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Characterization of Natural Killer Cell Phenotypes in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis

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Abstract

Objective: Natural Killer (NK) cells are classified into different phenotypes according to the expression of the surface markers CD56 and CD16. Each NK cell phenotype has a role in the immune response through cytotoxic activity or cytokine production. Reduced NK cell cytotoxic activity is a consistent finding in patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) and investigations into the potential causes of reduced NK cell cytotoxic activity have predominantly focused on total NK cells. The purpose of this study was to investigate and characterize four NK cell phenotypes in CFS/ME.

Methods: Twenty nine CFS/ME patients (mean age ± SEM=48.28 ± 2.63) meeting the 1994 Fukuda definition and 27 healthy controls (mean age ± SEM=49.15 ± 2.51) were included in this study. Flow cytometric protocols identified CD56^{bright}CD16$^{-/dim}$, CD56^{dim}CD16$^-$, CD56^{dim}CD16$^+$ or CD56$^-$CD16$^+$ NK cells for the measurement of surface markers including adhesion molecules CD2, CD18, CD11a, CD11b and CD11c, natural cytotoxicity receptors, Killer Immunoglobulin Like Receptors, signalling lymphocytic activation molecules and cell maturation (CD57). Following stimulation, NK cell phenotype expression of CD107a and CD107b was measured as a marker for degranulation. Intracellular staining measured lytic proteins including perforin, Granzyme A and Granzyme B in the four NK cell phenotypes.

Results: In the CFS/ME group, CD56^{bright}CD16$^{-/dim}$ NK cell co-expression of adhesion molecules CD2 and CD18 was significantly reduced. Granzyme B was significantly decreased in CD56^{dim}CD16$^+$ and CD56$^-$CD16$^+$ NK cells from CFS/ME patients. CD57 expression on CD56^{dim}CD16$^+$ NK cells from CFS/ME patients was significantly increased.

Conclusion: This is the first study to characterize four NK cell phenotypes in CFS/ME by investigating surface and intracellular molecules necessary for NK cell effector function. The data suggests that a combination of impairments in CD56^{dim}CD16$^+$ NK cells from CFS/ME patients may contribute to reduced cytotoxic activity of this phenotype.

Introduction

Immune cells from the innate and the adaptive systems mediate responses to protect against and clear invading pathogens or tumors. Natural Killer (NK) cells are effector cells of the innate immune system which remove target cells by cytotoxic activity and produce cytokines to regulate the immune response [1]. In the peripheral blood, NK cells comprise 15-20% of total lymphocytes and consist of different phenotypes according to the surface expression of the neutral cell adhesion molecule (CD56) and the Fcγ receptor III (CD16) which include CD56^{bright}CD16$^{-/dim}$, CD56^{dim}CD16$^-$, CD56^{dim}CD16$^+$ and CD56$^-$CD16$^+$ NK cells [2,3]. Ten percent of circulating NK cells are CD56^{bright}CD16$^{-/dim}$ cells and when activated, produce cytokines including interferon-gamma (IFN-γ), tumour necrosis factor-β (TNF-

β), granulocyte-macrophage colony-stimulating factor, interleukin (IL)-10 and IL-13 [4,5]. Cytokine production by CD56^{bright}CD16$^{-/dim}$ NK cells regulates and allows bi-directional communication between immune cells which is important for the transition from the innate to the adaptive immune response [4,6]. CD56^{bright}CD16$^-$ NK cells are considered immature cells which are pre-cursors for the mature CD56^{dim}CD16$^+$ NK cells [2].

The majority of NK cells in the peripheral blood are CD56^{dim}CD16$^+$ cells which are responsible for the cytotoxic lysis of target cells infected with viruses, bacteria, parasites or cells which have been malignantly transformed [3,5]. Activation of cytotoxic activity is dependent on a number of steps including contact, adhesion, activation, granule polarization and degranulation [5,7-10]. Target cell adhesion to the NK cell and ligation of the activating receptor/s initiates an intracellular signalling cascade [11]. NK cell cytotoxic granules polarize towards the plasma membrane to degranulate, releasing lytic proteins including perforin, Granzyme A and Granzyme

B into the immune synapse formed between the NK cell and the target cell [7,12]. Perforin assembles as a pore on the target cell membrane to facilitate delivery of the granzymes which fragment the cell's DNA, inducing apoptosis [13].

$CD56^{dim}CD16^-$ and $CD56^-CD16^+$ cells are a minority of circulating NK cells and whilst the function of the immature $CD56^{dim}CD16^-$ NK cells is largely unknown, $CD56^-CD16^+$ cells are functional through CD16 mediated antibody-dependent cellular cytotoxicity [2]. Activation of NK cell effector function is tightly regulated and each NK cell phenotype has functionally distinct roles in the immune response [4,14,15]. Changes in the expression of adhesion molecules, receptors or the mechanisms responsible for NK cell effector function may significantly affect the ability of NK cells to efficiently and effectively remove target cells [11,14,16-18].

Reduced NK cell cytotoxic activity is a consistent finding in patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) and aberrant function of lymphocytes in the immune system may contribute to the pathogenesis of CFS/ME [19-24]. Whilst studies have suggested that altered gene and protein expression of perforin and granzymes, increased expression of NK cell inhibitory receptors or a lack of cytokines required to regulate NK cell activity may contribute to reduced NK cell cytotoxic activity, these studies have mostly focused on the function of total CD56 NK cells [19,21,25-27]. This is the first study to characterize four NK cell phenotypes in CFS/ME to identify whether phenotype expression of adhesion molecules, receptors, lytic proteins, degranulation or cell maturation may contribute to reducing the cytotoxic activity of $CD56^{dim}CD16^+$ NK cells in CFS/ME patients.

Methods

Study participants and sample collection

Twenty-nine CFS/ME patients (mean age ± SEM = 48.28 ± 2.63) and 27 healthy controls (mean age ± SEM=49.15 ± 2.51) were included in this study which was conducted with approval from the Griffith University Human Research Ethics Committee. All participants provided informed consent and completed an online questionnaire which identified CFS/ME patients according to the 1994 Fukuda definition whilst the healthy controls had no reports of fatigue [28,29]. Participants donated whole blood samples which were collected in the morning from the antecubital vein into lithium heparinized and EDTA tubes. The blood samples were analyzed within five hours of collection and routine blood parameters including a full count of red blood cells and lymphocytes were measured on all samples by Pathology Queensland.

NK cell phenotypes

NK cells were isolated from heparinized whole blood using RosetteSep Human NK cell enrichment cocktail (Stem Cell Technologies, Vancouver, BC). Four NK cell phenotypes were identified by CD56-PE-Cy7, CD16-BV711 and CD3-BV510 fluorochrome-conjugated monoclonal antibodies from BD Biosciences (San Diego, CA) on a LSR-Fortessa X20 flow cytometer (Becton Dickinson). Forward and side scatter properties of lymphocytes identified the NK cells and surface density expression of CD56 and CD16 classified NK cells as $CD56^{bright}CD16^{-/dim}$, $CD56^{dim}CD16^-$, $CD56^{dim}CD16^+$ or $CD56^-CD16^+$ cells (Supplementary Figure 1) [2].

NK cell adhesion molecules and receptors

NK cell adhesion molecules, natural cytotoxicity receptors (NCRs), Killer Immunoglobulin-Like Receptors (KIR) and signalling lymphocytic activation molecules (SLAMs) were assessed on isolated NK cells from heparinized whole blood using RosetteSep Human NK cell enrichment cocktail. NK cells were stained with monoclonal antibodies for CD56, CD16, CD3, adhesion molecules (CD2, CD18, CD11a, CD11b and CD11c), NCRs (NKp30, NKp46 and NKp80), KIRs (KIR3DL1/DL2, KIR2DL5, CD94, KIR2DS4, NKG2D, KIR3DL1, KIR2DL1, KIR2DL2/DL3 and KIR2DL1/DS1) and SLAMs (2B4 and CD150) (Supplementary Table 1) [30,31]. NK cells were CD3- and $CD56^{bright}CD16^{-/dim}$, $CD56^{dim}CD16^-$, $CD56^{dim}CD16^+$ and $CD56^-CD16^+$ NK cell expression of the adhesion molecules and receptors were analyzed on the flow cytometer.

NK cell degranulation, lytic protein and cell maturation measurement

Peripheral blood mononuclear cells (PBMCs) isolated from EDTA blood by density gradient centrifugation with Ficoll-Hypaque (GE Health Care, Uppsala, UP) were used for NK cell degranulation, lytic proteins and cell maturation measurement. NK cell degranulation was measured by the expression of CD107a and CD107b following stimulation with either K562 cells at a ratio of 25:1, 1 µg/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and 1 µg/ml of ionomycin (Sigma-Aldrich, St. Louis, MO) or 1 µg/ml Phytohemagglutinin (PHA) (Gibco Life Technologies, Grand Island, NY) [7]. Monoclonal antibodies CD107a-PE and CD107b-FITC (BD Biosciences, San Diego, CA), monensin and brefeldin A (BD Biosciences, San Diego, CA) were added to the cells which were incubated for six hours at 37°C with 5% CO_2. Following incubation, CD56-PE-Cy7, CD16-BV711 and CD3-BV510 monoclonal antibodies stained NK cells in order to identify $CD56^{bright}CD16^{-/dim}$, $CD56^{dim}CD16^-$, $CD56^{dim}CD16^+$ and $CD56^-CD16^+$ NK cell expression of CD107a and CD107b on the flow cytometer.

NK cell lytic proteins including perforin, Granzyme A and Granzyme B were measured by intracellular staining of PBMCs (1×10^7cells/ml) and the surface expression of CD57 was measured as a marker for NK cell maturation [30,32]. Monoclonal antibodies for CD56-PE-Cy7, CD16-BV711, CD3-BV510 and CD57-PE-CF594 stained the NK cells which were then washed, permeabilized with BD cytofix (BD Biosciences, San Diego, CA), washed with perm wash buffer (BD Biosciences) and incubated with monoclonal antibodies for perforin-PE, Granzyme A-FITC and Granzyme B-BV421 (BD Biosciences, San Diego, CA). $CD56^{bright}CD16^{-/dim}$, $CD56^{dim}CD16^-$, $CD56^{dim}CD16^+$ and $CD56^-CD16^+$ NK cell expression of the lytic proteins and CD57 were then analysed on the flow cytometer.

Statistical analysis

Statistical analysis of the data was conducted on SPSS software (Version 21) and GraphPad Prism (Version 6). All of the data was tested for a Gaussian distribution by the Shaprio-Wilk test and the non-parametric Mann Whitney test was used to identify significance in the NK cell parameters measured between the CFS/ME and control groups. Two-way ANOVA with Tukey's multiple comparisons test was used to identify significant differences in NK cell phenotype expression of the parameters within the CFS/ME and control groups. Significance was set at an alpha level of $p<0.05$.

Results

No significant differences in participant blood parameters

White blood cell and red blood cell parameters were measured in the CFS/ME and control groups and no significant differences were observed between the two groups (Table 1).

	CFS/ME (n=29)	Controls (n=27)	P value
White blood cells (10^9/L)	5.39 ± 0.24	5.83 ± 0.22	0.15
Lymphocytes (10^9/L)	1.69 ± 0.09	1.92 ± 0.08	0.06
Monocytes (10^9/L)	0.31 ± 0.02	0.3 ± 0.02	0.57
Neutrophils (10^9/L)	3.2 ± 0.19	3.45 ± 0.18	0.31
Eosinophils (10^9/L)	0.15 ± 0.01	0.13 ± 0.01	0.33
Basophils (10^9/L)	0.03 ± 0.003	0.03 ± 0.003	0.60
Platelets (10^9/L)	241.34 ± 10.72	254.19 ± 12.59	0.50
Red blood cells (10^{12}/L)	4.67 ± 0.09	4.62 ± 0.07	0.46
Haemoglobin (g/L)	140.21 ± 2.48	139.92 ± 2.44	0.72
Haematocrit	0.41 ± 0.007	0.41 ± 0.005	0.76
Mean cell volume (fl)	88.31 ± 0.82	89.23 ± 0.75	0.33
Erythrocyte Sedimentation Rate (mm/Hr)	9.86 ± 1.47	11.84 ± 1.94	0.22

Table 1: Blood parameters measured in the CFS/ME and healthy control groups. Results from the white and red blood cell parameters measured in the CFS/ME and control groups. Comparisons of the blood parameters between the CFS/ME and control groups revealed no significant differences. Data is presented as the mean ± SEM.

Significant decrease in CFS/ME NK cell adhesion molecules

A significant decrease in the co-expression of CD2 and CD18 on CD56^{bright}CD16$^{-/dim}$ NK cells was observed when the CFS/ME group was compared to the control group (Figure 1A). No significant differences between the CFS/ME and control groups were observed on CD56^{dim}CD16$^-$, CD56^{dim}CD16$^+$ or CD56$^-$CD16$^+$ NK cell co-expression of CD2 and CD18. In the control group, a significant increase in the co-expression of CD2 and CD18 was observed when CD56^{dim}CD16$^+$ NK cells were compared to the CD56^{bright}CD16$^{-/dim}$ NK cells. Expression of the β2-Integrin adhesion molecules CD18, CD11a, CD11b and CD11c on NK cells were measured in the CFS/ME and control groups and no significant differences were observed between the groups (data not shown). CD18 associates with CD11a, CD11b and CD11c to form the functional β2-Integrin adhesion molecule and CD56^{dim}CD16$^-$ NK cells from the CFS/ME group had a significant decrease in the expression of CD18$^+$CD11a$^+$CD11c$^+$ (Figure 1B).

CD56^{bright}CD16$^{-/dim}$ and CD56^{dim}CD16$^-$ NK cell degranulation significantly increased

Degranulation was measured by the expression of CD107a and CD107b on NK cells following stimulation with K562 cells, PMA/I and PHA (Figure 2). No significant differences between the CFS/ME and control groups were observed in the expression of CD107a and CD107b in the unstimulated and stimulated NK cell phenotypes. In all of the stimulated NK cells from the CFS/ME and control groups, CD56^{bright}CD16$^{-/dim}$ NK cells expressed significantly increased amounts of CD107a and CD107b in comparison to CD56^{dim}CD16$^+$ NK cells. CD56^{dim}CD16$^-$ NK cells from CFS/ME and control groups stimulated with K562 cells also had a significant increase of CD107a and CD107b expression in comparison to CD56^{dim}CD16$^+$ NK cells. PHA stimulated CD56^{dim}CD16$^-$ NK cells from the control group had a significant increase in CD107a and CD107b expression in comparison to the CD56^{dim}CD16$^+$ NK cells.
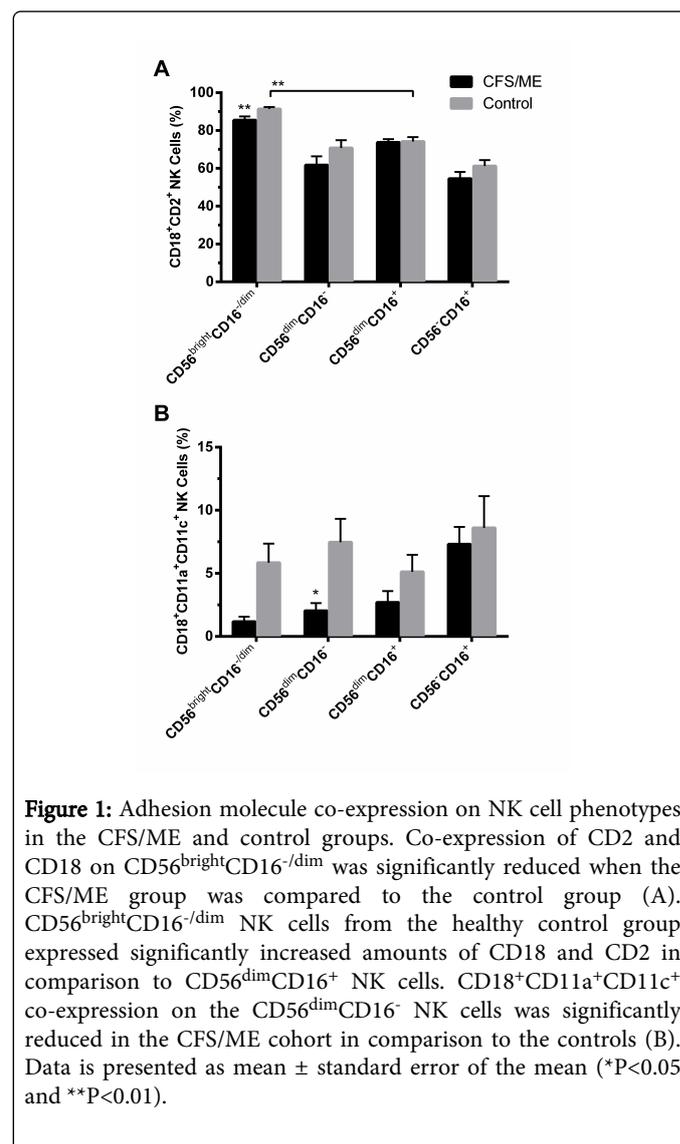

Figure 1: Adhesion molecule co-expression on NK cell phenotypes in the CFS/ME and control groups. Co-expression of CD2 and CD18 on CD56^{bright}CD16$^{-/dim}$ was significantly reduced when the CFS/ME group was compared to the control group (A). CD56^{bright}CD16$^{-/dim}$ NK cells from the healthy control group expressed significantly increased amounts of CD18 and CD2 in comparison to CD56^{dim}CD16$^+$ NK cells. CD18$^+$CD11a$^+$CD11c$^+$ co-expression on the CD56^{dim}CD16$^-$ NK cells was significantly reduced in the CFS/ME cohort in comparison to the controls (B). Data is presented as mean ± standard error of the mean (*P<0.05 and **P<0.01).

Granzyme B significantly reduced in CFS/ME CD56^{dim}CD16$^+$ and CD56$^-$CD16$^+$ NK cells

Intracellular staining measured lytic proteins in four NK cell phenotypes from the CFS/ME and control groups. Granzyme B was significantly reduced in CD56^{dim}CD16$^+$ and CD56$^-$CD16$^+$ NK cells from CFS/ME patients in comparison to the controls (Figure 3A). Measurement of perforin and Granzyme A revealed no significant differences between the two groups. In both the CFS/ME and control

group, $CD56^{dim}CD16^+$ and $CD56^-CD16^+$ NK cells expressed significantly increased amounts of perforin and Granzyme B in comparison to the $CD56^{bright}CD16^{-/dim}$ and $CD56^{dim}CD16^-$ NK cells.

$CD56^{dim}CD16^+$ NK cells in comparison to $CD56^{bright}CD16^{-/dim}$ NK cells expressed significantly increased amounts of Granzyme A in both groups.
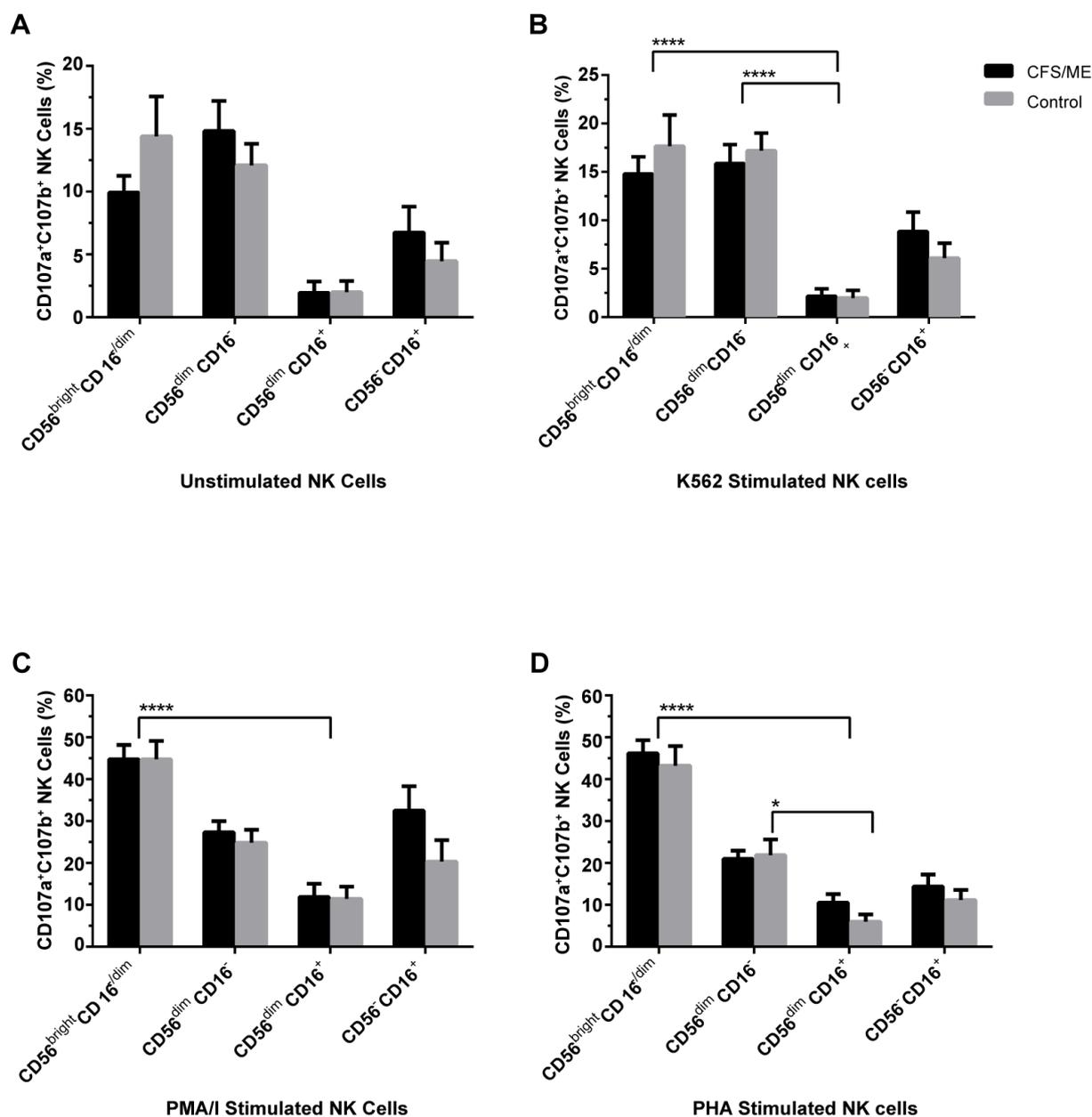

Figure 2: NK cell degranulation was measured by the expression of CD107a and CD107b in unstimulated and stimulated NK cells. Comparison of the CFS/ME and control group NK cell degranulation revealed no significant differences in the unstimulated **(A)** and K562 stimulated cells **(B)**, PMA/I stimulated cells **(C)** or PHA stimulated cells **(D).** Significantly increased amounts of CD107a and CD107b were observed when the $CD56^{bright}CD16^{-/dim}$ NK cells were compared to the $CD56^{dim}CD16^+$ NK cells stimulated with K562 cells (B), PMA/I (C) and PHA (D) in both the CFS/ME and control groups. $CD56^{dim}CD16^-$ NK cells stimulated with K562 cells also had a significant increase in CD107a and CD107b expression in comparison to $CD56^{dim}CD16^+$ NK cells from both groups (B). In the PHA stimulated cells, $CD56^{dim}CD16^-$ NK cells from the control group also expressed significantly increased amounts of CD107a and CD107b in comparison in comparison to $CD56^{dim}CD16^+$ NK cells (D). Data is presented as mean ± standard error of the mean (*P<0.05 and ****P<0.0001).
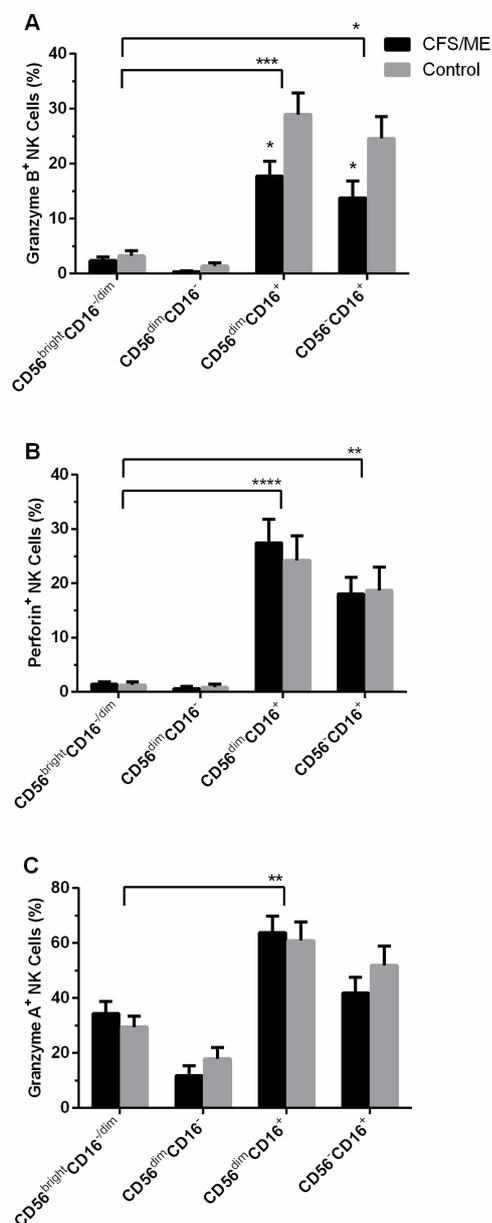
Figure 3: NK cell lytic proteins in CFS/ME and control groups. Granzyme B expression was significantly reduced in CD56^{dim}CD16$^+$ and CD56$^-$CD16$^+$ NK cells from CFS/ME patients in comparison to the controls **(A)**. CD56^{dim}CD16$^+$ and CD56$^-$CD16$^+$ NK cells from the CFS/ME and control groups expressed significantly increased amounts of Granzyme B **(A)** and perforin **(B)** in comparison to CD56^{bright}CD16$^{-/dim}$ NK cells. Granzyme A expression was significantly increased in CD56^{dim}CD16$^+$ NK cells compared to CD56^{bright}CD16$^{-/dim}$ NK cells in both the groups **(C)**. Data is presented as mean ± standard error of the mean (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001).

CD57 significant increase in CFS/ME CD56^{dim}CD16$^+$ NK Cells

CD57 is a surface marker for mature cells which was measured in four NK cell phenotypes from the CFS/ME and control groups. CD57 expression on CD56^{dim}CD16$^+$ NK cells was significantly increased in the CFS/ME patient group in comparison to the controls (Figure 4). In both groups, CD56^{dim}CD16$^+$ NK cells in comparison to CD56^{bright}CD16$^{-/dim}$ and CD56^{dim}CD16$^-$ NK cells expressed significantly increased amounts of CD57.

No significant differences in NK cell phenotypes or receptors

Comparison of NK cell phenotypes and expression of the KIRs, NCRs and SLAM receptors between the CFS/ME and control groups revealed no significant differences (Supplementary Figures 2-4).
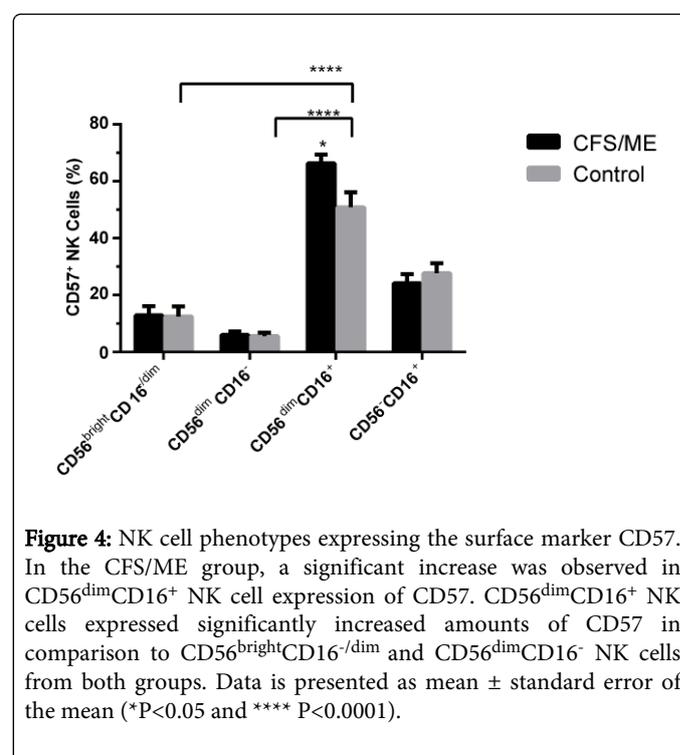

Figure 4: NK cell phenotypes expressing the surface marker CD57. In the CFS/ME group, a significant increase was observed in CD56^{dim}CD16$^+$ NK cell expression of CD57. CD56^{dim}CD16$^+$ NK cells expressed significantly increased amounts of CD57 in comparison to CD56^{bright}CD16$^{-/dim}$ and CD56^{dim}CD16$^-$ NK cells from both groups. Data is presented as mean ± standard error of the mean (*P<0.05 and **** P<0.0001).

Discussion

This is the first study to simultaneously measure and characterize NK cell adhesion molecules, receptors, degranulation, lytic proteins and cell maturation on four NK cell phenotypes from CFS/ME patients. These parameters are important for NK cell effector function and significant reductions in the co-expression of adhesion molecules and Granzyme B, as well as significant increases in mature NK cells may be associated with functional deficits which may contribute to reduced cytotoxic activity of CD56^{dim}CD16$^+$ NK cells from CFS/ME patients.

The decrease observed in the co-expression of the adhesion molecules CD2 and CD18 on CD56^{bright}CD16$^{-/dim}$ NK cells and CD18, CD11a and CD11c on CD56^{dim}CD16$^-$ NK cells from CFS/ME patients may affect the migratory ability of these cells [1,2]. Expression of adhesion molecules are enhanced on CD56^{bright}CD16$^{-/dim}$ NK cells which is important for facilitating migration to secondary lymphoid

organs. In secondary lymphoid organs, CD56^{bright}CD16$^{-/dim}$ NK cells produce cytokines such as IFN-γ, TNF-β, granulocyte-macrophage colony-stimulating factor, IL-10 and IL-13 [1,33]. In the lymph node, the presence of specific monocyte-derived cytokines (monokines) determines whether NK cells produce type 1 cytokines (eg. IFN-γ or TNF-β) or type 2 cytokines (eg. IL-10 or IL-13) [4]. CD56^{bright}CD16$^{-/dim}$ production of cytokines in the lymph node is a regulatory mechanism as the cytokines may influence the subsequent development of a type 1 or type 2 T helper response [4]. CD56^{bright} NK cell cytokine production is also important as IFN-γ acts in an autocrine manner on NK cells to augment the cytotoxic lysis of target cells by the death receptor pathway [1]. Decreased expression of the adhesion molecules on CD56^{bright} NK cells from CFS/ME patients may therefore impair the ability of these cells to migrate into the secondary lymphoid organs where they produce cytokines to influence the T helper adaptive immune response [4]. Activation related changes may contribute to the decreased expression of adhesion molecules on NK cells from CFS/ME patients. The cytokine environment can regulate and prime ligation of the adhesion molecules and IL-2 production has been found to be increased in CFS/ME patients [20,34]. Whilst IL-2 is traditionally associated with enhanced potency for target cell lysis, long term activation with IL-2 may have undesired effects due to the down-regulation of CD11c/CD18 expression [20,31,34].

Degranulation is associated with the release of the lytic proteins from cytotoxic CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} NK cells and to our knowledge, this is the first study to report on degranulation in four NK cell phenotypes from CFS/ME patients. Following stimulation, CFS/ME CD56^{-}CD16^{+} NK cells expressed increased amounts of the markers for degranulation. CD56^{-}CD16^{+} NK cells are potent mediators of antibody-dependent cell-mediated cytotoxicity (ADCC) due to high expression of CD16 which binds to target cells coated with IgG [35]. NK cell polarization of the lytic granule and degranulation are controlled by separate receptors where CD18/CD11a initiates the signals required for NK cell polarization whilst engagement of CD16 or synergistic activation of co-receptors induces NK cell degranulation [8,36]. The frequency of NK cell degranulation can be significantly increased by the expression of co-receptors such as 2B4, which lowers the activation threshold required to initiate the cytotoxic effector function of CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} NK cells [8]. Further investigations are required to identify mechanisms in CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} NK cells which may contribute to increased degranulation in CFS/ME patients.

It was expected that degranulation would be highest in CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} NK cells due to the exocytosis of the lytic proteins required for NK cell cytotoxic activity [7,15]. However, following stimulation, CD56^{bright}CD16$^{-/dim}$ and CD56^{dim}CD16^{-} NK cells had significantly increased amounts of CD107a and CD107b. The loss of CD16 from the CD56^{dim}CD16^{+} NK cells by a process known as ectodomain shedding by A Disintegrin and A Metalloproteinase 17 (ADAM17) may explain the reduction of NK cell degranulation in the CD56^{dim}CD16^{+} NK cell phenotype [37]. During the six hour incubation, ADAM17 may have cleaved CD16 from the surface of CD56^{dim}CD16^{+} NK cells undergoing degranulation and when the different NK cell phenotypes were identified post-incubation, a shift from CD56^{dim}CD16^{+} NK cells to CD56^{bright}CD16$^{-/dim}$ or CD56^{dim}CD16^{-} NK cells was observed.

Consistent with our previous findings from total NK cells, this study identified that Granzyme B was significantly reduced in CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} NK cells from CFS/ME patients

[38]. Granzyme B is crucial for rapid and effective target cell lysis as knockout mice experiments have demonstrated that deficiencies in Granzyme B result in delayed DNA fragmentation and the persistence of viral infections [10,39,40]. Reduced Granzyme B expression in CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} NK cells may therefore contribute to the reduced NK cell cytotoxic activity reported in CFS/ME patients. Reduction of NK cell Granzyme B in CFS/ME patients may be attributed to the presence of microRNAs (miRNAs) which promote degradation and/or prevention of target messenger RNA translation, silencing protein expression of Granzyme B [41]. Our research group has previously found that CFS/ME patients have a significant reduction in miR-223 [42]. miR-223 has been implicated in the regulation of murine Granzyme B during NK cell activation, suggesting that miR-223 may have a similar effect on human NK cells [43]. Additional miRNAs including miR-23a and miR-27a have also been suggested to influence cytotoxic effector function of NK cells by regulating expression of Granzyme B [44,45].

CD56^{dim}CD16^{+} NK cells from CFS/ME patients expressed significantly increased amounts of CD57 which suggests that this phenotype is mature and terminally differentiated [46]. CD56^{dim}CD16^{+} NK cell expression of CD57 is associated with more potent cytotoxic activity due to the presence of lytic proteins perforin, Granzyme A and Granzyme B [32,46]. However, reduced NK cell cytotoxic activity is consistently reported in CFS/ME patients and Granzyme B was significantly reduced in the CD56^{dim}CD16^{+} NK cells from our CFS/ME patients. CD57 expression on NK cells increases with immunosenescence, however there was no significant difference in the ages between the CFS/ME and control groups [47]. Expression of CD57 on CD8 T cells is associated with shorter telomere lengths, low telomerase activity and reduced expression of the cell-cycle associated genes [47-49]. Heightened levels of CD57 expression therefore indicate that the cells have undergone multiple divisions and as a consequence, have a reduced proliferative capacity [47]. Increased expression of CD57 on CD56^{dim}CD16^{+} NK cells from CFS/ME patients may suggest that this NK cell phenotype has undergone multiple cell divisions which may be in response to in vivo immune activation by pathogens or cytokines [46,47]. Expression of T-cell immunoglobulin- and mucin domain-containing (Tim)-3 has been associated with matured CD56^{dim}CD16^{+} NK cells [50]. Similar to CD57, Tim-3 expression is associated with potent cytotoxic activity, however, cross-linking of the activating receptors CD16 and NKG2D inhibits NK cell mediated cytotoxic activity as a mechanism to suppress the immune response [50]. Tim-3 expression on the matured CD56^{dim}CD16^{+} NK cells may therefore contribute to reduced cytotoxic potential of NK cells from CFS/ME patients.

Investigation of four NK cell phenotypes in CFS/ME has identified significant differences in adhesion molecules, degranulation, lytic proteins and cell maturation in comparison to the healthy control group. The combination of impairments including reduced Granzyme B and increased cell maturation in CD56^{dim}CD16^{+} NK cells from CFS/ME patients suggests that these differences may significantly affect the cytotoxic effector function of this phenotype. As this study was conducted on a small sample size, further replicates in a larger population are necessary to determine if these markers are consistent and representative of CFS/ME.

Acknowledgements

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