

Analysis of the Relationship between Immune Dysfunction and Symptom Severity in Patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME)

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Abstract

Objective: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a disabling illness, characterised by persistent, debilitating fatigue and a multitude of symptoms. Immunological alterations are prominent in CFS/ME cases, however little is known about the relationship between CFS/ME severity and the extent of immunological dysfunction. The purpose of this study was to assess innate and adaptive immune cell phenotypes and function of two groups of CFS/ME patients, bedridden (severe) and mobile (moderate).

Methods: CFS/ME participants were defined using the Centres for Disease Prevention and Control (1994 CDC) Criteria for CFS/ME. Participants were grouped into healthy controls (n=22, age=40.14 ± 2.38), moderate/mobile (n=23; age=42.52 ± 2.63) and severe/bedridden (n=18; age=39.56 ± 1.51) CFS/ME patients. Flow cytometric protocols were used to examine neutrophil, monocyte, dendritic cells (DCs), iNKT, Treg, B, $\gamma\delta$ and CD8⁺ T cell phenotypes, NK cytotoxic activity and receptors.

Results: The present data found that CFS/ME patients demonstrated significant decreases in NK cytotoxic activity, transitional and regulatory B cells, $\gamma\delta$ T cells, KIR2DL1/DS1, CD94⁺ and KIR2DL2/L3. Significant increases in CD56⁺CD16⁺NKs, CD56^{dim}CD16⁻ and CD56^{bright}CD16^{-/dim} NKs, DCs, iNKT phenotypes, memory and naive B cells were also shown in CFS/ME participants. Severe CFS/ME patients demonstrated increased CD14⁺CD16⁺ DCs, memory and naive B cells, total iNKT, iNKT cell and NK phenotypes compared to moderate CFS/ME patients.

Conclusion: This study is the first to determine alterations in NK, iNKT, B, DC and $\gamma\delta$ T cell phenotypes in both moderate and severe CFS/ME patients. Immunological alterations are present in innate and adaptive immune cells and sometimes, immune deregulation appears worse in CFS/ME patients with more severe symptoms. It may be appropriate for CFS/ME patient severity subgroups to be distinguished in both clinical and research settings to extricate further immunological pathologies that may not have been previously reported.

Keywords: Chronic Fatigue Syndrome, Severity, $\gamma\delta$ T cells, Immune, Natural Killer Cell, iNKT, Cytotoxic Activity, Adaptive

Introduction

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a severe physically and cognitively incapacitating illness diagnosed by symptom-specific criteria [1-3]. CFS/ME presents as a multifactorial illness that varies greatly in the nature of onset and severity of symptom presentation [1,2,4,5]. A key characteristic is debilitating fatigue that lasts for a period of 6 or more months that has a critical effect on a patient's daily activities are critically affected [1-3,6]. The severity of symptoms can vary greatly in CFS/ME. For example patients with moderate symptoms are able to maintain some normal daily activities with slight reduced mobility while those severely affected by CFS/ME experience high levels of daily fatigue and are therefore typically housebound [7].

Currently, there is no known cause for CFS/ME although research has demonstrated consistent immunological dysfunction associated with the illness [8-12]. We have previously been the only research group to have examined Natural Killer (NK) cell function, phenotype and receptors in housebound severe patients in comparison to a healthy control group [13]. Housebound severe patients had significantly reduced NK cell cytotoxic activity when compared with the moderately affected patients and there was an increase in the Killer Immunoglobulin-like Receptor (KIRs) KIR3DL1 in the moderate

patients, highlighting that differing levels of severity may also have varying levels of immune perturbation [13].

The most consistent immunological finding in CFS/ME is significantly reduced NK cell cytotoxic activity [10,13-15]. This study is one of the first to assess those housebound and severe CFS/ME patients in comparison to a moderate and mobile CFS/ME patient subgroup. Segregation of patients into moderate/mobile and severe/housebound CFS/ME subgroups may elucidate further immunological markers that may explain the pathomechanism of the illness. Hence, the purpose of this study was to investigate phenotypic and functional parameters of innate (NK cells, neutrophils, monocytes, dendritic cells (DCs)) and adaptive (gamma delta ($\gamma\delta$), iNKT, CD8⁺T cells, B cells)

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immune cells to compare moderate and severe CFS/ME patients.

Methods

Ethical clearance

Ethical approval for this research was granted after review by the Griffith University Human Research Ethics Committee (GU Ref No: MSC/23/12/HREC).

Participant recruitment

Participants were recruited from Queensland and New South Wales areas of Australia through CFS/ME support groups, email advertisements and social media. All participants were between 20 and 65 years old. All CFS/ME patients had the illness for a period of at least 6 months prior to the study and questionnaires were used to define CFS/ME using the Centre for Disease Prevention and Control (CDC) criteria for CFS. The 1994 CDC was used for CFS/ME in the absence of a biomarker or diagnostic test for CFS/ME. After CFS/ME patients were identified as either mobile or housebound, their 'moderate' and 'severe' status was confirmed using the 1994 CDC in conjunction with an extensive questionnaire to assess symptomology, health status, quality of life, severity and mobility in all participants.

Participants were excluded if they were previously diagnosed with an autoimmune disorder, psychosis, heart disease or thyroid-related disorders or if they were pregnant, breast feeding, a smoker, or experiencing symptoms of CFS/ME that did not conform to the CDC criteria for CFS/ME.

A total of 63 participants were initially recruited for the study. Participants (n=63) included in the study were either moderately (n=23) or severely (n=22) affected by CFS/ME as well as a non-fatigued control group (n=18). Those in the severe CFS/ME group were housebound and displayed significantly worsened symptoms. The Fatigue Severity Scale (FSS), Dr Bell's Disability Scale, the FibroFatigue Scale and the Karnofsky Performance Scale (KPS), were used in the questionnaire as a determinant of severity.

Sample preparation and routine measures

A non-fasting blood sample of 50 mL was collected from the antecubital vein of participants into lithium heparinised and ethylenediamine tetraacetic acid (EDTA) tubes. Blood was collected between 8:30 am and 11:30 am and samples were analysed within 12 hours of collection. Initial full blood count assessment was undertaken by Pathology Queensland to determine levels of white blood cell and red blood cell markers.

Natural killer cell cytotoxic activity analysis

NK cell cytotoxic activity was performed as described previously [9,13]. Density gradient centrifugation using Ficoll-hypaque (Sigma, St Louis, MO) was used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood. Isolated PBMCs were labelled with 0.4% PKH-26 (Sigma, St Louis, MO) and incubated with K562 cells for 4 hours at the following effector (NK cell) to target (K562) ratios, 12.5:1, 25:1 and 50:1. Cell death was analysed using Annexin-V-FITC and 7-AAD reagents (BD Biosciences, San Diego, CA) and the ability of NK cells to lyse target K562 cells was measured on the flow cytometer (Becton Dickinson Immunocytometry Systems).

Intracellular analysis

Density gradient centrifugation using Ficoll-hypaque (Sigma,

St Louis, MO) was used to isolate PBMCs from EDTA whole blood. PBMCs were adjusted to 1×10^7 cells/mL and stained with monoclonal antibodies for Tregulatory cell (Treg) phenotypes, NK lytic proteins and CD8 lytic proteins as described [9,13] (Supplementary Table 1). The Treg phenotypes were assessed as PBMCs were permeabilised and fixed with buffers containing diethylene glycol and formaldehyde before being stained with FOXP3. After washing with Phosphate Buffered Saline (PBS) (GibcoBiocult, Scotland), cells were analysed on the flow cytometer (Becton Dickinson Immunocytometry Systems) where the expression of FOXP3⁺Tregs was determined on CD4⁺CD25⁺CD127^{low}T cells [9]. NK and CD8 T cell lytic proteins were assessed as previously described [9]. Cells were incubated for 30 mins in Cytofix then permwash was added. Perforin, granzyme A and granzyme B monoclonal antibodies were added to cells and incubated for 30 minutes in the dark at room temperature. Cells were washed and analysed on the flow cytometer where perforin, granzyme A and granzyme B expression was measured in NK and CD8 T cells.

NK phenotype and KIR analysis

NK cells were isolated from whole blood cells using a negative selection system RosetteSep Human Natural Killer Cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC). Isolated NK cells were labelled with CD56, CD16, CD3 (BD Biosciences, San Diego, CA) and monoclonal antibodies for KIR receptors (Supplementary Table 1) (MiltenyiBiotec). Cells were analysed on the flow cytometer (Becton Dickinson Immunocytometry Systems). NK lymphocytes were gated using CD56, CD16 and CD3 and KIR receptors were analysed based on their appropriate antibodies (Supplementary Table 1) [13]. NK cell phenotypes CD56^{dim}CD16⁺, CD56⁺CD16⁺, CD56^{dim}CD16⁻ and CD56^{bright}CD16^{-dim} were assessed (Supplementary Figure 4).

Whole blood analysis

Appropriate antibodies (Supplementary Table 1) were added to whole blood samples and incubated for 30 minutes. Following which cells were lysed, washed, fixed and analysed on the flow cytometer. Neutrophil, monocyte, DC, B cell and $\gamma\delta$ T cell phenotypes were assessed using appropriate antibodies (Supplementary Table 1) and gating strategies on the flow cytometer (Supplementary Figures 1, 2 and 5).

iNKT phenotype analysis

PBMCs were isolated using density gradient centrifugation as described above. PBMCs were labelled with monoclonal antibodies to assess expression of 6B11, CD3, CD4, CD8, CD8a, CD45RO, CD28, CCR7 (C-chemokine receptor type 7), SLAM (signalling lymphocytic activation molecule), CD56 and CD16 (Supplementary Table) (BD Biosciences, San Diego, CA). Cells were fixed with stabilising fixative (BD Biosciences, San Diego, CA) for analysis on the flow cytometer (Becton Dickinson Immunocytometry Systems) (Supplementary Figure 3) [16].

Data and statistical analysis

Statistical analysis was performed using SPSS statistical software version 21.0. All experimental data represented in this study are reported as plus/minus the standard error of the mean (\pm SEM). Comparative assessments among the three participant groups (control, moderate CFS/ME and severe CFS/ME) were performed with the analysis of variance test (ANOVA). The LSD Post Hoc test was used to determine *p* values of significance and statistical significance was set at an alpha criterion at *p*<0.05. Pearsons correlation was conducted on

significant parameters to determine correlates where significance was accepted as $p < 0.01$. Outliers were identified using a boxplot technique on SPSS whereas extreme outliers were highlighted based on lying beyond the plot's whiskers [17]. Outliers were handled by eliminating particular data points from the analysis [17].

Results

Participants

Data were available for a total of 63 participants (22 control, 23

Parameter	Control (n=22)	Moderate (n=23)	Severe (n=18)	p value
Age in years (Mean ± SEM)	40.14 ± 2.38	42.52 ± 2.63	39.56 ± 1.51	0.631
Gender Female, Male	14, 8	16, 7	15, 3	0.391

Age data is represented as Mean ± SEM in control (n=22), moderate CFS/ME (n=23) and severe CFS/ME (n=18). *signifies $p < 0.05$ between participant groups. There were no significant differences in age or gender within the research groups. CFS/ME: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis; Standard Error of the Mean.

Table 1: Participant characteristics and comparisons of the age (mean ± standard deviation) and gender distribution of each participant group (control, moderate and severe).

moderate CFS/ME and 18 severe CFS/ME). The mean ages for the control, moderate CFS/ME and severe CFS/ME were 40.14 ± 2.38 , 42.52 ± 2.63 and 39.56 ± 1.51 respectively. There was no statistical difference for age between participant groups. All CFS/ME participants from the moderate and severe CFS/ME participant groups satisfied the CDC criteria for CFS/ME. The severe participant group demonstrated significantly worsened scores for symptoms in the FSS, Dr Bell's Disability Scale, the FibroFatigue Scale and the KPS (data not shown) when compared with the moderate CFS/ME group. Participant groups were also age and gender matched and there were no significant ($p < 0.05$) differences between these parameters (Table 1). The three participant groups consisted of predominantly females with the control, moderate CFS/ME and severe CFS/ME having 64%, 70% and 83% female participants and this was not statistically different between groups (Table 1).

Blood pressure, pulse, temperature and routine full blood counts were measured in all participants (Table 2). There were no statistically significant differences in any clinical or routine full blood count parameters between the participant groups.

Reduced NK cytotoxic activity

In all three effector cell target ratios (12.5:1, 25:1, 50:1), there was

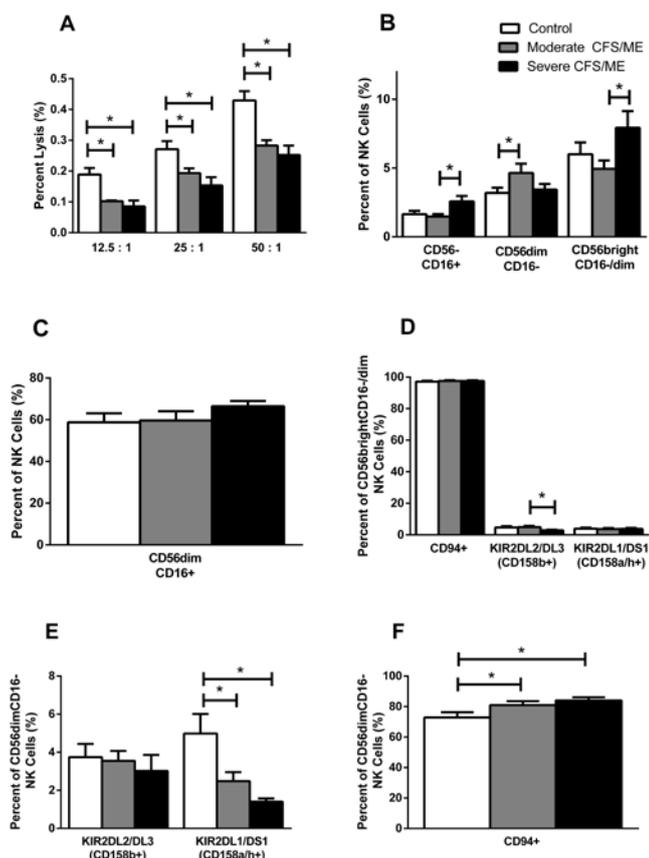


Figure 1: The profile of NK cells in control, moderate and severe CFS/ME. (A) K cell cytotoxic activity was measured at 12.5:1, 25:1 and 50:1 effector to target cell ratios. Data is presented as percentage of K562 target cells lysed by NK cells. (B) NK cell phenotypes (CD56⁻CD16⁺, CD56^{dim}CD16⁻ and CD56^{bright}CD16^{-/dim}) presented as a percentage of total NK cells. (C) NK cell phenotype CD56^{dim}CD16⁺ presented as a percentage of total NK cells. (D) CD56^{bright}CD16^{-/dim} NK cells expressing KIR receptors CD94⁺, KIR2DL2/DL3 and KIR2DL1/DS1 are represented as a percentage of total CD56^{bright}CD16^{-/dim} NK cells. (E) CD56^{dim}CD16⁻ NK cells expressing the KIR receptors KIR2DL2/DL3 and KIR2DL1/DS1 are represented as a percentage of CD56^{dim}CD16⁻ NK cells. (F) CD56^{dim}CD16⁻ NK cells expressing the KIR receptor CD94⁺ represented as a percentage of CD56^{dim}CD16⁻ NK cells. All data is represented as mean ± SEM. *represents results that were significantly different where $p < 0.05$. NK: Natural Killer; KIR: Killer Immunoglobulin-like Receptor; CFS/ME: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis; SEM: Standard Error of the Mean.

Parameter	Control (n=22)	Moderate (n=23)	Severe (n=18)	p value
Blood Pressure (Systolic)	119.32 ± 3.97	114.13 ± 2.86	111.39 ± 3.57	0.281
Blood Pressure (Diastolic)	76.36 ± 2.01	71.52 ± 1.15	74.44 ± 2.88	0.214
Pulse bpm	66.00 ± 1.73	70.56 ± 1.80	67.28 ± 1.64	0.152
Temperature (°C)	36.43 ± 0.09	36.33 ± 0.08	36.55 ± 0.12	0.291
Haemoglobin g/L	138.81 ± 3.52	140.52 ± 2.71	137.00 ± 3.80	0.763
White Cell Count (x10 ⁹ /L)	5.73 ± 0.26	6.17 ± 0.36	6.60 ± 0.52	0.289
Platelets (x10 ⁹ /L)	237.91 ± 10.34	250.57 ± 9.72	248.17 ± 18.16	0.741
Haematocrit (x10 ⁹ /L)	2.17 ± 1.75	0.41 ± 0.01	0.40 ± 0.01	0.398
Red Cell Count (x10 ⁹ /L)	4.64 ± 0.09	4.66 ± 0.09	4.39 ± 0.10	0.110
MCV (fL)	89.23 ± 0.92	89.13 ± 0.58	90.94 ± 1.25	0.320
Neutrophils (x10 ⁹ /L)	3.31 ± 0.22	3.58 ± 0.27	3.88 ± 0.32	0.351
Lymphocytes (x10 ⁹ /L)	1.86 ± 0.12	2.05 ± 0.13	11.87 ± 9.72	0.277
Monocytes (x10 ⁹ /L)	0.40 ± 0.2	0.35 ± 0.02	0.40 ± 0.04	0.371
Eosinophils (x10 ⁹ /L)	0.14 ± 0.02	0.16 ± 0.02	0.16 ± 0.03	0.754
Basophils (x10 ⁹ /L)	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.430
ESR (mm/Hr)	7.32 ± 0.66	8.39 ± 1.69	10.28 ± 1.46	0.325
Sodium (mmol/L)	138.62 ± 0.43	138.48 ± 0.40	137.56 ± 0.88	0.392
C-Reactive Protein (mg/L)	3.07 ± 0.49	3.29 ± 0.79	4.00 ± 0.97	0.688

All data is represented as Mean ± SEM in control (n=22), moderate CFS/ME (n=23) and severe CFS/ME (n=18). *signifies p<0.05 between participant groups. CFS/ME: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis; Standard Error of the Mean.

Table 2: Clinical and full blood count results for the control, moderate and severe CFS/ME participant groups.

a significant reduction in cytotoxic activity of NK cells in moderate ($p=0.001, 0.000, 0.017$) and severe CFS/ME participants ($p=0.000, 0.000, 0.001$) (Figure 1A). Cytotoxic activity was further reduced in the severe CFS/ME group for all three ratios although there was no statistical significance between the moderate and severe CFS/ME groups (Figure 1A). NK cytotoxic activity at with a target cell ratio of 12.5:1 was positively correlated with the target cell ratios of 25:1 and 50:1 and the 25:1 ratio was also positively correlated to the ratio of 50:1 ($p<0.01$) (Supplementary Table 3).

No differences to lytic proteins in CD8⁺ T cells and NK cells

NK and CD8⁺ T cell Granzyme A, Granzyme B and perforin as well as CD25⁺CD127^{low}CD4⁺FOXP3⁺Tregs were not significantly different between the groups (Data not shown).

Increased NK phenotypes and reduced KIR receptors in CFS/ME

There was a significant increase in the number of CD56^{bright}CD16^{-dim} and CD56^{dim}CD16⁺ NK cells in severe CFS/ME compared with moderate ($p=0.023, 0.049$) and CD56^{dim}CD16⁻ NK cells were significantly increased in moderate CFS/ME compared with controls ($p=0.045$) (Figures 1B and 1C). There was also a significant reduction in the percentage of CD56^{bright}CD16^{-dim}CD158b⁺ NK cells in the severe CFS/ME group compared with the moderate CFS/ME group ($p=0.027$) (Figure 1D). Moderate CFS/ME patients had significantly reduced CD56^{dim}CD16⁻CD158a/h⁺ NK cell expression compared with the control group ($p=0.003, 0.004$). There was also a significant increase in the expression of CD94⁺ on CD56^{dim}CD16⁻ NK cells in both

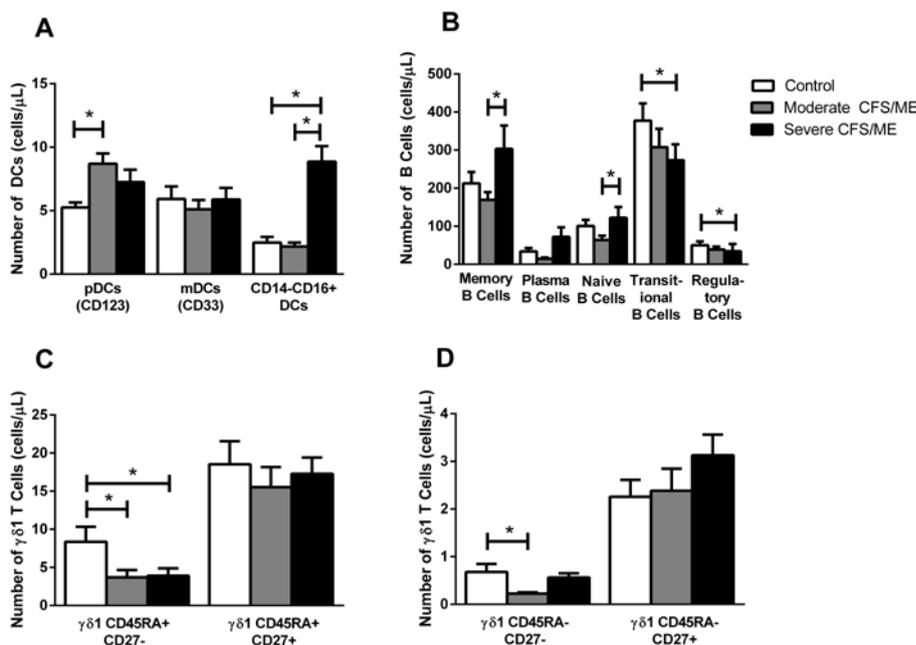


Figure 2: Alterations in DC, B and γδ1 T cell phenotypes in control, moderate and severe CFS/ME participant groups. (A) DC phenotypes, including pDCs, mDCs and CD14-CD16⁺ DCs where data is represented as the total number of cells (cells/μL). (B) B cell phenotypes (memory, plasma, naive, transitional and regulatory B cells) in control, moderate and severe CFS/ME groups represented as total number of cells (cells/μL). (C) γδ1 T cell CD45RA⁺CD27⁻ and CD45RA⁺CD27⁺ phenotypes are represented as total number of cells (cells/μL). (D) γδ1 T cell phenotypes CD45RA⁻CD27⁻ and CD45RA⁻CD27⁺ are represented as total number of cells (cells/μL). Data are represented as mean ± SEM. *represents results that were significantly different where p<0.05. DC: Dendritic Cell; CFS/ME: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis; pDC: Plasmacytoid Dendritic Cell; mDC: Myeloid Dendritic Cells; SEM: Standard Error of the Mean.

moderate ($p=0.042$) and severe ($p=0.007$) CFS/ME patients (Figures 1E and 1F). $CD56^{dim}CD16^{-}CD158a/h^{+}$ was positively correlated to plasma B cell and naïve B cell phenotypes and $CD56^{bright}CD16^{-/dim}$ NK cells were positively correlated to memory B cells ($p<0.01$) (Supplementary Table 2).

Whole blood phenotypes

Plasmacytoid DCs (pDCs) were significantly higher in the moderate CFS/ME group ($p=0.002$). Myeloid DCs (mDCs) were not statistically different between any of the groups and $CD14^{-}CD16^{+}$ DCs were higher

in the severe CFS/ME group compared with the moderate CFS/ME ($p=0.000$) and control group ($p=0.000$) (Figure 2A). The number of memory and naïve B cells (cells/ μ L) was significantly increased in the severe CFS/ME group compared with the moderate CFS/ME group ($p=0.025, 0.026$). There was also a significantly reduced number of transitional B cells and B regulatory cells (Bregs) in the severe CFS/ME participants compared with the control CFS/ME participants ($p=0.047, 0.041$) (Figure 2B). There was a significant reduction in the number of $\gamma\delta 1$ T cells (cells/ μ L) with the phenotype $CD45RA^{+}CD27^{-}$ in the moderate and severe CFS/ME groups ($p=0.007$ and 0.018). $\gamma\delta 1$ T cells

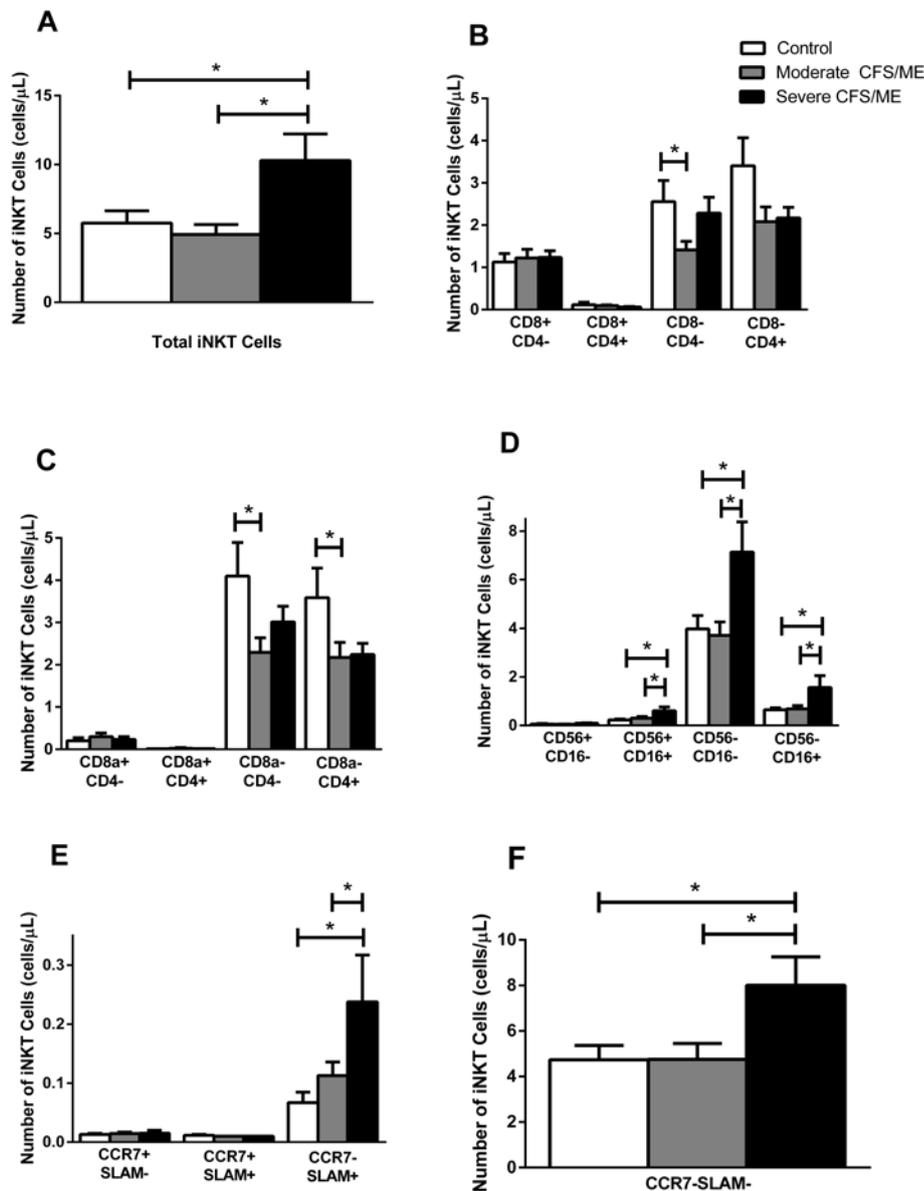


Figure 3: Perturbations in iNKT cell phenotypes and receptors in control, moderate and severe CFS/ME. (A) Total iNKT cell numbers are represented as a total number of cells (cells/ μ L) in control, moderate and severe CFS/ME groups. (B) CD8 and CD4 iNKT cell phenotypes represented as total number of cells (cells/ μ L). (C) CD8a and CD4 iNKT cell phenotypes are represented as total number of cells (cells/ μ L). (D) CD56 and CD16 iNKT cell phenotypes are represented as total number of cells (cells/ μ L). (E) CCR7 and SLAM iNKT cell receptor expression (CCR7⁺SLAM⁺; CCR7⁺SLAM⁻ and CCR7-SLAM⁺) is shown as total number of cells (cells/ μ L). (F) CCR7 and SLAM iNKT cell receptor expression (CCR7⁺SLAM⁻) is represented as total number of cells (cells/ μ L). All data is represented as mean \pm SEM. *represents results that were significantly different where $p<0.05$ CFS/ME: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis; CCR7: C Chemokine Receptor Type 7; SLAM: Signaling Lymphocytic Activation Molecule; SEM: Standard Error of the Mean.

(cells/ μ L) with the phenotype CD45RA⁺CD27⁻ were also significantly reduced in the moderate CFS/ME group ($p=0.0142$) (Figures 2C and 2D). $\gamma\delta 2$ T cells (cells/ μ L) were not significantly different between the groups (Data not shown).

No significant differences in total neutrophil numbers, CD177^{bright} or CD177^{dim} neutrophils, true monocytes, proinflammatory, intermediate or classical monocytes, total CD8⁺ T cells or CD8⁺ T cell phenotypes (cells/ μ L) between the control, moderate CFS/ME or severe CFS/ME groups were found ($p>0.05$) (Data not shown).

iNKT Phenotypes

Total iNKT numbers (cells/ μ L) were significantly increased in severe CFS/ME compared with controls ($p=0.012$) and moderate CFS/ME ($p=0.004$) (Figure 3A). There was a significantly reduced number of 6B11⁺CD3⁺CD8⁻CD4⁻, 6B11⁺CD3⁺CD8a⁻CD4⁻ and 6B11⁺CD3⁺CD8a⁻CD4⁺iNKT cells (cells/ μ L) in the moderate CFS/ME group ($p=0.031$, 0.026, 0.047) (Figures 3B and 3C). 6B11⁺CD3⁺CD56⁺CD16⁺, 6B11⁺CD3⁺CD56⁻CD16⁻ and 6B11⁺CD3⁺CD56⁻CD16⁺iNKT cells were significantly increased in the severe CFS/ME group compared with the control ($p=0.045$, 0.009, 0.025) and moderate groups, ($p=0.014$, 0.005, 0.031) (Figure 3D). 6B11⁺CD3⁺CCR7⁺SLAM⁺iNKT cells were significantly higher in the severe group compared with the moderate and control groups ($p=0.012$, 0.011) and 6B11⁺CD3⁺CCR7⁺SLAM⁺iNKT cells were also significantly increased in the severe CFS/ME group compared with controls ($p=0.012$) (Figures 3E and 3F). iNKT cell markers were significantly correlated in a positive manner with subsequent iNKT cell markers and subsets ($p<0.01$) (Supplementary Table 3).

Discussion

This is the first study to examine monocytes, neutrophils, DCs, CD8 T cells, $\gamma\delta$ T cells, iNKT cells, Tregs and B cells in moderate CFS/ME patients compared to severe CFS/ME patients and this is the first study to assess iNKT cells in CFS/ME. Previous literature has assessed immunological function in CFS/ME patients in relation to NK cells, monocytes, neutrophils, DCs, CD8 T cells, $\gamma\delta$ T cells, Tregs and B cells [13-15,18-21] however only one other study has examined immune parameters in moderate and severe CFS/ME patients [13].

This investigation confirmed previous findings that NK cell cytotoxic activity is consistently reduced in CFS/ME patients [12-14,20]. Reductions in cytotoxic activity may be associated with differential levels of cytotoxic molecules, including perforin, granzyme A and granzyme B which have been shown to be varied in cytotoxic NK cells and CD8⁺ T cells in CFS/ME [22,23]. NK cell cytotoxic activity can also be regulated by CD56^{bright} and CD56^{dim}NK cell phenotypes which have equivocal levels in CFS/ME patients [24-26]. This study also confirmed increases CD56^{bright}CD16⁻/^{dim} and CD56⁻CD16⁺NK cells in CFS/ME [25,26]. CD56^{dim} and CD56^{bright} NK cell phenotypes are primarily responsible for cytotoxic activity and cytokine release respectively. [27]. CD56^{bright} NK cells secrete immunoregulatory cytokines, such as IFN- γ , TNF- β , IL-10, IL-13 and GM-CSF which initiate an immune response and cytotoxic activities in other cells, including NK cells, B cells and DCs. CD56^{bright} NK cells also exhibit potent lymphokine-activated killer (LAK) activity which stimulates other lymphocytes to kill target cells, while CD56^{dim} NK cells are predominantly responsible for cytotoxic activity and antibody-dependent cellular cytotoxicity [27]. The increase in CD56^{bright} NK cells may serve as a regulatory mechanism to improve the reduced NK cytotoxic activity in CFS/ME. This study has shown that NK cell dysfunction may vary between

CFS/ME patient subgroups, with significant differences between the moderate and severe CFS/ME patients. The mechanism causing the reduced NK cytotoxic activity and increased NK phenotypes in CFS/ME patients is unknown although potential genetic defects may affect NK cell function in CFS/ME [28].

KIR2DS1 is an activating NK cell receptor which promotes NK cytotoxic activity in a human leukocyte antigen (HLA) class-1 dependent manner [29]. In psoriasis, the *KIR2DS1* gene is a strong predictor of disease where psoriasis patients demonstrate reduced *KIR2DS1* genetic frequency [30]. This study was the first to find reduced expression of the KIR2DS1 in moderate and severe CFS/ME patients. This may be associated with the reduced ability of NK cells to successfully lyse target cells [24,29-31]. Similarly, reductions in the corresponding inhibitory receptors KIR2DL1 and KIR2DL2/DL3 in CFS/ME may trigger reduced alloreactive KIR NK cell cytotoxic activity [29,30,32-34]. Thus, changes in KIR receptors may be an important component in the CFS/ME illness mechanism. In our previous studies KIR3DL1 was increased in CFS/ME patients, however we were unable to confirm this finding and this may be related to the heterogeneity of CFS/ME [13].

CD94 is a cytokine induced receptor complex on NK cells which can also either activate or inhibit cell-mediated cytotoxicity in NK cells, dependent on NKG2 protein association which recruits SHP-1 tyrosine phosphatase and couples to tyrosine kinase [35]. CD94 NK receptors recognise HLA-E which presents HLA class 1 molecule-derived peptides and inhibits NK cell-mediated lysis [36]. An increase in CD94 expression in CD56^{dim}CD16⁻ NK cells of both moderate and severe CFS/ME may be associated with an upregulated expression of HLA-E, which protects target cells from NK cell lysis and hence possibly reducing overall NK cell cytotoxic activity [24,37].

iNKT cells regulate disease conditions including type I diabetes and cancer via communication with a number of innate and adaptive immune cells [38,39]. Cellular interaction with iNKT cells results in the release of IL-10, causing reduced IL-12 in DCs and self-destructive ability in both T and B cells [38]. An increased number of iNKT cells in the severe CFS/ME participants may indicate the further promotion of iNKT cell proliferation in the bone marrow [40] and maybe related to an enhanced cell-mediated regulation of immunity in these patients [38]. The CD4⁺ and CD8a⁺iNKT cell subsets provide immunoregulation by releasing cytokines including IL-4, IL-5, IL-13, TNF- α and IFN- γ while the CD8⁻CD4⁻iNKT subset produces only IL-9 and IL-10 and induce cytotoxic activity [38,41-43]. Reduced CD8⁻CD4⁻, CD8a⁻CD4⁻ and CD8a⁻CD4⁺iNKT subsets in CFS/ME may reduce cytokine secretion required for maturation and activation of NK cells, DCs, B and T cells [42].

Increases in the CCR7⁺iNKT cells in CFS/ME may affect the trafficking of B and T cell trafficking to secondary lymphoid organs, as CCR7 plays a role in immunosurveillance [44,45]. SLAM is a surface receptor essential for iNKT cell development and the production of cytokines, particularly IFN- γ [46-48]. Increased iNKTCCR7⁺SLAM⁺ and reduced CCR7⁺SLAM⁺ expression in both moderate and severe CFS/ME compared to controls may be associated with a reduced ability of iNKT cells to interact and activate DC and T cells during an immune response [46]. Circulating iNKT cells also have a variable expression of the NK markers, CD56 and CD16, although little is known about the functional significance of these markers on the surface of iNKT cells [16]. Increases in CD56 and CD16 iNKT cell phenotypes in CFS/ME patients may be related to the altered expression of CD56 and CD16

NK cell phenotypes in the illness as iNKT cells are often dependent on NK cells, which are dysfunctional in CFS/ME. Similarly to NK cells, the alterations in iNKT cell markers displayed between moderate and severe CFS/ME patients may be due to NK or iNKT gene influencing an individual's susceptibility and extent of dysfunction [49].

pDCs are also responsible for modulating NK, T and B cell immune responses through antigen presentation and the release of cytokines and chemokines [50]. pDCs are particularly important in modulating and activating NK cell cytotoxic activity in response to a host viral infection through their secretion of IFN- α [51]. Increased pDCs in the moderate CFS/ME patients may be associated with increased NK cell activation and effector cell functioning. Increased pDCs in conjunction with reduced NK cytotoxic activity may highlight a reduced efficiency in cell-cell crosstalk and immune dysregulation in CFS/ME [51]. Elevated pDCs may also be linked to pDCs having an increased ability to become readily infected than mDCs, as found in HIV. This could potentially explain why there was no significant difference between mDC phenotypes in controls, moderate or severe CFS/ME [52]. Another DC phenotype, cytokine-producing CD14⁺CD16⁺ DCs were also significantly increased in the severe CFS/ME patients compared to both controls and the moderate CFS/ME subgroup, potentially suggesting dysfunction in the secretion of inflammatory cytokines. This supports studies which have shown IL4, IL-10 and IL-12, primarily produced by CD14⁺CD16⁺ DCs are found to be increased in CFS/ME [53-57]. These cytokines are important in the neutralisation of the Th1/Th2 cytokine shift which is also altered in CFS/ME patients [56,57].

$\gamma\delta$ T cells are sentinel cytotoxic cells involved in the elimination of bacterial infection, delayed-type hypersensitivity reactions, wound repair, antigen presentation and immunoregulation [58]. Effector memory $\gamma\delta$ T cells are responsible for cell migration to sites of inflammation and demonstrate NK-like functions such as the detection of abnormal MHC expression. Effector memory $\gamma\delta$ T cells also have potential for greater cytotoxic activity, tissue homing and rapid innate-like target recognition than central memory and naïve $\gamma\delta$ T cell subsets [58]. Reduced $\gamma\delta$ 1 effector memory in both moderate and severe CFS/ME and reduced $\gamma\delta$ 1 naïve phenotypes in moderate CFS/ME may potentially be a reflection of the consistently reduced NK cytotoxic activity in the illness as these cells are similar to NK cytotoxic cells.

It has been previously suggested that B cell activation may be increased in CFS/ME [59]. The increased naïve and memory B cells shown in severe CFS/ME compared to moderate CFS/ME patients may be consistent with amplified B cell activation, particularly as B cells are regulated by T and NK cells which also demonstrate dysfunction in CFS/ME. The increased naïve and memory B cells were shown in only the severe CFS/ME group, potentially highlighting a significant difference between CFS/ME severity subgroups. This confirms previous studies where CFS/ME patients have demonstrated increased numbers of naïve and transitional memory B cells [60]. The generation of memory B cells from naïve B cells is promoted by IL-4, IL-5, IL-13 and IL-10, secreted from CD4⁺ T cells. This suggests that potential increases in these cytokines, previously found in CFS/ME, may be triggering the increase in these B phenotypes [61]. Interestingly, transitional B cells are reduced in CFS/ME, indicating that the T cell-mediated extrinsic signals that drive B cell progression into transitional B cells may be abnormal [62,63]. Reduced Bregs are associated with reduced interactions with pathogenic T cells via cell-cell contact which are important in suppressing inflammatory T cells as well as regulatory cytokines (such as IL-10, TGF- β) [64,65]. The anti-inflammatory cytokine IL-10 in particular, has been reported as increased in CFS/

ME patients and is often related to enhanced production and survival of B cells [66].

Overall, immunological dysfunction in CFS/ME patients occurs as a consequence of changes in cytotoxic activity, NK cell phenotypes, KIR receptors, iNKT, DCs, $\gamma\delta$ T cells and B cell phenotypes. The severe CFS/ME subgroup of patients also experienced further immunological function in some instances. These findings suggest that immune perturbations may be further persistent in CFS/ME patients who experience more severe CFS/ME symptoms and hence may potentially dictate illness severity, as occurs in other diseases, such as rheumatoid arthritis (RA) [67-69].

Conclusion

This study is the first to assess a wide range of innate and adaptive immune cells in CFS/ME patients subgrouped by severity. Immune dysregulation was found in both moderate and severe patients with a consistent reduction in NK cytotoxic activity, alterations in B and iNKT cell phenotypes and an increase in CD14⁺CD16⁺ DCs. Interestingly, CD14⁺CD16⁺ DCs, total iNKTs and iNKT cell phenotypes differed between the moderate and severe CFS/ME patient subgroups. The findings of this study demonstrate that immune dysfunction appears to be related to the level of severity experienced by the patient hence severity subgroups may be important in identifying a specific disease mechanism in CFS/ME. Severity subgrouping of CFS/ME need to be considered in future studies as they may have implications for diagnosis and developing therapeutic strategies.

Competing Interests

The authors declare that they have no competing interests.

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