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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 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; Killer Cells, Natural; Cytoplasmic Granules; Preforin; Flow Cytometry

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 persons in the United States, although most are not diagnosed [2, 3]. Persons with CFS are often sick for many years [4]. 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 etiology 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 NK cells (NKCC) *in vitro* [6, 7, 8, 9]. Often, the absolute count of NK cells is also low. However, we showed reduced

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killing when effector cell concentration was defined as the number of CD56+CD3- lymphocytes in the assay and cytotoxic activity was expressed as % 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, 5 male; mean age years \pm s.d.: 46 ± 10) with symptoms that met the CDC's 1994 case definition for CFS [1]. The mean age of onset was 36 ± 12 years. Twenty-five subjects reported acute onset of the illness while four reported gradual onset. The mean duration of illness was 10 ± 7 years. The comparison group consisted of 19 apparently healthy but sedentary (no regular exercise program) controls (16 female, 3 male; mean age years \pm s.d.: 43 ± 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

Ethylene diamine tetraacetic acid (EDTA) anticoagulated 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, CD26-PE, and isotype controls, in 4 color combinations for 15 min, 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 Beckman Coulter Corporation, Hialeah, Florida. 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 are published [11]. Heparinized whole blood (1 ml) was fixed with 1 ml 4% p-formaldehyde (p-fma) for 15 minutes, followed by the addition of 1ml 17.5% BSA, 0.1% NaN₃ in phosphate buffered saline (PBS) for 10 minutes to stop fixation. The sample was washed 2 times with PBS containing 0.1% bovine serum albumin, 0.1% NaN₃. Fixed samples were then re-suspended in PBS containing 0.1 % saponin (Sigma, St Louis, MO) for 10 minutes and washed 2 times in PBS with 0.1% saponin. Aliquots of fixed and permeabilized cells (50 ul) were surface stained 15 minutes with optimal concentrations of CD8-FITC, CD3-ECD and CD56-PC5 antibodies (Beckman Coulter, Hialeah, FL). Isotypic control or anti-perforin-PE antibodies (Pharmingen, San Diego, CA) were then added to separate aliquots of the surface stained cells for 30 minutes, followed by 2 saponin washes and 1 PBS wash. Samples were analyzed 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 CD3-CD56+ (NK) cells or CD3+CD8+ (cytotoxic T) cells. The anti-perforin-PE fluorescence intensity was measured in the flow cytometer for each of these subsets and recorded as median the fluorescence channel. Semi-quantitative determinations of cell associated perforin were then derived from these median fluorescence intensity values. Through the use of QuantiBRITE fluorescence standards (Becton Dickinson, San Jose, CA), a standard curve was calculated. These standards consist of a mixture of beads which are impregnated with graded levels of phycoerythrin and each bead set comes with defined values (molecules PE / bead). Once these beads are analyzed 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 anti-perforin-PE binding to relative molecules perforin (rMol P)/cell.

Natural killer 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 done in triplicate at four target to effector cell ratios. Heparinized blood was collected before noon and transported to lab at ambient temperature so that the assay was started within 4 hours of draw. An EDTA tube was collected for the complete blood count that was needed for lymphocyte count. Target cells for the assay were the K562 cell line which was grown to log phase and then labeled with ^{51}Cr . Labeled target cells at final concentrations of 2, 1, 0.5, and 0.25×10^6 cells/ml were prepared and placed in triplicate wells of 96 well flat bottom plate and 150 μl of whole blood were added. The spontaneous release control was target cells plus 150 μl of assay medium and total release control was target cells plus 150 μl of 1% Triton X-100. The covered microtiter plate was centrifuged 10 minutes at room temperature at $400 \times g$; and incubated for 4 hours at 37°C in 5% CO_2 humidified atmosphere. Incubation was terminated by dispensing 100 μl chilled (4°C) assay medium to each well. The plate was centrifuged for 10 minutes at $400 \times g$. Supernatant fluid (100 μl) 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 color flow cytometry were done to determine the number of $\text{CD}56+\text{CD}3-$ cells. Calculation of percentage cytotoxicity (CYT) for each dilution of labeled target cells was done as follows:

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

where ER = mean 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 ($\text{CD}56+\text{CD}3-$ lymphocytes) held constant, a regression analysis was performed to calculate NKCC (the percent cytotoxicity at an effector to target ratio of 1:1).

Statistics

All statistical comparisons were made using the Students T Test. Results are presented as mean \pm standard error of the mean (SEM). 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 $\text{CD}3-\text{CD}56+$ (NK) cells/ μL of blood compared to the control group (122 ± 10 and 184 ± 27 , respectively; $p = 0.04$). No differences were found between the subject groups for the percent $\text{CD}3-\text{CD}56+$, $\text{CD}3+\text{CD}4+$, $\text{CD}3+\text{CD}8+$, $\text{CD}19+$, $\text{CD}3+$ or $\text{CD}2+$ lymphocytes. The lymphocyte (T cells + NK cells) compartment of the CFS group was found to be activated as evidenced by a significant elevation in percent $\text{CD}2+\text{CD}26+$ subset when compared to healthy controls ($58\% \pm 3$ and $38\% \pm 3$, respectively; $p = 0.001$; Fig. 1). The number of $\text{CD}2+\text{CD}26+$ lymphocytes/ μL of blood was also elevated in CFS patients (1069 ± 122 and 779 ± 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 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 ± 313 and 6051 ± 956 rMol P/NK cell respectively, $p = 0.01$ (Fig.4). two representative analyses demonstrating the difference in fluorescence intensity of anti-perforin binding is shown in Fig. 5 wherein the CFS subject had a lower level of perforin binding than the healthy control. Analysis of the perforin content of the CD3+CD8+ cytotoxic T cell subset revealed a reduction of intracellular perforin content in the CFS group relative to controls that approached statistical significance (270 ± 69 and 899 ± 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 to 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 elevation of lymphocyte surface activation markers [6,14], expression of pro-inflammatory cytokines and evidence of Th2 cytokine increase [15, 16, 17]. In order to determine if the cohort of CFS patients in this study had evidence of immune activation we elected to analyze 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, costimulatory 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 significantly elevated percent and absolute count of CD26+ lymphocytes.

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, methodologic 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+CD3-) per volume of blood, the diminished cytotoxicity was due to a decreased functional capacity of the NK cells, since the percent killing in the ^{51}Cr release assay was calculated for both subject groups at a 1:1 target to CD56+CD3 - 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 tumor 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 TNF- α [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-MHC-restricted fashion without the need for previous in vitro or in vivo activation, and only NK cells constitutively express 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 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 of these 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 pathologic 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 tumors [24-25]. These mice had increased numbers of activated CD8 cells [26] and altered cytokine production with elevated expression of IL-1, IFN γ and TNF α [27-29]. In the absence of perforin, the granule protein granzyme A was pro-inflammatory, and induced IL6 and IL8 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 hemophagocytic 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 γ , TNF α , 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 since a gene dosage effect is seen in mice [36] and humans [34]; those who are heterozygous for the deficiency display an intermediate phenotype. Second, 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. Third, lymphocytes have multiple mechanisms to mediate killing. The

perforin pathway of cytotoxicity is distinct from that mediated by Fas or TNF α . 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.

CONCLUSION

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 likely multi-factorial, and variations in susceptibility and inciting stimuli may account for the constellation of symptoms seen in CFS. Perforin deficiency may prove useful as a biologic marker in CFS - perhaps one that will help define a subgroup with a common pathogenesis.

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REFERENCES

1. Fukuda K, Straus SE, Hickie I, et al. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. *Ann Int Med* 1994;121:953–9.
2. Reyes M, Nisenbaum R, Hoaglin DC, et al. Prevalence and incidence of chronic fatigue syndrome in Wichita, Kansas. *Arch Intern Med* 2003;163:1530–36. [PubMed: 12860574]
3. Jason LA, Richman JA, Rademaker AW, et al. A community-based study of chronic fatigue syndrome. *Arch Intern Med* 1999;159:2129–37. [PubMed: 10527290]
4. Reyes M, Dobbins JG, Nisenbaum R, Subedar NS, Randall B, Reeves WC. Chronic fatigue syndrome progression and self-defined recovery: evidence from the CDC surveillance system. *J Chronic Fatigue Syndr* 1999;5:17–27.
5. Hill NF, Tiersky LA, Scavalla VR, Laviertes M, Natelson BH. Natural history of severe chronic fatigue syndrome. *Arch Phys Med Rehabil* 1999;80:1090–94. [PubMed: 10489014]
6. Klimas NG, Salvato FR, Morgan R, Fletcher MA. Immunologic abnormalities in chronic fatigue syndrome. *J Clin Micro* 1990;28:1403–10.
7. Maher, K.; Klimas, NG.; Fletcher, MA. Immunology. In: Jason, L.; Fennell, P.; Taylor, R., editors. *Handbook of Chronic Fatigue Syndrome*. John Wiley and Sons; Hoboken, New Jersey: 2003. p. 124.-51.
8. Caligiuri M, Murray C, Buchwald D, et al. Phenotypic and functional deficiency of natural killer cells in patients with chronic fatigue syndrome. *J Immunol* 1987;139:3306–13. [PubMed: 2824604]
9. Fletcher MA, Maher K, Klimas NG. Natural killer cell function in chronic fatigue syndrome. *Clin Appl Immunol Rev* 2002;2:129–39.
10. 1997 revised guidelines for performing CD4+ T-cell determinations in persons infected with human immunodeficiency virus (HIV). Centers for Disease Control and Prevention. *Morb Mortal Wkly Rep* 1997;46:1–29.
11. Maher KJ, Klimas NG, Hurwitz B, Schiff R, Fletcher MA. Quantitative Fluorescence Measures for the Determination of Intracellular Perforin Content. *Clin Diag Lab Immunol* 2002;9:1248–52.
12. Patarca, R.; Fletcher, MA.; Podack, E. Cytotoxic lymphocytes. In: Rose, N.; deMacario, E.; Folds, J.; Lane, HC.; Nakamura, R., editors. *Manual of Clinical Laboratory Immunology*. 5th Edn. ASM Press; Washington, D.C.: 1997. p. 29.-303.
13. Plioplys, A. Differential diagnosis in medical assessment. In: Jason, L.; Fennell, P.; Taylor, R., editors. *Handbook of Chronic Fatigue Syndrome*. John Wiley and Sons; Hoboken, New Jersey: 2003. p. 26.-41.

14. Landay AL, Jessop C, Lennette ET, Levy JA. Chronic fatigue syndrome: clinical condition associated with immune activation. *Lancet* 1991;338(8769):707–12. [PubMed: 1679864]
15. Skowera A, Cleare A, Blair D, Bevis L, Wessely SC, Peakman M. High levels of type 2 cytokine-producing cells in chronic fatigue syndrome. *Clin Exp Immunol* 2004;135:294–302. [PubMed: 14738459]
16. Gupta S, Aggarwal S, See D, Starr A. Cytokine production by adherent and non-adherent mononuclear cells in chronic fatigue syndrome. *J Psych Res* 1997;31:149–56.
17. Patarca R, Klimas NG, Lugtendorf S, et al. Dysregulated expression of tumor necrosis factor in chronic fatigue syndrome: interrelations with cellular sources and patterns of soluble immune mediator expression. *Clin Inf Dis* 1994;18:S147–53.
18. Kameoka J, Tanaka T, Nojima, et al. Direct association of adenocine diaminase with T cell activation antigen, CD26. *Science* 1994;261:466–70. [PubMed: 8101391]
19. Boonacker E, Van Noorden CJ. The multifunctional or moonlighting protein CD26/DPPIV. *Eur J Cell Biol* 2003;82:53–73. [PubMed: 12647932]
20. Moretta L, Bottino D, Pende MC, et al. Human natural killer cells: their origin, receptors and function. *Eur J Immunol* 2002;32:1205–11. [PubMed: 11981807]
21. Barry M, Bleackley R. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol* 2002;2:401–409. [PubMed: 12093006]
22. Steinau M, Unger ER, Vernon SD, et al. Differential-display PCR of peripheral blood for biomarker discovery in chronic fatigue syndrome. *J Mol Med* 2004;82:750–55. [PubMed: 15490094]
23. Kagi D, Ledermann B, Burki K, et al. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 1994;369:31–37. [PubMed: 8164737]
24. Kagi D, Ledermann B, Burki K, et al. CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur J Immunol* 1994;24:3068–72. [PubMed: 7805735]
25. Tang Y, Hugin AW, Gies NA, et al. Control of immunodeficiency and lymphoproliferation in mouse AIDS: studies of mice deficient in CD8+ T cells or perforin. *J Virol* 1997;71:1808–13. [PubMed: 9032310]
26. Kagi D, Odermatt B, Mak TW. Homeostatic regulation of CD8+ T cells by perforin. *Eur J Immunol* 1999;29:3262–72. [PubMed: 10540338]
27. Sambhara S, Switzer I, Kurichh A, et al. Enhanced antibody and cytokine responses to influenza viral antigens in perforin-deficient mice. *Cellular Immunol* 1998;187:13–8. [PubMed: 9681998]
28. Kehren J, Desvignes C, Krasteva M, et al. Cytotoxicity is mandatory for CD8(+) T cell-mediated contact hypersensitivity. *J Expl Med* 1999;189:779–86.
29. Binder D, van den Broek MF, Kagi D, et al. Aplastic anemia rescued by exhaustion of cytokine-secreting CD8+ T cells in persistent infection with lymphocytic choriomeningitis virus. *J Exp Med* 1998;187:1903–20. [PubMed: 9607930]
30. Sower LE, Klimpel GR, Hanna W, et al. Extracellular activities of human granzymes. I. Granzyme A induces IL6 and IL8 production in fibroblast and epithelial cell lines. *Cellular Immunol* 1996;171:159–63.
31. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 1999;286:1957–9. [PubMed: 10583959]
32. Henter JI, Elinder G, Soder O, et al. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. *Blood* 1991;78:2918–22. [PubMed: 1954380]
33. Arico M, Janka G, Fischer A, et al. Hemophagocytic lymphohistiocytosis. Report of 122 children from the International Registry. FHL Study Group of the Histiocyte Society. *Leukemia* 1996;10:197–203. [PubMed: 8637226]
34. Sullivan KE, Delaar CA, Douglas S, et al. Defective natural killer cell function in patients with hemophagocytic lymphohistiocytosis and in first degree relatives. *Ped Res* 1998;44:465–8.
35. Par G, Rukavina D, Podack E, et al. Decrease in CD3-negative-CD8dim and V δ /V γ 9 TcR + peripheral blood lymphocyte counts, low perforin expression and the impairment of natural killer cell activity is associated with chronic hepatitis C virus infection. *J Hep* 2002;37:514–522.

36. Matloubian M, Suresh M, Glass A, et al. A role for perforin in downregulating T-cell responses during chronic viral infection. *Journal of Virology* 1999;73:2527–36. [PubMed: 9971838]
37. Henter JI, Ehrnst A, Andersson J, et al. Familial hemophagocytic lymphohistiocytosis and viral infections. *Acta Paediatrica* 1993;82:369–72. [PubMed: 8391350]

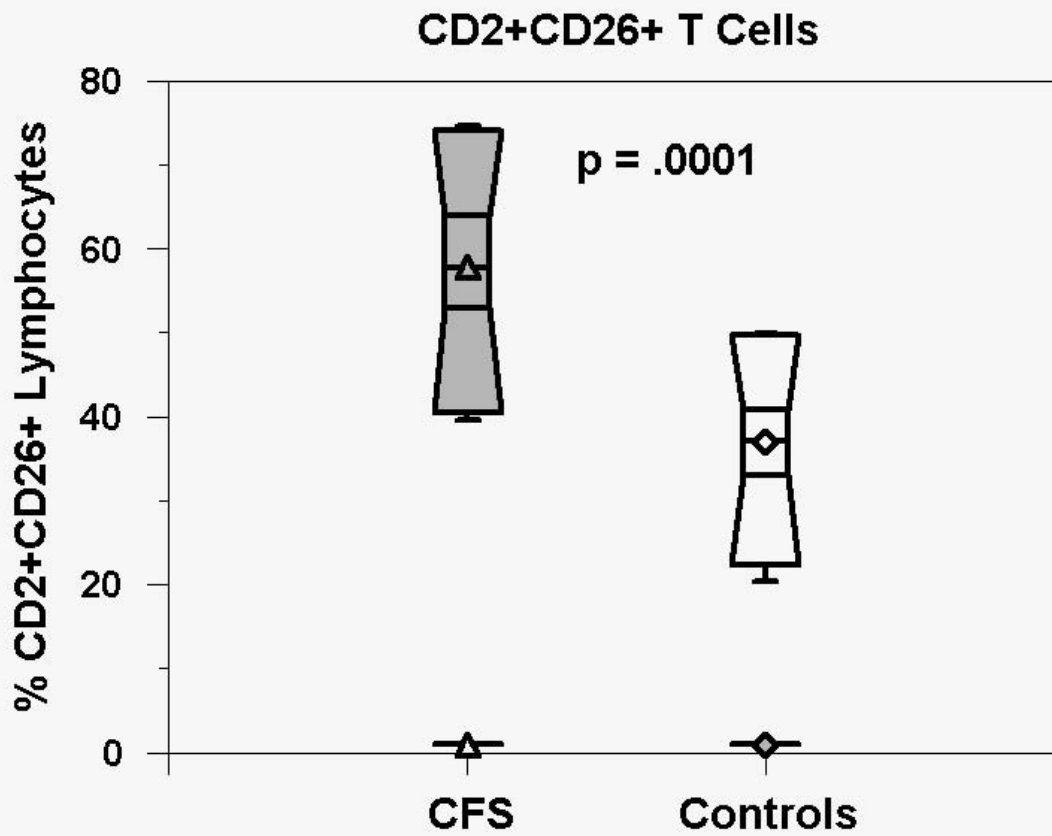


Figure 1. Flow cytometry indicated that the percentage of lymphocytes (T cells and NK cells) co-expressing surface CD2 and CD26 was significantly elevated in the CFS patients compared to controls. The results are shown as a box-whisker plot. The horizontal lines mark the minimum and maximum, and the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

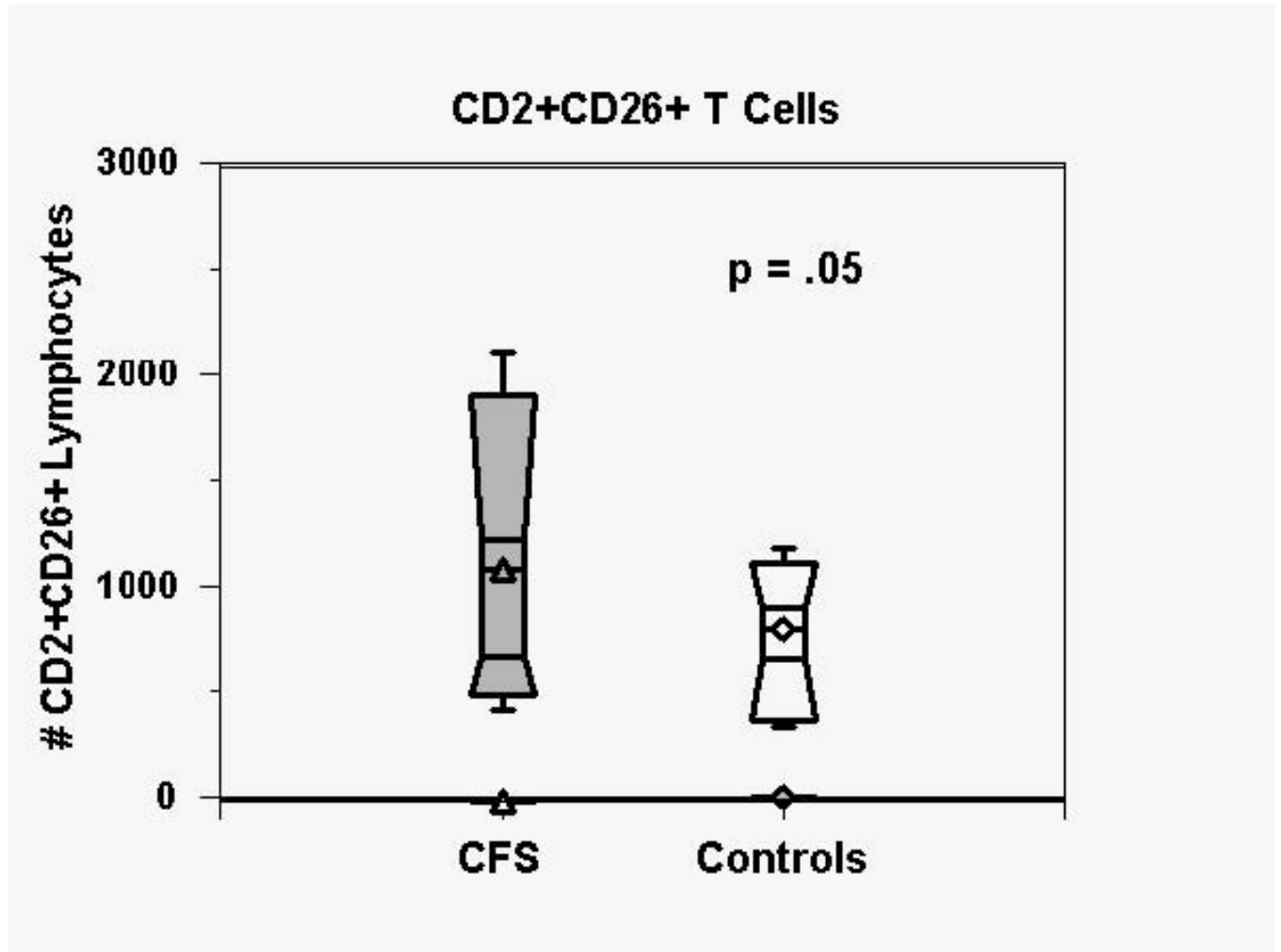


Figure 2. Flow cytometry indicated that the number of CD2+ lymphocytes (T cells and NK cells)/ μ L of blood expressing surface CD26 was elevated in the CFS patients compared to controls. The results are shown as a box-whisker plot. The horizontal lines mark the minimum and maximum, and the 10th, 25th, 50th, 75th, and 90th percentile points.

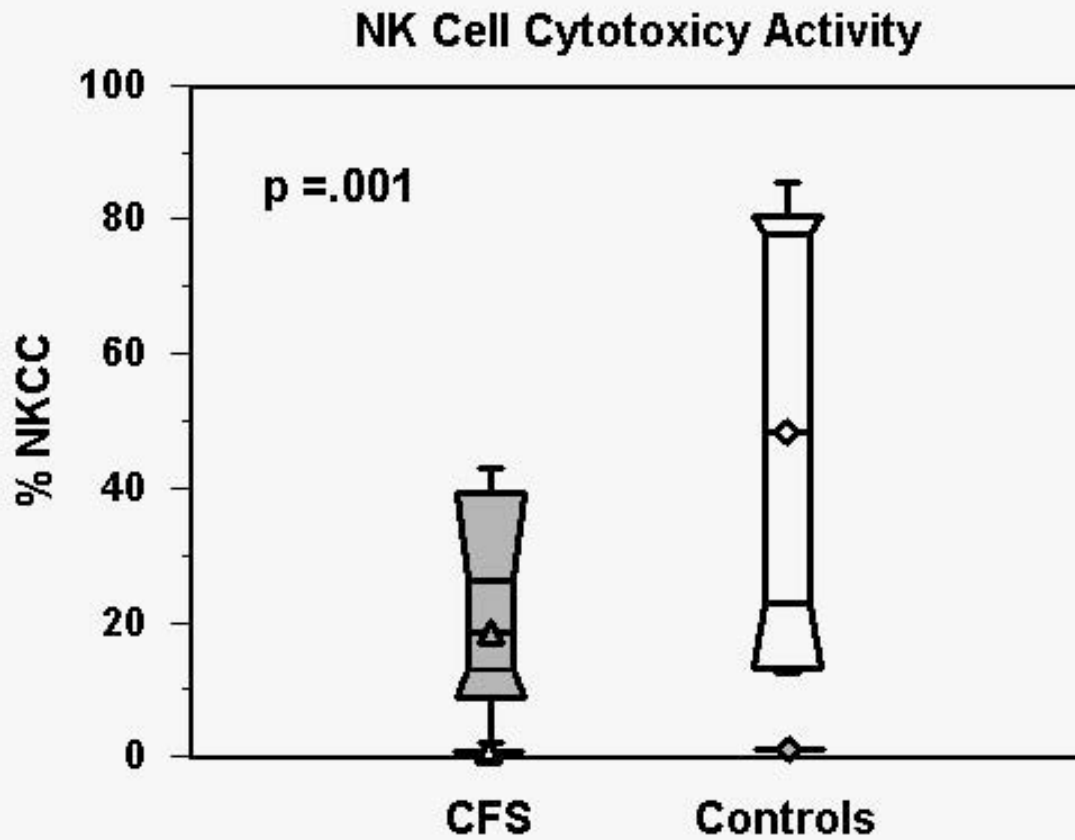


Figure 3. Subjects with chronic fatigue syndrome had significantly lower NK cytotoxicity (NKCC) as determined by the ^{56}Cr release assay, when compared to healthy controls. The results are expressed as % of target cells (K562 cells) killed in the 4 hour assay at an effector cell (CD56 +CD3- lymphocytes) 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.

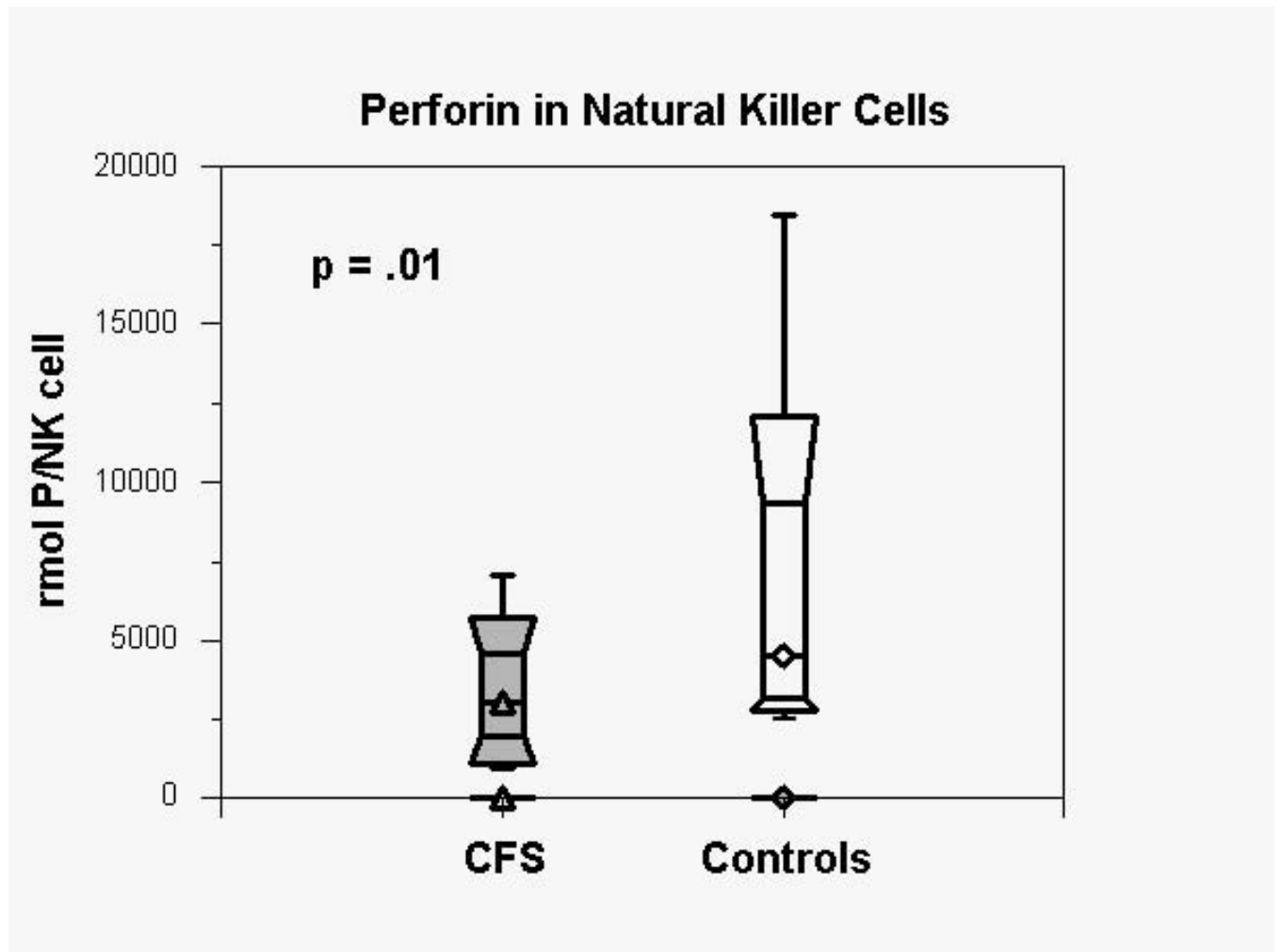
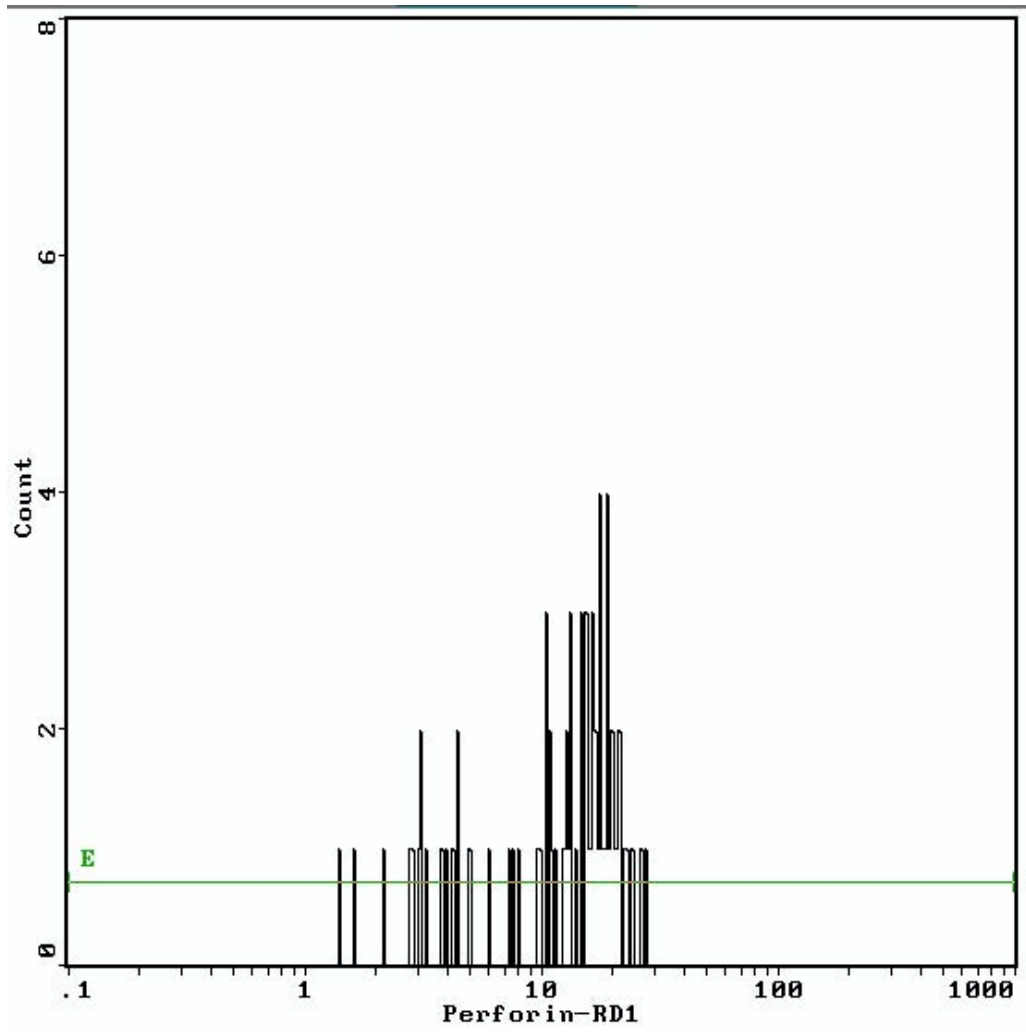


Figure 4. Subjects with chronic fatigue syndrome had significantly lower intracellular perforin in NK cells as determined by quantitative fluorescence. Results are expressed as relative number of molecules of perforin (rMol P).per CD56+CD3- lymphocyte. The results are shown as a box-whisker plot. The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



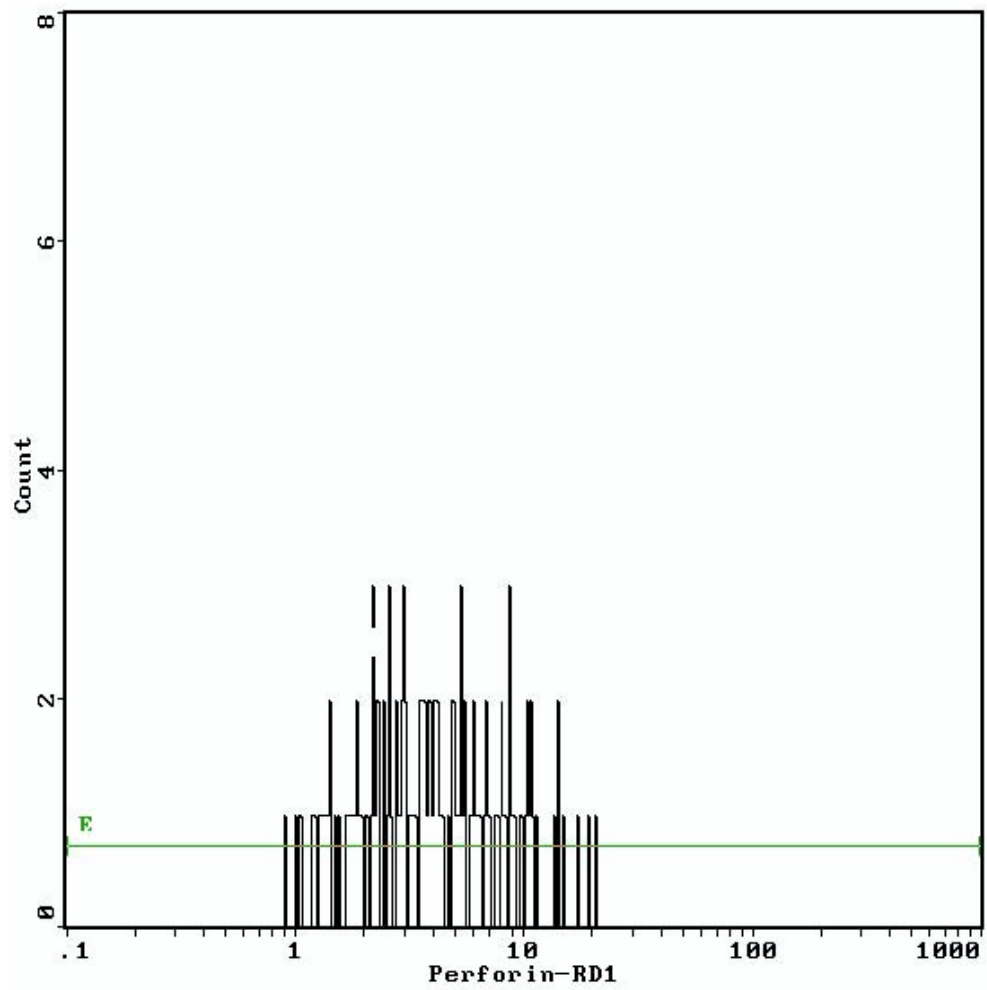


Figure 5. Representative histograms showing the fluorescence intensity of the anti-perforin antibody binding in NK cells. The x-axis corresponds to channel number and the y-axis corresponds to relative frequency of events. 5a. Healthy control 222641, median fluorescence intensity = 13.2; rMoP per NK cell = 9348. 5b. CFS patient 234467, median fluorescence intensity = 3.7; rMoP per NK cell = 3404.

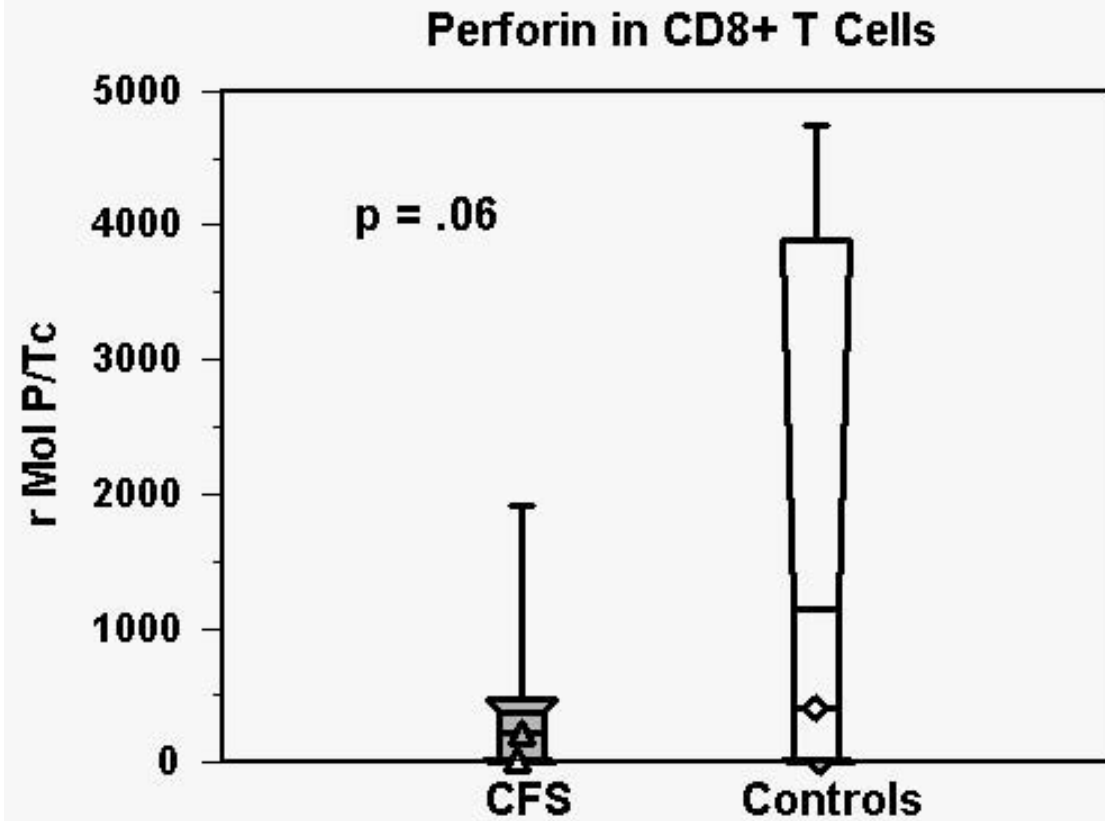


Figure 6. Subjects with chronic fatigue syndrome had a lower intracellular cytotoxic T cell perforin level as determined by quantitative fluorescence that bordered on statistical significance. Results are expressed as relative number of molecules of perforin (rMolP) per CD8+CD3+ lymphocyte. The results 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.

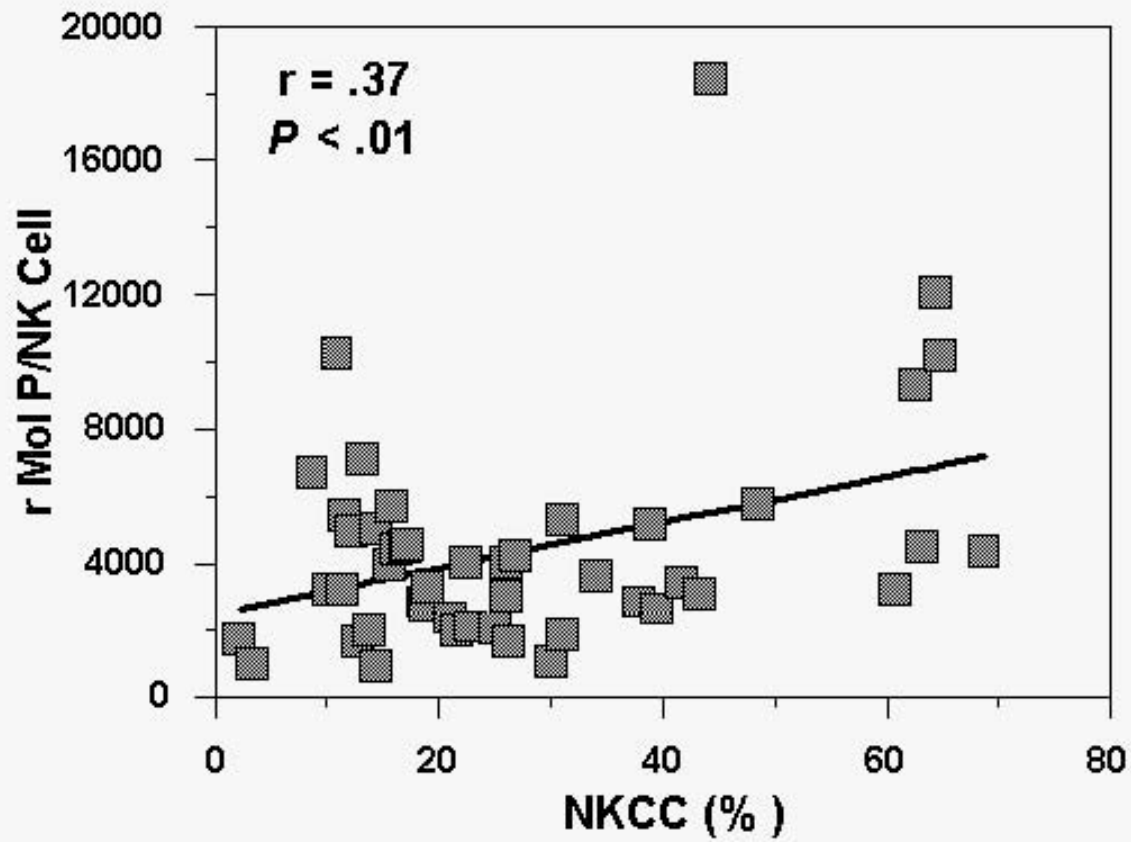


Figure 7. There was a significant correlation between NK cell activity (NKCC) and amount of intracellular perforin (rMolP). Filled symbols represent the individual CFS subjects and control subjects in this study. The line represents regression line.