) is an illness characterized by unexplained fatigue that persists for greater than 6 months, is not alleviated by rest, and is associated with at least four of eight case-defining symptoms such as impaired memory or concentration, sore throat or lymph nodes, myalgia or arthralgia [1]. Other conditions that could explain the illness must be excluded. The syndrome is estimated to affect over 800 000 peoples in the United States, although most are not diagnosed [2,3]. People with CFS are often sick for many years [4]; the prognosis for recovery is poor [4,5]. The lack of pathognomonic signs or accepted biomarkers contributes to the difficult nature of the study and clinical management of CFS. A consensus regarding the aetiology and pathophysiology of CFS has not yet developed. However, the concept of immune dysfunction remains actively debated, with reports of alterations in lymphocyte function, activation and subset distributions common [6,7]. Frequently, patients with chronic fatigue are found to have reduced cytotoxic activity of natural killer (NK) cells (NKCC) *in vitro* [6–9]. Often, the absolute count of NK cells

is also low. However, we showed reduced killing when effector cell concentration was defined as the number of CD56<sup>+</sup> CD3<sup>+</sup> lymphocytes in the assay and cytotoxic activity was expressed as percentage killing of target cells at an effector to target cell ratio of 1 : 1 [6,9]. Factors that contribute to reduced NKCC have not been studied in the context of CFS. The purpose of the present study was to investigate NK cell function and intracellular concentration of the lytic granule protein perforin in NK cells in a cohort of patients with CFS compared to matched sedentary but healthy controls. The importance of perforin in cytotoxic function led to its selection for study. Because of the decreased lytic activity of NK cells from patients with CFS, we hypothesized that the intracellular perforin content of NK cells would be reduced in CFS patients.

## Materials and methods

### Patients

The CFS study population consisted of 30 individuals (25 female, five male; mean age years  $\pm$  s.d.  $46 \pm 10$ ) with

symptoms that met the Centers for Disease Control and Prevention (CDC)'s 1994 case definition for CFS [1]. The mean age of onset was  $36 \pm 12$  years. Twenty-five subjects reported acute onset of the illness, while four reported gradual onset. The mean duration of illness was  $10 \pm 7$  years. The comparison group consisted of 19 apparently healthy but sedentary (no regular exercise programme) controls (16 female, three male; mean age years  $\pm$  s.d.  $43 \pm 10$ ). All subjects signed the informed consent and the University of Miami's Internal Review Board approved this protocol.

### Cell surface phenotyping by flow cytometry

Ethylenediamine tetraacetic acid (EDTA) anti-coagulated whole blood was surface-stained with optimal dilutions of CD45-FITC, CD14-PC5, CD19-ECD, CD2-FITC, CD3-FITC, CD56-PE, CD4-ECD, CD8-PC5 and CD26-PE and isotype controls in four colour combinations for 15 min at 25°C. Samples were then fixed and lysed with Optilyse-C reagent, followed by analysis on an XL-MCL flow cytometer. All reagents and instrumentation were from the Beckman Coulter Corporation (Hialeah, FL, USA). The accuracy and precision of analyses were optimized through the adherence to the CDC's recommendations for flow cytometric analyses [10].

### Flow cytometric assessment of intracellular perforin

We have developed a flow cytometric method for the semi-quantitative assessment of intracellular perforin. The details of this method have been published previously [11]. Heparinized whole blood (1 ml) was fixed with 1 ml 4% p-formaldehyde (p-fma) for 15 min, followed by the addition of 1 ml 17.5% bovine serum albumin (BSA), 0.1%  $\text{NaN}_3$  in phosphate buffered saline (PBS) for 10 min to stop fixation. The sample was washed twice with PBS containing 0.1% BSA, 0.1%  $\text{NaN}_3$ . Fixed samples were then resuspended in PBS containing 0.1% saponin (Sigma, St Louis, MO, USA) for 10 min and washed twice in PBS with 0.1% saponin. Aliquots of fixed and permeabilized cells (50  $\mu\text{l}$ ) were surface-stained for 15 min with optimal concentrations of CD8-FITC, CD3-ECD and CD56-PC5 antibodies (Beckman Coulter). Isotypic control or anti-perforin-phycoerythrin (PE) antibodies (PharMingen, San Diego, CA, USA) were then added to separate aliquots of the surface stained cells for 30 min, followed by two saponin washes, one PBS wash and one p-fma wash (0.1%). Samples were analysed on an XL-MCL flow cytometer. Analyses consisted of a sequential gating strategy whereby lymphocytes, defined by forward- and side-scatter properties, were then phenotyped as  $\text{CD3}^+ \text{CD56}^+$  (NK) cells or  $\text{CD3}^+ \text{CD8}^+$  (cytotoxic T) cells. The anti-perforin-PE fluorescence intensity was measured in the flow cytometer for each of these subsets and recorded as the median of the fluorescence channel. Semiquantitative deter-

minations of cell-associated perforin were then derived from these median fluorescence intensity values. Through the use of QuantiBRITE fluorescence standards (Becton Dickinson), a standard curve was calculated. These standards consist of a mixture of beads which are impregnated with graded levels of PE and each bead set comes with defined values (molecules PE/bead). Once these beads are analysed in the flow cytometer, a defined number of molecules of PE can be assigned to the median channel for the bead. From these channel numbers and assigned values, a standard curve is generated and used to convert median fluorescence intensity values for antiperforin-PE binding to relative molecules perforin (rMol P)/cell.

### NK cell cytotoxicity assay

The whole blood assay used relates the cytolytic activity of the blood sample to the number of cells in the sample that are phenotypically NK cells, as determined by flow cytometry and lymphocyte count [12]. The assay was performed in triplicate at four target to effector cell ratios. Heparinized blood was collected before noon and transported to the laboratory at ambient temperature so that the assay was started within 4 h of draw. An EDTA tube was collected for the complete blood count that was needed for the lymphocyte count. Target cells for the assay were the K562 cell line, which was grown to log phase and then labelled with  $^{51}\text{Cr}$ . Labelled target cells at final concentrations of 2, 1, 0.5 and  $0.25 \times 10^6$  cells/ml were prepared and placed in triplicate wells of 96-well flat-bottomed plates and 150  $\mu\text{l}$  of whole blood were added. The spontaneous release control was target cells plus 150  $\mu\text{l}$  of assay medium and total release control was target cells plus 150  $\mu\text{l}$  of 1% Triton X-100. The covered microtitre plate was centrifuged for 10 min at room temperature at 400 g and incubated for 4 h at 37°C in 5%  $\text{CO}_2$  humidified atmosphere. Incubation was terminated by dispensing 100  $\mu\text{l}$  chilled (4°C) assay medium to each well. The plate was centrifuged for 10 min at 400 g. Supernatant fluid (100  $\mu\text{l}$ ) was transferred to counting tubes and counted in a gamma counter. To relate the cytotoxic activity of the blood sample to the cells in the sample that were phenotypically NK cells, the lymphocyte count and four-colour flow cytometry were performed to determine the number of  $\text{CD56}^+ \text{CD3}^-$  cells. Calculation of percentage cytotoxicity (CYT) for each dilution of labelled target cells was performed as follows:

$$\text{CYT} = ((\text{ER}-b) - (\text{SR}-b)/(\text{TR}-b) - (\text{SR}-b)) \times 100$$

where ER = mean counts per minute (cpm) of experimental release, SR = mean cpm of spontaneous release, TR = mean cpm of total release and b = gamma counter instrument background in cpm.

From the CYT of target cells killed and the number of target cells in the well, with the number of effector cells ( $\text{CD56}^+ \text{CD3}^-$  lymphocytes) held constant, a regression

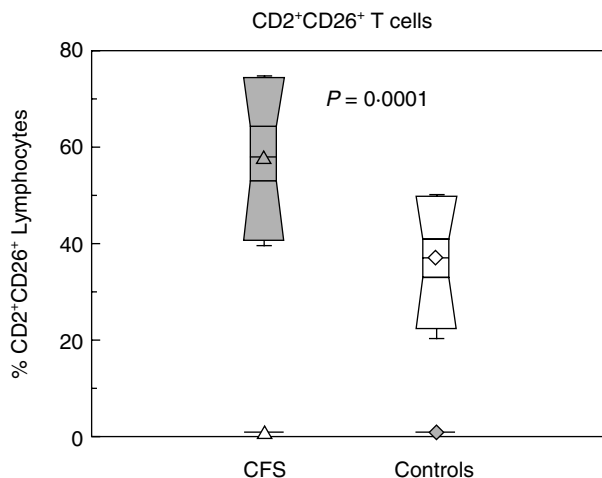
analysis was performed to calculate NKCC (the percentage of cytotoxicity at an effector to target ratio of 1 : 1).

### Statistics

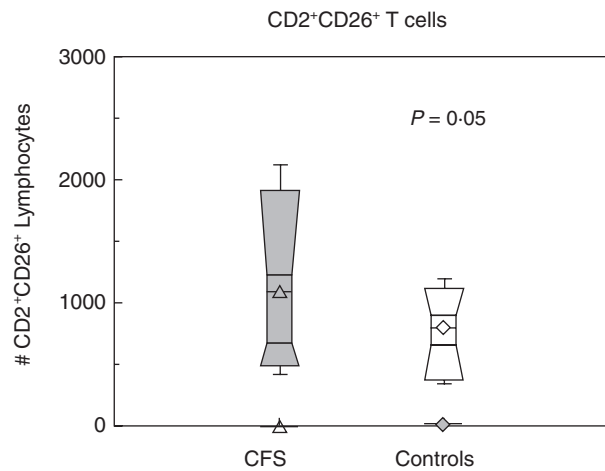
All statistical comparisons were made using Student's *t*-test. Results are presented as mean  $\pm$  standard error of the mean (s.e.m.). Correlations were made using the Pearson product-moment test.

### Results

Standard surface flow cytometric phenotyping demonstrated that the CFS group had a significantly reduced number of CD3<sup>+</sup> CD56<sup>+</sup> (NK) cells/ $\mu$ l of blood compared to the control group ( $122 \pm 10$  and  $184 \pm 27$ , respectively;  $P = 0.04$ ). No differences were found between the subject groups for the percentage of CD3<sup>+</sup> CD56<sup>+</sup>, CD3<sup>+</sup> CD4<sup>+</sup>, CD3<sup>+</sup> CD8<sup>+</sup>, CD19<sup>+</sup>, CD3<sup>+</sup> or CD2<sup>+</sup> lymphocytes. The lymphocyte (T cells + NK cells) compartment of the CFS group was found to be activated, as evidenced by a significant elevation in the percentage of CD2<sup>+</sup> CD26<sup>+</sup> subset when compared to healthy controls ( $58\% \pm 3$  and  $38\% \pm 3$ , respectively;  $P = 0.001$ ; Fig. 1). The number of CD2<sup>+</sup> CD26<sup>+</sup> lymphocytes/ $\mu$ l of blood was also elevated in CFS patients ( $1069 \pm 122$  and  $779 \pm 66$ , respectively;  $P = 0.05$ ; Fig. 2). Consistent with the preponderance of previous reports in the literature, the cytotoxic activity of natural killer cells from subjects with CFS ( $21\% \pm 2$  of K562 cells killed at an effector to target cell ratio of 1 : 1) compared to healthy controls ( $39\% \pm 5$ ) was significantly lower ( $P = 0.001$ ), as shown in Fig. 3. In order to begin our study of NK function on the molecular level, we

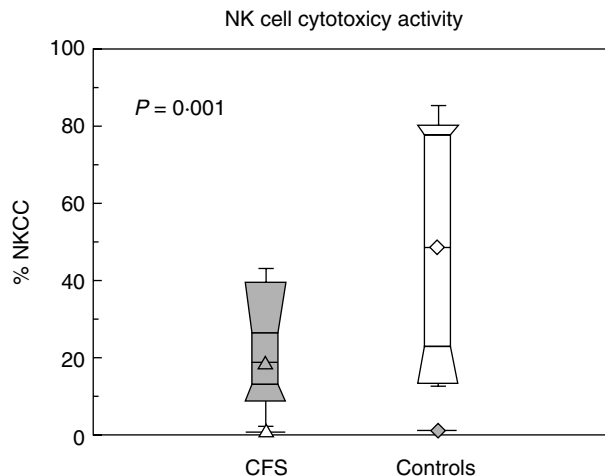


**Fig. 1.** Flow cytometry indicated that the percentage of lymphocytes [T cells and natural killer (NK) cells] co-expressing surface CD2 and CD26 was significantly elevated in the chronic fatigue syndrome (CFS) patients compared to controls. The results are shown as a box-whisker plot. The horizontal lines mark the minimum and maximum, and the 10th, 25th, 50th, 75th and 90th percentile points.

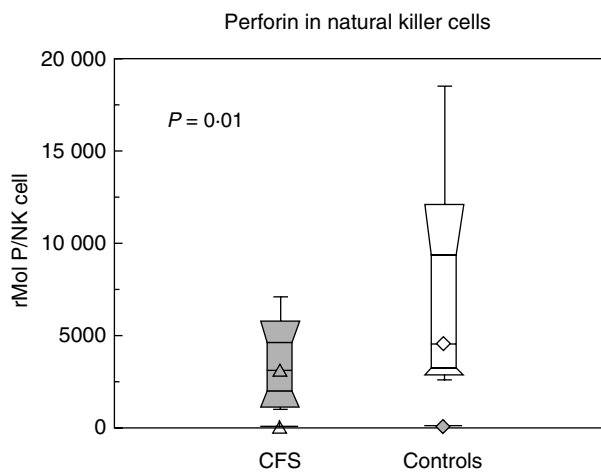


**Fig. 2.** Flow cytometry indicated that the number of CD2<sup>+</sup> lymphocytes [T cells and natural killer (NK) cells]/ $\mu$ l of blood expressing surface CD26 was elevated in the chronic fatigue syndrome (CFS) patients compared to controls. The results are shown as a box-whisker plot. The horizontal lines mark the minimum and maximum, and the 10th, 25th, 50th, 75th and 90th percentile points.

used intracellular staining techniques to make quantitative fluorescence measures of intracellular perforin. Perforin in NK cells was found to be significantly reduced in CFS subjects when compared to controls ( $3320 \pm 313$  and  $6051 \pm 956$  rMol P/NK cell, respectively,  $P = 0.01$  (Fig. 4). Two representative analyses demonstrating the difference in fluorescence intensity of antiperforin binding is shown in Fig. 5 wherein the CFS subject had a lower level of anti-perforin binding



**Fig. 3.** Subjects with chronic fatigue syndrome had significantly lower natural killer (NK) cytotoxicity (NKCC) as determined by the <sup>51</sup>Cr release assay, when compared to healthy controls. The results are expressed as a percentage of target cells (K562 cells) killed in the 4 h assay at an effector cell (CD56<sup>+</sup>CD3<sup>+</sup> lymphocytes) to target cell ratio of 1 : 1. Results are shown as a box-whisker plot. The horizontal lines mark the minimum and maximum, and the 10th, 25th, 50th, 75th and 90th percentile points.



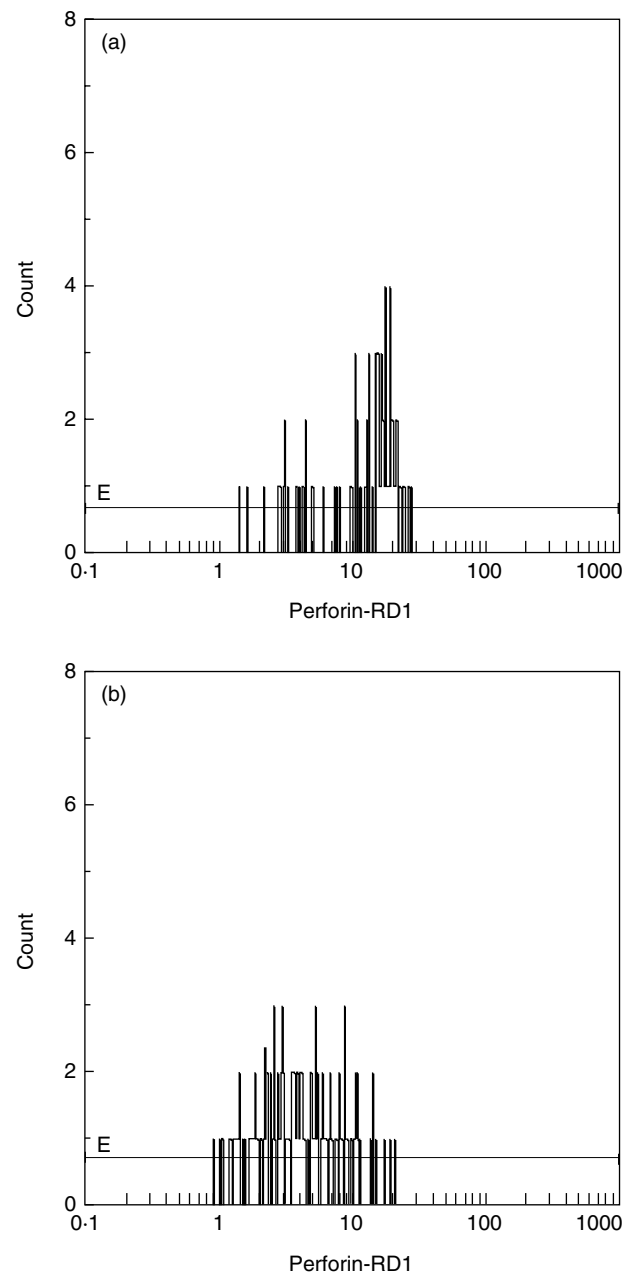
**Fig. 4.** Subjects with chronic fatigue syndrome had significantly lower intracellular perforin in natural killer (NK) cells as determined by quantitative fluorescence. Results are expressed as relative number of molecules of perforin (rMol P) per CD56<sup>+</sup> CD3<sup>+</sup> lymphocyte. The results are shown as a box-whisker plot. The horizontal lines mark the minimum and maximum, and the 10th, 25th, 50th, 75th and 90th percentile points.

than the healthy control. Analysis of the perforin content of the CD3<sup>+</sup> CD8<sup>+</sup> cytotoxic T cell subset revealed a reduction of intracellular perforin content in the CFS group relative to controls that approached statistical significance ( $270 \pm 69$  and  $899 \pm 309$  rMol P/Tc cell, respectively,  $P = 0.06$  (Fig. 6). The correlation of rMol P/NK cell and NKCC at an effector to target cell ratio of 1 : 1 for all individuals studied (CFS subjects plus controls) was significant ( $P = 0.01$ ), as shown in Fig. 7.

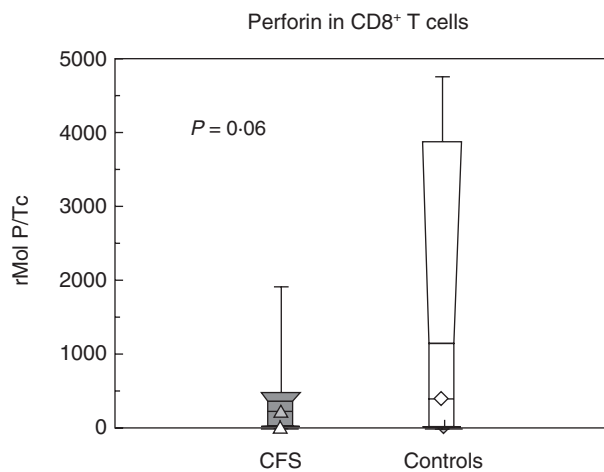
## Discussion

Many of the symptoms of CFS are inflammatory in nature (myalgia, arthralgia, sore throat, tender lymphadenopathy), and have prompted a theory of infection induced illness. CFS often presents with acute onset of illness (reported in 60–80% of published samples) with systemic symptoms similar to influenza infection that do not subside [13]. However, reports of associated microbial infection or of latent virus reactivation have been inconsistent. Whether associated with a known antigen (e.g. a specific infection) or not, there is a considerable literature describing immune activation in CFS. These reports have described the elevation of lymphocyte surface activation markers [6,14], the expression of proinflammatory cytokines and evidence of Th2 cytokine increase [15–17]. In order to determine if the cohort of CFS patients in this study had evidence of immune activation we elected to analyse the surface marker, CD26 (dipeptidyl peptidase IV). This ectoenzyme is known to increase upon cell activation [18]. DPPIV/CD26 is a multifunctional molecule that is a proteolytic enzyme, receptor, co-stimulatory protein, and is involved in adhesion and apoptosis. CD26 is associated on

T cells with adenosine deaminase (ADA), and plays a major role in immune response. Abnormal expression is found in autoimmune diseases, HIV-related diseases and cancer [19]. Compared to controls, the CFS patients we studied had a significantly elevated percentage and absolute count of CD26<sup>+</sup> lymphocytes.

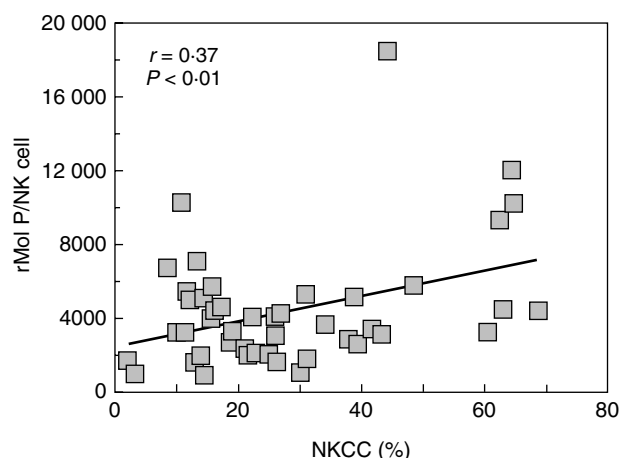


**Fig. 5.** Representative histograms showing the fluorescence intensity of the antiperforin antibody binding in natural killer (NK) cells. The x-axis corresponds to channel number and the y-axis corresponds to relative frequency of events. (a) Healthy control 222641, median fluorescence intensity = 13.2; rMolP per NK cell = 9348. (b) Chronic fatigue syndrome (CFS) patient 234467, median fluorescence intensity = 3.7; rMolP per NK cell = 3404.



**Fig. 6.** Subjects with chronic fatigue syndrome had a lower intracellular cytotoxic T cell perforin level as determined by quantitative fluorescence that bordered on statistical significance. Results are expressed as relative number of molecules of perforin (rMolP) per CD8<sup>+</sup> CD3<sup>+</sup> lymphocyte. The results are shown as a box-whisker plot. The horizontal lines mark the minimum and maximum, and the 10th, 25th, 50th, 75th and 90th percentile points.

The laboratory finding reported with the greatest consistency in CFS patients is that of reduced NKCC [7,9]. In the few studies that failed to find depressed NK activity in CFS subjects, methodological problems may have been responsible [7]. In the present study, consistent with previous reports, the NK-mediated cytotoxicity of CFS subjects against the K562 cell targets was significantly lower than that of controls. Although the CFS subjects in this cohort also had significantly lower numbers of NK cells (CD56<sup>+</sup> CD3<sup>-</sup>) per volume of blood, the diminished cytotoxicity was due to a decreased functional capacity of the NK cells, as the percent-



**Fig. 7.** There was a significant correlation between natural killer (NK) cell activity (NKCC) and amount of intracellular perforin (rMolP). Filled symbols represent the individual CFS subjects and control subjects in this study. The line represents the regression line.

age of killing in the <sup>51</sup>Cr release assay was calculated for both subject groups at a 1 : 1 target to CD56<sup>+</sup> CD3<sup>-</sup> effector cell ratio. NK cells are critical for immune surveillance against fungal, bacterial and viral infections. They also play a vital role in cellular resistance to malignancy and tumour metastasis [20]. NK cells can destroy their target cells by calcium-dependent release of cytolytic granules, by activation of the Fas (CD95) pathway or through contact with tumour necrosis factor (TNF)- $\alpha$  [21]. Perforin is released along with granzymes, particularly granzyme B, from intracellular vesicles of cytolytic effector cells and facilitates passage of these molecules through target cell membranes, which then activate the apoptotic pathways of the caspases [21]. NK cells differ from the other cytotoxic effector cell types (CTL) in two major ways: they kill the target cells in a non-major histocompatibility complex (MHC)-restricted fashion without the need for previous *in vitro* or *in vivo* activation, and only NK cells express constitutively the lytic machinery [20,21].

There was a recent suggestion in the literature of perforin reduction in CFS. Steinhau *et al.* [22] used differential display polymerase chain reaction (PCR) to search for candidate biomarkers in CFS. RNA expression profiles of one subject with CFS and an age- and sex-matched control showed differential expression of 10 genes. Of these, five were down-regulated and one was perforin. In the present report, we found that the relative number of molecules of perforin per NK cells from CFS patients was significantly below that found in matched healthy controls. This finding added support to the concept of an NK-associated immune deficit in CFS and suggested that the decrease in cytolytic potential of NK cells might be associated with a reduction in the cell-associated concentrations of the effector molecule perforin. A similar finding, which approached statistical significance ( $P = 0.06$ ), was a deficit in the perforin content of the cytotoxic T subset. The observed decrease of perforin in T cells was of an even greater magnitude than that seen in NK cells (~30% and 55% of control levels for Tc and NK, respectively). To our knowledge, this is the first report of evidence to suggest a deficit in the cytotoxic T cell compartment of CFS patients. These findings have considerable significance in providing a potential mechanism in the pathogenesis of CFS.

Two areas of research provided important insight in the role of perforin in normal and pathological conditions. The perforin knockout mouse, a model of perforin deficiency, demonstrated the role of perforin as a cytotoxic effector molecule through decreased cytotoxicity against virus infected and allogeneic targets [23] and reduced clearance of intracellular pathogens and tumours [24,25]. These mice had increased numbers of activated CD8 cells [26] and altered cytokine production with elevated expression of interleukin (IL)-1, interferon (IFN)- $\gamma$  and TNF- $\alpha$  [27–29]. In the absence of perforin, the granule protein granzyme A was proinflammatory, and induced IL-6 and IL-8 expression [30].



A second area of investigation that pointed to the significance of perforin in health and disease comes from studies of humans with the condition known as familial haemophagocytic lymphohistiocytosis (FHL). This rare and fatal disorder of early childhood was associated with a genetic mutation that, in the homozygous state, resulted in the absence of perforin expression [31]. Individuals with FHL were found to suffer from an extensive immune activation, expansion and infiltration of activated lymphocytes throughout the body, increased expression of proinflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-1 and IL-6) and severely impaired cytotoxic abilities [32–34].

Given the deficiency of perforin among patients with CFS reported here and the role of perforin in immune surveillance and immunomodulation, we suggest that decreased intracellular perforin content may play a role in the pathogenesis of CFS, an illness reported to be associated with increased immune activation, inflammatory cytokine levels, herpes virus reactivation and decreased cytotoxicity. The mechanism responsible for the reduced perforin in CFS remains unknown. The mechanisms may include genetic deficiency or chronic microbial activation leading to perforin consumption and exhaustion. An example of the latter mechanism is found in the report of reduced NKCC and reduced intracellular perforin in patients with chronic hepatitis C virus infection [35]. CFS is an illness whose symptomatic expression is variable and any hypothesis regarding the development of CFS should account for this variability. It can be envisioned that either mechanism mentioned above could be subject to variability. The diversity of symptoms may be due to the degree of perforin deficiency, as a gene dosage effect is seen in mice [36] and humans [34]; those who are heterozygous for the deficiency display an intermediate phenotype. Secondly, exposure to activating stimuli may be necessary for the evolution of symptomatic CFS in that perforin deficiency in both children and C57BL/6 perforin knockout mice is not associated with pathology until a viral infection stimulates an uncontrolled immune activation [36,37]. Thus, superimposition of immune activation, either microbial, autoimmune or even allergic, may vary between those susceptible and alter the course of illness. Thirdly, lymphocytes have multiple mechanisms to mediate killing. The perforin pathway of cytotoxicity is distinct from that mediated by Fas or TNF- $\alpha$ . Perforin is essential for both lytic and granzyme-mediated apoptotic killing but not Fas-mediated killing [21]. Because these systems provide a similar function, varying levels of redundancy among patients may explain varying degrees of susceptibility to developing symptomatic illness.

## Conclusions

We have presented evidence of a significant reduction in the intracellular content of perforin among patients with chronic fatigue syndrome. This molecule plays an important

role in immune surveillance against microbes and neoplasia as well as in immune homeostasis. Such a deficiency is likely to be associated with altered immune function and we would propose that this finding may be important in the pathogenesis of CFS. However, this pathogenic process is probably multifactorial, and variations in susceptibility and inciting stimuli may account for the constellation of symptoms seen in CFS. Perforin deficiency may prove useful as a biological marker in CFS – perhaps one that will help define a subgroup with a common pathogenesis.

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