



Examination of Innate and Adaptive Immune Cells in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Patients with Varying Degrees of Symptom Severity

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**Examination of Innate and Adaptive Immune
Cells in Chronic Fatigue Syndrome/Myalgic
Encephalomyelitis Patients with Varying Degrees
of Symptom Severity**

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Bachelor of Biomedical Science (Hons1)

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ABSTRACT

The immune system has a critical influence on the maintenance of physiological homeostasis. To date, immunological dysfunction, particularly reduced natural killer (NK) cell cytotoxic activity in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients has been consistently observed. CFS/ME is a severely debilitating illness, with no known pathomechanism and diagnosis is made according to symptom specific criteria. CFS/ME is characterised by persistent and unexplained fatigue, alongside a range of symptoms, including: post-exertional neuroimmune exhaustion, neurological, immune, gastrointestinal, genitourinary and energy metabolism impairments. However, a symptom specific criterion provides complications for diagnosis, particularly as symptoms may be qualitative. CFS/ME is also a heterogeneous illness, with patients experiencing moderate to severe symptoms. CFS/ME patients with moderate symptoms are those who have reduced mobility and ability to perform their routine daily activities. CFS/ME patients with severe symptoms are usually homebound and/or restricted to a wheelchair. The debilitating nature of CFS/ME creates an economic burden and contributes largely to health resources, affecting CFS/ME patients as well as the wider community. In Australia, the annual cost to the community per CFS/ME patient, with a prevalence rate of 0.2% is \$729.3 million (based on 2012 estimates and earlier prevalence studies).

This thesis research aimed to further current knowledge of CFS/ME by assessing aspects of the innate and adaptive immune systems that may be associated with the potential pathomechanism of the illness. This research provided an analysis of innate

and adaptive immune systems in CFS/ME patients in moderate CFS/ME versus severe CFS/ME patients, particularly assessing cell markers, receptors and functions at baseline (week 0) and six months (week 24).

The study initially comprised 63 participants, including: 22 non-fatigued healthy controls, 23 moderate CFS/ME patients and 18 severe CFS/ME patients at baseline (week 0). At the six months (week 24) follow up, participants included 18 non-fatigued healthy controls, 12 moderate and 12 severe CFS/ME patients. All groups were age and sex matched. CFS/ME participants were defined using the 1994 Fukuda criterion for CFS/ME, which is the most commonly used criterion for CFS/ME diagnosis and research. Flow cytometry was utilised at baseline (week 0) to assess NK cell, neutrophil, monocyte, dendritic cell (DC), invariant natural killer T (iNKT), T regulatory cell (Treg), B cell, gamma delta ($\gamma\delta$) T and CD8⁺T cell phenotypes. NK cell cytotoxic activity, NK cell killer immunoglobulin-like (KIR) receptors and lytic proteins in NK and CD8⁺T cells were also assessed at baseline. Stored Serum Separation Tube (SST) serum from baseline (week 0) was used for a human cytokine 27-plex immunoassay to detect levels of IL-1 β , IL-1ra (interleukin 1 receptor agonist), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17, basicFGF (basic fibroblast growth factor), eotaxin (CCL11), G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte macrophage colony-stimulating factor), IFN- γ (interferon gamma), IP-10 (interferon gamma-induced protein 10, CXCL10), PDGF-BB (platelet-derived growth factor-BB), RANTES (regulated on activation, normal T cell expressed and secreted, CCL5), TNF- α (tumor necrosis factor alpha), MCP1 (monocyte chemotactic protein 1), MIP1a (macrophage inflammatory protein alpha), MIP1b (macrophage inflammatory protein beta), and VEGF (vascular endothelial growth

factor) in participant groups. At six months (week 24), flow cytometry was used to assess DC, monocyte and neutrophil function as well as lytic proteins in iNKT, Tregs, NK, CD8⁺T and $\gamma\delta$ T cells. NK, T and B cell receptors (BCRs) and phenotypes of iNKT, Tregs, DC, B, $\gamma\delta$ T, CD8⁺T and NK cells.

At baseline (week 0), both moderate and severe CFS/ME patients demonstrated significantly reduced NK cell cytotoxic activity, CD56^{dim}CD16⁻ KIR2DL1/DS1 NK cells, CD45RA⁺ effector memory $\gamma\delta$ 1T cells. Additionally, at baseline both moderate and severe CFS/ME patients demonstrated significant increases in CD94⁺ CD56^{dim}CD16⁻ NK cells and iNKT cell numbers. The moderate CFS/ME patient cohort showed significantly increased CD56^{dim}CD16⁻ NK cells and plasmacytoid DCs along with reduced effector memory $\gamma\delta$ 1T cells, CD8⁻CD4⁻, CD8a⁻CD4⁻ and CD8a⁻CD4⁺ iNKT cells when compared with controls at baseline (week 0). In comparison to the moderate CFS/ME patients at baseline (week 0), severe CFS/ME patients had significantly increased CD56^{bright}CD16^{dim} and CD56^{dim}CD16⁺ NK cells, memory and naïve B cells as well as reduced CD56^{bright}CD16^{dim} KIR2DL2/DL3, transitional and regulatory B cells (Bregs). Severe CFS/ME patient's baseline (week 0) analysis had significantly increased CD14⁻CD16⁺ DCs and CD56⁺CD16⁺, CD56⁻CD16⁻, CD56⁻CD16⁺, CCR7⁻SLAMF⁻ and CCR7⁻SLAMF⁺ iNKT cells compared with both moderate CFS/ME patients and controls. IL-6 and RANTES were significantly increased in moderate CFS/ME patients compared with non-fatigued healthy controls and severe CFS/ME patients. IL-7 and IL-8 were significantly increased in the severe CFS/ME group compared with controls and moderate CFS/ME patients. Baseline (week 0) IFN- γ was significantly increased in severe CFS/ME patients compared with moderately

affected patients. Serum IL-1 β was significantly reduced in severe CFS/ME compared with moderate patients at baseline (week 0).

At six months (week 24), total, effector memory and CD45RA⁺ effector memory $\gamma\delta$ T cells, CD94⁻CD11a⁻, CD62L⁺CD11a⁻ and CD62L⁺CD11a⁺ $\gamma\delta$ T cells were significantly higher in severe CFS/ME patients compared with controls and moderate CFS/ME patients. CD62L⁺CD11a⁻ $\gamma\delta$ T cells and CD62L⁺CD11a⁻ $\gamma\delta$ 1T cells were significantly lower in severe CFS/ME patients compared with other participant groups. Severe CFS/ME patients then demonstrated significantly increased CD94⁻CD11a⁺ $\gamma\delta$ 2T cells and reduced CD56^{dim}CD16⁻ KIR2DL2/DL3 NK cells, CD56^{bright}CD16^{dim} NK cell NKG2D, CD94⁻CD11a⁻ $\gamma\delta$ 1T cells and CD62L⁺CD11a⁻ $\gamma\delta$ 1T cells at six months (week 24) compared with controls. Also at six months (week 24), naïve CD8⁺T cells, CD8⁻CD4⁻ and CD56⁻CD16⁻ iNKT phenotypes, $\gamma\delta$ 2T cells and effector memory subsets were significantly increased in severe CFS/ME patient compared with controls.

Only the severe CFS/ME patients had a significant increase in CD56^{bright}CD16⁺ KIR2DL1 over the six month (week 24) time period. Over six months (week 24), a significant increase was shown in CD56^{bright}CD16^{dim} KIR3DL1/DL2 and CD56^{bright}CD16⁺ KIR2DL2/DL3 and KIR2DS4 in both the controls and moderate CFS/ME patients. CD62L⁺ iNKT cells were significantly increased in moderate CFS/ME at the six month (week 24) time point compared with baseline (week 0).

Firstly, the results from this research have validated the presence of immune abnormalities in CFS/ME patients. This research was the first to examine iNKT and $\gamma\delta$ T

cells in CFS/ME patients and identified alterations in these cells which may provide potential avenues for future research into the illness. Significant immunological differences have been found in this research in severe CFS/ME patients compared with moderate CFS/ME patients, highlighting that it may be important to identify severity subgroups for improved specificity in future CFS/ME research. This research also showed potential differences in immunological parameters which may possibly correspond to differences in the aetiology between CFS/ME severity subgroups. Overall, this thesis research also demonstrated significant immune abnormalities in CFS/ME patients that can be further investigated in future studies to improve the understanding of the illness, this may lead to the discovery of potential pathomechanisms and biomarkers that may be used for diagnosis.

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STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Sharni Hardcastle

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ABBREVIATIONS

$\delta\gamma$	Gamma delta
AAD	Aminoacetinomycin D
ADCC	Antibody-dependent cell-mediated cytotoxicity
ANOVA	Analysis of variance test
APC	Antigen-presenting cell
BAFF	B cell activating factor
BC	British Columbia
BCL	B cell lymphoma
BCR	B cell receptor
BD	Becton Dickinson
Blimp	B lymphocyte induced maturation protein
Breg	B regulatory cell
BTLA	B and T lymphocyte attenuator
CA	California
CCR7	CC-chemokine receptor 7
CD	Cluster of differentiation

cDC	Classical dendritic cell
CFS	Chronic Fatigue Syndrome
CPRS	Comprehensive Psychopathological Rating Scale
CRACC	CD2-like Receptor Activating Cytotoxic Cell
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DP	Double positive
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
FasL	Fas-ligands
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FL	FLT3-ligand
FM	Fibromyalgia
FOXP3	Forkhead box protein
FSS	Fatigue Severity Scale

G-CSF	Granulocyte colony-stimulating factor
GHQ	General health questionnaire
GM-CSF	Granulocyte macrophage colony-stimulating factor
GP	General practitioner
GU	Griffith University
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
ICAM	Intracellular adhesion molecule
ICC	International Consensus Criteria
iGb3	Isoglobotrihexosylceramide
IL	Interleukin
iNKT	Invariant natural killer T cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer Immunoglobulin-like Receptor

KPS	Karnofsky Performance Scale
LAK	Lymphokine-activated killer
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
LSD	Least significant difference
MAC	Macrophage-1 antigen
mDC	Monocyte-derived or myeloid dendritic cell
ME	Myalgic Encephalomyelitis
MHC	Major histocompatibility complex
MFTQ	ME/CFS Fatigue Types Questionnaire
MO	Missouri
MS	Multiple Sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NCNED	National Centre for Neuroimmunology and Emerging Diseases
NCR	Natural cytotoxicity receptor
NETs	Neutrophil extracellular traps
NK	Natural Killer

PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	aphosphate buffered saline
pDC	Plasmacytoid dendritic cell
PRR	Pattern-recognition receptors
PSGL	P-selectin glycoprotein ligand
RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T cell expressed and secreted, CCL5
rcf	Relative centrifugation force
ROS	Reactive oxygen species
SAP	SLAM associated protein
SD	Standard deviation
SEM	Standard error of the mean
SF-36	Short Form 36 Health Survey
SHP	Scr homology domain containing tyrosine phosphatase
SIP	Sickness Impact Profile
SLAM	Signalling lymphocytic activation molecule

SLE	Systemic Lupus Erythematosus
SST	Serum separating tubes
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Th	T helper
TGF	Transforming growth factor
TLR	Toll like receptor
TNF	Tumour necrosis factor
TNFR	Ligand-activated tumour-necrosis factor receptor
Tr	Type 1 T regulatory cell
TRAIL	Tumour necrosis factor-related apoptosis-induced ligand
Treg	T regulatory cell
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
WHO DAS	WHO Disability Assessment Schedule

PUBLICATIONS

Acknowledgement of Papers included in this Thesis

Section 9.1 of the Griffith University Code for the Responsible Conduct of Research (“Criteria for Authorship”), in accordance with Section 5 of the Australian Code for the Responsible Conduct of Research, states:

To be named as an author, a researcher must have made a substantial scholarly contribution to the creative or scholarly work that constitutes the research output, and be able to take public responsibility for at least that part of the work they contributed. Attribution of authorship depends to some extent on the discipline and publisher policies, but in all cases, authorship must be based on substantial contributions in a combination of one or more of:

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- Accept or decline offers of authorship promptly in writing.
- Include in the list of authors only those who have accepted authorship
- Appoint one author to be the executive author to record authorship and manage correspondence about the work with the publisher and other interested parties.
- Acknowledge all those who have contributed to the research, facilities or materials but who do not qualify as authors, such as research assistants, technical staff, and advisors on cultural or community knowledge. Obtain written consent to name individuals.

Included in this thesis are papers in **Chapters 1.2.5, 1.3.6, 3, 4, 5 and 6** which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details (if published or accepted for publication)/status (if prepared or submitted for publication) for these papers, including all authors, are:

Chapter 1.2.5: Review Paper One: Severity scales for use in primary health care to assess Chronic Fatigue Syndrome/Myalgic Encephalomyelitis

Hardcastle, SL., Brenu, EW., Johnston, S., Staines, DR., Marshall-Gradisnik, S. (2014) Review: Severity Scales for Use in Primary Health Care to Assess Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. Health Care for Women International, 0:1-16.

Chapter 1.3.6: Review Paper Two: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis and the potential role of T cells

Hardcastle, SL., Brenu, EW., Staines, DR., Marshall-Gradisnik, S. (2014) Review: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis and the potential role of T cells. Biological Markers and Guided Therapy. 1(1), 25-38.

Chapter 3: Project One: Analysis of the relationship between immune dysfunction and symptom severity in patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME)

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Kaur, M., Ramos, S., Salajegheh, A., Staines, D., Marshall-Gradisnik, S. (2014). Analysis of the relationship between immune dysfunction and symptom severity in patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). Journal of Clinical and Cellular Immunology, 5(1), 190.

Chapter 4: Project Two: Serum immune proteins in moderate and severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Ramos, S., Salajegheh, A., Staines, D., Marshall-Gradisnik, S. (2015). Serum immune proteins in moderate and severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. *International Journal of Medical Sciences*, 12(10), 764-772.

Chapter 5: Project Three: Characterisation of cell functions and receptors in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME)

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Wong, N., Ramos, S., Staines, D., Marshall-Gradisnik, S. (2015). Characterisation of cell functions and receptors in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). *BMC Immunology*, 16(35).

Chapter 6: Project Four: Longitudinal analysis of immune abnormalities in varying degrees of Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Ramos., Staines, D., Marshall-Gradisnik, S. (2014). Longitudinal analysis of immune abnormalities in varying severities of Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients. *Journal of Immunology Research*, 13:299.

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Supervisor: Sonya Marshall-Gradisnik

Journal Publications Generated from this Thesis

Hardcastle, SL., Brenu, EW., Johnston, S., Staines, DR., Marshall-Gradisnik, S. (2014) Review: Severity Scales for Use in Primary Health Care to Assess Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. *Health Care for Women International*, 0:1-16.

Hardcastle, SL., Brenu, EW., Staines, DR., Marshall-Gradisnik, S. (2014) Review: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis and the potential role of T cells. *Biological Markers and Guided Therapy*. 1(1), 25-38.

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Kaur, M., Ramos, S., Salajegheh, A., Staines, D., Marshall-Gradisnik, S. (2014). Analysis of the relationship between immune dysfunction and symptom severity in patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). *Journal of Clinical and Cellular Immunology*, 5(1), 190.

Hardcastle, SL., Brenu, EW., Wong, N., Johnston, S., Nguyen, T., Huth, T., Ramos, S., Salajegheh, A., Staines, D., Marshall-Gradisnik, S. (2014). Serum immune proteins in moderate and severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. *International Journal of Medical Sciences*.

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Wong, N., Ramos, S., Staines, D., Marshall-Gradisnik, S. (2014). Characterisation of cell functions and receptors in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). *BMC Immunology*.

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Ramos., Staines, D., Marshall-Gradisnik, S. (2014). Longitudinal analysis of immune abnormalities in varying severities of Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients. *Journal of Immunology Research*.

Conference Presentations Generated from this Thesis

1. **Hardcastle, SL.**, Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Ramos, S., Staines, D., Marshall-Gradisnik, S. Gamma delta T cell abnormalities in severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. Submitted for presentation at the 4th European Congress of Immunology in Vienna, September 2015.
2. **Hardcastle, SL.**, Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Ramos, S., Staines, D., Marshall-Gradisnik, S. Longitudinal changes in natural killer cell receptors in moderate versus severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. Submitted for presentation at the 4th European Congress of Immunology in Vienna, September 2015.
3. **Hardcastle, SL.**, Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Wong, N., Hawthorn, A., Ramos, S., Staines, D., Marshall-Gradisnik, S. Perturbations in adhesion molecules and receptors in moderate versus severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients. Poster presentation at the Australasian Society for Immunology in Sydney, Australia, December 2014.
4. **Hardcastle, SL**, Brenu, EW, Wong, N, Johnston, S, Nguyen, T, Huth, TK, Hawthorn, A, Passmore, R, Ramos, S, Salajegheh, A, Staines, DR & Marsall-Gradisnik, SM, Serum cytokines in patients with moderate and severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, International Cytokine and Interferon Society, Melbourne, Australia, October, 2014.
5. **Hardcastle, SL**, Brenu, EW, Johnston, S, Nguyen, T, Huth, TK, Ramos, S, Salajegheh, A, Staines, DR & Marsall-Gradisnik, SM, Alterations in innate and

- adaptive immune cells in moderate versus severely affected Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, International Student Research Forum, Odense, Denmark, June 2014.
6. **Hardcastle, SL**, Brenu, EW, Johnston, S, Huth, TK, Ramos, S, Staines, DR & Marshall-Gradisnik, SM, Innate immune cells in severe and moderate Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, 11th International Association for Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Biennial International Research and Clinical Conference, San Francisco, United States of America, March 2014.
 7. **Hardcastle, SL**, Brenu, EW, Johnston, S, Huth, TK, Ramos, S, Staines, DR & Marshall-Gradisnik, SM, Assessment of severity scales and their use in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, 11th International Association for Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Biennial International Research and Clinical Conference, San Francisco, United States of America, March 2014.
 8. **Hardcastle, SL**, Brenu, EW, Johnston, S, Huth, TK, Nguyen, T, Ramos, S, Staines, DR & Marshall-Gradisnik, SM, Immune cells in severe and moderate Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, 11th International Association for Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Biennial International Research and Clinical Conference, San Francisco, United States of America, March 2014.
 9. **Hardcastle, SL**, Brenu, EW, Johnston, S, Nguyen, T, Kaur, M, Huth, TK, Fuller, K, Ramos, SB, Staines, DR & Marshall-Gradisnik, SM, Analysis of innate immune cells in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis

- patients with varying severities, 9th International Congress on Autoimmunity, Nice, France, March, 2014.
10. **Hardcastle, SL**, Brenu, EW, Johnston, S, Nguyen, T, Kaur, M, Huth, TK, Fuller, K, Ramos, SB, Staines, DR & Marshall-Gradisnik, SM, Adaptive immune cells in severe and moderate Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, 9th International Congress on Autoimmunity, Nice, France, March, 2014.
 11. **Hardcastle, SL**, Brenu, EW, Staines, DR & Marshall-Gradisnik, SM, Assessment of natural killer cell receptors and activity in severe and moderate Chronic Fatigue Syndrome, 15th International Congress of Immunology, Milan, Italy, August 2013.
 12. **Hardcastle, SL**, Brenu, EW, Staines, DR & Marshall-Gradisnik, SM, Analysis of neutrophil function in severe and moderately affected Chronic Fatigue Syndrome subjects, 15th International Congress of Immunology, Milan, Italy, August 2013.
 13. **Hardcastle, SL**, Brenu, EW, van Driel, M, Staines, DR, Kreijkamp-Kaspers, S & Marshall-Gradisnik, SM, 2012, Natural Killer cells in patients with severe Chronic Fatigue Syndrome, 8th International Congress on Autoimmunity, Granada, Spain, May, 2012.

Additional Journal Publications Generated from this Research

Brenu, EW, Huth, TK, **Hardcastle, SL**, Fuller, K, Kaur, M, Johnston, S, Ramos, S, Staines, DR & Marshall-Gradisnik, SM, (2014) Role of adaptive and innate immune cells in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, *International Immunology*, doi: 10.1093/intimm/dxt068.

Huth, TK, Brenu, EW, Nguyen, T, **Hardcastle SL**, Johnston SC, Ramos SB, Staines DR, Marshall-Gradisnik SM. (2014). Characterisation of Natural Killer Cell Phenotypes in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, *Journal of Clinical and Cellular Immunology – Neuroinflammatory Diseases Special Issue*, 5:223, doi: 10.4172/2155-9899.1000223.

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Brenu, EW., **Hardcastle, SL**, Atkinson, GM., van Driel, ML., Kreijkamp-Kaspers, S., Staines, DR., Marshall-Gradisnik, SM. (2013). Natural killer cells in patients with severe Chronic Fatigue Syndrome. *Autoimmunity Highlights*.

Brenu, EW, van Driel, ML., Staine, DR., Kreijkamp-Kaspers, S., **Hardcastle, SL**, Marshall-Gradisnik. (2012) The effects of influenza vaccination on immune function in

patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. *International Journal of Translational Medicine*.

Brenu, EW. van Driel, ML., Staines, DR., Ashton, KJ., **Hardcastle, SL.**, Keane, J., Tajouri, T., Peterson, D., Ramos, S., Marshall-Gradisnik, S. (2012). Longitudinal investigation of natural killer cells and cytokines in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. *Journal of Translational Medicine*.

Additional Conference Presentations Generated from this Research

1. Wong, N, Brenu, EW, **Hardcastle, SL**, Johnston, S, Nguyen, T, Huth, TK, Hawthorn, A, Passmore, R, Ramos, Salajegheh, A, Broadley, S, Staines, DR & Marsall-Gradisnik, SM, Cytokine profiles of Chronic Fatigue Syndrome patients and Multiple Sclerosis patients, International Cytokine and Interferon Society, Melbourne, Australia, October, 2014.
2. Marshall-Gradisnik, SM, Brenu, EW, Huth, TK, **Hardcastle, SL**, Kapur, M, Johnston, S, Nguyen, T, Ramos, S, & Staines, DR, Chronic fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME): Immunological features and possible pathomechanisms, 2nd Asian Clinical Congress, Kyoto, Japan, April 2014.
3. Huth, TK, Brenu, EW, Fuller, K, **Hardcastle, SL**, Johnston, S, Staines, DR & Marshall-Gradisnik, SM, Natural killer cell degranulation and lytic proteins in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, 11th International Association for Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Biennial International Research and Clinical Conference, San Francisco, United States of America, March 2014.
4. Johnston, S, Brenu, EW, **Hardcastle, SL**, Huth, TK, Fuller, K, Ramos, SB, Staines, DR, Marshall-Gradisnik, SM, Immunological, physical and social functioning in varying cases of Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, 11th International Association for Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Biennial International Research and Clinical Conference, March 2014, San Francisco, United States of America.

5. Nguyen, T, Brenu, EW, Ramos, S, Huth, TK, **Hardcastle, SL**, Fuller, K, Johnston, S & Marshall-Gradisnik, SM, Degranulation of cytotoxic T lymphocytes in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, 9th International Congress on Autoimmunity, Nice, France, March, 2014.
6. Fuller, K, Brenu, EW, Huth, TK, **Hardcastle, SL**, Johnston, S, Nguyen, T, Staines, DR & Marshall-Gradisnik, SM, Comprehensive analysis of peripheral B cell phenotypes in Chronic Fatigue Syndrome, 9th International Congress on Autoimmunity, Nice, France, March, 2014.
7. Ramos, S, Brenu, EW, Huth, TK, **Hardcastle, SL**, Johnston, S, Nguyen, T, Fuller, K, Staines, DR & Marshall-Gradisnik, SM, Immunoglobulin secretion in Chronic Fatigue Syndrome, 9th International Congress on Autoimmunity, Nice, France, March, 2014.
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- Fatigue Syndrome, Autoimmunity Congress Asia, Hong Kong, November, 2013.
12. Brenu, EW, Ashton, KJ, Tajouri, L, **Hardcastle, SL**, Huth, TK, Cosgrave, LM, Batovska, J, Atkinson, GM, van Driel, M, Staines, DR & Marshall-Gradisnik, SM. Gene expression changes following influenza vaccination in patients with Chronic Fatigue Syndrome, Immunological Mechanisms of Vaccination, Ottawa, Canada, December 2012.
 13. Marshall-Gradisnik, SM, Ashton, KJ, Tajouri, L, **Hardcastle, SL**, Huth, TK, Cosgrave, LM, Batovska, J, Atkinson, GM, van Driel, M, Staines, DR & Brenu, EW 2012, The role of vaccination on the immune function in patients with Chronic Fatigue Syndrome, Immunological Mechanisms of Vaccination, Ottawa, Canada, December 2012.
 14. Marshall-Gradisnik, SM, van Driel, M, Ashton, KJ, **Hardcastle, SL**, Staines, DR & Brenu, EW, 2012, Adaptive and innate immune markers as potential biomarkers for Chronic Fatigue Syndrome, International Conference on Translational Medicine, San Antonio, United States of America, September, 2012.
 15. Brenu, EW, Ashton, KJ, **Hardcastle, SL**, Huth, TK, Cosgrave, LM, Batovska, J, Atkinson, GM, van Driel, M, Staines, DR & Marshall-Gradisnik, SM, 2012, Differential expression of mRNAs and miRNAs in Patients with Chronic Fatigue Syndrome, International Conference on Translational Medicine, San Antonio, United States of America, September, 2012.
 16. Marshall-Gradisnik, SM, Staines, DR, Ashton, KJ, **Hardcastle, SL**, Huth, TK, Batovska, J, Cosgrave, LM, van Driel, M & Brenu, EW, 2012, Immunological

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17. **Hardcastle, SL**, Brenu, EW, Staines, DR, van Driel, M, Peterson, D & Marshall-Gradisnik, SM, 2011, Assessment of Natural Killer cell receptors in severe and moderate Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, 10th International Association for Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Biennial International Research and Clinical Conference, Ottawa, Canada, September, 2011.

CHAPTER 1: INTRODUCTION AND OVERVIEW

1.1 CHRONIC FATIGUE SYNDROME/MYALGIC ENCEPHALOMYELITIS

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is classified clinically as a condition where a patient suffers from severe debilitating fatigue for a period of at least six months, as well as suffering a combination of other symptoms (Carruthers et al., 2011; Fukuda et al., 1994b). The illness is complex and multifactorial, allowing for potential misconceptions and inconsistency in the diagnosis of CFS/ME (Carruthers et al., 2011; Fukuda et al., 1994a; Hutchinson et al., 2002). The most common age of onset for CFS/ME ranges from early twenties to mid-forties although CFS/ME has been diagnosed in children as young as five years old (Hutchinson et al., 2002). CFS/ME is also an illness which typically affects women at a higher rate than men (Hutchinson et al., 2002). CFS/ME patients may experience varying degrees of severity in their symptoms, depending on the level of activity a patient is able to undertake. Consequently, CFS/ME patients may be grouped into either moderate or severe CFS/ME. Moderate CFS/ME patients have reduced mobility and their ability to perform normal daily activities is lessened. Severe CFS/ME patients are usually bedridden or restricted to a wheelchair (Hutchinson et al., 2002; Wiborg et al., 2010b).

The most widely used diagnostic criteria for CFS/ME is the 1994 Fukuda case definition (Fukuda et al., 1994a). This primarily defines CFS/ME patients as those who have persistent chronic fatigue which is independent of significant physical exertion and

cannot be alleviated by rest (Fukuda et al., 1994a). The Fukuda case definition takes into consideration the prevalence of symptoms prior to diagnosis and requires patients to have experienced a substantial reduction in the levels of occupation, education, social and personal activities (Hutchinson et al., 2002). To fulfil the Fukuda case definition for CFS/ME, symptoms must have prevailed for at least six months, with the presence of at least four of the following symptoms, including: post exertional malaise, impaired memory, unrefreshing sleep, muscle pain, joint pain without redness, tender lymph nodes, sore throat and/or headaches (Fukuda et al., 1994a). Some patients may experience symptoms with lesser severity while others may be affected by symptoms to the extent whereby they are habitually bedridden.

The 2003 Canadian definition for CFS/ME increased the specificity of CFS/ME diagnostic criteria by outlining symptoms from the immune, autonomic, neurological and neuroendocrine body systems (Carruthers et al., 2003). The Canadian definition was revised to contribute to the 2011 International Consensus Criteria for ME (ICC), which considered the most up to date symptomatology for CFS/ME. The ICC was created to enhance the clarity and specificity of the criteria while providing guidelines for the interpretation of symptoms. The ICC guidelines are specific and more detailed, particularly concerning particular types of fatigue, neurological, gastro-intestinal, immune impairments and energy production as well as considering symptom severity and stability (Carruthers et al., 2011).

Biological systems, including: neurological (Rampello et al., 2012), endocrine (Cleare, 2003; Roberts et al., 2004) and digestive, have been examined in CFS/ME with equivocal outcomes. Similarly, immunological investigations of the innate and adaptive

system, including: perforin and granzyme expression in natural killer (NK) cells (Brenu et al., 2011; Maher et al., 2005), NK cell receptor expression (Brenu et al., 2013a), neutrophil death receptor expression (Kennedy et al., 2004), neutrophil respiratory burst (Brenu et al., 2010), mitochondrial function in neutrophils (Myhill et al., 2009), monocyte adhesion molecule expression (Natelson et al., 2002), T cell subset phenotypes (Klimas et al., 1990) and T cell cytokine production (Fletcher et al., 2009; Patarca-Montero et al., 2001; Visser et al., 1998) have predominantly been investigated in the moderate CFS/ME cohort, however results are inconsistent. Currently, the most consistent immunological finding is significantly reduced NK cell cytotoxic activity in CFS/ME (Brenu et al., 2013a; Brenu et al., 2010; Caligiuri et al., 1987; Fletcher et al., 2009; Klimas et al., 2012; Klimas et al., 1990; Patarca-Montero et al., 2001; Visser et al., 1998).

Collectively, these studies suggest that changes in the innate and adaptive immune system may be an important component of the pathomechanism of CFS/ME; however this is yet to be explored extensively and forms the basis of this research. Also, the majority of previous CFS/ME immune examinations were performed in CFS/ME patients who were only moderately affected by the illness. Severe CFS/ME patients were often not included as they are mostly confined to their homes and may have difficulty travelling for research. As immunological studies on severe CFS/ME patients are very limited, there is an imperative need for further investigations examining the severity of CFS/ME to describe whether severity correlates with larger perturbations in immune function in patients with CFS/ME.

Prior to this thesis research, only one study had examined NK cell function, phenotype and receptors in bedridden severely affected CFS/ME patients in comparison with a non-fatigued healthy control group (Brenu et al., 2013a). Brenu et al found that severe CFS/ME patients had significantly reduced NK cell cytotoxic activity when compared with moderate CFS/ME patients (Brenu et al., 2013a). This thesis research is supported by Brenu et al's (2013a) study, where significant changes in NK cell Killer Immunoglobulin-like Receptor (KIR) expression and NK cell cytotoxic activity were shown in severe CFS/ME patients. The results of the Brenu et al (2013a) study and this thesis research, highlighted the importance for future studies to examine CFS/ME patients who are severely affected by their symptoms and supported the notion that differing levels of severity may also influence immune perturbations in CFS/ME (Brenu et al., 2013a).

An analysis of the immune system in CFS/ME patients with varying severities may benefit international CFS/ME research. This is the first study to examine potential differences in the innate and adaptive immune system in moderate CFS/ME patients compared with severe CFS/ME patients. This research may further current research by assessing potential immunological pathomechanisms which may be linked to the consistently reduced NK cell cytotoxic activity already shown in CFS/ME (Brenu et al., 2013a; Brenu et al., 2010; Caligiuri et al., 1987; Fletcher et al., 2009; Klimas et al., 2012; Klimas et al., 1990; Patarca-Montero et al., 2001; Visser et al., 1998). This thesis will provide an understanding of the influence of CFS/ME on a patient's immune system in relation to severity. This thesis research will therefore also contribute to the possibility of immunological markers for identification and diagnostic potential for CFS/ME in the future. Essentially, this research may also assist future diagnosis by

outlining the need for medical services to be directed to those who are severely affected by CFS/ME, where interventions for this subgroup may then be tailored.

1.2 SEVERITY CLASSIFICATIONS OF CFS/ME

Assessments of patient physical functioning are commonly used to supplement medical information in order to characterise the impact of an illness on a patient (Mor et al., 1984). Measures of both physical and mental capacity are important in determining the severity of CFS/ME patient symptoms and essentially form a necessary aspect of research into differing levels of CFS/ME immune dysfunction. Assessment of symptom severity may be important in analysing severity variation and determining if patients are moderately or severely affected by CFS/ME. The physical and mental status and severity of an illness can be measured using a variety of scales.

1.2.1 Sickness Impact Profile

An important measure of health status based on behaviour is the Sickness Impact Profile (SIP) (Bergner et al., 1981). The SIP is sensitive enough to determine changes in health status, illness progression and severity based on changes over time (Busija et al., 2011; Heins et al., 2011; Wadden & Phelan, 2002; Wiborg et al., 2010b). The clinical validity of the SIP was confirmed after studies on the relationship between the SIP and clinical measures of an illness found the SIP was accurate in determining basic health status and changes based on behaviours (Bergner et al., 1981).

The SIP was widely used in CFS/ME studies as a measure of a person's functional performance, assisting researchers in categorising CFS/ME patients into severity subgroups (Busija et al., 2011; Heins et al., 2011; Wadden & Phelan, 2002; Wiborg et al., 2010b). The SIP has often been used alongside a number of similar severity scales of both physical and emotional function to give an in-depth analysis of functional and mental status of patients. The SIP was used to effectively confirm severity subgroups of CFS/ME patients in conjunction with other severity scales (Wiborg et al., 2010b).

1.2.2 Fatigue Severity Scale

A commonly used measurement of fatigue is the self-rating scale for fatigue severity, the Fatigue Severity Scale (FSS) (Herlofson & Larsen, 2002; Krupp et al., 1989), is a reliable and valid measure of fatigue severity (Hewlett et al., 2011). This scale comprises a list of 14 questions relating to both physical and mental fatigue, with the options 'better than usual', 'no more than usual', 'worse than usual' and 'much worse than usual' (Chalder et al., 1993). Based on a bimodal response system or general health questionnaire (GHQ) method for scoring, the method allows errors to be eliminated from the data based on 'end users' and 'middle users'. Weighting of scores can also be applied to the FSS and each method has the advantage of minimising the effects of outliers in data. This scale may be used to detect cases of fatigue, being especially useful in conjunction with the other scales of mobility or performance (Chalder et al., 1993; Herlofson & Larsen, 2002; Hewlett et al., 2011; Krupp et al., 1989).

1.2.3 Karnofsky Performance Scale

The Karnofsky Performance Scale (KPS) is an 11-point rating scale ranging from 0 through to 100, where 100 represents normal functioning (Hutchinson et al., 1979). The KPS is commonly used in research to rank patients on a one-dimensional numerical scale, regardless of the multifactorial functional and symptom status. The KPS score measures performance based on a value of combined physical and mental functioning. The KPS has gained widespread acceptance as an effective and reliable way of rating the functional status of a patient (Mor et al., 1984; Wiborg et al., 2010b). The KPS scale was also used as the predominant measure of functional status in patients in a clinical trial examining 234 patients classified as being in a group of severe CFS/ME (Strayer et al., 2012). A number of functional status and quality of life scales, including the KPS, have been used in a study analysing the symptom severity subgroups in CFS/ME patients (Wiborg et al., 2010b).

1.2.4 Short Form 36 Health Survey

The Short Form 36 Health Survey (SF-36) is a multipurpose measure of the overall health status of a person with an illness. The form consists of eight scaled scores covering vitality, physical functioning, bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning and mental health, which are rated on a scale of 0 to 100 (Ware Jr & Sherbourne, 1992).

The SF-36 is an international measure of quality of life, providing useful information on the health status of patients with CFS/ME (Contopoulos-Ioannidis et al., 2009; Fulcher

& White, 1997; Myers & Wilks, 1999; Powell et al., 2001; Reeves et al., 2005). The SF-36 is the most extensively validated scale and is frequently used for assessing quality of life, with applications in a range of studies from population health surveys to biological research into illnesses (Contopoulos-Ioannidis et al., 2009; Doll et al., 2000; Fulcher & White, 1997; Myers & Wilks, 1999; Powell et al., 2001; Reeves et al., 2005; Yarlal et al., 2011). The interpretation and significance of using SF-36 is consistent with results from other quality of life and health survey measures (Contopoulos-Ioannidis et al., 2009).

Clinical research studies have highlighted the uses for severity scales in research, including in cases of CFS/ME (Strayer et al., 2012; Wiborg et al., 2010b). Following from these studies and the heterogeneous nature of the illness, the use of severity scales in CFS/ME may be important in distinguishing severity subgroups. The identification of severity subgroups in clinical and research settings may improve the understanding of CFS/ME and potential variations in the illness pathomechanism and aetiology. The review paper below provides an analysis of severity scales and their potential use in CFS/ME.

1.2.5 REVIEW PAPER ONE: SEVERITY SCALES FOR USE IN PRIMARY HEALTH CARE TO ASSESS CHRONIC FATIGUE SYNDROME/MYALGIC ENCEPHALOMYELITIS

Hardcastle, SL., Brenu, EW., Johnston, S., Staines, DR., Marshall-Gradisnik, S. 2014. Severity Scales for Use in Primary Health Care to Assess Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. Health Care for Women International.

Author contributions:

Hardcastle was the principle contributor to this manuscript. Hardcastle was responsible for contributions to the manuscript design, literature review and analysis as well as the primary drafting of the manuscript. Brenu, Johnston, Staines and Marshall-Gradisnik contributed to the manuscript design and critical manuscript revisions.

1.2.5.1 ABSTRACT

CFS/ME is a physical and cognitive disabling illness, characterised by severe fatigue and a range of physiological symptoms that primarily affects women. The immense variation in clinical presentation suggests differences in severity based on symptomatology, physical and cognitive functional capacities. In this review paper, we examined a number of severity scales used in assessing severity of patients with CFS/ME and the clinical aspects of CFS/ME severity subgroups. The use of severity scales may be important in CFS/ME as it permits the establishment of subgroups which may improve accuracy in both clinical and research settings.

1.2.5.2 INTRODUCTION

CFS/ME is a physically and cognitively disabling illness which affects a multitude of bodily systems, including: neurological, gastrointestinal, cardiovascular and immunological systems (Chia & Chia, 2008; Demitrack & Crofford, 2006; Fukuda et al., 1995; Gupta & Vayuvegula, 1991a; Jason et al., 2003a; Klimas & Koneru, 2007; Lyall et al., 2003). CFS/ME affects millions worldwide, with a disproportionately high number of women sufferers (Tuck, 2000). Common symptoms of CFS/ME include cognitive impairment, physical fatigue, headaches, dizziness, muscle and joint pain, palour, abdominal pain and bowel symptoms, nausea, swollen or tender lymph nodes and aversion to noise and light. These symptoms can be very diverse and vary greatly over time and in severity (Brenu et al., 2013a; Carruthers et al., 2011; Cox & Findley, 1998; Fukuda et al., 1994b; Fukuda et al., 1995; Jason et al., 2003a; Straus, 1992; Wiborg et al., 2010b). The pathogenesis of CFS/ME is unknown, therefore diagnosis is based on a series of symptom specific criteria (Carruthers et al., 2011). It can be difficult for practitioners in primary health care to assess patients with CFS/ME in a short consultation due to the varying nature of symptom severity in the illness. It may be important for severity scales of mobility and symptoms to be widely used by those in primary health to assist in accurately understanding a patient's individual condition. In this review, we assess severity scales that may be used in clinical primary health care and research settings to assess severity of CFS/ME patients. The use of these severity scales may also have potential to accompany illness diagnosis for patients internationally that are debilitated by the symptoms of CFS/ME.

Criteria have also been developed to allow the assessment of symptom severity of symptoms in patients with CFS/ME based on their mobility, level of self-management and daily abilities (Straus, 1992). CFS/ME patients may further be categorised into mild, moderate, severe or very severely affected by their illness. Mild CFS/ME patients are mobile and often still employed, moderate CFS/ME patients have reduced mobility and are restricted in daily tasks, such as household chores, severe CFS/ME patients are only able to perform minimal necessary hygiene-related tasks and are wheelchair dependant while those with very severe CFS/ME are unable to carry out any daily task for themselves and are essentially bedridden (Straus, 1992). The ICC is the most recent and accurate set of criteria used for CFS/ME diagnosis and contains reference to these severity subgroups of CFS/ME patients, although it is not a necessary component of the guidelines (Carruthers et al., 2011). It may be necessary for primary health care professionals to use the ICC and severity scales to categorise the severity of a CFS/ME patient for a better understanding of their condition.

A variety of severity scales that measure symptoms, quality of life and functional disability can be effectively used in CFS/ME research to assess patient's levels of severity. Clinically distinct severity subgroups have increasing significance as differences are being found both clinically and physiologically in the illness and supporting the idea of defining such distinct severity subgroups (Baraniuk et al., 2013; Brenu et al., 2013a; Friedberg & Krupp, 1994; Härle et al., 2006; Joyce et al., 1997; Kerr et al., 2008; Peckerman et al., 2003a; Rangel et al., 2000; Stringer et al., 2013). A combination of severity scales was used to distinguish a clinically distinct severity subgroup of CFS/ME patients as 'housebound' and confirmed the significance of using severity scales in CFS/ME research (Wiborg et al., 2010b).

It is possible that the lack of recognition or inclusion of these severity subgroups may be a causative factor underlining the inconsistency in research findings. Patients with varying severities or symptomatology in CFS/ME are typically clustered into a single patient group hence precluding the specificity and success of clinical maintenance or assistance and also in research settings (Zaturenskaya et al., 2009). In this review, we provide an assessment of severity scales in CFS/ME that may be used to assess patients' severity in clinical primary health care and research settings, focusing on the importance of clinical and physiological studies that have examined CFS/ME patient severity. The implementation of severity scales in CFS/ME may also assist the diagnosis and assessment of many patients suffering with the illness internationally.

1.2.5.2.1 Determination of Symptom Severity based on Fukuda and ICC

The 1994 Fukuda definition for CFS/ME is widely used to diagnose CFS/ME patients (Fukuda et al., 1995). This definition requires patients to have persistent chronic fatigue lasting longer than six months independent of significant physical exertion that is not alleviated by rest (Cox & Findley, 1998). The Fukuda definition requires a CFS/ME patient to have at least 4 of the following symptoms, including: post-exertional malaise, impaired memory, unrefreshing sleep, muscle pain, joint pain without redness, tender lymph nodes, sore throat and/or headaches (Cox & Findley, 1998). This does not consider some patients who may experience symptoms with lesser severities while others can be relentlessly affected, such that they are habitually bedridden and very severe. In response to the vagueness and commonality of symptoms in the 1994 Fukuda

definition for CFS/ME, further criteria for CFS/ME have been developed, the most recent being in 2011 (Carruthers et al., 2011; Straus, 1992).

The ICC definition was developed in 2011 to provide enhanced clarity and specificity in CFS/ME diagnosis (Carruthers et al., 2011). The 2011 ICC guidelines incorporate more specific and detailed symptomatology, including: fatigue, neurological, gastrointestinal, immune impairments and energy production. The application of the 2011 ICC also allows an assessment of symptom severity and impact based on stages of symptom severity and it is suggested that symptom severity may frequently fluctuate (Carruthers et al., 2011; Cox & Findley, 1998).

The ICC is a tool that can be utilised to acknowledge severity subgroups of CFS/ME patients, referring to mild as those with reduced activity, moderate as those with a 50% reduction to activity levels, severe as being housebound and very severe as those who are bedbound and requiring assistance with daily functions (Carruthers et al., 2011). The recognition of such CFS/ME severity subgroups in research outlines the importance of severity scales in CFS/ME.

The Fukuda definition for CFS/ME is still the most commonly used CFS/ME definition regardless of the new enhanced development of the ICC. As a result, those who suffer from severe CFS/ME symptoms are typically pooled as a single CFS/ME cohort regardless of severity as they are difficult to access and typically unable to maintain regular appointments (Jason et al., 2009a). It is recommended that the ICC definition for CFS/ME is used in primary health care situations and research as it may increase specificity and identify severe cases of the illness according to extensive new criteria

(Carruthers et al., 2011). The best assessment of CFS/ME can be made by using the ICC in conjunction with other valid severity scales to permit the classification of severe CFS/ME patients and patient subgroups according to level of severity.

1.2.5.2.2 Severity Scales and CFS/ME

Symptom severity subgroups have been recognised in CFS/ME although these have not been strictly outlined. Illness severity can be assessed using measures of both physical and cognitive capacities and this is essential for analysing illness progression and variations in severity. Alongside CFS/ME case definitions, assessments of CFS/ME patients physical functioning are often used to supplement medical information in order to characterise the impact of an illness on a patient and assess variations in symptom severity (Mor et al., 1984). A patient's severity status can be measured using a variety of scales.

The Karnofsky Performance Scale (KPS) was constructed in 1948 in the absence of a generic scale for clinical characteristics as an assessment tool for performance status in oncology (Abernethy et al., 2005; Mor et al., 1984; Wiborg et al., 2010a).

The KPS scale comprises of a single 11-point rating scale ranging from 0 to 100 where patients are ranked a number interval of 10, with 100 representing normal functioning and 0 representing dead. The KPS allows assessors to rank patients mobility and condition based on a one-dimensional numerical scale (Mor et al., 1984; Wiborg et al., 2010a).

The reliability of KPS has gained widespread acceptance and it is therefore a valuable tool for rating the functional status of a patient (Mor et al., 1984; Wiborg et al., 2010a). While the KPS has limited sensitivity due to the restricted range of scores available, it has been used clinically to establish levels of disability in patients and may be effective in further differentiating severity of CFS/ME (Clapp et al., 1999; Strayer et al., 2012). The use of the KPS has been deemed reliable in evaluating the degree of disability in CFS/ME patients (Clapp et al., 1999). The KPS scale is still used as a predominant measure of functional status in patients, particularly in a recent clinical trial examining 234 patients classified as CFS/ME (Strayer et al., 2012). These severely classified patients scored 40 to 60 on the KPS scale, indicating that they required some daily assistance similar to those who were 'disabled' (Strayer et al., 2012). The KPS has also been used to determine impairment of daily activities (a KPS score of below 80) associated with CFS/ME (Sharpe et al., 1996). The KPS as a focal measure of severity highlights its efficiency in classifying severity subgroups of CFS/ME, in particular, this short scale may be beneficial in primary health settings.

The Sickness Impact Profile (SIP) was developed in 1975. It is a generic measure of health status based on changes in behaviour that are consequential of illness-related qualities of life (Bergner et al., 1981; Gilson et al., 1975). The SIP allows measures of physical, mental and social aspects of health-related functions using 6 subscales, including: somatic autonomy, mobility control, mobility range, social behaviour, emotional stability and psychological autonomy/communication, which contribute to an overall total score (WM Post, 2001). Clinically, the validity and accuracy of the SIP was confirmed following a study of the relationship between SIP and clinical measures of illness (Bergner et al., 1981; Gilson et al., 1975). Based on the generic nature of the SIP

it is used for a wide range of illnesses, the SIP is typically applied in conjunction with other measures of health or functioning (Gilson et al., 1975). A number of items assessed in the SIP, including: sleep and rest, daily work, mobility and bodily movement, allow the SIP to be a beneficial measure of functional performance of a patient with illness, specifically CFS/ME, based on changes in health status, illness progression and severity over time (Busija et al., 2011; Gilson et al., 1975; Heins et al., 2011; Wadden & Phelan, 2002; Wiborg et al., 2010a). Alongside other severity scales of both physical and emotional function, the SIP has been used in CFS/ME studies to provide an in-depth analysis of functional and cognitive status of patients (Gaab et al., 2002; Petrie et al., 1995; Vercoulen et al., 1994). The SIP has been applied in conjunction with other severity scales to effectively confirm severity subgroups of CFS/ME patients, hence highlighting the ability of those in health care to use the SIP to assess health status and severity of patients with CFS/ME (Wiborg et al., 2010a).

One of the most widely used measures of fatigue is the Fatigue Severity Scale (FSS) (Herlofson & Larsen, 2002; Krupp et al., 1989; Malagoni et al., 2010). The FSS was generated in 1993 to measure the severity of fatigue-related symptoms of those with CFS/ME. The scale comprises a list of 14 questions related to both physical and cognitive fatigue, with a scale from 1 to 7 correlating with the possible options of “better than usual”, “no more than usual”, “neutral”, “worse than usual” and “much worse than usual” (Chalder et al., 1993). The use of the FSS provides assessors with a total final score, with higher scores indicating more severe fatigue-related symptomatology (Jason et al., 2011b). A proposed limitation of using the FSS is the range of 7 optional responses which cannot easily be used as distinctions between fatigue categories and it has been suggested that reducing the range of options to three

(disagree, neutral, agree) may improve the scale overall (Burger et al., 2010). According to Jason et al., the FSS is the most efficient fatigue-related scale in differentiating CFS/ME patients from non-fatigued healthy controls based on fatigue (Jason et al., 2011a). The FSS is also more accurate and comprehensive in comparison with most fatigue scales when used to assess fatigue-related severity and disability of CFS/ME symptoms (Taylor et al., 2000). Overall, this self-reporting scale has been effectively applied when detecting cases of fatigue and is therefore a valid measure of fatigue severity (Chalder et al., 1993; Friedberg & Krupp, 1994; Herlofson & Larsen, 2002; Jason et al., 2009a; Krupp et al., 1989; Krupp et al., 1994; Malagoni et al., 2010). The recommended 'high' level of fatigue using the FSS has been suggested as an average FSS score above 4 or 5 although further validation is required (Lerdal et al., 2005). In future, the FSS may be beneficial in assisting with the characterisation of severe cases of CFS/ME into a number of distinct subgroups, including: mild, moderate, severe and very severe.

Dr Bell's CFS Disability Scale was developed to clinically assess patients and their response to treatments (Bell, 1995). The scale is a modified version of the KPS, also designed to allow the examination of both physical and cognitive activity alongside a measure of wellness to essentially outline a level of disability. Like the KPS, the scale itself is a numerical score between 0 (severe symptomatology, bedridden and unable to care for self) and 100 (no symptoms at rest). Scores are allocated based on symptom severity, the degree of activity impairment both at rest and during activity and a functional ability regarding full-time work (Bell, 1995). The scale has been effectively applied to assess the disability of CFS/ME patients based on their physical functioning and also to further distinguish severity subgroups. Severity subgroups can be

distinguished using this 10-point rating scale which allows physicians to assess patient's activity levels. A score of 100 on the KPS is related to a person with normal ability and physical functioning. Moderate CFS/ME patients tend to score between 40 and 70 on the KPS, severe CFS/ME patients tend to score 30 and very severe CFS/ME patients score below 20 on the scale (Myhill et al., 2009). The severity groups we defined and used in this study are similar to the severity subgroups described by the ICC (Carruthers et al., 2011). In effect, the Dr Bell's Disability Scale has been utilised to examine functional ability and severity of CFS/ME participants, supporting the notion that it is a simple and adequate tool for assessing the illness (Bell, 1995; Myhill et al., 2009; Tiersky et al., 2001).

The FibroFatigue Scale was developed according to items from the neurasthenia subscale of the Comprehensive Psychopathological Rating Scale (CPRS) and is comprised of 12 observer-rated items (Zachrisson et al., 2002). This neurasthenia subscale contains 15 items regarding symptomatology, such as: aches and pain, fatigability, reduced sleep, muscular tension and concentration difficulties and was found to be useful when evaluating differences in symptomatology severity in Fibromyalgia (FM) and CFS/ME patients (Andersson et al., 1998; Zachrisson et al., 2002). This FibroFatigue Scale has since been used as an efficient measure of illness severity in FM and CFS/ME patients in a number of studies (Lucas et al., 2006; Maes et al., 2006; Maes et al., 2007; Maes et al., 2012). An analysis of the FibroFatigue scale also found that it is reliable in determining severity of symptoms in both FM and CFS/ME patients and it does require a trained administrator for use which makes it less appropriate for research studies although it has high potential for health care consultations (Shahid et al., 2012).

The ME/CFS Fatigue Types Questionnaire (MFTQ) was developed in 2009 following an analysis of fatigue types in CFS/ME patients which determined distinct variations in fatigue-related symptoms among patients (Jason et al., 2009b). CFS/ME patients were examined using a combination of fatigue measures, including the FSS and a 1993 Fatigue Scale, to categorise different types of fatigue-related sensations and symptoms. The MFTQ generated is a 22-item scale designed to measure fatigue duration, severity and frequency using a number of specific dimensions, including: lack of energy, overstimulation of the mind or body and abnormal exhaustion following physical activity (Jason et al., 2009b). The MFTQ has been used to categorise distinct clusters of fatigue state patterns within CFS/ME patients, with results suggesting heterogeneous fatigue patterns in CFS/ME patients that can be classified into fatigue subgroups of low, moderate and severe (Jason et al., 2010a) although the MFTQ has not yet been used to assess CFS/ME. Use of the MFTQ for CFS/ME would require accompanying functional and disability scales as it focuses entirely on fatigue which is only one of many CFS/ME symptoms experienced by patients, hence the MFTQ is not ideal for primary health.

1.2.5.2.3 Clinical Severity of CFS/ME

A subgroup of CFS/ME patients have been identified as housebound patients. These patients experience a high level of daily fatigue, somatic disturbances and low level activity. Importantly, the housebound CFS/ME group are less likely to hold a paying job due to the significant impairment in their physical functioning and higher levels of daily fatigue compared with other CFS/ME patients (Wiborg et al., 2010b). It has been

suggested that severity may play a role in differentiating dysfunctions in CFS/ME patients and be important for primary health assessment of the illness (Brenu et al., 2013a; Rangel et al., 2000; Strayer et al., 2012; Wiborg et al., 2010b).

Fatigue severity has been outlined as one of the most consistent and essential predictors of illness severity with prognostic outcome for a CFS/ME patient (Jason et al., 2005; Joyce et al., 1997). It has similarly been observed when examined clinically, that CFS/ME patients with less severe illness and fatigue were more likely to get a positive prognosis than those who were more severe (Jason et al., 2005).

The rather vague 1991 Oxford criteria for CFS were used in an interview-based study of childhood CFS/ME severity. Severity was assessed using a 0-10 point scale encompassing scales for impairment of school attendance, family and friend relationships, sleep patterns and physical symptoms (Rangel et al., 2000). The majority of patients recovered within 45 months although interestingly, those who maintained the CFS/ME symptomatology had significantly further severe differences in fatigue, physical symptoms and handicap compared with those who recovered (Rangel et al., 2000).

Significant differences have also been examined in CFS/ME compared with other fatigue-related illnesses based on self-reported symptom profiles. It is suggested that the next step is to assess CFS/ME symptom profiles in severity-related subgroups to further determine the pathophysiological mechanisms (Baraniuk et al., 2013). Post-exertional fatigue and infectious-related symptoms are the most characteristic in patients with severe CFS/ME (Peckerman et al., 2003b). Similarly, CFS/ME patients who experience

more than the required 4 symptoms are most likely further affected in their functional ability. There has also been a correlation between the number of severe CFS/ME related symptoms and measures of functional disability, again suggesting potential CFS/ME severity subgroups (Jason et al., 2003b).

Severity in CFS/ME symptoms may fluctuate where some patients are able to maintain full time to part time jobs while others may be severely affected by symptoms and are completely bedridden. Homebound CFS/ME patients have demonstrated higher levels of fatigue and somatic disturbances combined with significantly lower levels of activity, physical functioning and ability to maintain a job compared with other CFS/ME patients (Wiborg et al., 2010b). Undoubtedly, this homebound subgroup of CFS/ME patients is important to CFS/ME research as it accounts for 25% of the CFS/ME patient population. In the majority of studies, classifications of patient variation have been ignored or severe patients were specifically excluded due to patients' difficulties keeping appointments (Hooper, 2007; Jason et al., 2009a).

1.2.5.3 DISCUSSION

Variation in symptom severity in CFS/ME patients has been established and a number of severity scales have proven to be effectively applied when assessing CFS/ME patients' severity (Baraniuk et al., 2013; Brenu et al., 2013a; Fletcher et al., 2010; Jason et al., 2009b; Jason et al., 2010a; Jason et al., 2005; Jason et al., 2011a). Inconsistency in symptom presentation in CFS/ME advocates the use of severity scales to assess functional ability and quality of life to form an accurate measure of an individual patient's condition. Illnesses such as cancer, Multiple Sclerosis (MS) and FM have

distinct stages of severity based on symptoms and it is likely that CFS/ME patients also require such severity assessments and classification.

MS is a severe inflammatory demyelinating disease that is typically assessed based on stages, predominantly starting with an 'attack', followed by a relapsing-remitting stage and a secondary chronic-progressive stage. The severity of symptoms and activity pattern exhibited in CFS/ME individuals also fluctuates over time, with patients seemingly experiencing improved symptoms or 'relapses' (Meeus et al., 2011).

FM is also commonly acknowledged similarly to CFS/ME as it is characterised by chronic widespread pain in conjunction with fatigue, sleep disturbances and joint stiffness with occasional cognitive dysfunction, bowel and bladder abnormalities and difficulty swallowing (Saa'd et al., 2012). Unlike the severity of CFS/ME which is not recognised or assessed in clinical or research settings, FM is usually assessed and diagnosed with the use of severity scales. Various scales have been used to examine FM patients and assess the severity of the illness, these scales include: the FM Survey Diagnostic Criteria and Severity Scale, Mindful Attention Awareness Scale, Psychological Inflexibility in Pain Scale, Widespread Pain Index, Symptom Severity scale and the FibroFatigue scale (Cebolla et al., 2013; Fitzcharles et al., 2012; Rodero et al., 2013; Wolfe et al., 2011). The usefulness and benefits of applying a severity scale to assess FM patients in primary health care denotes similarly the importance for CFS/ME patients to be examined in the same way.

The SIP is an efficient quality of life scale although it is widely used and does not specifically adhere to CFS/ME. Similarly, the FSS and FibroFatigue Scales are

effectively used when analysing the symptoms of fatigue however they do not include assessment of other important symptoms associated with CFS/ME. The FibroFatigue Scale, SIP and FSS allow assessment of severity in CFS/ME patients, although should be used in conjunction with other scales such as the KPS which assess a broad range of symptoms to encompass CFS/ME (Burger et al., 2010; Gaab et al., 2002; Petrie et al., 1995; Vercoulen et al., 1994; Wiborg et al., 2010b).

The KPS is widely used in a number of illnesses as a measure of functional performance, providing a simple and practical method of scaling a person's functional abilities and mobility (Clapp et al., 1999; Sharpe et al., 1996; Strayer et al., 2012). The KPS or similarly the adapted Dr Bell's Disability Scale may potentially be effective in relation to the severity ranges proposed by the ICC. The numerical scale of the KPS or Dr Bell's Disability Scale can be subdivided to correlate with mild, moderate, severe and very severe descriptions of physical ability in CFS/ME for an overall analysis of a patient's symptom severity (Carruthers et al., 2011). The simple numerical scale of the KPS or Dr Bell's Disability Scale also allows for a quick assessment of a CFS/ME patient's severity that may easily be used by primary health care professionals as well as in research settings.

CFS/ME patients' symptom severity has been recognised and acknowledged in the 2011 ICC for ME although such important severity subgroups continue to be ignored in most research instances (Carruthers et al., 2011; Jason et al., 2010b). A number of scales, such as the FSS, KPS and FibroFatigue scale, can be accurately applied and successful in assessing levels of fatigue, disability and health status in CFS/ME patients. It is recommended that such scales are utilised in primary health and research to assess

patient severity in correspondence with the severity categories suggested in the ICC (mild, moderate, severe and very severe) for specific distinction and understanding of the multifaceted illness.

1.2.5.4 CONCLUSION

CFS/ME is a serious illness that predominantly affects women and requires a more accurate assessment to assist those suffering. According to the ICC, CFS/ME severity subgroups are present and range between the categories mild, moderate, severe and very severe (Carruthers et al., 2011). There is an imperative need for these CFS/ME severity subgroups to be widely recognised and consistently distinguished for assessments in clinical primary health care and in research. Severity scales are available and efficient when used to assess CFS/ME patients based on symptoms, quality of life and functional disability. This is important as it is possible that, like other disorders such as cancer, there are distinct subgroups of CFS/ME (Zaturenskaya et al., 2009). The use of a functional severity scale alongside a simple numerical mobility scale such as the KPS or Dr Bell's Disability Scale is highly recommended for use by health care professionals and in research settings to assess a CFS/ME patient's individual severity and condition.

1.3 THE IMMUNE SYSTEM

The immune system consists of the adaptive and innate immune systems. In CFS/ME, evidence suggests that the innate immune system is compromised owing to the significant reduction in NK cell function (Brenu et al., 2013a; Brenu et al., 2010; Brenu et al., 2012c; Brenu et al., 2011; Klimas et al., 1990; Levine et al., 1998; Ojo-Amaize et al., 1994; Patarca-Montero et al., 2001).

The innate immune system consists of a variety of myeloid and lymphoid cells, including: NK cells, neutrophils, monocytes and dendritic cells (DCs). Innate immune cells exhibit rapid effector functions and act as a first line of defence to protect the host from infection or pathogen invasion (Burg & Pillinger, 2001; Vivier et al., 2011). Innate immune cells function to identify foreign particles, recruit cells to inflammatory sites, activate complement to promote viral clearance and activate the adaptive immune system (Burg & Pillinger, 2001; Vivier et al., 2011). CFS/ME patients typically present with a compromised immune system (Klimas et al., 1990; Patarca-Montero et al., 2001) including reduced NK cell cytotoxic activity (Brenu et al., 2013a; Brenu et al., 2010; Brenu et al., 2012c; Brenu et al., 2011; Klimas et al., 1990; Levine et al., 1998; Ojo-Amaize et al., 1994; Patarca-Montero et al., 2001). Nonetheless, other innate immune cells such as neutrophils, monocytes and DCs may be affected in the illness; hence the importance of investigating these cells in CFS/ME patients.

The adaptive immune system is predominantly characterised by T and B cells, both expressing site-specific antigen receptors, T cell receptors (TCRs) and B cell receptors (BCRs) (Bluestone & Abbas, 2003; Iwasaki & Medzhitov, 2010). Parenthetical cells

such as B1, gamma delta ($\gamma\delta$) T and NKT cells have both innate and adaptive immune characteristics. The adaptive immune system is in constant communication with the innate immune system. These Cell-cell interactions include antigen presentation, cytokines and chemokines secretion to induce target cell lysis or suppression in response to infection, inflammation or foreign particles (Bluestone & Abbas, 2003; Iwasaki & Medzhitov, 2010; Vivier et al., 2011; Zotos et al., 2010). Breakdowns in innate immune function may affect the adaptive immune cells and related immune activities (Bluestone & Abbas, 2003; Iwasaki & Medzhitov, 2010; Vivier et al., 2011; Zotos et al., 2010). Therefore, reduced NK cell function in CFS/ME patients (Brenu et al., 2013c; Brenu et al., 2010; Brenu et al., 2011; Caligiuri et al., 1987; Klimas et al., 1990) may suggest potential immune perturbations in the adaptive immune system.

1.3.1 Natural Killer Cells

NK cells are large granular lymphocytes belonging to the hematopoietic progenitor cells (HPC)s expressing cluster of differentiation 34 (CD34⁺) in the bone marrow (Caligiuri et al., 1987; Carson et al., 1997; Farag et al., 2002; Robertson & Ritz, 1990). NK cells represent approximately 15% of the total lymphocyte population in the human body (Cooper et al., 2001a; Robertson & Ritz, 1990). Mature NK cells recognise and lyse pathogens such as tumour cells, viruses, bacteria and parasites within three days of initial pathogen infiltration or infection (Baume et al., 1992; Caligiuri, 2008; Carson et al., 1997; Cooper et al., 2001a; Farag et al., 2002). NK cells can be distinguished from other lymphocytes based on particular surface antigens, CD56 and CD16 (Baume et al., 1992; Caligiuri, 2008; Cooper et al., 2001a; Farag et al., 2002; Robertson & Ritz, 1990).

1.3.1.1 Natural Killer Cell Phenotypes

There are four distinct subsets of human NK cells, categorised based on their density of CD56 and CD16 expression and the absence of CD3 and CD14. The majority of NK cells are CD56^{dim} (~90%) with a low expression of CD56 and variable CD16 expression (Baume et al., 1992). The remaining ~10% of NK cells are CD56^{bright}, expressing a high density of CD56 (CD56^{bright}) (Baume et al., 1992; Cooper et al., 2001a; Robertson & Ritz, 1990).

CD56^{dim} NK cells are abundant with cytolytic granules (such as perforin and granzymes) compared with CD56^{bright} NK cells. Consequently, these cells are more cytotoxic, expressing higher levels of NK cell receptors compared with the CD56^{bright}

NK cells (Caligiuri, 2008; Cooper et al., 2001a; Vivier et al., 2011). CD56^{bright} NK cells have low natural cytolytic properties and are a major source of activation-induced cytokines (tumour necrosis factor (TNF)- α , interferon (IFN)- γ) and chemokines following stimulation (Cooper et al., 2001a; Fehniger et al., 2003). CD56^{bright} NK cells function predominantly using cytokine secretion and also express a number of cytokine and chemokine receptors, including: the IL-2 receptor (IL-2R α), c-kit, CC-chemokine receptor 7 (CCR7), C-type lectin CD94/NKG2 NK cell receptors and IL-10 receptors. CCR7 and L-selectin are responsible for trafficking immune cells (such as T cells and DCs) to the lymph nodes (Cooper et al., 2001a; Fehniger et al., 2003).

1.3.1.2 Natural Killer Cell Function

NK cells require molecular and cellular interactions, or 'priming', as they are not naturally active killers (Bryceson et al., 2006; Ganal et al., 2012; Lucas et al., 2007). Priming stimulates NK cells to enhance cytotoxic activity, ligand interactions and the production of IFN- γ (Long, 2007; Lucas et al., 2007). After NK cells are primed, interactions between macrophage, DC or endothelial cell-derived chemokines (such as CXCL8 and CX3CL1) and NK cell chemokine receptors (CXCR1 and CX3CR1) recruit NK cells to infected tissues (Campbell et al., 2001; Moretta et al., 2008; Vitale et al., 2004). During inflammation, NK cells secrete chemokines CCL2 (MCP-1), CCL3 (MIPI- α), CCL4 (MIPI- β), RANTES (regulated on activation, normal T cell expressed and secreted, CCL5), lymphotactin (XCL1) and IL-8 (CXCL8) necessary for NK cell colocalization with other hematopoietic cells such as DCs (Vivier et al., 2011).

NK cells can also initiate an immune response by recognising self-antigens, human leukocyte antigen (HLA) ligands, known as major histocompatibility complex (MHC), on infected cells (Cooper et al., 2001a). Inhibitory receptors (such as KIRs) on NK cells recognise MHC-I alleles which identify self-antigens (HLA-A, HLA-B, HLA-C) on non-self, infected or altered cells (Smyth et al., 2005; Vivier et al., 2008).

Microbes and tumours have evolved the ability to down-regulate the presentation of MHC-I molecules on infected cells as they internalise the MHC-I via endocytosis to avoid detection by cytotoxic T cells and prevent the associated T cell mediated immune response (Bartee et al., 2004; Smyth et al., 2005; Vivier et al., 2008). To prevent the evasion of the immune response, NK cell inhibiting receptors detect the loss of the MHC-I molecule via the absence of inhibitory ligands (such as HLA-A, HLA-B, HLA-C) and generate NK cell mediated apoptosis (Raulet & Vance, 2006; Smyth et al., 2005; Vivier et al., 2008; Yawata et al., 2008).

NK cells are critical to host defence as they are an important source of innate immunoregulatory cytokines (Caligiuri, 2008; Cooper et al., 2001a; Cooper et al., 2001b; Smyth et al., 2005). Once infected cells are opsonised, cytotoxic processes are triggered in NK cells, which also induce cytokine production, including: IFN- γ , TNF- α and IL-10 (Cooper et al., 2001a). Growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and IL-3 are also released from mature NK cells, acting on other cells (such as monocytes and neutrophils) to promote cell proliferation (Cooper et al., 2001a; Farag et al., 2002; Vivier et al., 2011).

IFN- γ is the principal NK cell cytokine that shapes the T helper (Th)1 immune response, activates macrophages to kill pathogens via phagocytosis and has antiproliferative effects on infected or transformed cells (Caligiuri, 2008). Production of IFN- γ by NK cells requires signals by monocyte-derived IL-2 (Wang et al., 2000) and IL-1, IL-15, IL-18, ligand binding of an activating receptor (such as CD16) or binding to NKG2D. Secreted IFN- γ acts predominantly on antigen-presenting cells (APCs) and T cells in conjunction with IL-12, from monocytes, macrophages and/or DCs (Caligiuri, 2008; Cooper et al., 2001b; Fehniger et al., 2003). IFN- γ activates APCs to upregulate MHC-I expression on target cells, resulting in an increase in APC cytokine secretion. Interactions between IFN- γ , APCs and T cells are imperative to the immune response (Caligiuri, 2008).

Following the priming and recognition of NK cells, various cytotoxic pathways are activated in NK cells to promote target cell death. The granule dependent pathway includes the ADCC (antibody-dependent cell-mediated cytotoxicity) pathway. The granule independent pathway incorporates death receptor domains such as Fas or TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) receptors, which induce caspase cascades that lead to target cell death (Smyth et al., 2005).

The NK cell granule dependent pathway promotes target cell lysis, utilising granules which contain proteins such as perforin (a membrane-disrupting cytolytic protein) and granzymes (a family of serine proteases with substrate specificities). These granules are used to induce cell death, owing to the degradative properties characteristic of lysosomes (Smyth et al., 2005; Trapani & Smyth, 2002; Warren & Smyth, 1999).

The granule-independent target cell death pathway of NK cells involves several death receptors on the surface of NK cells, characterised by an intracellular ‘death’ domain. These death receptors include CD95 Fas-Ligands (FasL) and TRAIL-1, which function to induce the apoptosis of target cells expressing a TNF receptor molecule (Warren & Smyth, 1999). Cytotoxic signalling transmitted via the death domains of these death receptors then promotes apoptotic cell death characterised by cytoplasmic and nuclear condensation and deoxyribonucleic acid (DNA) fragmentation within hours of target cell recognition via MHC-I (Warren & Smyth, 1999).

1.3.1.3 Natural Killer Cell Receptors

NK cells have a class of receptors which have developed the ability to recognise self-MHC-I or class I-like molecules while inhibiting or activating NK cell killing (Table 1) (Farang et al., 2002; Smyth et al., 2005). A number of inhibitory NK cells specific to the classical (eg. HLA-A, HLA-B, HLA-C) and non-classical (eg. HLA-E, HLA-G) class I molecules have been recognised to protect target cells expressing normal levels of MHC-I on their surface. Upon ligation of NK cell activating receptors with the membrane-bound molecules of target cells, NK cells induce cytokine production, cytotoxic activity and migration (Andre & Anfossi, 2008; Caligiuri, 2008; Farang et al., 2002; Smyth et al., 2005). Receptor families on the surface of NK cells can include both activating and/or inhibitory receptors (Table 1).

Table 1: Major natural killer cell receptor families of humans.

Receptors	Activating	Inhibitory	Ligand Family
CD94:NKG2	+	+	MHC-Ib molecule, HLA-E
NCR	+	-	NKp30, NKp44, NKp46
KIR	+	+	MHC-I

Table 1 shows the major families of NK cell receptors and whether receptors are activating, inhibitory or both. The associated ligand families for the receptors are also given. '+' refers to positive for either activating or inhibitory functions where '-' refers to negative for inhibitory functions (Andre & Anfossi, 2008).

CD94 and NKG2 genes are responsible for encoding type II transmembrane proteins from the C-type lectin-like family. CD94 can be expressed as a disulphide-linked heterodimer with NKG2A and NKG2C. The CD94:NKG2 family have both activating and inhibitory receptors, and are found on most NK cells, $\gamma\delta$ T cells and on a subset of memory CD8⁺ $\alpha\beta$ T cells (Lanier, 2001, 2005). All CD94:NKG2 receptors on NK and T cells are regulated by cytokines, with IL-15, transforming growth factor (TGF)- β and IL-12 having the ability to induce the receptors (Lanier, 2001, 2005). In humans, inhibitory CD94:NKG2A and activating CD94:NKG2C receptors function following recognition of HLA-E (Lanier, 1998, 2001, 2005) (Figure 1).

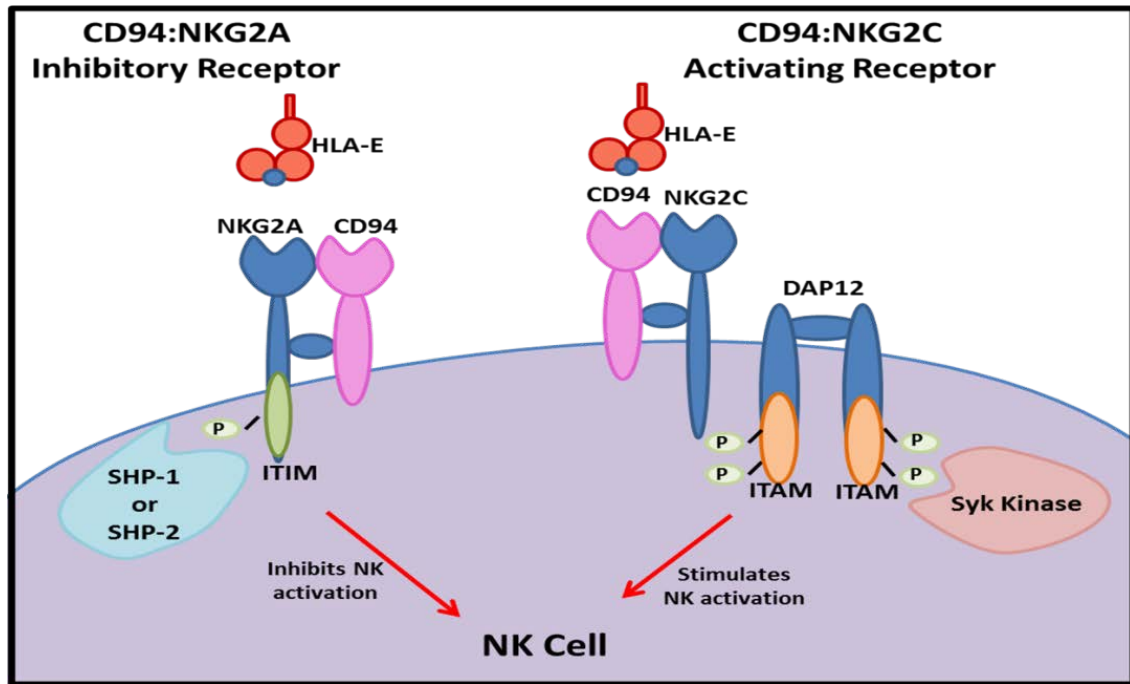


Figure 1: CD94:NKG2 inhibitory and activating receptor structures.

The CD94:NKG2A inhibitory receptor structure contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that is phosphorylated by kinase Src homology domain containing tyrosine phosphatase (SHP)-1 or SHP-2 when HLA-E ligand binding this inhibits NK cell activation. The CD94:NKG2C activating receptor structure interacts with DAP12 to phosphorylate immunoreceptor tyrosine-based activation motif (ITAM) complexes with Syk kinase to promote NK cell activation (Lanier, 1998, 2001, 2005).

Natural Cytotoxicity Receptors (NCRs) are receptors expressed on some resting NK cells, identified according to their role in natural cytotoxic activity towards tumour cells (Bryceson et al., 2006; Moretta et al., 2001). Human NCRs are coupled to signal transducing adapter proteins, including: CD3 ζ , Fc ϵ RI γ and KARAP/DAP12 (Moretta et al., 2001). NKp30, NKp44 and NKp46 are NCRs which function when monoclonal antibodies bind and block the NK cell mediated lysis of target cells. This stimulates the anti-tumour activity of human NK cells through cell-mediated lysis and also allows the release of IFN- γ (Moretta et al., 2001; Trapani & Smyth, 2002).

KIRs are part of the Ig family, specifically able to recognise MHC-I alleles, such as HLA-A, HLA-B and HLA-C on cells. There are two types of KIRs, the most common are those that act as inhibitory and activating. KIRs have identical extracellular domains and bind to identical ligands, however differences in their transmembrane and intracellular domains control their ability to either signal an inhibitory or activating response after they are bound to the MHC-I allele (Farag et al., 2002; McMahon & Raulet, 2001).

There are 12 main KIRs, 6 inhibitory and 6 activating receptors. These include single chain receptors with 2 or 3 immunoglobulin-like domains (KIR2D and KIR3D respectively) and long or short cytoplasmic tails. KIRs with long cytoplasmic tails (such as KIR2DL and KIR3DL) induce an inhibitory signal while the short tailed cytoplasmic receptors (such as KIR2DS and KIR3DS) are activating receptors (Figure 2). Short tailed receptors (such as KIR2DS in Figure 2) require the adaptor protein DAP12, which bears immunoreceptor tyrosine-based activation motifs (ITAM)s to induce activating signals when phosphorylated by Syk Kinase (Farag et al., 2002; Kane et al., 2001). The cytoplasmic domains of long tail receptors (such as KIR2DL) contain ITIMs, which produce inhibitory signals when phosphorylated by SHP-1 (Farag et al., 2002; Kane et al., 2001).

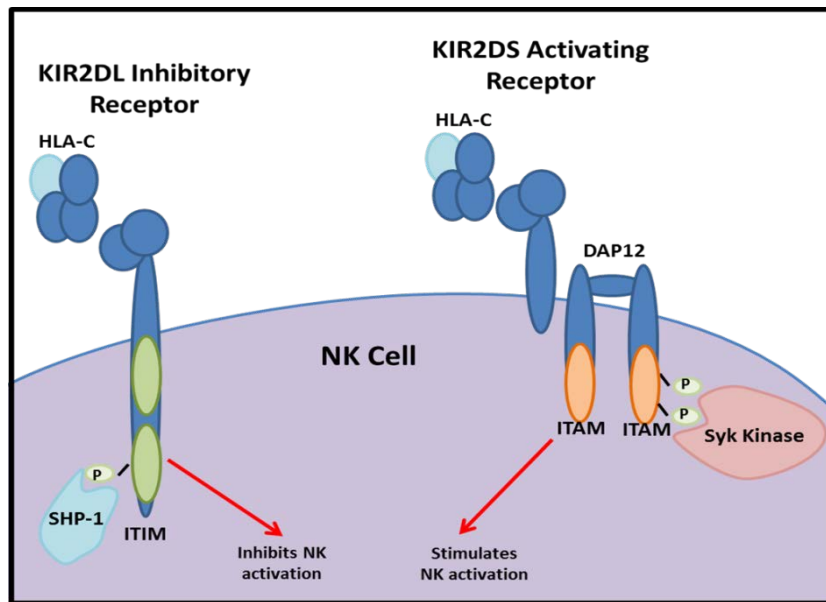


Figure 2: Inhibitory and activating KIR receptors.

When HLA-C ligands bind to the KIR2DL inhibitory receptor, SHP-1 is required for the inhibition of NK cell cytotoxic activity. KIR2DS activating receptor interacts with DAP12 complex and requires Syk kinase stimulation for NK activation (Bryceson et al., 2006).

KIR2DS, KIR3DS and NKG2C (CD159c) are activating receptors containing ITAM-containing adapter proteins which transmit activation signals through the recruitment of tyrosine kinases syk and ζ -associated protein (Bryceson et al., 2006). In this ITAM associated class of receptors, KIR2DS, KIR3DS and NKG2C are rapidly evolving receptors. These receptors bind to the ligand HLA class I with less affinity than inhibitory KIRs (such as KIR3DL1 and KIR3DL2) (Bryceson et al., 2006; Lanier, 2005).

KIR2DL4 is a long chain activating receptor expressed on all resting NK cells. The KIR2DL4 receptor is primarily found in intracellular vesicles where it induces the

production of cytokines from resting NK cells after it is activated through the binding of its HLA-G ligand (Bryceson et al., 2006).

Some tumour, transformed, stressed or infected cells express ligands that are structurally related to MHC. These ligands bind to the lectin-like NKG2D receptors, such as MHC I chain-related gene A and MHC I chain-related gene B. These NKG2D receptors are key in innate and adaptive immune responses, with ligands that generally induce DNA damage such as genotoxic stress or stalled DNA replication (Bryceson et al., 2006; Gonzalez et al., 2006).

1.3.1.4 Natural Killer Cells in CFS/ME

NK cell numbers and phenotypes have been assessed in CFS/ME (Brenu et al., 2010; Brenu et al., 2011; Klimas et al., 1990; Morrison et al., 1991; Robertson et al., 2005), with CFS/ME patients demonstrating differences in overall NK cell numbers and phenotypes (Brenu et al., 2010; Brenu et al., 2011; Klimas et al., 1990; Morrison et al., 1991; Robertson et al., 2005; Tirelli et al., 1994). Reductions in CD56^{bright}CD16⁻ NK cell phenotypes have been shown in CFS/ME patients as well as in rheumatoid arthritis (RA) patients (Brenu et al., 2011; Villanueva et al., 2005). As CD56^{bright}CD16⁻ NK cells regulate CD56^{dim}CD16^{bright} cells, this reduction may suggest a negative effect on cytotoxic activity. The reduction of CD56^{bright}CD16⁻ NK cells in the periphery of some CFS/ME patients may also reflect active recruitment to inflammatory sites (Villanueva et al., 2005). In contrast, some studies have found increases in the in CD56^{bright}CD16⁻ NK cell phenotype in some CFS/ME or fatigued-like patients, suggesting potentially a consequential decrease in the CD56^{dim}CD16⁺ NK cell phenotype and hence the

possibility of a reduced capacity of ADCC activity, consistent with persistent viral infections (Morrison et al., 1991; Tirelli et al., 1994).

Many CFS/ME studies show significant decreases in NK cell cytotoxic activity (Brenu et al., 2013a; Brenu et al., 2010; Brenu et al., 2012c; Brenu et al., 2011; Klimas et al., 1990; Levine et al., 1998; Ojo-Amaize et al., 1994; Patarca-Montero et al., 2001). Decreased cytotoxic activity of NK cells in CFS/ME patients may be associated with compromised granule-mediated cell death pathways involving perforin and granzymes (Maher et al., 2005; Swanink et al., 1996). Measures of perforin and granzymes in CFS/ME have also demonstrated significant reductions in CFS/ME patients (Brenu et al., 2011; Maher et al., 2005; Saiki et al., 2008), highlighting the potential significance of a compromised NK cell cytotoxic activity and its role in the pathomechanism of CFS/ME.

It is possible that reduced cytotoxic activity in CFS/ME may be influenced by activating or inhibitory NK cell receptors. Importantly, increased expression of the inhibitory KIR3DL1 receptor was found in CFS/ME patients (Brenu et al., 2013a). Increased inhibitory KIR3DL1 in CFS/ME patients may contribute to a decrease in cytotoxic activity of NK cells thus increasing the prevalence of infectious cells in CFS/ME patients (Brenu et al., 2013a; Brenu et al., 2010; Brenu et al., 2011; Broderick et al., 2010; Caligiuri et al., 1987; Klimas et al., 1990).

NK cells also have roles in the mediation and activation of other immune cells, including: neutrophils, monocytes and DCs (Costantini & Cassatella, 2011; Thorén et al., 2012).

1.3.2 Monocytes

Monocytes are a heterogeneous population of circulating blood cells, constituting 10% of peripheral leukocytes in the human blood system (Geissmann et al., 2003; Yona & Jung, 2010). These cells are important in development and homeostasis, with cellular functions aiding in the removal of apoptotic cells and foraging toxic compounds (Auffray et al., 2009). Monocytes also function as a systemic reserve of myeloid precursors with the ability to develop into tissue macrophages and DCs. The development of monocytes into DCs in particular predominantly occurs during an inflammatory immune response, such as an active infection (Auffray et al., 2009).

1.3.2.1 Monocyte Phenotypes

Human peripheral monocytes have three distinct subsets, defined based on phenotype and cytokine production (Auffray et al., 2009). The classic monocyte subset accounts for 80-90% of blood monocytes, phenotypically distinguished by their expression of CD14 and lack of CD16. Classic monocytes have greater phagocytic functions and lower cytokine production than other monocyte subsets. The remainder of monocytes, classified as pro-inflammatory ($CD14^{dim}CD16^+$) or intermediate ($CD14^+CD16^+$), are reportedly found in higher numbers in patients with infectious diseases or acute inflammation (Auffray et al., 2009; Ziegler-Heitbrock, 2007). Intermediate monocytes express Fc receptors CD64 and CD32 and have phagocytic activities (Auffray et al., 2009). Pro-inflammatory monocytes lack Fc receptors and phagocytic abilities as they have high expression of pro-inflammatory cytokines (Auffray et al., 2009; Ziegler-Heitbrock, 2007).

1.3.2.2 Monocyte Function

The role of monocytes in the immune system is to induce phagocytosis, present antigens, migrate to inflammatory sites and secrete cytokines (Vega & Corbí, 2006; Ziegler-Heitbrock, 2007). Upregulation of CCR2 and CD62L expression on the surface of monocytes is important for rapid recruitment of additional monocytes to sites of inflammation (Ziegler-Heitbrock, 2007). Once recruited, monocyte adhesion to the vascular endothelial lining is upregulated via activation of the endothelium by inflammatory cytokines TNF- α , IL-1 and IL-4. Activated endothelium expresses monocyte-binding proteins, such as intracellular adhesion molecule (ICAM)-1, ICAM-2, vascular cell adhesion molecule (VCAM)-1, E-selectin, L-selectin and P-selectin (Wójciak-Stothard et al., 1999). E-selectin, L-selectin, P-selectin, α 4 (CD49d) and β 2 integrins (CD11/CD18) mediate the rolling and attachment of monocytes to the activated endothelium (Macedo et al., 2007; Wójciak-Stothard et al., 1999). ICAM-1 and VCAM-1 allow the stable adhesion of leukocytes to the endothelium, successive spreading and diapedesis through the endothelial wall (Wójciak-Stothard et al., 1999). At the site of inflammation, monocytes induce phagocytosis and release cytokines to recruit cells of the adaptive immune system (Banchereau & Steinman, 1998; Wójciak-Stothard et al., 1999).

Monocyte phagocytosis involves the uptake of microbes and foreign particles or pathogens which are subsequently digested and destroyed (Capsoni et al., 1995; Geissmann et al., 2003). Phagocytosis in monocytes is regulated by cytokines IFN- γ , IL-4 and IL-10, which modulate membrane expression and function of phagocytic complement and Fc receptors (Capsoni et al., 1995; Pricop et al., 2001).

1.3.2.3 Monocytes in CFS/ME

Monocyte adhesion molecules have been assessed in CFS/ME patients. CFS/ME patients have a significantly increased density of ICAM-1 and lymphocyte function-associated antigen (LFA)-1 with a reduced response to IFN- γ (Gupta & Vayuvegula, 1991b). Enhanced ICAM-1 and LFA-1 binding in monocytes is suggestive of a possible increase in monocyte adhesion to endothelial cells in patients with CFS/ME. Similarly, L-selectin expression in monocytes is also enhanced in CFS/ME patients (Macedo et al., 2007). Amplified monocyte adhesion molecules may demonstrate an excessive number of monocytes migrating towards sites of inflammation, indicating potential inflammation or prolonged pathogen presence alongside a compromised innate immune ability of pathogen and viral clearance (Rains & Jain, 2011). Augmented monocyte adhesion is present in a number of inflammatory disease conditions and may result in the over-infiltration of monocytes into the circulatory system and promote further vascular problems and associated symptoms (Rains & Jain, 2011).

Monocyte elastase activity and lysozyme, a marker of monocyte/macrophage activity have been examined in CFS/ME, with both measures increased in CFS/ME patients (Alegre et al., 2013). It was suggested that CFS/ME patients may have had overactive pro-inflammatory levels and perhaps that CFS/ME may be accompanied by a low grade chronic inflammatory response based on increases in a number of inflammatory markers (Alegre et al., 2013; Maes et al., 2012). Few studies have examined monocytes in CFS/ME, particularly directly relating to phenotypes or activity in peripheral blood therefore this should be examined to assist in outlining the potential role of monocytes in CFS/ME.

1.3.3 Neutrophils

Neutrophils form an essential part of the innate immune system, representing 50-60% of circulating leukocytes and acting as the first line of defence against foreign substances that penetrate the body's physical barriers (Burg & Pillinger, 2001; Smith, 1994). These phagocytic cells are the first to respond to acute inflammation as they undergo chemotaxis and migrate towards sites of infection or inflammation. Reduced functioning of neutrophils may reduce their ability to eliminate foreign pathogens or undergo viral clearance. Hence, it is important to assess neutrophil function in CFS/ME patients as an increase in viral load or inflammation may be prevalent in the illness. Further studies examining neutrophil respiratory burst and phagocytosis may also be important in moderate and severe CFS/ME patients.

1.3.3.1 Neutrophil Function

During pathogenesis or inflammation, neutrophils migrate towards the endothelial cell layer. Chemotaxis is the process whereby circulating neutrophils move towards the site of infection upon interactions with chemoattractant molecules such as IL-8. The process includes rolling, firm adhesion and diapedesis where neutrophils pass through the endothelial surface to the site of infection (Figure 3) (Amulic et al., 2012; Burg & Pillinger, 2001; Muller, 2003).

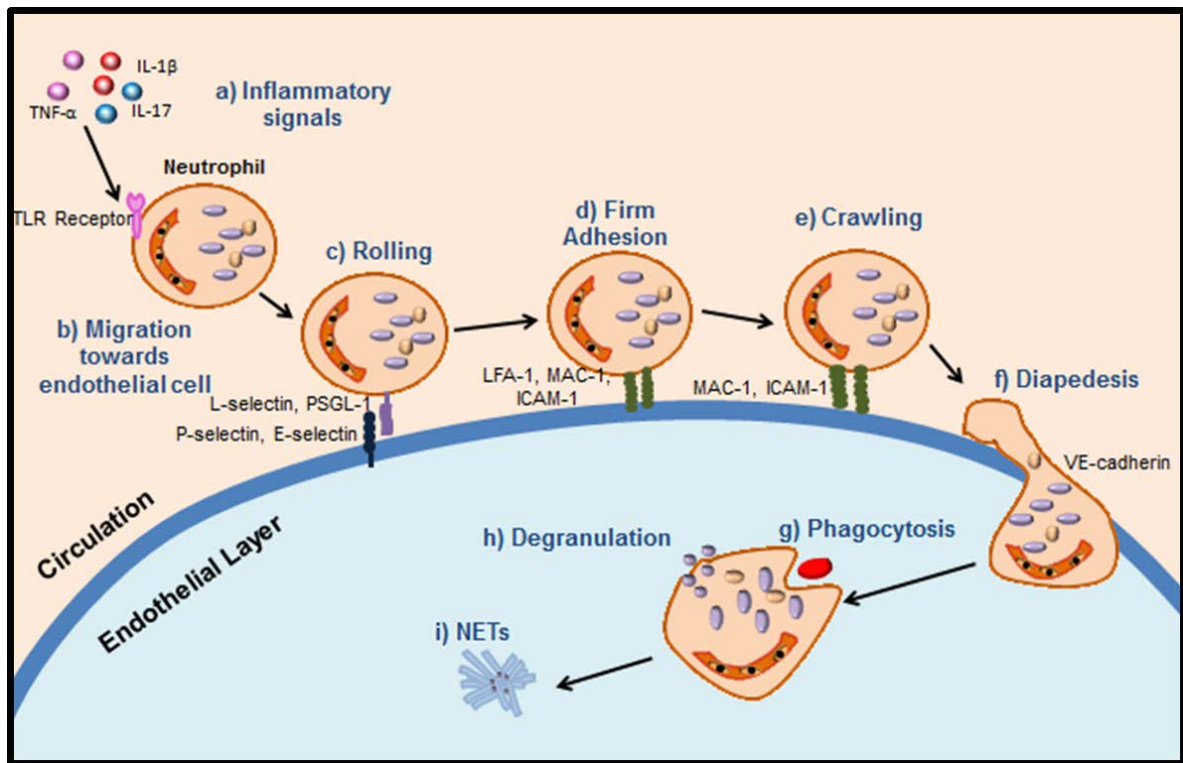


Figure 3: Neutrophil recruitment to inflammatory sites.

a) Neutrophils receive inflammatory signals from cytokines (IL-1 β , IL-17 and TNF- α) produced by mast cells and macrophages. b) Neutrophils migrate towards the surface of the endothelial layer. c) Neutrophils roll along the endothelial surface while L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) interact with P-selectin and E-selectin. d) Neutrophils bind via LFA, MAC (macrophage-1 antigen)-1 and ICAM-1 in firm adhesion. e) Chemoattractants, cytokines and integrins prepare for diapedesis. Neutrophils 'crawl' until an extravascular site of transmigration is found. f) Diapedesis occurs as the neutrophil traverses through the endothelium and arrives at the site of inflammation. g) Phagocytosis allows the neutrophil to engulf foreign microbes. h) Granules and antimicrobial molecules are released and the nicotinamide adenine dinucleotide phosphate (NADPH) respiratory burst pathway provides an antimicrobial environment unsuitable for pathogens. i) Neutrophil extracellular traps (NETs) are formed as the neutrophil nucleus condenses and allows the pathogen and cell debris to be eradicated (Amulic et al., 2012; Muller, 2003).

Rolling is the first process in neutrophil recruitment as they interact with vessel walls and postcapillary venules to converge with endothelial cells that have been stimulated

during an inflammatory response (Amulic et al., 2012; Burg & Pillinger, 2001). Host cells (including mast cells and macrophages) produce bacterial-derived lipopolysaccharide (LPS) and cytokine (TNF- α , IL-1 β and IL-17) inflammatory signals to stimulate endothelial cells. L-selectin and PSGL-1 allow interactions with other neutrophils by interacting with P- and E-selectin on the endothelial surface to promote selectin-mediated tethering of neutrophils to the vessel wall (Amulic et al., 2012; Burg & Pillinger, 2001). Adhesion then occurs where cytosolic domains of variable α subunits (CD11a, CD11b, CD11c) and common β subunits (CD18) interact with the cytoskeleton to firmly adhere the neutrophil to the endothelium (Amulic et al., 2012).

Diapedesis refers to neutrophil transmigration where the cytoskeleton is disassembled to cross the endothelial border and reassembled on the abluminal side of the endothelium. Neutrophils release soluble cationic proteins to trigger endothelial cytosolic free calcium and promote the phosphorylation of myosin light chain kinase to unfold myosin II and facilitate actin-myosin contractions, endothelial-cell retraction and neutrophil passage (Muller, 2003).

At the site of inflammation or infection, phagocytosis can be direct, with recognition of pathogen-associated molecular patterns (PAMPS) by pattern-recognition receptors, or opsonin mediated (Amulic et al., 2012; Bianchi, 2007). Phagosomes mature into a phagolysosome upon granule fusion to the phagosome before the phagosomal membrane coincides with NADPH oxidase creation and ROS (reactive oxygen species) production to combine phagocytosis and respiratory burst, creating the most harmful environment for pathogens (Amulic et al., 2012).

Oxidative burst is stimulated as granules move to the neutrophil surface to fuse with the plasma membrane or phagolysosome, allowing interactions with chemoattractants and extracellular matrix proteins in the respective environment (Almkvist et al., 2002; Amulic et al., 2012). The oxidation of super peroxides by NADPH triggers ROS production in the phagolysosome and outside the cell, creating an antimicrobial environment which is defensive to invading pathogens. Neutrophils then lyse pathogens and foreign cells via respiratory burst, the rapid release of ROS from neutrophils (Almkvist et al., 2002; Burg & Pillinger, 2001; Smith, 1994). NETs are activated when IL-8 or LPS prompts the release of granule proteins with chromatin from neutrophils to form extracellular fibril matrixes and they then facilitate pathogen removal and reduce host damage (Almkvist et al., 2002; Amulic et al., 2012; Burg & Pillinger, 2001).

1.3.3.2 Neutrophils in CFS/ME

Neutrophils in some CFS/ME patients may be abnormally apoptotic, with increased levels of TGF- β ligand and TNFR (ligand-activated tumour-necrosis factor receptor)-1 death receptor molecules (Kennedy et al., 2004).

TGF- β downregulates regulatory cytokines during the inflammatory response and can stimulate apoptosis. Augmented expression of TNFR1 death receptor molecules in CFS/ME patients imply that extrinsic factors are promoting accelerated apoptosis in neutrophils as a result of apoptotic pathways. Inflammatory signals and pro-inflammatory mediators have the ability to delay apoptosis in neutrophils which would result in altered mitochondrial potential and a prolongation of neutrophil function and activity (El Sakka et al., 2006). Reduced neutrophil apoptosis is associated with

increased respiratory burst. This presupposes that the decreased respiratory burst shown in CFS/ME may indicate an increase in the apoptotic ability of neutrophils, also demonstrated in CFS/ME (Brenu et al., 2010; El Sakka et al., 2006; Kennedy et al., 2004).

Neutrophil mitochondrial energy availability is also reduced in CFS/ME patients. This decline in energy availability in the mitochondria of neutrophils was significantly correlated to severity of CFS/ME symptoms, where patients with more severe symptoms had significantly reduced mitochondrial energy (Myhill et al., 2009). The mitochondrion is a major source of energy for all physiological functions. It was suggested that in conditions such as CFS/ME, where this energy production is reduced, symptoms such as muscle pain, fatigue and exhaustion may be related to this decline in 'energy profile'. Thus the energy profile of neutrophils may potentially be a hallmark of symptom severity in CFS/ME patients (Myhill et al., 2009).

1.3.4 Dendritic Cells

DCs are in the periphery and operate as messengers between the innate and adaptive immune systems. Functioning as APCs, DCs process antigens presented by MHC-I proteins and present them on their cell surface for recognition by other immune cells (Banchereau et al., 2000; Banchereau & Steinman, 1998; Shortman & Naik, 2006).

DCs are predominant in tissue with direct contact to the external environment, including: the skin, nose, lungs, stomach and intestines. Immature DCs can also be found in the blood stream (Banchereau et al., 2000; Banchereau & Steinman, 1998).

Following activation, DCs migrate into the lymphatic system to initiate the adaptive immune response by interacting with T cells and B cells via cell-cell contact or cytokine stimulation (Banchereau & Steinman, 1998; Shortman & Naik, 2006).

Classical DCs (cDCs), monocyte-derived or myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) are each distinguished based on location, migratory pathways, function and surface markers (Colonna et al., 2004; Hoene et al., 2006; León et al., 2005; Shortman & Naik, 2006). These DC subsets can also be identified by their variable expression of CD33, CD123 and CD11c (Henriques et al., 2012).

1.3.4.1 Dendritic Cell Phenotypes

All DCs originate from CD34⁺ haematopoietic stem cells (HSCs), although subsets of DCs differ in location and migratory pathways. Transcription factors FLT3-ligand (FL) and c-kit-ligand with the presence of GM-CSF or IL-3 assist in differentiation of DCs. GM-CSF enhances myeloid DC differentiation while IL-3 supports pDC and cDC development (Gilliet et al., 2002; Shortman & Naik, 2006). cDCs can also be distinguished by the surface expression of MHC-II molecules, CD40, CD80 and CD86 as well as CD123, CD33 and CD11c (Henriques et al., 2012; Shortman & Naik, 2006).

mDCs are imperative for the initiation of the adaptive immune response. Immature mDCs circulate in the blood stream, lymph and tissues, capturing soluble and particulate antigens for processing and subsequent surface presentation (Hoene et al., 2006). IL-4 and GM-CSF promote mDC differentiation by allowing their development from early progenitor cells into immature DCs, which are then characterised by low

expression of MHC-II and costimulatory molecules (Hoene et al., 2006; Shortman & Naik, 2006). During pathogenesis or inflammation, T cells costimulate mDCs via CD40 ligands to enhance mDC secretion of inflammatory cytokines, including IL-12 which then activates naïve T cell differentiation from the Th1 lineage (León et al., 2005). mDCs induce B cell proliferation, antibody production and T regulatory cell (Treg) activity while inducing tumour cell apoptosis and NK cell activation (Hoene et al., 2006).

pDCs are circulating cells which produce 100 to 1000 times more type I IFNs, such as IFN- α , than any other cell after inflammatory stimulation (Hoene et al., 2006). Structurally, pDCs express toll like receptor (TLR)7, TLR9, IL-3Ra, Fc γ , CD4, CD45RA and ILT3. pDCs function to enhance NK cell-mediated cytotoxic activity and promote the migration of IFN- γ inducible chemokines CXCL9 and CXCL10 (Colonna et al., 2004).

1.3.4.2 Dendritic Cell Function

DCs in the blood stream are less mature than those in tissues and have no dendrites although they still perform complex functions. TLRs on the surface of DCs recognise chemical signatures on pathogens prior to immature DCs phagocytosing and presenting antigens. Once antigen activated, DCs become mature and migrate towards the lymph nodes to stimulate an immune response (Banchereau & Steinman, 1998). During migration to the lymph nodes, immature DCs phagocytose pathogens and degrade their proteins to present MHC molecules upon DC maturation (Banchereau et al., 2000). This process involves the upregulation of surface receptors acting as coreceptors, such as

CD80, CD86 and CD40, which are able to enhance their ability to activate T-cells. CD80 and CD86 are expressed by human DCs and are critical to T cell activation due to the costimulation signals which lead to IL-1 and cytokines that induce IFN- γ (Buelens et al., 1995). CD80 is almost absent in immature DCs and CD86 is expressed in low numbers. During an immune response, activated T cells contact DCs and upregulate the expression of both CD80 and CD86 (Mellman & Steinman, 2001).

The chemotactic receptor CCR7 is also upregulated to induce the migration of DCs through the lymphatic system acting as APCs, activating helper T cells, killer T cells and B cells (Banchereau & Steinman, 1998).

1.3.4.3 Dendritic Cells in CFS/ME

DC derived IL-2 secreted by mDCs has been significantly increased in moderate CFS/ME patients (Fletcher et al., 2009). IL-12 is the predominant Th1 inducing cytokine and it leads to the production of IFN- γ , IL-2 and TNF- α during an immune response. IFN- γ , IL-2 and TNF- α have also been analysed in the plasma of CFS/ME patients with no differences in the illness when compared with non-fatigued healthy controls (Fletcher et al., 2009; Hoene et al., 2006).

DC derived IL-15 plays an important role in the survival and proliferation of NK cells as it interacts with IL-2R to assist in both NK cell cytotoxic activity and production of IFN- γ (Lucas et al., 2007). IL-15 has been significantly reduced in the blood of CFS/ME patients, potentially suggesting reduced NK cell activation in the illness (Fletcher et al., 2009).

DCs share similar functions with other innate immune cells, including antigen presentation and cytokine release, which are necessary for adaptive cell recruitment and pathogen clearance. Prior to this research, there were no studies on the direct role of DCs in CFS/ME or in CFS/ME severity subgroups that is moderate and severe CFS/ME patients. Reduced pDCs have since been shown in moderate CFS/ME patients (Brenu et al., 2013c). These reduced pDCs may be suggestive of lower levels of type I IFNs which may impede the effective elimination of pathogens in CFS/ME patients (Brenu et al., 2013c). In cases where pathogens are infecting DCs, it may lead to a reduced maturation and cytokine release from T cells because DCs play a vital role in T cell activation through constant cell-cell interactions between the innate and adaptive immunity. Examining DCs in CFS/ME patients, in particularly moderate and severe subgroups of patients, may confirm whether overall immunological abnormalities in the innate immune system are present in CFS/ME.

1.3.5 T Cells

T cells are lymphocytes of the adaptive immune system that play an important role in cell-mediated immunity. They are recruited during an immune response by the release of soluble proteins (including cytokines and chemokines) from DCs, macrophages and neutrophils in immune responses. T cells act against antigens released during inflammation or tumour invasion (Broere et al., 2011; Fontenot et al., 2003).

T cells express the surface protein CD3 and TCRs which determine the specificity of antigens present in the body through the MHC complex (Schmitt & Zúñiga-Pflücker,

2002; Weaver et al., 2006). All T cells originate from the bone marrow and populate the thymus as HPCs which differentiate into immature thymocytes (Schmitt & Zúñiga-Pflücker, 2002; Weaver et al., 2006). Mature T cells can be either CD3⁺CD4⁺ or CD3⁺CD8⁺ and selectively recognise and bind to MHC-II and I respectively (Starr et al., 2003; Zhu & Paul, 2008). The manuscript below provides a review of T cells with specific focus on CD4⁺, CD8⁺ phenotypes, and their potential role in CFS/ME.

1.3.6 REVIEW PAPER TWO: CHRONIC FATIGUE SYNDROME/MYALGIC ENCEPHALOMYELITIS AND THE POTENTIAL ROLE OF T CELLS

Hardcastle, SL., Brenu, EW., Staines, DR., Marshall-Gradisnik, S. 2014. Chronic Fatigue Syndrome/Myalgic Encephalomyelitis and the Potential Role of T Cells. *Biological Markers and Guided Therapy*.1:1, 25-38.

Author contributions:

Hardcastle was the principle contributor to this manuscript. Hardcastle was responsible for contributions to the manuscript design, literature review and analysis as well as the primary drafting of the manuscript. Brenu, Staines and Marshall-Gradisnik contributed to the manuscript design and critical manuscript revisions.

1.3.6.1 ABSTRACT

CFS/ME is a multifactorial disorder defined by symptom-specific criteria and characterised by severe and prolonged fatigue. CFS/ME typically affects a variety of bodily systems, including the immune system. Patients with CFS/ME exhibit significantly reduced NK cell cytotoxic activity suggesting immune abnormalities which may be hallmarks of changes in the adaptive immune system, potentially including T cell subsets and function. The principal purpose of T cells is to regulate immune responses and maintain immune homeostasis. These regulatory measures can often be compromised during illness and may present in a number of diseases, including CFS/ME. This review paper examines the role of T cells in CFS/ME and the potential impact of T cells on CFS/ME immune profiles with an evaluation of the current literature.

1.3.6.2 INTRODUCTION

The purpose of T cells is to regulate the immune responses of both innate and adaptive immune cells by maintaining immunological homeostasis, which may often be compromised during illness. Some immunological disorders have also been associated with deficiencies or dysfunction in subtypes of T cells, such as Tregs (Sakaguchi, 2005; Shevach, 2002). Dysfunction in T cells and their pro- or anti-inflammatory cytokines can reduce the ability of these cells to maintain cytokine homeostasis, promote autoimmunity or respond to pathogens (Murphy & Reiner, 2002; Visser et al., 1998; Weaver et al., 2007; Yamane et al., 2005; Zhu & Paul, 2008). Imbalances in Th1/Th2/Th17 cytokine profiles have been related to autoimmune diseases, such as MS and RA (Drulovic et al., 2009; Nevala et al., 2009; Peakman et al., 2006; Singh et al., 2007; Yamane et al., 2005; Zabrodskii et al., 2007).

CFS/ME is a serious illness with consistent immune perturbations (Brenu et al., 2010; Brenu et al., 2011; Broderick et al., 2010; Fletcher et al., 2009; Klimas et al., 2012; Klimas et al., 1990; Skowera et al., 2004; Swanink et al., 1996). Patients diagnosed with CFS/ME primarily experience persistent fatigue, physically and mentally, for a period of at least six months. Other symptoms include headaches, dizziness, muscle pain, pallor, abdominal pain, nausea and swollen lymph nodes (Fukuda et al., 1994a; Jason et al., 2004). CFS/ME patients may in some cases present with altered susceptibility for infections, indicative of chronic low-grade inflammation and potential dysregulation in T cells (Maes et al., 2007). Currently, many T cell studies in CFS/ME have inconsistent results and it remains to be determined if these cells have a possible role in the pathology of CFS/ME patients (Brenu et al., 2010; Brenu et al., 2011; Broderick et al.,

2010; Fletcher et al., 2009; Klimas et al., 2012; Klimas et al., 1990; Skowera et al., 2004; Swanink et al., 1996), hence this review aims to examine T cells in CFS/ME.

1.3.6.3 T CELLS

T cells are lymphocytes of the adaptive immune system that play an important role in cell-mediated immunity as they respond to antigens released during inflammation or tumour invasion after being recruited by soluble proteins presented by DCs, macrophages and neutrophils (Broere et al., 2011; Reinherz & Schlossman, 1980). T cell subsets can be identified based on the expression of surface markers and specific cytokine secretion (Curotto de Lafaille & Lafaille, 2009; Harrington et al., 2006; Jonuleit & Schmitt, 2003; Weaver et al., 2006).

All T cells originate from the bone marrow and populate the thymus as HPCs which differentiate into immature thymocytes (Schmitt & Zúñiga-Pflücker, 2002; Weaver et al., 2006). Thymic lymphoid progenitors can develop into either $\alpha\beta$ or $\gamma\delta$ T cells as a result of TCR chain rearrangement, with the majority of heterodimers forming the $\alpha\beta$ T cell lineage (~98%) (Caccamo et al., 2005). $\alpha\beta$ T cells then develop into $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells which selectively recognise and bind to molecules MHC-II and I respectively or NKT cells (Starr et al., 2003; Zhu & Paul, 2008; Zúñiga-Pflücker & Lenardo, 1996).

1.3.6.4 CD4⁺T CELLS

CD4⁺T cells coordinate the activity of both innate and adaptive immune systems (Harrington et al., 2006). Naive CD4⁺ effector T cells differentiate into distinct lineages following activation by NK cells and DCs and differentiate into Tregs, Th1, Th2 and Th17 subsets (Harrington et al., 2006; Mosmann & Coffman, 1989; Parish & Liew, 1972).

Th1 effector CD4⁺T cells are responsible for cell-mediated immunity and identified predominantly based on their production of pro-inflammatory cytokines, IFN- γ , LT- α /TNF- β and IL-2 (Murphy & Reiner, 2002; Weaver et al., 2007; Zabrodskii et al., 2007; Zhu & Paul, 2008). IFN- γ stimulates macrophages to phagocytose pathogens (Huenecke et al., 2010; Mocikat et al., 2003; Patarca-Montero et al., 2001; Rham et al., 2007) and IL-2 importantly regulates and induces the differentiation and proliferation of T cells, memory T cells and NK cells (Huenecke et al., 2010; Mocikat et al., 2003; Patarca-Montero et al., 2001; Rham et al., 2007).

Th17 cells also secrete pro-inflammatory cytokines, IL-17A, IL-17F, IL-21, IL-22, IL-26 and TNF- α (Murphy & Reiner, 2002; Zhang et al., 2012; Zhu & Paul, 2008) and enhance host protection against extracellular bacteria, fungi and microbes as well as improving the clearance of intracellular pathogens (Weaver et al., 2007). Th17 cells also secrete chemokines CCL2, CCL3 and CCL20 to allow for the migration of monocytes, T cells and neutrophils towards necessary sites for inflammatory responses (Murphy & Reiner, 2002; Zhang et al., 2012; Zhu & Paul, 2008). IL-17 from Th17 cells is involved in the development of immune-related diseases, such as autoimmune arthritis

(Komiyama et al., 2006; Röhn et al., 2006) and MS (Tzartos et al., 2008), and is also amplified in patients with asthma and RA (Mocikat et al., 2003; Rham et al., 2007). Regulation of pro- and anti-inflammatory cytokines is important for immune-related responses and cytokine shift either towards a Th1/Th17 or Th2 cytokine profile may underlie certain disorders, including CFS/ME (Nevala et al., 2009; Patarca-Montero et al., 2001; Visser et al., 1998).

Th2 cells are responsible for extracellular pathogen immunity, producing anti-inflammatory cytokines, including: IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 (Mosmann & Coffman, 1989; Murphy & Reiner, 2002; Weaver et al., 2006; Zhu & Paul, 2008). IL-5 and IL-9 are important in immune response to allergic reactions while IL-4 and IL-10 regulate inflammatory responses (Zhu & Paul, 2008). Shifts towards a Th2 mediated immune response may encourage chronic inflammation, as observed in disorders such as MS, RA and Gulf War Illness (Brenu et al., 2010; Klimas et al., 1990; Nevala et al., 2009; Peakman et al., 2006; Singh et al., 2007; Yamane et al., 2005).

1.3.6.5 T REGULATORY CELLS

Tregs are a subset of CD4⁺T cells, distinguished by their functional ability to suppress immune responses and prevent autoimmunity (Bluestone & Abbas, 2003; Hori et al., 2003; Weaver et al., 2006). There are two main CD4⁺ Tregs, iTregs which develop from naïve CD4⁺T cells in peripheral lymphoid tissues and intrathymic nTregs (Hori et al., 2003; Weaver et al., 2006). Forkhead box protein (FOXP)3 is an important transcription factor in iTregs and nTregs, which regulate pro-inflammatory factors by suppressing both IL-2 and IFN- γ (Yang et al., 2008; Zhang et al., 2012). IL-4, IL-10 and TGF- β

induce the generation of iTreg cells from naïve CD4⁺T cells (Jonuleit & Schmitt, 2003). The iTreg subset of T cells includes further subsets such as type 1 Tregs (Tr1) and Th3 cells which variably express FOXP3 (Curotto de Lafaille & Lafaille, 2009; Jonuleit & Schmitt, 2003). iTregs mediate inhibitory function by producing anti-inflammatory cytokines, IL-5, IL-10, TGF- β and IFN- γ (predominantly IL-10 and TGF- β), which may indirectly contribute to the suppression of cell functioning or differentiation (Bluestone & Abbas, 2003; Jonuleit & Schmitt, 2003). Impairments in Treg development and function, including diminished FOXP3 expression, can also be related to autoimmune diseases (Nouri-Aria, 2010).

1.3.6.6 CD8⁺T CELLS

CD8⁺T cells are functionally important for both innate and adaptive immunity (Berg & Forman, 2006). CD8⁺T cells protect the body from foreign or invading microorganisms by recognising diverse antigens presented by MHC-I peptides. This stimulates CD8⁺T cell proliferation, cytokine (IFN- γ and TNF) and chemokine (IL-8) secretion and lysis of infected cells (Belz & Kallies, 2010; Berg & Forman, 2006). CD8⁺T cells also produce lytic proteins (such as granzyme B and perforin) (Belz & Kallies, 2010).

Cytotoxic CD8⁺T cells express high quantities of granzymes, perforin, cytokines and chemokines (Appay et al., 2008; Belz & Kallies, 2010; Berg & Forman, 2006; Mempel et al., 2006). The cytotoxic pathways of CD8⁺T cells allow defence against virus-infected or transformed cells through MHC-I recognition (Deckert et al., 2010; Trapani & Smyth, 2002). Perforin and granzymes exocytose from CD8⁺T cells to induce apoptosis of target cells (Trapani & Smyth, 2002). Perforin is a membrane-disrupting

protein, secreted during an immune response, which enters the membrane of a target cell, allowing granzymes to enter. Once inside, granzymes cleave caspases and degrade the DNA of target cells, promoting an apoptotic cascade (Trapani & Smyth, 2002). Aside from perforin and granzyme secretion, target-cell death receptors, such as Fas (CD95) can also induce caspase-dependent apoptosis in target cells (Trapani & Smyth, 2002). The role of CD8⁺T cells and their maintenance of inflammation may be associated with autoimmune disease (Blanco et al., 2005; Deckert et al., 2010), incidentally, increases in CD8⁺T cells, perforin and granzyme B, may be related to diseases such as Lupus (Blanco et al., 2005).

1.3.6.7 T CELLS IN CFS/ME

CFS/ME is a diverse multisystem illness with varied symptom severity that can substantially affect a person's way of life (Fukuda et al., 1994a). There are substantial costs associated with CFS/ME worldwide and there is no known cure, successful treatments and/or useful diagnostic method. Most patients with CFS/ME are incapable of maintaining full-time occupations while the more severe cases require constant daily assistance (Toulikidis, 2002).

The estimated prevalence rate of CFS/ME is 0.2-0.7% (Toulikidis, 2002) with women being more greatly affected by CFS/ME than men by up to 80% (Reeves et al., 2005). Of those diagnosed with CFS/ME, approximately 83% report gradual onset of the disorder while 17% experience sudden onset, highlighting potential subgrouping based on the onset of CFS/ME (Reeves et al., 2005). Immune investigations in CFS/ME have identified variations in immune cell numbers, significantly reduced lymphocyte

cytotoxic activity, decreased neutrophil respiratory burst, fluctuations in cytokine distribution with particular shifts in Th1/Th2 related cytokines and altered expression of immune related genes (Brenu et al., 2010; Brenu et al., 2012c; Brenu et al., 2011; Broderick et al., 2010; Caligiuri et al., 1987; Fletcher et al., 2009; Klimas et al., 2012; Klimas et al., 1990; Skowera et al., 2004; Swanink et al., 1996).

Many studies have examined T cells in CFS/ME, particularly overall T cell numbers, CD4⁺ and CD8⁺T cells, with inconsistent results. Some studies found decreases in the number of CD4⁺T cells (Klimas et al., 1990; Lloyd et al., 1989), while others concluded that there were increases (Hanson et al., 2001; Klimas et al., 1990). Similar variations in results have been discovered regarding the CD8⁺ subset of T cells where decreases in the number of CD8⁺T cells were found in some studies (Barker et al., 1994; Hanson et al., 2001; Lloyd et al., 1989) while another found increases in CD8⁺T cell subsets (Nijs et al., 2003).

Only one study has examined cytokine production in isolated CD4⁺T cells in CFS/ME patients and it was found that IFN- γ was significantly reduced in CFS/ME patients (Visser et al., 1998). In CFS/ME patients, variable levels of Th1 cytokines and IFN- γ may potentially explain the constant infections and increased inflammation experienced by CFS/ME patients (Klimas et al., 1990; Patarca-Montero et al., 2001). The potential increase in secretion or presence of IFN- γ specifically may lead to autoimmune related immune responses (Fukuda et al., 1994a; Klimas et al., 1990; Patarca-Montero et al., 2001; Peakman et al., 2006; Visser et al., 1998).

Isolated CD4⁺T cells and subsets may provide definitive results thereby reducing the interference from other potential producers of cytokines. Most CFS/ME cytokine studies did not use this technique hence the cytokine data is representative of the whole peripheral blood mononuclear cell (PBMC) population and a number of studies on these cytokines have been measured in CFS/ME patients (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF- α , TNF- β , IFN- γ and TGF- β), producing inconsistent results (Lloyd & Pender, 1992; Patarca, 2001; Visser et al., 1998). These studies have highlighted potential dysregulation in the CFS/ME cytokine profile by demonstrating significant shifts in cytokines in CFS/ME. Although the majority of these studies measured cytokines in PBMCs, irrespective of this, cytokine production in CFS/ME may be indicative of the cytokine profile of T cells in CFS/ME and potential shifts between Th1/Th2/Th17 regulations.

IL-2 levels have been inconsistent in CFS/ME participants. IL-2 plays an important role in the maintenance of natural immunological self-tolerance with impairments in IL-2 leading to autoimmune gastritis, early onset diabetes and T-cell mediated autoimmune diseases such as thyroiditis and severe neuropathy (Fletcher et al., 2009; Setoguchi et al., 2005). IL-2 also contributes to the induction of NK cell cytotoxic activity (Henney et al., 1981; Siegel et al., 1987), therefore alterations in pro-inflammatory IL-2 levels may potentially correlate with consistently significant reduced NK numbers and activity in CFS/ME (Blanco et al., 2005; Brenu et al., 2012c; Broderick et al., 2010; Caligiuri et al., 1987; Karupiah et al., 1990; Maes et al., 2007; Reeves et al., 2005).

The levels of cytokines TGF- β and IL-6 are sometimes raised in patients with CFS/ME (Brenu et al., 2012c; Yang et al., 2008; Zhou et al., 2009). Elevated levels of TGF- β and

IL-6 in CFS/ME patients promote the production of Th17 cells by inducing signal transducer and activator of transcription (STAT)3, necessary for Th17 cell differentiation (Qin et al., 2009). Th17 cells produce IL-17 which contributes to disease pathogenesis by acting as a potent pro-inflammatory mediator and is inconsistent in CFS/ME (Wynn, 2005). IL-17 enhances autoimmune inflammation by acting on APCs to signal IL-1, IL-6, IL-23 and TGF- β , factors for pathogenic Th17 development and resulting in exacerbation of autoimmunity (Barker et al., 1994; Cua & Tato, 2010). An increased expression of IL-17 cytokines (such as IL-17A), has been linked to a number of autoimmune, immune and inflammatory related diseases, including: RA, Lupus and Asthma (Barker et al., 1994; Fletcher et al., 2009; van den Berg & Miossec, 2009). Similarly, a decrease in Th17 and particularly IL-17 may be related to a reduced host protection mechanism and clearance of pathogens (Weaver et al., 2007) .

Th2 T cells are responsible for the secretion of anti-inflammatory cytokines, such as IL-4 and IL-10. When isolated CD4⁺T cells were analysed in CFS/ME patients, no significant changes were found in IL-4 cytokine levels (Visser et al., 1998). In whole blood, CFS/ME patients have significantly increased expression of the anti-inflammatory cytokine IL-10. IL-10 stimulates the production and survival of B cells as well as antibody production and down regulating Th1/Th17 pro-inflammatory cytokines (Lee et al., 1996). Infection is the primary promoter of the production of IL-10 producing cells, suggesting that an increase in IL-10 in CFS/ME patients could reflect the chronic infection typically experienced by CFS/ME patients (Couper et al., 2008; Klimas et al., 2012). IL-10 also influences signalling of T cells with B cells and may alter T cell responses necessary to autoimmune diseases (Lee et al., 1996). Assessment

of such cytokines in isolated T cells in CFS/ME patients can provide further insight into dysregulation and cytokine profiles in the disorder.

TGF- β is the only cytokine examined unique to iTregs and has been up regulated in CFS/ME patients (Qin et al., 2009). TGF- β is primarily an immunosuppressive cytokine which down regulates the inflammatory response through the inhibition of pro-inflammatory cytokines (Aoki et al., 2005; Letterio et al., 1996). TGF- β deficiencies may promote excessive lymphocyte activation and differentiation, cell adhesion molecule expression, Treg functioning and cell apoptosis. Therefore in CFS/ME it is possible that increases in TGF- β may reflect an increase in Treg suppression or Treg related activities in CFS/ME (Aoki et al., 2005). Incidentally, significantly increased levels of CD4⁺CD25⁺FOXP3⁺ cells have been found in CFS/ME patients (Baumgarth et al., 2005). Regulation and maintenance of immunological tolerance and inflammatory responses can be maintained by Tregs (Mempel et al., 2006; Sakaguchi, 2005; Shevach, 2002). Hence, deficiencies or dysfunctions of Tregs or the subtypes of Tregs may promote autoreactive immune responses resulting in autoimmune diseases (Sakaguchi, 2005; Shevach, 2002). Increased FOXP3⁺ is typically observed in various forms of cancer (Lu, 2009).

Significantly reduced cytotoxic activity is an important hallmark of CFS/ME, with many CFS/ME patients demonstrating significant reductions NK cell cytotoxic activity (Brenu et al., 2010; Brenu et al., 2011; Klimas et al., 2012; Klimas et al., 1990). Recent studies have identified significant reductions in the cytotoxic activity of isolated CD8⁺T cells (Brenu et al., 2011). Although, the underlying causal factor stimulating this effect is unknown, it presupposes that CFS/ME patients are potentially compromised due to

failures in this cytotoxic mechanism, possibly relatable to the function of cytotoxic granules and subsequent cytokines in these T cells. Incidentally, reductions in perforin and granzymes have been reported in CFS/ME (Brenu et al., 2011; Saiki et al., 2008; Trapani & Smyth, 2002). Perforin and granzymes are lytic proteins that ensure effective lysis of viral or microbial pathogens (Chattopadhyay et al., 2009). Reductions in perforin lead to significant declines in apoptosis of target cells (Capitini et al., 2013; Chattopadhyay et al., 2009). Perforin levels are severely decreased in systemic juvenile idiopathic arthritis, instigating defective cytotoxic activity in T cells (Wulffraat et al., 2003). In contrast, elevated concentrations of perforin are reported in chronic inflammatory disorders with autoimmune features, such as MS and autoimmune thyroid disease. Perforin is significantly increased in inflammatory disorders although the role in these disorders is undefined, it may be indicative of increased cytolytic activity or an immune reaction aimed at removing inflammatory cells (Arnold et al., 2000).

Alterations in the levels of perforin can incidentally affect the release of other lytic proteins, such as granzymes. Granzyme A expression is significantly decreased in CD8⁺T cells in CFS/ME patients (Brenu et al., 2011). Granzyme A specifically induces the breakage of single-strand DNA and the nuclear lamina. Therefore, decreases in granzyme A in CFS/ME patients may lead to a reduced ability of the cells to induce target cell death (Brenu et al., 2012a; Wu et al., 2007).

T cell perturbations may potentially be attributing to alterations T cell subtypes, fluctuations in T cell cytokine production, decreases in cytotoxic activity and differential expression of immune related genes in CFS/ME patients.

1.3.6.8 CONCLUSION

A number of studies have assessed T cells in CFS/ME, although further studies are required to obtain consistency and validation of results. Assessment of T cell cytokines in CFS/ME patients based on PBMCs is not the most appropriate method of assessing these cells as they are not specific to subsets of T cells that vary in cytokine secretion. Similarly, assessment of CD8⁺ and CD4⁺T cells and cytokine profiles may highlight specific cells that may be affected in CFS/ME patients. In particular, Tregs and their regulatory activities may deserve closer investigation. Subgrouping of CFS/ME patients may be necessary in the future to determine whether T cell subsets and function differs among CFS/ME patients based on their variation of disorder onset or severity.

1.3.7 Gamma Delta T Cells

In thymic selection of T cell development, the $\gamma\delta$ TCR chain represents a small population of T cells, alongside the common $\alpha\beta$ TCR chains. $\gamma\delta$ TCR chain expression on T cells forms innate-like subsets which can be activated by cytokines or TCR stimulation (Carding, 1998; Carding & Egan, 2002; Girardi, 2006). $\gamma\delta$ T cells are located predominantly in epithelial tissues such as the skin and mucosal surfaces where they are involved in the anti-tumour response, providing initial responses to microbial invasions as well as secreting cytokines for immune response regulation (Girardi, 2006).

In humans, there are two subsets of $\gamma\delta$ T cells. $\gamma\delta 1$ T cells are the first to emerge from the thymus and populate epithelial tissues such as the intestines and airways, leaving only a minor portion of in the blood stream while $\gamma\delta 2$ T cells constitute around 80% of $\gamma\delta$ T cells in the adult blood stream (Girardi, 2006).

$\gamma\delta$ T cells may be considered as part of the innate or adaptive immune system as they have pattern recognition receptors and have some cytotoxic activities while also developing a memory phenotype (Girardi, 2006; Raulet, 1989). Therefore, both subsets of $\gamma\delta$ T cells can then be further divided into four memory phenotypes using the expression of CD45RA and CD27, similar to the characterisation of CD4⁺ and CD8⁺T cell phenotypes (Anane et al., 2010). Naïve $\gamma\delta$ T cells are CD45RA⁺CD27⁺ and they lack cytotoxic effector functions and have high levels of adhesion molecules to enable migration of cells to lymph nodes. The remaining phenotypes all have memory components, central memory (CD45RA⁻CD27⁺), effector memory (CD45RA⁻CD27⁻ and CD45RA⁺ effector memory (CD45RA⁺CD27⁻). Central memory $\gamma\delta$ T cells have low

cytotoxic activity and have homing potential while the effector memory phenotypes demonstrate NK cell-like functions. Effector memory $\gamma\delta$ T cells can detect aberrant MHC expression and also have greater cytotoxic capacities than other $\gamma\delta$ T cell phenotypes (Anane et al., 2010).

IFN- γ is secreted by $\gamma\delta$ T cells during an immune response to enhance antimicrobial, antitumour and functional properties of NK and $\alpha\beta$ T cells and promote integrity (Girardi, 2006). The $\gamma\delta$ T cells also then function in 'microscopic wound healing', where growth factors (such as fibroblast growth factor-VII, FGF-VII and keratinocyte growth factor-1) stimulate the proliferation of healthy keratinocytes to replace those that have been eliminated (Girardi, 2006).

There have been no examinations of $\gamma\delta$ T cells in CFS/ME patients therefore it may be beneficial to assess, as these cells have features of both innate and adaptive immune cells, which are abnormal in the illness and hence $\gamma\delta$ T cells may play a role in bridging the innate and adaptive cell responses in CFS/ME (Brenu et al., 2010; Brenu et al., 2011; Girardi, 2006).

1.3.8 NKT Cells

NKT cells are T cells that share characteristics with NK cells (Godfrey et al., 2000; Godfrey et al., 2004; Kronenberg, 2005). NKT cells develop from T cell precursors in T cell lineage although once mature; NKT cells contain perforin and granzymes to undergo cytotoxic activities, like those of NK cells. In mice, NKT cells are found most frequently in the liver (30-50% of NKT cells), bone marrow (20-30% of NKT cells) and

thymus (10-20% of NKT cells) (Godfrey et al., 2000; Kronenberg, 2005). Little is known about the distribution of NKT cells in humans although studies suggest that NKT cells are located in the same locations with approximately 10 times less the frequency (Bendelac et al., 2007; Godfrey et al., 2000). NKT cells recognise CD1d MHC-I-like molecules, presenting to glycolipid antigens (Brennan et al., 2013; Godfrey et al., 2000; Godfrey et al., 2004; Ruffell et al., 2010).

NKT cells develop in the thymus from T cell precursors as part of the $\alpha\beta$ TCR T cell lineage, prior to TCR expression (Bendelac et al., 2007; Brennan et al., 2013; Godfrey & Berzins, 2007; Godfrey et al., 2004). During T cell thymocyte development, particularly at the CD4⁺CD8⁺ double positive (DP) stage, NKT cells express a TCR α chain (Bendelac et al., 2007; Godfrey & Berzins, 2007). The TCR α chain interacts with glycolipid antigen ligands (such as isoglobotrihexosylceramide (iGb3) and α -GalCer) presented by CD1d on DP thymocytes to deviate from thymocyte development into NKT lineage (Bendelac et al., 2007; Brennan et al., 2013; Godfrey & Berzins, 2007). Some TCR chains of NKT cells display a high affinity for self-ligand-CD1d complexes, potentially allowing self-reactivity and NKT cells are negatively selected in response to high levels of CD1d (Brennan et al., 2013; Godfrey & Berzins, 2007). Ligand α -GalCer binds efficiently to CD1d and the glycolipid complex as well as the TCR on the NKT cell, often allowing accurate identification of NKT cells in both humans and mice (Godfrey et al., 2004).

Initially characterised by a TCR and a C-lectin type NK cell receptor (NK1.1, NKR-P1 or CD161c), human Type I NKT cells are identified by expression of invariant V α 24J α 18 rearrangement coupled V β 11 (Bendelac et al., 2007; Coquet et al., 2008;

Godfrey & Berzins, 2007; Godfrey et al., 2000; Kronenberg, 2005). A second subset of NKT cells, Type II NKT cells, has been identified with diverse TCR V α chains. Little is known about type II NKT cells therefore, the focus of this review is on Type I iNKT cells (Bendelac et al., 2007; Coquet et al., 2008; Godfrey & Berzins, 2007; Godfrey et al., 2000; Kronenberg, 2005).

NKT cells contain perforin, granzymes and Fas ligands, giving them cytotoxic functions dependent on TCR recognition of antigens (Kronenberg, 2005). However, based on the rapid production of cytokines following a TCR signal, NKT cells primarily function as part of both the innate and adaptive immune systems through interactions with an array of leukocytes (Figure 4) (Bendelac et al., 2007; Godfrey et al., 2000; Ruffell et al., 2010). NKT cells function to enhance microbial immunity and tumour rejection, suppress autoimmune disease and promote tolerance (Figure 4) (Brennan et al., 2013; Godfrey & Berzins, 2007).

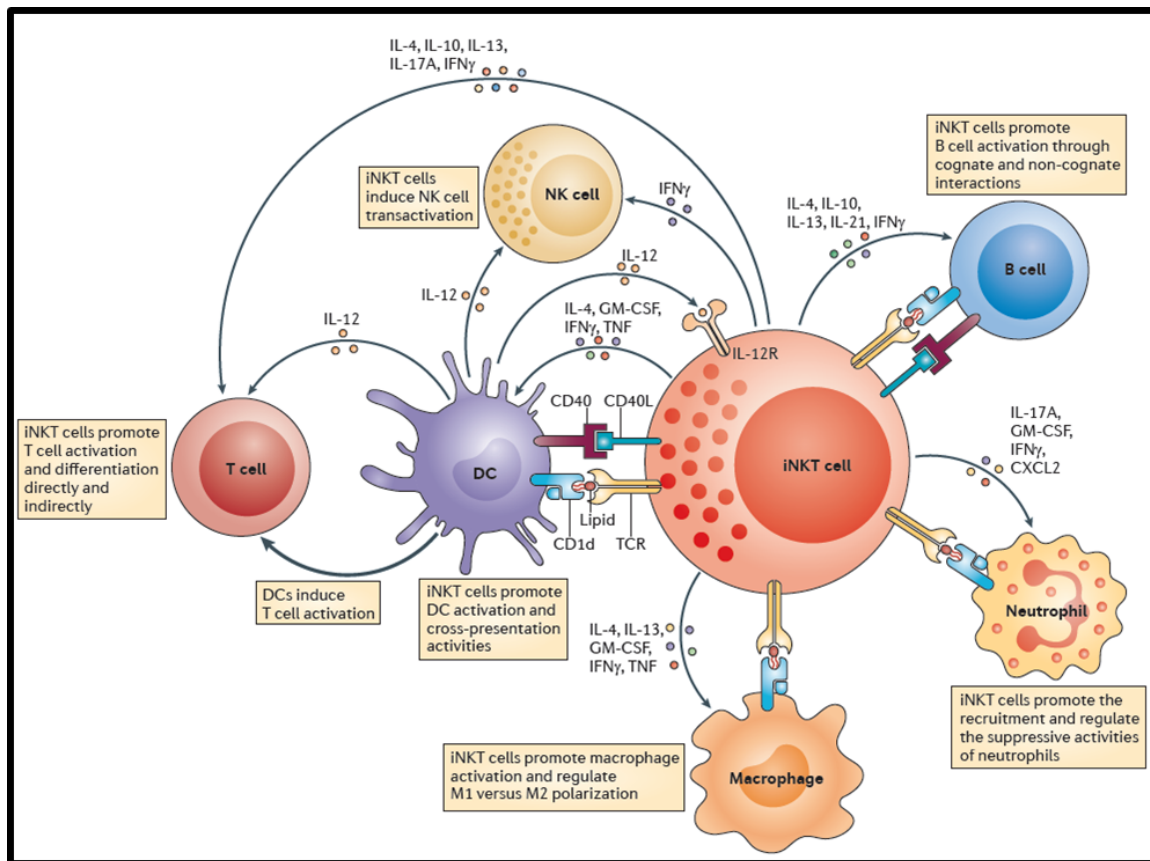


Figure 4: iNKT cell interactions with other leukocytes.

Cytokines and ligands allow interactions between iNKT cells and NK cells, B cells, T cells, DCs, macrophages and neutrophils (Brennan et al., 2013)

iNKT cells can have bidirectional interactions with DCs. During an immune response, pattern-recognition receptors (PRRs) promote IL-12 secretion from DCs. DCs also upregulate their production of stimulatory lipid antigens by surface CD1d for presentation to iNKT cells in CD40-CD40L (ligand) interactions (Brennan et al., 2013; Kronenberg, 2005). These interactions promote the production of IFN- γ by iNKT cells, further IL-12 production by DCs and enhance NK cell transactivation, protein antigen responses by CD4⁺ and CD8⁺T cells and DC cross-presentation (Brennan et al., 2013; Kronenberg, 2005). iNKT cells can also influence B cell functions through iNKT cell production of IL-4, IL-5, IL-6, IL-13 and IL-21 and their expression of the CD40

ligands (Brennan et al., 2013). iNKT cells can assist B cells with cognate help when iNKT stimulatory lipid antigens are linked specifically to B cell epitopes, allowing B cells to internalise and present it to CD1d. B cell early IgM and IgG responses during infections can be enhanced through cognate help from iNKT cells during an immune response (Brennan et al., 2013; Kronenberg, 2005). Macrophages mediate iNKT cell activation as specialised macrophage populations of Kupffer and stellate cells can present particulate antigens to iNKT cells, promoting iNKT cell activation (Brennan et al., 2013). Similarly, activated iNKT cells have the ability to influence macrophage phenotypes as iNKT cell-produced IFN- γ enhances phagocytosis and bacterial clearance by pulmonary macrophages (Brennan et al., 2013).

Cytotoxic activity of NK cells and CD8⁺T cells are significantly reduced in CFS/ME (Brenu et al., 2011; Caligiuri et al., 1987; Klimas et al., 1990) and other immune discrepancies, such as reduced neutrophil phagocytosis and increased Tregs are present in the illness (Brenu et al., 2010; Brenu et al., 2011). NKT cells share characteristics and functions with both NK and T cells and interact with immune cells, including: DCs, macrophages, B cells, neutrophils, NK cells and T cells. Hence an assessment of iNKT cells in CFS/ME may provide insight into relationship between iNKT cells and other cytotoxic cells in subgroups of CFS/ME patients.

1.3.9 B Cells

B cells are lymphocytes of the adaptive immune system and are precursors of plasma cells characterised by the presence of a BCR. B cells perform a number of

immunological functions, including: antibody production, supporting T cell differentiation and cytokine secretion (Mauri & Bosma, 2012; Vaughan et al., 2011).

B cells originate from pluripotent HSCs and progress from pre-B, pre-pro-B, early pro-B, late pro-B, pre-B, immature B and mature B cells. These stages are distinguishable based on distinct surface phenotypes (LeBien & Tedder, 2008; Vaughan et al., 2011). IgM_s and IgD are anchored in B cell membranes through a C-terminal domain of the IgH chain, allowing them to function as antigen-specific receptors (LeBien & Tedder, 2008). During the process of development from precursor to a mature B cell, there are a number of phenotypic changes that distinguish the stages of development (Hardy & Hayakawa, 2001; LeBien & Tedder, 2008; Vaughan et al., 2011).

1.3.9.1 B Cell Phenotypes

Naïve B cell survival is dependent on BCRs and the receptor for B cell activating factor (BAFF) (Cabatingan et al., 2002). The identification of naïve B cells can be determined based on the positive expression of IgM, IgD and negative expression of CD38 and CD27 (Baumgarth et al., 2005; Jackson et al., 2008). CD81 is cell-surface tetraspanin expressed on human B cells, known to form a costimulatory complex with CD19 and CD21. Costimulation of BCRs with this complex lowers the threshold involved in BCR-mediated B cell proliferation, consequently enhancing proliferation of naïve B cells. Hence, the expression of CD81 can also be used as a measure of naïve B cell activation (Reichardt et al., 2007; Rosa et al., 2005). Naïve B cells have the ability to undergo negative selection so that self-reactive B cells are removed (Vaughan et al., 2011).

Memory B cells are effective during recurring antigen pathogenesis because memory B cells lack effector function and require the recurrence of an antigen before memory is formed (Yoshida et al., 2010). Evidence suggests that there is substantial heterogeneity in the surface phenotype of memory B cells. Surface markers expressed on memory B cells can include CD38, CD21, CD24, CD19, B220, CD80, CD86, CD95 FcRH4 and CD25 and these cells lack IgD and CD38 expression (Jackson et al., 2008; Sanz et al., 2008; Yoshida et al., 2010). Functions of memory B cells involve enhanced specific antigen presentation, cytokine production, isotype switching, affinity maturation and lymphoid tissue organisation. Once activated, memory B cells can also differentiate into plasma B cells (Yoshida et al., 2010).

Once activated, B cells enter the spleen and lymph nodes and develop into antibody-secreting plasmablasts and plasma B cells (Calame, 2001). Plasma B cells, or effector B cells, are large B cells formed to secrete a number of antibodies to assist the destruction of microbes (Jackson et al., 2008). Plasma B cells reside mainly in organs or the bone marrow, representing less than 1% of all cells in lymphoid organs and are responsible for all antibody secretion (Fairfax et al., 2008; Minges Wols, 2006). B lymphocyte-induced maturation protein-1 (Blimp-1) is a transcriptional repressor and regulator of plasma cell development. Plasma cells express CD138, CD44, CXCR4 and very late antigen-4 (VLA-4). CD138 binds to fibronectin, collagen and basic fibroblast growth factors. Plasma B cells secrete immunoglobulins approximately 8-10 days after an immune response is activated. During the primary immune response, low-affinity antibodies are secreted as an early defence mechanism against immunogens. When a secondary immune response occurs, plasma B cells have a greater affinity for the antigen and hence a greater response (Minges Wols, 2006). The antibodies secreted bind

to the microbes and allow them to be targeted by phagocytes as well as activating the complement system (Janeway et al., 2001).

B regulatory cells (Bregs) are a functional subset of B cells that terminate excess inflammatory responses, in particular during autoimmune diseases (Mauri & Bosma, 2012). Human CD19⁺ Bregs express high levels of CD1d and may be identified as CD1d^{hi}CD21^{hi}CD23^{lo}CD24^{hi}IgM^{hi}IgD^{lo} Bregs (Mauri & Bosma, 2012). Breg maturation, antibody and cytokine production occurs following CD40 cross-linking by a CD40 ligand (CD154) on CD4⁺T cells (Bouaziz et al., 2008; Mauri & Bosma, 2012; Ozaki et al., 2004). Bregs express TLRs which stimulate B cells to promote cytokine production when engaged. Upon activation, Bregs predominantly function by secreting IL-10 to inhibit pro-inflammatory cytokines and support the differentiation of Tregs (Bouaziz et al., 2008; Chen et al., 2003; Mauri & Bosma, 2012). IL-10 and costimulatory molecules are necessary for the differentiation and regulation of Tregs and the inhibition of Th17 and Th1 cell lineages, highlighting the role of B cells in T cell maintenance in the periphery (Bouaziz et al., 2008; Mauri & Bosma, 2012).

1.3.9.2 B Cells in CFS/ME

In an illness such as CFS/ME where recurring pathogen infection may occur it is important to assess B cell memory as this is important in ensuring rapid elimination of pathogens during secondary exposure to antigens (Mauri & Bosma, 2012). A number of studies have confirmed adverse variation in B cells in CFS/ME patients, although a B cell inhibitor has successfully been used to treat CFS/ME patients in a small study

(Fluge et al., 2011). Hence, this suggests potential B cell perturbations in CFS/ME patients and highlights the importance examining B cell subsets in this illness.

B cell numbers in some CFS/ME patients are elevated compared with non-fatigued healthy controls (Klimas et al., 1990; Patarca-Montero et al., 2001). In particular, CD20⁺CD21⁺ and CD20⁺CD5⁺ B cells were highly expressed in CFS/ME patients (Klimas et al., 1990). An increase in CD20⁺CD21⁺ and CD20⁺CD5⁺ B cells are indicative of elevations in the inflammatory response and activation of B cells respectively (LeBien & Tedder, 2008), this may explain the persistence of CFS/ME symptoms and the duration of the illness. Increases in B cell activation and B cell production of pro-inflammatory cytokines may play a role in chronic inflammation, which is often experienced by CFS/ME patients (Gupta et al., 2013; Klimas et al., 1990; Patarca-Montero et al., 2001). Evidence of this potential increase in B cell activation in CFS/ME patients was demonstrated when a B cell inhibitor, Rituximab, significantly improved CFS/ME patients clinical responses and self-rated levels of fatigue when given to patients as a treatment (Fluge et al., 2011).

The regulation of B cell activity is also controlled by T cells, as well as NK cells. Importantly, CFS/ME patients may demonstrate low CD4⁺ CD45RA⁺T inflammatory cell subsets as well as reduced NK cell cytotoxic activity, which may promote an overreactive B cell activation in CFS/ME (Abruzzo & Rowley, 1983). It is unknown whether these atypical B cell presentations in CFS/ME are subject to illness severity hence further studies may be required to confirm evidence for hyper B cell activation in severe CFS/ME patients.

1.4 SUMMARY OF LITERATURE

Despite the plethora of immune investigations on CFS/ME, the most consistent finding is the reduction of NK cell cytotoxic activity in CFS/ME patients (Brenu et al., 2010; Caligiuri et al., 1987; Klimas et al., 1990; Lloyd et al., 1989). Fluctuations in NK cell phenotypes also demonstrate immune dysfunction in the illness (Brenu et al., 2011). Increases in FOXP3 expression in Tregs suggest an enhanced suppression of pro-inflammatory cytokines and a potential suppression of cytotoxic activity in resting NK cells (Ralainirina et al., 2007). A decreased respiratory burst in neutrophils is symbolic of a reduced ability of CFS/ME patients to completely degrade internalised particles, potentially indicating that other phagocytic cells, such as monocytes, may display a similar pattern of phagocytosis in CFS/ME patients. While monocyte studies are inconsistent in CFS/ME, prospective increases in monocyte adhesion in CFS/ME may confirm an increase in pathogen presence in the illness (Gupta & Vayuvegula, 1991b; Macedo et al., 2007; Rains & Jain, 2011). Inconsistent alterations in cytokines in CFS/ME may be related to illness severity and should be investigated to determine the profile of cytokines in CFS/ME patients of varying severities (Brenu et al., 2012c; Broderick et al., 2010; Fletcher et al., 2009). Similarly, some CFS/ME patients may have increases in B cell numbers and B cell inhibition has proven successful in reducing symptoms of the illness in some patients (Fluge et al., 2011).

The innate and adaptive immune systems are constantly engaged in cellular interactions that result in cell proliferation, cytokine secretion, pathogen clearance and immunological homeostasis. Hence, aberrations in either the innate or adaptive components may have severe consequences on physiological processes and health. In

CFS/ME, compromises to the innate cell function may interfere with adaptive immune cell processes, which may translate into shifts in cytokine patterns that are either anti-inflammatory or pro-inflammatory. Additionally, deficiencies in antigen presentation may affect recruitment of adaptive immune cells to sites of infection, while persistent increases in suppression may suggest an increase in viral load and an inability to clear pathogens. Considerable reductions in important immune processes, such as cytotoxic activity and phagocytosis have been shown in CFS/ME patients; therefore further research is required to determine the extent of the immune perturbations in the illness, particularly in both moderate and severe CFS/ME patients.

Prior to this thesis research, there were no studies examining innate and adaptive immune cells in moderate and severe subgroups of CFS/ME patients. Therefore, an examination of innate and adaptive immune components in this illness will benefit the international knowledge of CFS/ME and potentially assist in determining the pathomechanism of the illness.

1.5 SIGNIFICANCE

CFS/ME is a severely debilitating multifactorial illness that affects large numbers of people worldwide, typically women are more highly affected than men (Fletcher et al., 2010; Reeves et al., 2007). The prevalence rate for CFS/ME varies in studies, ranging from 0.02% to as high as 6.41% (Johnston et al., 2013), with around 0.0371% in Australia (Lloyd et al., 1990). Of those diagnosed with CFS/ME, approximately 83% of patients report gradual onset of the illness while 17% experience sudden onset, highlighting the incidence of different modes of CFS/ME and variability among patients (Reeves et al., 2007).

There are substantial costs associated with CFS/ME worldwide for both individuals affected by the illness and the government, as there is currently no known cure, successful treatments or a useful diagnostic method. In Australia, the estimated economic impact of CFS/ME based on the annual cost to the community per CFS/ME patient, at a prevalence of 0.2% relative to 2012, is \$729.3 million (Toulikidis, 2002). This represents an extensive loss in economic funds and a significant financial burden to individuals, their families and the community (Lloyd & Pender, 1992; Toulikidis, 2002).

It is acknowledged that patients with CFS/ME may display variations in symptom severity (Brenu et al., 2013a; Carruthers et al., 2011; Strayer et al., 2012; Wiborg et al., 2010b). In CFS/ME, immunological dysfunction has been reported, including: reduced NK cell cytotoxic activity and neutrophil respiratory burst, aberrant Tregs and cytokines. A B cell inhibitor has shown potential as a treatment for the illness (Brenu et al., 2011; Fletcher et al., 2009; Fluge et al., 2011; Klimas et al., 1990). Alterations in

cell numbers and function may affect cellular interactions between innate and adaptive immune cells. This demonstrates potential for immune abnormalities in innate cells, such as NK cells, antigen presenting cells, such as DCs and monocytes, as well as other immune cells, including subtypes of T cells. It is possible that the extent of immune dysfunction may be associated with variations in symptom severity experienced among CFS/ME patients.

This research will provide insight into the immunological dysfunction in CFS/ME patients with varying degrees of severity. Importantly, this may determine whether patient symptom severity is associated with the level of immune perturbations experienced. The results from this research may also potentially assist in providing a basis for the development of a pathomechanism for CFS/ME by contributing to the knowledge of prospective biomarkers for the illness. Consequently, this may assist by contributing to reducing costs for CFS/ME patients in the future as the illness becomes more widely understood and management strategies may be implemented.

1.6 AIMS

Prior to this research, examinations of innate and adaptive immune systems in CFS/ME were limited by studies focusing on moderately affected CFS/ME patients. This study aimed to assess moderate and severely affected CFS/ME patients, primarily aiming to:

1. Provide an examination of innate immune cells, including: NK cells, neutrophils, monocytes and DCs in moderate and severe CFS/ME patients compared with a non-fatigued healthy control group.
2. Measure adaptive immune cells, including: T, NKT and B cells in moderate and severe CFS/ME patients, compared with a non-fatigued healthy control group.
3. Assess the potential role of immune cell phenotypes of NK cells, monocytes, DCs, T and B cells in moderate and severe CFS/ME.
4. Measure serum cytokines and immunoglobulins in moderate and severe CFS/ME patients compared with a non-fatigued healthy control group.
5. Longitudinally assess NK cell receptors, iNKT, DC and $\gamma\delta$ T, CD8⁺T cell and B cell phenotypes, Tregs, NK cell and CD8 T cell lytic proteins between moderate CFS/ME, severe CFS/ME and non-fatigued healthy controls.
6. Determine new immune parameters which had not previously been assessed and which may assist in the future development of potential biomarkers for CFS/ME.
7. Assist in enhancing the current research on CFS/ME by providing further knowledge into whether severe CFS/ME patients have the greatest immune perturbations or whether severe CFS/ME patients demonstrate a different immunological aetiology compared to moderate CFS/ME patients.

1.7 HYPOTHESES

The null hypotheses for this research are that between or among the moderate and severe CFS/ME patients and non-fatigued healthy control groups:

1. NK cell phenotypes, activity and receptors are not statistically different.
2. Monocyte and neutrophil function is not statistically different.
3. Phenotype expression of monocytes does not differ significantly.
4. DC phenotypes and functions are not statistically different.
5. T cell phenotypes do not vary significantly.
6. Phenotypes of $\gamma\delta$ T cells, iNKT cells and B cells do not significantly differ.
7. Serum cytokines and immunoglobulins are not altered significantly.
8. There are no significant changes in any immunological parameters over time.

CHAPTER 2: METHODOLOGY

2.1 PROJECT DESIGN

This research study was designed to extensively investigate the innate and adaptive immune system in CFS/ME patients of varying severities. Literature suggests that CFS/ME patients demonstrate immunological dysfunction although no studies had assessed immune parameters of the illness in relation to symptom severity. Hence, this study assessed a number of immunological aspects comparing moderate CFS/ME to severe CFS/ME patients and non-fatigued healthy controls.

This project contained two components as described below. The first project provided an analysis of innate and adaptive immune cell phenotypes, markers and function in controls, moderate and severe CFS/ME. This was the first study of its kind and also provided baseline (week 0) measures for the subsequent project. Serum analysis was also conducted following this study for an assessment of serum proteins between the participant groups.

In the second project, participants were invited for a six month follow up blood sample to provide a longitudinal analysis of immune parameters. This part of the project also investigated immune function and receptor analysis which had not been previously assessed. Further details of these projects are discussed in the sections that follow. The specific methods used in each study are outlined in a methods section within each project.

2.1.1 Ethical Clearance

This research was reviewed by the Griffith University Human Research Ethics Committee and was granted approval to proceed (GU Ref No: MSC/23/12/HREC). All participants in this study were provided with the participant information and provided signed consent as approved by the Griffith University Human Research Ethics Committee.

2.2 PARTICIPANT RECRUITMENT

Participants recruited for the study were located in Northern New South Wales or Southern Queensland in Australia and aged between 20 and 65 years of age. Recruitment strategies included contacting CFS/ME support groups, sending email advertisements and via social media. The recruitment of non-fatigued healthy controls was often assisted by participants inviting relatives, spouses or friends to also take part in the research or staff working in the vicinity of collection sites. Participants interested in volunteering in the study contacted the investigator via telephone or email and were provided with an overview of the respective study, explaining requirements and outcomes of their participation.

Prior to the study, participant details were obtained, including: name, gender, date of birth and home address for those in the severe CFS/ME patient group. 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 1994 Fukuda criterion for CFS/ME.

Moderate CFS/ME and non-fatigued healthy control participants visited a location in Northern New South Wales or South East Queensland that was most convenient to them to participate in the research, either Tweed Heads Hospital Pathology, National Centre for Neuroimmunology and Emerging Diseases (NCNED) at Griffith University, Logan Hospital Pathology or Royal Brisbane Women's Hospital Pathology. Severe CFS/ME participants have limited mobility and were housebound; therefore they were visited in their home by the investigator, a phlebotomist and a general practitioner (GP).

Upon the visit, all participants were taken through participant information (Appendix 1) by the investigator and were given the opportunity to ask any questions before signing the informed consent form (Appendix 2). A short questionnaire was undertaken in person with all participants (Appendix 3) and then an online link was provided for a questionnaire (Appendix 4). The questionnaires were used to assess symptomatology, health status, quality of life, severity and mobility in all participants. A qualified phlebotomist collected blood from all participants at each time point. At the baseline (week 0) collection, blood pressure, temperature and pulse were measured by the GP.

After all participants had their appointments and data analysed, they were contacted as a follow up to allow them to provide feedback on their experience participating in the study. Individual routine full blood count results were compiled and emailed with an accompanying information letter to all participants for their records. Baseline (week 0) participants were then contacted and given the opportunity to participate in the six

month (week 24) follow up study, which was conducted in the same way as the baseline (week 0) collection, although without collecting blood pressure, temperature and pulse. Some original participants were not able to participate in the six month (week 24) follow up. Reasons for this included moving out of the city, availability and work commitments and recovery from CFS/ME. Participants excluded in the baseline (week 0) study were not invited for the six month (week 24) follow up.

2.2.1 Participant Questionnaire

Questionnaires used in the research were important for analysis of participants' individual conditions and assisted in segregating severity groups for the study. The questionnaire that was undertaken in person with participants included scales to assess the activity of the person in the week leading to the blood collection (Appendix 3). The FSS, KPS and Dr Bell's CFS Disability Scale described previously were included in this questionnaire, allowing analysis to confirm control, moderate CFS/ME and severe CFS/ME participant groups (Appendix 3).

The online questionnaire included 4 sections (A-D) (Appendix 4). Section A included questions of participant background information, including: date of birth, gender, location, questions about the onset of CFS/ME and questions directly associating with exclusion criteria. Section B of the online questionnaire asked participants questions about the presence, frequency and severity of any symptoms. In this section non-fatigued healthy controls were able to ignore questions unless they experienced symptoms and questions also allowed all participants to be assessed based on both the 1994 Fukuda and ICC criteria for CFS/ME. Section C of the questionnaire was the SF-

36 scale, described previously, this scale was used to analyse the overall health of all participants. The final section D of the questionnaire was the WHO Disability Assessment Schedule (DAS) 2.0 scales (Appendix 4), included questions about the previous 30 days prior to the blood collection to capture their functional ability in relation to their cognition, mobility, self-care, getting along, life activities and participation (Üstün, 2010). The online questionnaire data was used for both immunology and epidemiology research components for a number of research studies unrelated to this project.

2.3 LABORATORY METHODS

The project design consisted of a number of laboratory methods, including: assessment of whole blood phenotypes, PBMC isolation, intracellular Treg and lytic proteins and isolated NK cell phenotypes and receptors, outlined below. All specific methods and antibody combinations are then described in each project paper.

2.3.1 Sample Preparation and Routine Blood Characteristics

All blood samples were non-fasting. A maximum of 85mL was collected from the antecubital vein of participant's arm, filling lithium heparinised, Ethylenediaminetetraacetic acid (EDTA), serum-separating tubes (SST) and/or erythrocyte sedimentation rate (ESR) tubes. Blood collections occurred between 8am and 11:30am, and samples were analysed within 12 hours of blood collection. Initial full

blood count assessments were undertaken by Queensland Pathology to determine levels of white and red blood cell markers as well as inflammatory markers.

2.3.2 Whole Blood Phenotype Analysis

Whole blood analyses were undertaken using EDTA or lithium heparin whole blood depending on the cell types. Monoclonal antibodies (Becton Dickinson (BD) Pharminigen, San Diego, CA; Miltenyi Biotec, Bergisch-Gladbach, Germany) were added to whole blood and incubated for 30 minutes in the dark at room temperature. Blood was then diluted with FACS lysing solution (BD Biosciences, San Diego, California, CA) at a volume of 1mL lysing solution to 50 μ L whole blood and solution was then incubated in the dark at room temperature for 15 minutes. Solution was then washed twice with 2mL phosphate buffered saline (PBS) (Gibco Biocult, Scotland) before stabilising fixative (BD Biosciences, San Diego, CA) for analysis on the flow cytometer (BD Immunocytometry Systems).

2.3.3 Isolation of PBMCs

Isolation of PBMCs from whole blood was required for some further analyses using density gradient centrifugation using Ficoll-hypaque (Sigma, St Louis, Missouri, MO). Whole blood was diluted at a ratio of 1:2 with PBS and layered over a 1:1 whole blood ratio to Ficoll-hypaque and centrifuged for 30 minutes at 400 relative centrifugation force (rcf). After the centrifugation, four layers are formed in the tube, plasma with PBS, PBMCs, Ficoll-hypaque and red blood cells respectively from top to bottom. Using a transfer pipette, the PBMC layer was transferred into a new tube and washed

twice with 20mL and 10mL of PBS respectively. Typically PBMCs were then counted and ready for further assessments.

2.3.4 Intracellular Analysis

Intracellular analysis required PBMCs that had been isolated using density gradient centrifugation with Ficoll-hypaque as described previously. PBMC concentration was adjusted to 1×10^7 cells/mL and was then stained with monoclonal antibodies for Treg phenotypes, or lytic proteins as necessary. For Treg analysis, PBMCs were then permeabilised and fixed with buffers containing diethylene glycol and formaldehyde (BD Biosciences, San Diego, CA) before being stained with FOXP3 (BD Pharmingen, San Diego, CA). Cells were then washed with PBS and resuspended in stabilising fixative before being analysed on the flow cytometer. For lytic protein analysis, cells were incubated in Cytotfix (BD Biosciences, San Diego, CA) for 30 minutes then permwash (BD Biosciences, San Diego, CA) was added. Perforin, granzyme A and granzyme B (BD Pharmingen, San Diego, CA) were the lytic proteins measured in this research; therefore the appropriate monoclonal antibodies were added to the cells and incubated for 30 minutes in the dark at room temperature. Cells were then washed again with PBS and analysed on the flow cytometer.

2.3.5 Isolated Natural Killer Cell Phenotypes and Receptors

NK cell isolation for phenotype and receptor analysis was conducted using a negative selection system with RosetteSep Human Natural Killer Cell Enrichment Cocktail (StemCell Technologies, Vancouver, British Columbia, BC). Lithium heparinised

whole blood was added to RosetteSep with a volume of 1mL whole blood to 50µL RosetteSep and incubated in the dark at room temperature for 25 minutes. Blood solution was then diluted with PBS at a ratio of 1:2 and layered over a 1:1 whole blood ratio to Ficoll-hypaque before being centrifuged for 30 minutes at 400rcf. After the centrifugation, the NK cell layer was transferred into a new tube and washed with PBS. NK cells were then isolated ready for the addition of appropriate monoclonal antibodies (BD Pharminigen, San Diego, CA) for NK cell phenotype or receptor analysis. Cells with the monoclonal antibodies were incubated for 30 minutes in the dark at room temperature then fixed with stabilising fixative before being analysed on the flow cytometer.

CHAPTER 3: PROJECT ONE: ANALYSIS OF THE RELATIONSHIP BETWEEN IMMUNE DYSFUNCTION AND SYMPTOM SEVERITY IN PATIENTS WITH CHRONIC FATIGUE SYNDROME/MYALGIC ENCEPHALOMYELITIS (CFS/ME)

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Kaur, M., Ramos, S., Salajegheh, A., Staines, DR., Marshall-Gradisnik, S. 2014. Analysis of the relationship between immune dysfunction and symptom severity in patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). *Journal of Clinical and Cellular Immunology*, 5(1), 190.

Author contributions:

Hardcastle was the principle contributor to this manuscript. Hardcastle was responsible for contributions to study design, acquisition of data, analysis and interpretation of data and primary drafting of the manuscript. Brenu, Johnston, Nguyen, Huth, Kaur, Ramos and Salajegheh contributed to acquisition of data and manuscript revisions. Brenu, Staines and Marshall-Gradisnik contributed to the study conception and design, interpretation of data and critical manuscript revisions.

3.1 ABSTRACT

Objective: 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 1994 Fukuda Criterion for CFS/ME. Participants were grouped into non-fatigued 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, DCs, iNKT, Treg, B, $\gamma\delta$ and CD8⁺T cell phenotypes, NK cell cytotoxic activity and receptors.

Results: The present data found that CFS/ME patients demonstrated significant decreases in NK cell cytotoxic activity, transitional and Bregs, $\gamma\delta 1$ T cells, KIR2DL1/DS1, CD94⁺ and KIR2DL2/L3. Significant increases in CD56⁻CD16⁺ NK cells, CD56^{dim}CD16⁻ and CD56^{bright}CD16^{-/dim} NK cells, 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 naïve B cells, total iNKT, iNKT cell and NK cell phenotypes compared with 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.

3.2 INTRODUCTION

CFS/ME is a severe physically and cognitively incapacitating illness diagnosed by symptom-specific criteria (Carruthers et al., 2011; Fukuda et al., 1994a; Jason et al., 2005). CFS/ME presents as a multifactorial illness that varies greatly in the nature of onset and severity of symptom presentation (Baraniuk et al., 2013; Carruthers et al., 2011; Fukuda et al., 1994a; Zaturenskaya et al., 2009). A key characteristic is debilitating fatigue that lasts for a period of six or more months where patient's daily activities are critically affected (Carruthers et al., 2003; Carruthers et al., 2011; Fukuda et al., 1994a; Jason et al., 2005). 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 (Wiborg et al., 2010b).

Currently, there is no known cause for CFS/ME although research has demonstrated consistent immunological dysfunction associated with the illness (Barker et al., 1994; Brenu et al., 2011; Klimas et al., 1990; Ngonga & Ricevuti, 2009; Patarca-Montero et al., 2001). We have previously been the only research group to have examined NK cell cytotoxic activity, phenotype and receptors in housebound severe patients in comparison with a non-fatigued healthy control group (Brenu et al., 2013a). Housebound severe patients had significantly reduced NK cell cytotoxic activity when compared with the moderately affected patients and there was an increase in the KIR3DL1 in the moderate patients, highlighting that differing levels of severity may also have varying levels of immune perturbation (Brenu et al., 2013a).

The most consistent immunological finding in CFS/ME is significantly reduced NK cell cytotoxic activity (Brenu et al., 2013a; Brenu et al., 2012c; Klimas & Koneru, 2007; Klimas et al., 1990). This study is one of the first to assess those housebound and severe CFS/ME patients in comparison with 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, DCs) and adaptive ($\gamma\delta$, iNKT, CD8⁺T cells, B cells) immune cells to compare moderate and severe CFS/ME patients.

3.3 METHODS

3.3.1 Ethical Clearance

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

3.3.2 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 six months prior to the study and questionnaires were used to define

CFS/ME using the Fukuda criterion for CFS. The 1994 Fukuda 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 Fukuda in conjunction with an extensive questionnaire to assess symptomatology, 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 Fukuda criterion 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 healthy control group (n=18). Those in the severe CFS/ME group were housebound and displayed significantly worsened symptoms. The FSS, Dr Bell's Disability Scale, the FibroFatigue Scale and the KPS, were used in the questionnaire as a determinant of severity.

3.3.3 Sample Preparation and Routine Measures

A non-fasting blood sample of 50mL was collected from the antecubital vein of participants into lithium heparinised and EDTA tubes. Blood was collected between 8:30am and 11:30am 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.

3.3.4 Natural Killer Cell Cytotoxic Activity Analysis

NK cell cytotoxic activity was performed as described previously (Brenu et al., 2013a; Brenu et al., 2011). Density gradient centrifugation using Ficoll-hypaque (Sigma, St Louis, MO) was used to isolate 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-Fluorescein isothiocyanate (FITC) and 7-aminoactinomycin D (AAD) reagents (BD Biosciences, San Diego, CA) and the ability of NK cells to lyse target K562 cells was measured on the flow cytometer (BD Immunocytometry Systems).

3.3.5 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 Treg phenotypes, NK cell lytic proteins and CD8 lytic proteins (Appendix 5) as described by Brenu et al (Brenu et al., 2013a; Brenu et al., 2011). 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 PBS (Gibco Biocult, Scotland), cells were analysed on the flow cytometer (BD Immunocytometry Systems) where the expression of FOXP3⁺

Tregs was determined on CD4⁺CD25⁺CD127^{low} T cells (Brenu et al., 2011). NK and CD8 T cell lytic proteins were assessed as previously described (Brenu et al., 2011). Cells were incubated for 30 minutes in Cytofix then Permash 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.

3.3.6 Natural Killer Cell 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 (Appendix 5) (Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells were analysed on the flow cytometer (BD Immunocytometry Systems). NK cells were gated using CD56, CD16 and CD3 (Appendix 9) and KIR receptors were analysed based on their appropriate antibodies (Appendix 5) (Brenu et al., 2013a). NK cell phenotypes CD56^{dim}CD16⁺, CD56^{dim}CD16⁻, CD56^{bright}CD16^{-/dim} were assessed (Appendix 9).

3.3.7 Whole Blood Analysis

Appropriate antibodies (Appendix 5) 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 (Appendix 5) and gating strategies on the flow cytometer (Appendix 6, 7, 10).

3.3.8 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, SLAM (signalling lymphocytic activation molecule), CD56 and CD16 (Appendix 5) (BD Biosciences, San Diego, CA). Cells were fixed with stabilising fixative for analysis on the flow cytometer (Appendix 8) (Montoya et al., 2007).

3.3.9 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) or plus/minus the standard deviation (SD). 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 (Aguinis et al., 2013).

Outliers were handled by eliminating particular data points from the analysis (Aguinis et al., 2013).

3.4 RESULTS

3.4.1 Participants

Data were available for a total of 63 participants (22 controls, 23 moderate CFS/ME and 18 severe CFS/ME patients). The mean ages for the control, moderate and severe CFS/ME patients 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 Fukuda criterion 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 2). The three participant groups consisted of predominantly females with the control, moderate and severe CFS/ME patients having 64%, 70% and 83% female participants respectively, this was not statistically different between groups (Table 2).

Table 2: Participant characteristics and comparisons of the age (mean \pm SD) and gender distribution of each participant group (control, moderate and severe).

Parameter	Control (n=22)	Moderate (n=23)	Severe (n=18)	<i>p</i> value
Age in years (Mean \pm SD)	40.14 \pm 2.38	42.52 \pm 2.63	39.56 \pm 1.51	0.631
Gender Female, Male	14, 8	16, 7	15, 3	0.391

*Age data is represented as Mean \pm SD 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.*

Blood pressure, pulse, temperature and routine full blood counts were measured in all participants (Table 3). There were no statistically significant differences in any clinical or routine full blood count parameters between the participant groups.

Table 3: Clinical and full blood count results for the control, moderate and severe CFS/ME participant groups.

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 (x10⁹/L)	5.73 ± 0.26	6.17 ± 0.36	6.60 ± 0.52	0.289
Platelets (x10⁹/L)	237.91 ± 10.34	250.57 ± 9.72	248.17 ± 18.16	0.741
Haematocrit	0.41 ± 0.01	0.41 ± 0.01	0.40 ± 0.01	0.398
Red Cell Count (x10⁹/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 (x10⁹/L)	3.31 ± 0.22	3.58 ± 0.27	3.88 ± 0.32	0.351
Lymphocytes (x10⁹/L)	1.86 ± 0.12	2.05 ± 0.13	2.13 ± 0.21	0.277
Monocytes (x10⁹/L)	0.40 ± 0.2	0.35 ± 0.02	0.40 ± 0.04	0.371
Eosinophils (x10⁹/L)	0.14 ± 0.02	0.16 ± 0.02	0.16 ± 0.03	0.754
Basophils (x10⁹/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) patients. * signifies p <0.05 between participant groups.*

3.4.2 Reduced Natural Killer Cell Cytotoxic Activity

In all three effector cell target ratios (12.5:1, 25:1, 50:1), there was a significant reduction in cytotoxic activity of NK cells in moderate ($p < 0.0011$, 0.000, 0.017) and severe CFS/ME participants ($p < 0.0010$, 0.000, 0.001) (Figure 5A). 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 5A). NK cell 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 ($p < 0.01$) (Appendix 8).

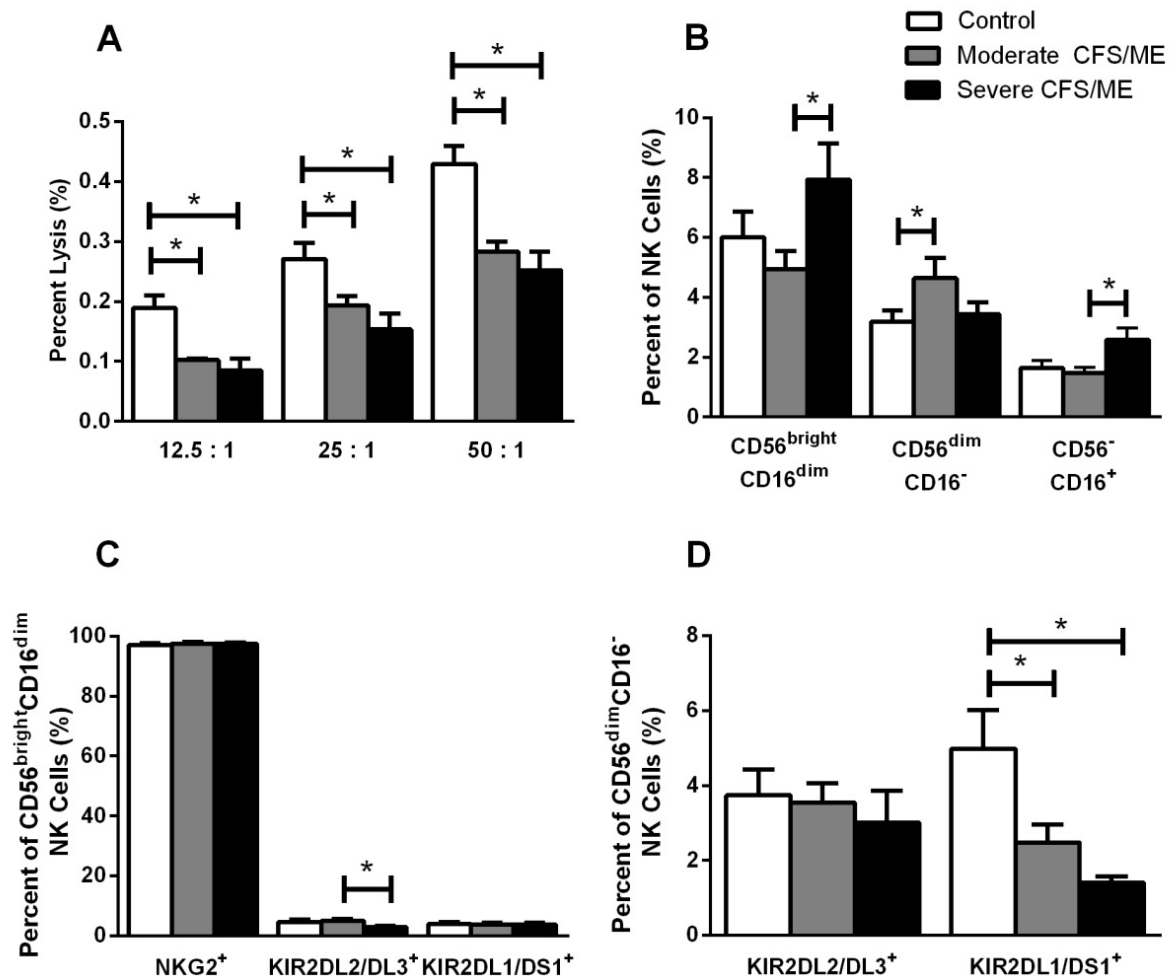


Figure 5: The profile of NK cells in control, moderate and severe CFS/ME patients.

A NK 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^{dim}CD16⁺, CD56⁻CD16⁺, CD56^{dim}CD16⁻ and CD56^{bright}CD16^{-/dim}) presented as a percentage of total NK cells. C 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. D CD56^{dim}CD16⁻ NK cells expressing the KIR receptors CD94⁺, KIR2DL2/DL3 and KIR2DL1/DS1 are

represented as a percentage of CD56^{dim}CD16⁻ NK cells. All data is represented as mean±SEM. * represents results that were significantly different where $p < 0.05$.

3.4.3 No Differences to Lytic Proteins in CD8⁺T Cells and Natural Killer 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).

3.4.4 Increased Natural Killer Cell Phenotypes and Reduced KIR Receptors in CFS/ME

There was a significant increase in the number of CD56^{bright}CD16^{-/dim} and CD56⁻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 ($p = 0.045$) (Figure 5B, C). There was also a significant reduction in the percentage of CD56^{bright}CD16^{-/dim} KIR2DL1/DL2⁺ NK cells in the severe CFS/ME group compared with the moderate CFS/ME group ($p = 0.027$) (Figure 5D). Moderate CFS/ME patients had significantly reduced CD56^{dim}CD16⁻ KIR2DL1/DS1⁺ NK cell expression compared with the control group ($p < 0.0013, 0.004$). There was also a significant increase in the expression of CD94 on CD56^{dim}CD16⁻ NK cells in both moderate ($p = 0.042$) and severe ($p < 0.0017$) CFS/ME patients (Figure 5E, F). CD56^{dim}CD16⁻ KIR2DL1/DS1⁺ 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 ($p < 0.01$) (Appendix 11).

3.4.5 Whole Blood Phenotypes

pDCs were significantly higher in the moderate CFS/ME group ($p < 0.0012$). 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 ($p < 0.0010$) and control group ($p < 0.0010$) (Figure 6A). 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 Bregs in the severe CFS/ME participants compared with the control CFS/ME participants ($p = 0.047, 0.041$) (Figure 6B). 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 ($p < 0.0017$ and 0.018). $\gamma\delta 1$ T cells (cells/ μ L) with the phenotype CD45RA⁻CD27⁻ were also significantly reduced in the moderate CFS/ME group ($p = 0.0142$) (Figure 6C, 2D). $\gamma\delta 2$ T cells (cells/ μ L) were not significantly different between the groups (data not shown).

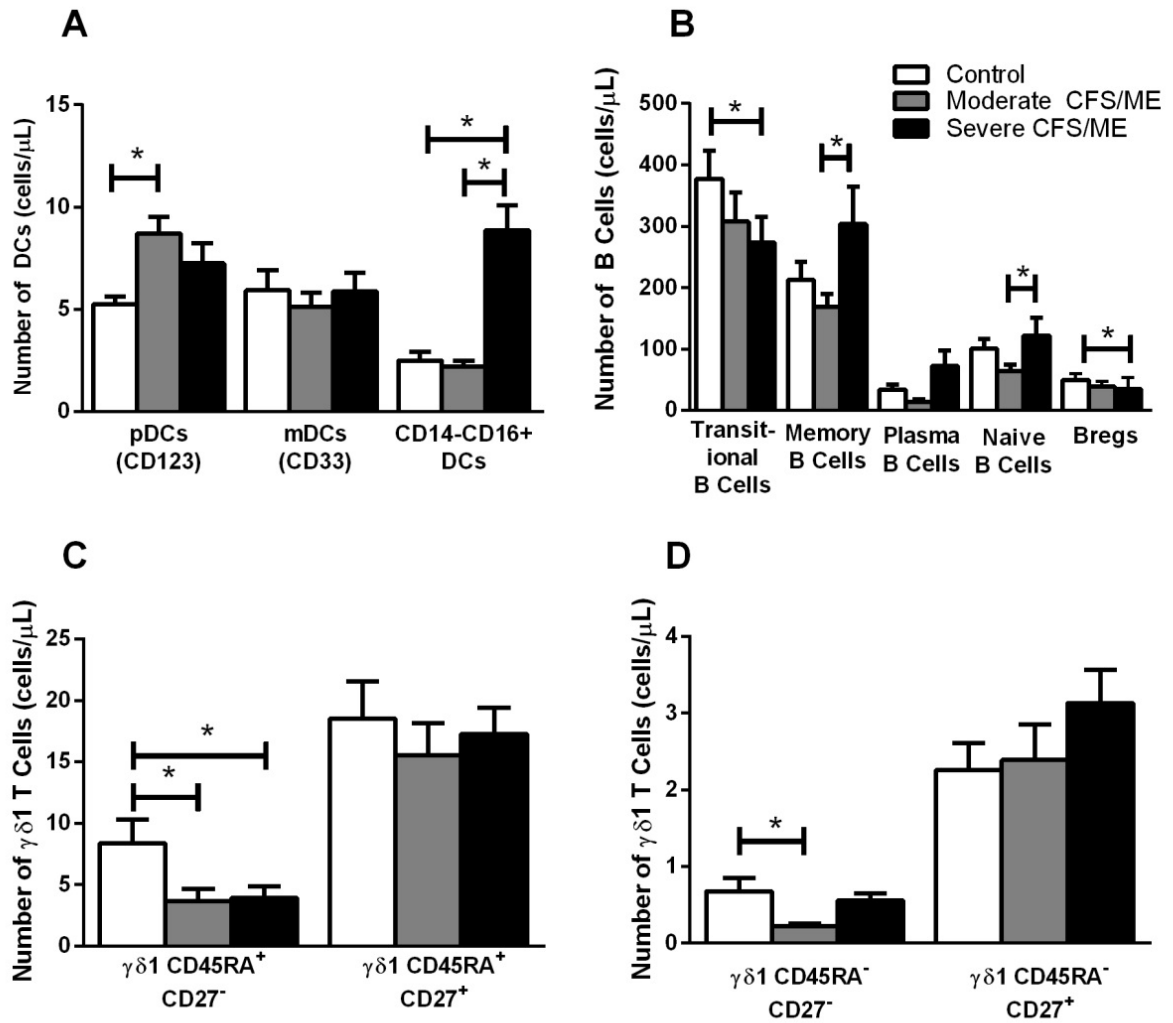


Figure 6: Alterations in DC, B and $\gamma\delta 1$ T cell phenotypes in control, moderate and severe CFS/ME participant groups.

A DC phenotypes, including: pDCs, mDCs and $CD14^+CD16^+$ DCs where data is represented as the total number of cells (cells/ μ L). *B* B cell phenotypes (memory, plasma, naive, transitional and Bregs) in control, moderate and severe CFS/ME groups represented as total number of cells (cells/ μ L). *C* $\gamma\delta 1$ T cell $CD45RA^+CD27^-$ and $CD45RA^+CD27^+$ phenotypes are represented as total number of cells (cells/ μ L). *D* $\gamma\delta 1$ T cell phenotypes $CD45RA^-CD27^-$ and $CD45RA^-CD27^+$ are represented as total number of cells (cells/ μ L). Data are represented as mean \pm SEM. * represents results that were significantly different where $p < 0.05$.

There were no significant differences in total neutrophil numbers, CD177^{bright} or CD177^{dim} neutrophils, true monocytes, pro-inflammatory, 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).

3.4.6 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 ($p < 0.0014$) (Figure 7A). 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 ($p = 0.031, 0.026, 0.047$) (Figure 7B, 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 7D). 6B11⁺CD3⁺CCR7⁻SLAMF⁻ 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⁻SLAMF⁺ iNKT cells were also significantly increased in the severe CFS/ME group compared with controls ($p = 0.012$) (Figure 7E,F). iNKT cell markers were significantly correlated in a positive manner with subsequent iNKT cell markers and subsets ($p < 0.01$) (Appendix 12).

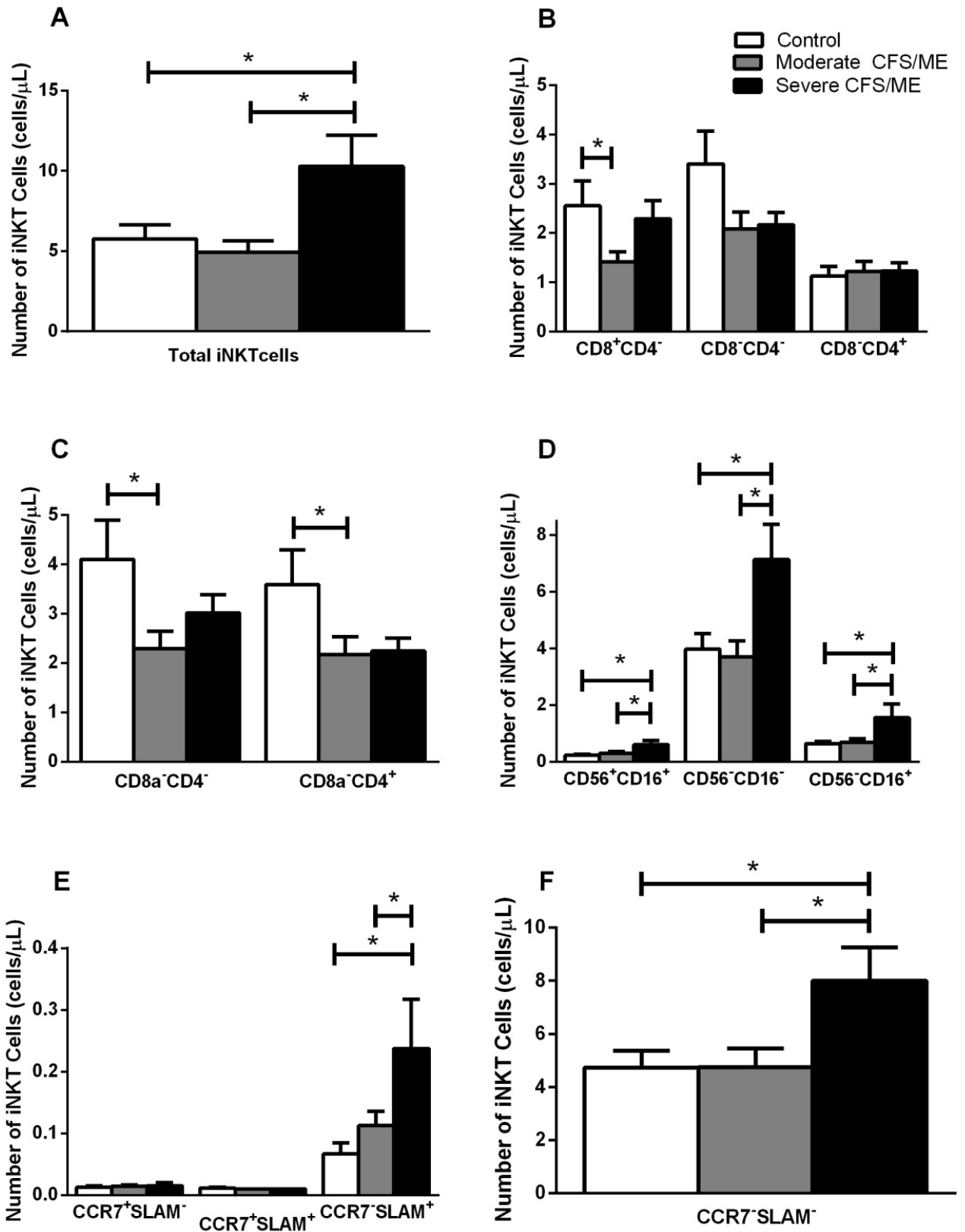


Figure 7: Perturbations in iNKT cell phenotypes and receptors in control, moderate and severe CFS/ME patients.

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 CCR7SLAM⁺) 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 $p < 0.05$.

3.5 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 with 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 (Brenu et al., 2013a; Brenu et al., 2010; Fletcher et al., 2009; Klimas & Koneru, 2007; Lloyd et al., 1989) however only one other study has examined immune parameters in moderate and severe CFS/ME patients (Brenu et al., 2013a).

This investigation confirmed previous findings that NK cell cytotoxic activity is consistently reduced in CFS/ME patients (Brenu et al., 2013a; Brenu et al., 2012c; Klimas et al., 2012; Patarca-Montero et al., 2001). 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 (Maher et al., 2005; Saiki et al., 2008). 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 (Lanier, 2005; Morrison et al., 1991; Tirelli et al., 1994). This study also confirmed increases CD56^{bright}CD16^{-/dim} and CD56⁻CD16⁺ NK cells in CFS/ME (Morrison et al., 1991; Tirelli et al., 1994). CD56^{dim} and CD56^{bright} NK cell phenotypes are primarily responsible for cytotoxic activity and cytokine release respectively. (Cooper et al., 2001a). 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 cytotoxic activity (Cooper et al., 2001a). The increase in CD56^{bright} NK cells may serve as a regulatory mechanism to improve the reduced NK cell cytotoxic activity in CFS/ME. This thesis 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 cell cytotoxic activity and increased NK cell phenotypes in CFS/ME patients is unknown although potential genetic defects may affect NK cell function in CFS/ME (Orange, 2002).

KIR2DS1 is an activating NK cell receptor which promotes NK cell cytotoxic activity in a HLA class 1 dependent manner (Stewart et al., 2005). In psoriasis, the *KIR2DS1* gene is a strong predictor of disease where psoriasis patients demonstrate reduced

KIR2DS1 genetic frequency (Płoski et al., 2006). 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 (Lanier, 2005; Płoski et al., 2006; Stewart et al., 2005; Yen et al., 2001). Similarly, reductions in the corresponding inhibitory receptors *KIR2DL1* and *KIR2DL2/DL3* in CFS/ME may trigger reduced alloreactive KIR NK cell cytotoxic activity (Kulkarni et al., 2008; Oliviero et al., 2009; Płoski et al., 2006; Stewart et al., 2005; Vitale et al., 2004). Thus, changes in KIR receptors may be an important component in the CFS/ME illness mechanism. In previous studies *KIR3DL1* was increased in CFS/ME patients, however this finding was not confirmed, which may be related to the heterogeneity of CFS/ME (Brenu et al., 2013a).

CD94 is a cytokine induced receptor complex on NK cells, which can either activate or inhibit cell-mediated cytotoxic activity in NK cells, dependent on NKG2 protein association which recruits SHP-1 tyrosine phosphatase and couples to tyrosine kinase (Liao et al., 2006). CD94 NK cell receptors recognise HLA-E which presents HLA class 1 molecule-derived peptides and inhibits NK cell-mediated lysis (Gumá et al., 2006). 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 reduces overall NK cell cytotoxic activity (Lanier, 2005; Tomasec et al., 2000).

iNKT cells regulate disease conditions, including type I diabetes and cancer via communication with a number of innate and adaptive immune cells (Lee et al., 2002; Mars et al., 2004). 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 (Mars et al., 2004). 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 (Godfrey et al., 2000) and may be related to an enhanced cell-mediated regulation of immunity in these patients (Mars et al., 2004). 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 induces cytotoxic activity (Cava & Kaer, 2006; Mars et al., 2004; Yamamura et al., 2007; Zeng et al., 2013). 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 (Yamamura et al., 2007).

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 (Campbell et al., 2001; Ohl et al., 2004). SLAM is a surface receptor essential for iNKT cell development and the production of cytokines, particularly IFN- γ (Baev et al., 2008; Hu et al., 2011; Quiroga et al., 2004). Increased iNKT CCR7⁻SLAM⁻ and reduced CCR7⁻SLAM⁺ expression in both moderate and severe CFS/ME compared with controls may be associated with a reduced ability of iNKT cells to interact and activate DC and T cells during an immune response (Baev et al., 2008). Circulating iNKT cells also have a variable expression of the NK cell markers, CD56 and CD16, although little is known about the functional significance of these markers on the surface of iNKT cells (Montoya et al., 2007). 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 (Chen et al., 2007).

pDCs are also responsible for modulating NK, T and B cell immune responses through antigen presentation and the release of cytokines and chemokines (Colonna et al., 2004). 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 (Tomescu et al., 2010). 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 cell cytotoxic activity may highlight a reduced efficiency in cell-cell cross talk and immune dysregulation in CFS/ME (Tomescu et al., 2010). Elevated pDCs may also be linked to pDCs having an increased ability to become readily infected than mDCs, as found in Human immunodeficiency virus (HIV). This could potentially explain why there were no significant difference between mDC phenotypes in controls, moderate or severe CFS/ME patients (Patterson et al., 2001). Another DC phenotype, cytokine-producing CD14⁻CD16⁺ DCs were also significantly increased in the severe CFS/ME patients compared with both controls and the moderate CFS/ME subgroup, potentially suggesting dysfunction in the secretion of inflammatory cytokines. This supports previous studies which have shown IL4, IL-10 and IL-12, primarily produced by CD14⁻CD16⁺ DCs are found to be increased in CFS/ME (Henriques et al., 2012; Nakamura et al., 2010; Patarca, 2001; Piccioli et al., 2007; Skowera et al., 2004). These cytokines are important in the neutralisation of the

Th1/Th2 cytokine shift which is also altered in CFS/ME patients (Henriques et al., 2012; Piccioli et al., 2007).

$\gamma\delta$ T cells are sentinel cytotoxic cells involved in the elimination of bacterial infection, delayed-type hypersensitivity reactions, wound repair, antigen presentation and immunoregulation (Anane et al., 2010). 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 (Anane et al., 2010). 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 cell cytotoxic activity in the illness as these cells are similar to NK cells.

It has been previously suggested that B cell activation may be increased in CFS/ME (Fluge et al., 2011). The increased naïve and memory B cells shown in severe CFS/ME compared with 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 (Bradley et al., 2013). 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 (Hutloff et al., 2004). 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 (Chung et al., 2003; Palanichamy et al., 2009). 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) (Agrawal et al., 2013; Lemoine et al., 2009). 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 (Visser et al., 1998).

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 RA (Baugh et al., 2002; Libraty et al., 2002; Vaughn et al., 2000).

3.6 CONCLUSION

This thesis 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 cell 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.

CHAPTER 4: PROJECT TWO: SERUM IMMUNE PROTEINS IN MODERATE AND SEVERE CHRONIC FATIGUE SYNDROME/MYALGIC ENCEPHELOMYELITIS (CFS/ME) PATIENTS

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Ramos, S., Staines, D and Marshall-Gradisnik, S. 2015. Serum immune proteins in moderate and severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients. *International Journal of Medical Sciences*, 12(10), 764-772

Author contributions:

Hardcastle was the principle contributor to this manuscript. Hardcastle was responsible for contributions to study design, acquisition of data, analysis and interpretation of data and primary drafting of the manuscript. Brenu, Johnston, Nguyen, Huth and Ramos contributed to acquisition of data and manuscript revisions. Brenu, Staines and Marshall-Gradisnik contributed to the study conception and design, interpretation of data and critical manuscript revisions.

4.1 ABSTRACT

Immunological dysregulation is present in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME), with recent studies also highlighting the importance of examining symptom severity. This research addressed this relationship between CFS/ME severity subgroups, assessing serum immunoglobulins and serum cytokines in severe and moderate CFS/ME patients. Participants included healthy controls (n= 22), moderately (n = 22) and severely (n=19) affected CFS/ME patients. The 1994 Fukuda Criteria defined CFS/ME and severity scales confirmed mobile and housebound CFS/ME patients as moderate and severe respectively. IL-1 β was significantly reduced in severe compared with moderate CFS/ME patients. IL-6 was significantly decreased in moderate CFS/ME patients compared with healthy controls and severe CFS/ME patients. RANTES was significantly increased in moderate CFS/ME patients compared to severe CFS/ME patients. Serum IL-7 and IL-8 were significantly higher in the severe CFS/ME group compared with healthy controls and moderate CFS/ME patients. IFN- γ was significantly increased in severe CFS/ME patients compared with moderately affected patients. This was the first study to show cytokine variation in moderate and severe CFS/ME patients, with significant differences shown between CFS/ME symptom severity groups. This research suggests that distinguishing severity subgroups in CFS/ME research settings may allow for a more stringent analysis of the heterogeneous and otherwise inconsistent illness.

4.2 INTRODUCTION

Immunological dysregulation can be caused or influenced by changes in serum immune protein levels, which play a key role in living cells by allowing specific interactions with other molecules (Pace et al., 2004). Many studies have assessed the levels of cytokines and immunoglobulin's in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) although even with conflicting and inconsistent results, this may suggest potential shifts in immune regulations in the illness (Klimas et al., 1990).

CFS/ME is a severely debilitating illness which causes patients to experience both physical and cognitive impairment alongside a range of accompanying symptoms (Carruthers et al., 2011; Fukuda et al., 1994a). CFS/ME has an unknown aetiology, with diagnosis made in accordance with symptom-specific criteria (Carruthers et al., 2011). While the pathomechanism of CFS/ME is unknown, the presence of immunological dysregulation is consistent in the illness; however validation is required as there are often inconsistencies found between studies. Studies have shown that CFS/ME patients demonstrate reduced natural killer (NK) cell cytotoxic activity (Barker et al., 1994; Brenu et al., 2011; Hardcastle et al., 2014a; Klimas et al., 1990; Ngonga & Ricevuti, 2009; Patarca-Montero et al., 2001), altered dendritic cell (DC) phenotypes (Hardcastle et al., 2014a), decreased CD8⁺T cell activity (Brenu et al., 2011) and potential B cell activation (Fluge et al., 2011). Compromises to innate immune cell functioning can interfere with, or reflect, adaptive processes and translate into shifts in cytokine patterns that are either pro- or anti-inflammatory (Harrington et al., 2006).

Cytokine imbalances in peripheral blood cells may have implications for physiological and psychological functions, with altered cytokine secretions impacting on endothelial, cognitive and cardiovascular body systems (Hill et al., 1997; McAfoose & Baune, 2009; Ziccardi et al., 2002). There are inconsistent alterations in cytokine patterns in CFS/ME for instance some studies demonstrated elevated IL-1 β (interleukin 1 beta), IL-4, IL-5 and IL-6 and reduced IL-8, while other research found no changes to cytokines in the illness (Lyall et al., 2003). NK cell dysfunction, namely reduced cytotoxic activity, is consistent in CFS/ME, therefore NK cell derived cytokines, such as IFN- γ , may demonstrate altered immunity patterns and associated cytokine shifts in the illness (Broderick et al., 2010; Chao et al., 1991; Fletcher et al., 2009; Klimas et al., 1990; Lauwerys et al., 2000; Lyall et al., 2003; Natelson et al., 2002; Patarca, 2001; Skowera et al., 2004).

Cytokine and/or immunoglobulin levels are often altered in diseased states, consequently leading to physiological symptoms (Maes et al., 2012). Immunoglobulin levels have also been inconsistently varied in CFS/ME patients (Neuberger, 2008; Schroeder Jr & Cavacini, 2010). Most studies demonstrate no change to immunoglobulins in CFS/ME although some studies have found low IgM, IgA and IgG levels in CFS/ME patients (Klimas et al., 1990; Lyall et al., 2003). Immunoglobulin levels may also be linked to the reduced NK activity shown in CFS/ME as intravenous immunoglobulin (IVIg) treatments have altered NK cell activity in immune-mediated diseases, such as Kawasaki disease, dermatomyositis and MS. Suppression of NK cell cytotoxic activity is also mediated by the antigen binding portion F(ab)₂ of immunoglobulin and hence IVIg infusion also improves antibody-dependent cell-mediated cytotoxicity (ADCC) (Kwak et al., 1996; Tha-In et al., 2008).

A clinically distinct housebound group of CFS/ME patients recently displayed reductions in NK cell cytotoxic activity, increased CD14⁺CD16⁺ DCs and altered B and iNKT cell phenotypes when compared with moderately affected patients, highlighting the importance of assessing severity subgroups in the illness (Hardcastle et al., 2014a). In an attempt to control for heterogeneity within a CFS/ME patient cohort, defining CFS/ME patients according to specific clinical characteristics allows for further analysis of the illness (Hornig et al., 2015). Symptom severity may be related to the extent of immune dysfunction found in CFS/ME patients, placing importance on the recognition of severity subgroups to allow an extensive analysis of the illness. Cytokine imbalances may be related to disease pathologies and therefore should also be assessed in severity subgroups of CFS/ME patients.

This study was the first to examine serum cytokines and immunoglobulins in severe CFS/ME patients in comparison to moderate CFS/ME patients and healthy controls. The purpose of this study was to further examine the relationship between CFS/ME severity subgroups by assessing IgA, IgM, IgG2, IgG4 and IgG_{Total} immunoglobulins and inflammatory cytokines in moderate and severe CFS/ME patients.

4.3 METHODS

4.3.1 Ethical Clearance

The Griffith University Human Research Ethics Committee granted ethical approval for this study after an extensive review (GU Ref No: MSC/23/12/HREC).

4.3.2 Participant Recruitment

All participants were aged between 20 and 65 years old and were recruited using CFS/ME support groups, social media, email advertisements and an expression of interest database. All those to be included in the study were briefed and provided written informed consent prior to participating.

All CFS/ME patients included in the research had the illness for at least 6 months prior to the study and were previously diagnosed with CFS/ME by a primary physician. The application of the 1994 Fukuda diagnostic criteria was then used in combination with an extensive questionnaire delineating patient's diagnosis history and symptoms. A General Practitioner also conducted clinical assessments on all research participants, including: patient symptoms, blood pressure, temperature and heart rate. Participants were subjected to stringent exclusion criteria to eliminate confounding co-morbidities or conditions that may influence the immunological data or the participant's illness state. Participants were excluded from the research if they had been diagnosed with an autoimmune, heart or thyroid-related disorder, psychosis, major depression, breast feeding, pregnant, a smoker, if they were taking strong hormone-related medications or if they experienced symptoms of CFS/ME but did not meet the 1994 Fukuda criteria for the illness.

The CFS/ME patients were classified as either mobile or housebound and these severities were translated to 'moderate' and 'severe' subgroups respectively. Moderate CFS/ME patients were those who were mobile, initially identified as those who were able to regularly leave the house unassisted and who had the potential to maintain a job,

even with reduced hours. Severe CFS/ME patients were those who were initially identified as housebound, unable to sustain a job due to the constraints of their symptoms and those who were not able to leave the house unassisted. These severity subgroups were then confirmed through the use of an extensive questionnaire containing routinely used severity scales in CFS/ME: the Fatigue Severity Scale (FSS), Dr Bell's Disability Scale, the FibroFatigue Scale and the Karnofsky Performance Scale (KPS) (Hardcastle et al., 2014a). CFS/ME patients who were severely affected by their symptoms were visited in their homes by a team with a mobile qualified phlebotomist and a General Practitioner. Moderate CFS/ME patients and healthy controls participated in the research at a designated collection site where they were met by the General Practitioner and qualified phlebotomist. There were 63 participants included in the study, consisting of: age and gender matched healthy controls (n=22), moderate CFS/ME (n=22) and severe CFS/ME (n=19) patients.

4.3.3 Sample Preparation

A morning non-fasting blood sample was collected from the antecubital vein of participants into serum separating tubes (SST). All participants' blood was collected between 8:30am and 11:30am and sample was kept in a cooler box until serum was obtained from centrifugation and snap frozen within 5 hours of the initial collection. Pathology Queensland also conducted an initial full blood count assessment on each participant to ensure that patients were within the ranges for the whole blood count parameters.

4.3.4 Immunoglobulin Analysis

Concentrations of the immunoglobulins IgA, IgM, IgG₂, IgG₄ and IgG_{Total} from stored SST serum were measured by flow cytometry using a BD CBA Human Immunoglobulin Flex Set system (BD Biosciences, San Diego, CA) as per manufacturer's instructions. FCap Array software (BD Biosciences, San Diego, CA) was then used for the construction of standard curves and for analysis of flow cytometric data.

4.3.5 Cytokine Analysis

Stored SST serum was thawed and a Bio-Plex Pro human cytokine 27-plex immunoassay kit (Bio-Rad Laboratories Inc, Hercules, CA) was used for the detection of inflammatory cytokines. Cytokines detected in the kit included IL-1 β , IL-1ra (interleukin 1 receptor agonist), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17, FGF (fibroblast growth factor), eotaxin (CCL11), G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte macrophage colony-stimulating factor), IFN- γ , IP-10 (interferon gamma-induced protein 10, CXCL10), PDGF-BB (platelet-derived growth factor-BB), RANTES (regulated on activation, normal T cell expressed and secreted, CCL5), TNF- α (tumor necrosis factor alpha), MCP1 (monocyte chemotactic protein 1), MIP1a (macrophage inflammatory protein alpha), MIP1b (macrophage inflammatory protein beta), and VEGF (vascular endothelial growth factor). The kit used magnetic beads to simultaneously detect cytokine levels and data was obtained in duplicates using the Bio-Plex system in

combination with Bio-Plex Manager software (Bio-Rad Laboratories Inc, Hercules, CA).

4.3.6 Data and Statistical Analysis

Statistical analyses were performed using SPSS statistical software version 22.0. All experimental data represented in this study are reported as plus/minus the standard error of the mean (\pm SEM) or plus/minus the standard deviation (SD) as specified. Comparative assessments among the three participant groups (control, moderate CFS/ME and severe CFS/ME patients) were performed using the Kruskal Wallis test of independent variables based on rank sums to determine the magnitude of differences between groups when parameters were not normally distributed. Normally distributed parameters were compared between groups using the ANOVA. The Mann-Whitney U or least significant difference (LSD) post hoc tests were used for nonparametric or parametric data respectively to determine significant differences between the groups where p values of statistical significance were set at an alpha criterion at $p < 0.05$. Spearman's correlation was conducted on parameters to determine correlates where statistical significance was accepted as $p < 0.01$. Outliers were identified using a boxplot technique on SPSS software where extreme outliers were highlighted if they presented beyond the plot's whiskers (Aguinis et al., 2013). Extreme outliers were identified as points beyond an outer fence, defined as the lower quartile - 1.5 x interquartile range (IQ) or the upper quartile plus 3 x IQ. These extreme outliers were then handled by eliminating particular data points from the analysis (Aguinis et al., 2013).

4.4 RESULTS

4.4.1 Participants

Data were available for 63 participants in total, including: 22 healthy controls, 22 moderate CFS/ME and 19 severe CFS/ME patients. The mean (\pm standard deviation) ages for the control, moderate CFS/ME and severe CFS/ME patients were 40.14 ± 2.38 , 42.09 ± 2.72 and 40.21 ± 1.57 respectively. There was no statistical difference in age or gender between participant groups ($p=0.598$, 0.324 respectively) (Table 4). The 1994 Fukuda criteria for CFS/ME were satisfied by all moderate and severe CFS/ME participants.

Severity scales used included: activity/ability level based on percentage of optimal functioning (0-100%), the FibroFatigue Scale, the FSS, the KPS and Dr Bell's Disability Scale. For all scales, there were statistically significant differences between all participant groups, with moderate and then severe CFS/ME participant groups displayed significantly worsened scores respectively (Table 4). There were no statistically significant differences between moderate and severe CFS/ME patient groups years since CFS/ME diagnosis, estimated visits to a general practitioner in the past 12 months or number of days in the past 30 days where symptoms presented difficulties for the patient (Table 4). Severe CFS/ME patients had a significantly increased number of days out of the 30 days prior to participation where 'usual' activities were not able to be carried out due to symptoms (Table 4).

There were no statistically significant differences in blood pressure, pulse, temperature or routine full blood count parameters between participants groups (data not shown).

Table 4: Participant and clinical characteristics for each participant group (control, moderate and severe).

	Control	Moderate CFS/ME patients	Severe CFS/ME patients	p Value between groups
Participant Characteristics				
Age in years (mean ± SD)	40.14 ± 11.17	42.09 ± 12.75	40.21 ± 6.84	0.598
Gender (Female, Male)	14, 8	15, 7	16, 3	0.324
CFS/ME Patient Clinical Data				
Years since CFS/ME diagnosis	NA	9.00 ± 8.870	13.071 ± 6.639	0.087
Estimated number of visits to a General Practitioner in the last 12 months	NA	10.12 ± 5.264	5.214 ± 4.263	0.174
Number of days in the past 30 days where symptoms/difficulties were present	NA	26.31 ± 8.396	29.50 ± 1.401	0.100
Number of days in the past 30 days where usual activities were not able to be carried out due to symptoms	NA	7.69 ± 8.875	14.57 ± 10.733	0.011
Activity/Ability Level Rating (0-100%)				
Typical good day	98.89 ± 3.333	60.45 ± 19.390	28.18 ± 14.013	0.000
Typical bad day	90.00 ± 11.180	25.00 ± 14.720	10.00 ± 0.000	0.000
Day of participation	97.78 ± 6.667	50.45 ± 19.875	14.55 ± 5.222	0.000
FibroFatigue Scale - Symptoms				
Fatigue	0.00 ± 0.000	3.32 ± 1.912	5.64 ± 0.674	0.000
Memory	0.11 ± 0.333	3.00 ± 1.718	3.00 ± 1.549	0.000
Concentration	0.00 ± 0.000	3.23 ± 1.572	3.73 ± 1.348	0.000
Sleep	0.56 ± 0.882	3.32 ± 1.555	4.00 ± 2.191	0.000
Headaches	0.00 ± 0.000	2.45 ± 1.920	3.36 ± 1.567	0.000
Pain	0.00 ± 0.000	3.32 ± 1.615	4.18 ± 1.471	0.000
Muscle Pain	0.11 ± 0.333	3.59 ± 1.652	4.36 ± 1.567	0.000
Infection	0.11 ± 0.333	2.50 ± 2.064	3.45 ± 1.753	0.000
Bowel	0.11 ± 0.333	1.86 ± 1.935	2.91 ± 2.071	0.000
Autonomic	0.00 ± 0.000	3.14 ± 1.612	2.73 ± 2.149	0.000
Irritability	0.00 ± 0.000	2.36 ± 1.649	1.55 ± 1.864	0.000
Sadness	0.00 ± 0.000	1.73 ± 1.778	1.36 ± 1.690	0.001
Severity Scales				
Fatigue Severity Scale	10.12 ± 1.219	52.32 ± 10.403	59.64 ± 2.649	0.000
Karnofsky Performance Scale	100.00 ± 0.000	72.27 ± 9.726	52.73 ± 12.721	0.000
Dr Bells Disability Scale	100.00 ± 0.000	49.09 ± 21.582	20.00 ± 10.000	0.000

Age data is represented as Mean \pm SD in control (n=22), moderate CFS/ME (n=22) and severe CFS/ME (n=19). *p* values were determined using the Kruskal-Wallis Test. * signifies *p* <0.05 between participant groups. There were no significant differences in age or gender within the research groups.

4.4.2 No significant differences to Immunoglobulins

Serum immunoglobulins, IgA, IgM, IgG2, IgG4 and IgG_{Total} were not significantly different between the control, moderate and severe CFS/ME groups (data not shown).

4.4.3 Cytokine Analyses

IL-1 β was positively correlated with IL-6 according to Spearman's Bivariate Correlation where *p* < 0.01. There was a significant increase in IL-1 β in moderate CFS/ME compared with severe CFS/ME (*p*=0.002) (Figure 8) and IL-6 was also significantly decreased in the moderate CFS/ME group compared with the healthy controls and severe CFS/ME group (*p*<0.001 and 0.001 respectively). IL-7 and IL-8 cytokines were significantly increased in the severe CFS/ME group compared with healthy controls and moderate CFS/ME (*p*<0.001, 0.001 and *p*=0.001, 0.001 respectively) (Figure 8). IL-7 was positively correlated with IFN- γ (*p* < 0.01) and IFN- γ was significantly increased in severe CFS/ME compared with the moderately affected CFS/ME group (*p*=0.025) (Figure 9). RANTES was significantly increased in moderate CFS/ME compared with healthy controls and severe CFS/ME (*p*=0.009 and 0.012 respectively) (Figure 9).

There was no statistical significance found between any of the groups in the cytokines IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, FGF, eotaxin, G-CSF, GM-CSF, IP-10, PDGF-BB, TNF- α and VEGF (Data not shown).

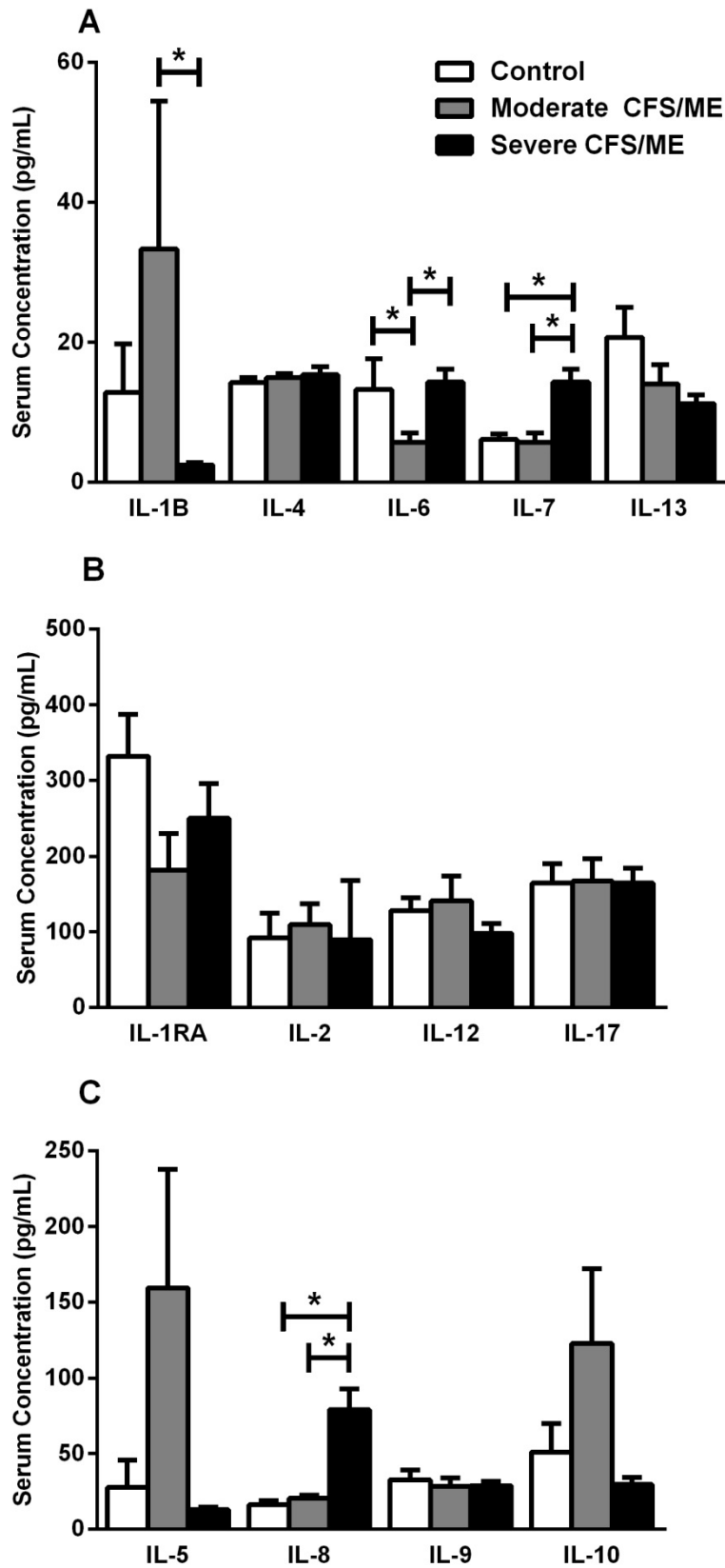


Figure 8: The profile of serum interleukin levels in control, moderate and severe CFS/ME.

*A IL-1 β , IL-4, IL-6, IL-7 and IL-13 serum concentrations where data is presented as serum concentration (pg/mL). B IL-1RA, IL-2, IL-12 and IL-17 serum concentrations where data is presented as serum concentration (pg/mL). C IL-5, IL-8, IL-9 and IL-10 serum concentrations where data is presented as serum concentration (pg/mL). All data is represented as mean \pm SEM. * represents results that were significantly different where $p < 0.05$.*

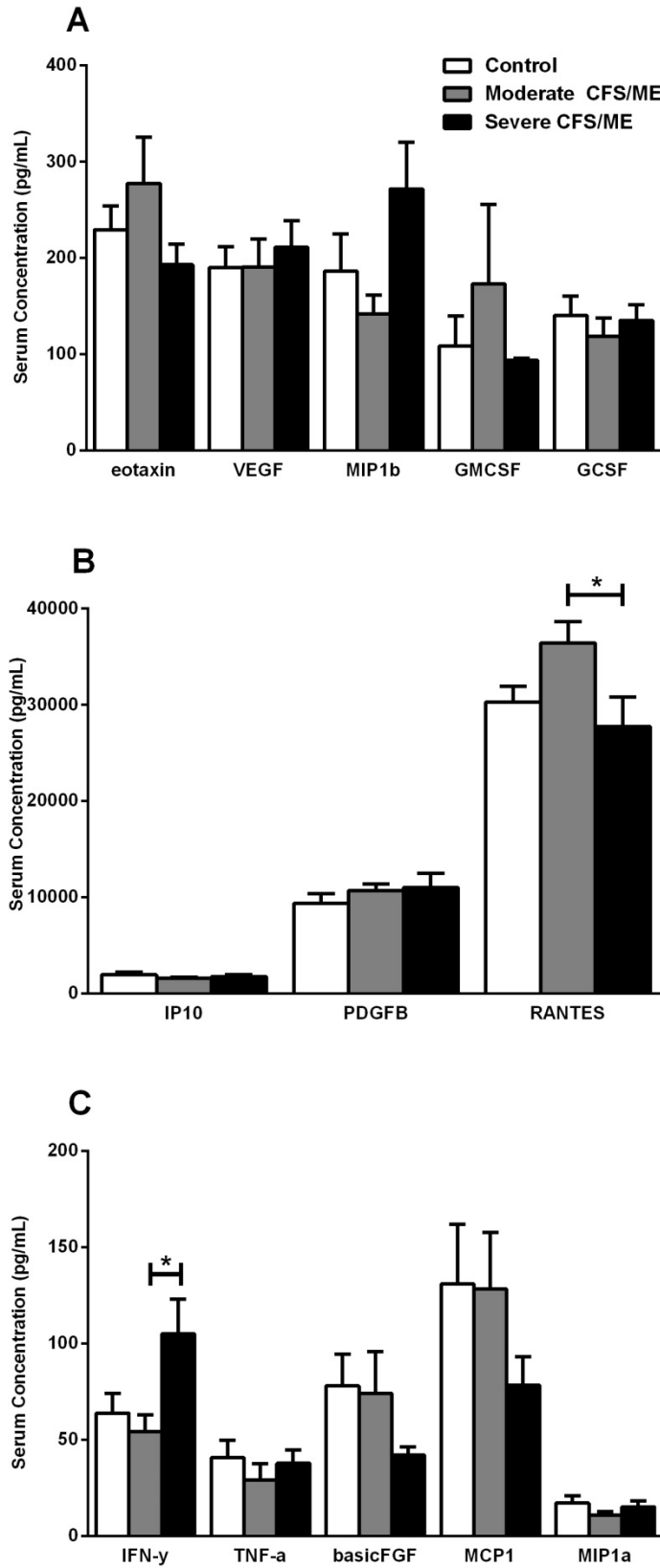


Figure 9: Serum cytokine levels in control, moderate and severe CFS/ME participant groups.

*A Eotaxin, VEGF, MIP1b, GM-CSF and G-CSF serum concentrations where data is presented as serum concentration (pg/mL). B IP10, PDGF-BB and RANTES serum concentrations where data is presented as serum concentration (pg/mL). C IFN- γ , TNF- α , basicFGF, MCP1 and MIP1a serum concentrations where data is presented as serum concentration (pg/mL). Data are represented as mean \pm SEM. * represents results that were significantly different where $p < 0.05$.*

4.5 DISCUSSION

This research was the first to assess immunoglobulins and inflammatory cytokines in severe CFS/ME patients compared with moderate CFS/ME patients and healthy controls. There were statistically significant differences in symptom severity and physical activities between controls, moderate and severe CFS/ME patients. According to severity scales, those in the severe CFS/ME patient group displayed the worst and most disabling symptoms compared to the moderate CFS/ME patients. Prior to this research, cytokine abnormalities and inconsistently altered immunoglobulin concentrations have been commonly found in both plasma and serum studies of CFS/ME (Broderick et al., 2010; Chao et al., 1991; Patarca, 2001; Skowera et al., 2004). However, literature had only assessed CFS/ME patients who were moderately affected by symptoms, leaving out those who are severely affected and subsequently housebound.

Cytokines and chemokines play a major role in many inflammatory diseases (Evereklioglu et al., 2002; Vivier et al., 2011). NK cell cytotoxic activity reflects a balance between activating and inhibitory signals which may be disturbed in CFS/ME

patients as they demonstrate consistently reduced NK cell cytotoxic activity compared to healthy controls (Brenu et al., 2012c; Hardcastle et al., 2014a; Klimas et al., 1990; Robertson, 2002). NK cell activation is triggered by inflammatory mediators, cytokines and chemokines, including: IL-2, IL-12, IL-15, IL-18 and RANTES, following recognition of stressed cells which leads NK cells to lyse target cells and secrete IFN- γ and TNF- α (Vivier et al., 2011). NK cells also have an influence on the generation of Th1 type pro-inflammatory responses, which consequently can result in a shift in the Th1/Th2 balance of cytokines. Previously, CFS/ME patients have shown evidence of a bias towards a Th2 immune response as IFN- γ and IL-4 were increased in CFS/ME patients compared with healthy controls (Skowera et al., 2004).

IFN- γ is secreted by T cells to form part of a Th1 immune response. The levels of IFN- γ in CFS/ME patients are inconsistent, with previous studies demonstrating a reduction of IFN- γ following mitogenic stimulation of PBMCs in the illness (Klimas et al., 1990; Visser et al., 1998), no difference in the levels of circulating plasma IFN- γ (Linde et al., 1992; Peakman et al., 1997; Visser et al., 1998) and increased production of IFN- γ by CD4⁺ and CD8⁺T cells (Skowera et al., 2004). NK-derived IFN- γ leads to the promotion of DC maturation and enhanced antigen presentation to T cells, hence improving or dampening T cell responses through IFN- γ (Robertson, 2002; Vivier et al., 2011). High amounts of IFN- γ are secreted by CD56^{bright} NK cells which exhibit weak cytotoxic activity (Robertson, 2002). Previously, plasma IFN- γ levels have significantly differed between CFS/ME patients based on illness duration, with increased plasma IFN- γ in patients who had a short illness duration (less than 3 years) (Hornig et al., 2015). The present study highlights that defining severity of CFS/ME may be important as the increase in serum IFN- γ in severe CFS/ME patients may be as a result of overactive

IFN- γ by CD56^{bright} NK cells as a mechanism to enhance the reduced NK cell cytotoxic activity in the illness (Patarca-Montero et al., 2001). This increased serum IFN- γ in severe CFS/ME patients may also be contributing to increased DC phenotypes, particularly CD14⁻CD16⁺ DCs which were increased in severe CFS/ME patients previously (Hardcastle et al., 2014a). Similarly, increased serum IFN- γ may promote heightened T cell responses, such as the increase in Tregs that is shown in CFS/ME patients (Brenu et al., 2013b).

IL-1 β is secreted by monocytes and tissue macrophages induced by Th1 cells and functions to regulate metabolic, immuno-inflammatory and reparative properties and it can be a mediator of some diseases (Chizzolini et al., 1997; Evereklioglu et al., 2002). IL-1 β gene polymorphisms may be a marker for the severity of joint destruction in rheumatoid arthritis (RA) (Buchs et al., 2001) as well as a determinant of disease course and severity in patients with inflammatory bowel disease, essentially contributing to the heterogeneity of the illness (Nemetz et al., 1999). IL-1 β is involved in the activation of T cells (Evereklioglu et al., 2002), a mechanism which is potentially defective in CFS/ME patients due to the suppression of early T cell activation and proliferation, possibly also contributing to the lowered inflammatory response in the illness (Maes et al., 2005). Increased serum IL-1 β in the moderate CFS/ME patients compared with severe CFS/ME patients suggests that patients experiencing moderate symptoms may have increased T cell activation as a result of enhanced serum IL-1 β compared with those who are more severely affected. Interestingly, severe CFS/ME patients' serum IL-1 β did not significantly differ from healthy controls, suggesting that in the case of severe CFS/ME, IL-1 β is not contributing to any deficiencies in T cell activation seen in CFS/ME. A previous study demonstrated significantly increased plasma IL-1 β in

CFS/ME patients with short illness duration (less than 3 years) when compared to CFS/ME patients with a greater illness duration (Hornig et al., 2015). The present study focused on illness severity, where increased cytokine abnormalities were reported in IFN- γ and IL-1 β , suggesting that illness severity and duration may play a role in cytokine profiles in CFS/ME.

IL-6 is part of the Th2 immune response and it synergizes with IL-1 in inflammatory reactions and may exacerbate the IL-1 β alterations demonstrated in CFS/ME (Patarca, 2001). There was a significant positive correlation between IL-6 and IL-1 β in this study. Increases in IL-1 β and IL-6 in the brain have been linked to 'sickness behaviours' that are exacerbated by pro-inflammatory cytokines and may be present in CFS/ME (Broderick et al., 2010; Vollmer-Conna et al., 2004).

IL-6 is a pro-inflammatory cytokine produced by monocytes, epithelial cells and fibroblasts. IL-6 plays a role in the regulation of immune responses, inducing cell proliferation and differentiation, promoting B cell activation and autoantibody production via T cell activation (Evereklioglu et al., 2002). Abnormal or overproduction of IL-6 has also been associated with some autoimmune diseases, such as MS, RA and experimental allergic encephalomyelitis (EAE) (Evans et al., 1998; Evereklioglu et al., 2002; Ishihara & Hirano, 2002; Kimura & Kishimoto, 2010). Increased IL-6 has been found in CFS/ME patients (Linde et al., 1992; Peakman et al., 1997), as well as elevated soluble IL-6 receptor (sIL-6R) which directly enhances the effects of IL-6 (Patarca et al., 1995). IL-6 is often linked with inflammation or increased inflammatory response (Evans et al., 1998) and acute infection is associated with elevated plasma concentrations of TNF, IL-6 and IL-8 (Evans et al., 1998). Increased IL-6 in severe

CFS/ME patients suggests that these patients may have an enhanced inflammatory response compared to those who have less severe symptoms. It has been speculated that increased IL-6 may influence the JAK-STAT3 signalling pathway or the molecular mechanism by which IL-6 regulates the Na⁺/K⁺ ATPase, suggesting that it may be beneficial to examine these pathways in CFS/ME patients (Ishihara & Hirano, 2002).

Chemokines are the largest family of cytokines and are critical to coordinating adaptive immune responses by binding to specific cell-surface receptors (Robertson, 2002). IL-8 (also known as CXCL8) is a chemokine and an important component of the pro-inflammatory immune response (Evans et al., 1998). Following activation, NK cells also produce increased amounts of IL-8 which may lead to the attraction of T cells, B cells and other NK cells (Robertson, 2002; Vivier et al., 2011). Increased serum IL-8 in severe CFS/ME patients in this research suggests that these patients may be experiencing a heightened pro-inflammatory immune response. Theoretically, increased IL-8 in severe CFS/ME patients may demonstrate increased NK, T and B cell recruitment although in CFS/ME, NK cells appear dysfunctional and there is often an imbalance in the activation of these cells (Barker et al., 1994; Brenu et al., 2012c; Hardcastle et al., 2014a; Maes et al., 2005).

IL-7 can also stimulate cytotoxic functioning in mature peripheral CD8⁺T cells, a function that appears to be reduced in CFS/ME patients (Maher et al., 2005). An increased serum IL-7 level in the severe CFS/ME patients may be related to an increase in T cell functioning or NK cell proliferation in the illness. T cell activation and cytotoxic activity have not been assessed in severe CFS/ME although moderate CFS/ME patients have previously demonstrated reduced numbers of T cells, including

both CD4⁺ and CD8⁺ subsets and decreased CD8⁺T cell activation (Curriu et al., 2013; Lloyd et al., 1989). Increased IL-7 should promote T cell function and NK cell proliferation in CFS/ME although both NK and T cell function have been reduced in the illness (Curriu et al., 2013; Lloyd et al., 1989). Therefore, it is possible that there is a defect downstream in the IL-7 pathway that prevents NK and T cell mechanisms in CFS/ME patients. IL-7 was also significantly and positively correlated to IFN- γ levels in the present study. Like IFN- γ , increased IL-7 in severely affected CFS/ME patients may be a response to the reduced NK cell cytotoxic activity typically associated with the illness (Hardcastle et al., 2014a), particularly because NK cell cytotoxic activity appears to be further reduced in severe CFS/ME patients who have higher levels of IFN- γ . It is also of interest that severe CFS/ME patients demonstrate increases in IFN- γ and IL-7, suggesting they may be involved in illness severity, as reported in other illnesses (Dengue infection) (Gunther et al., 2011; Ishihara & Hirano, 2002).

The present study found increased serum RANTES in moderate CFS/ME patients and reduced serum RANTES in severe CFS/ME patients. These findings may reflect patient diversity and heterogeneity in the illness. RANTES (or CCL5), is another inflammatory chemokine produced spontaneously by NK cells. RANTES is associated with lymphoid homing, activation of T cells and their apoptosis as well as resting migration, killing abilities and cytotoxic granule release by NK cells (Brenu et al., 2010; Curriu et al., 2013; Jonsson & Brun, 2010; Murooka et al., 2006; Robertson, 2002; Tyner et al., 2005; Vivier et al., 2011)..

Contrarily, the reduction in RANTES demonstrated in severe CFS/ME patients may suggest a decreased T cell activation, NK cell lysing abilities and granule release, as

shown in previous studies in moderate CFS/ME patients (Brenu et al., 2012c; Brenu et al., 2011; Hardcastle et al., 2014a; Klimas et al., 1990). These findings support previous studies which demonstrate further reduced NK cell cytotoxic activity in severe CFS/ME patients and perhaps highlights a link between some components of the immune response and the symptom presentation and severity experienced by patients (Brenu et al., 2013a; Hardcastle et al., 2014a).

In the current study, cytokine levels were marginally above those typically observed in human serum, however as CFS/ME is characterised by immune dysfunction, these elevated levels may represent illness severity presentation. The serum cytokine levels have not impacted the study as the statistical analysis conducted was used to identify differences between the groups. It is also acknowledged that some over-the-counter medications or vitamins were being taken by some participants at the time of this research and future studies with larger sample sizes may allow an analysis of specific medications or include a washout period so results are free from the influence of medication.

Further studies into severity subgroups in CFS/ME may be important to further explore the mechanisms behind such cytokine alterations. These cytokine changes in CFS/ME may be an indication of further immune dysregulation and the illness pathomechanism.

4.6 CONCLUSIONS

The present study is the first to assess serum immunoglobulin and cytokine levels in CFS/ME patients grouped according to symptom severity. All significant serum

cytokine differences shown were between moderate and severe CFS/ME patient groups. Interestingly, the moderate CFS/ME patient cohort displayed more extreme levels of some cytokines than the severe CFS/ME patient cohort, which did not significantly differ from healthy controls in IL-1, IL-6, IFN- γ and RANTES. On the other hand, the most severe clinical group had distinctively raised levels of IFN- γ , IL-7 and IL-8. Cytokine abnormalities have been associated with a number of diseases, particularly regarding severity and suggestive pathomechanism, highlighting the importance of the outcomes from the present study and the necessity for future expansions of this research.

Overall, the present study has demonstrated serum cytokine abnormalities in CFS/ME patients. These findings support the investigation of CFS/ME patient severity subgroups in both research and clinical settings. Further identification of different clinical groups in CFS/ME may influence diagnosis and development of therapeutic strategies in future.

CHAPTER 5: PROJECT THREE: CHARACTERISATION OF CELL FUNCTIONS AND RECEPTORS IN CHRONIC FATIGUE SYNDROME/MYALGIC ENCEPHALOMYELITIS (CFS/ME)

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Wong, N., Ramos, S., Staines, D and Marshall-Gradisnik, S. 2014. Characterisation of cell functions and receptors in moderate and severe Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). *BMC Immunology*, 16(35).]

Author contributions:

Hardcastle was the principle contributor to this manuscript. Hardcastle was responsible for contributions to study design, acquisition of data, analysis and interpretation of data and primary drafting of the manuscript. Johnston, Nguyen, Huth, Wong and Ramos contributed to acquisition of data and manuscript revisions. Brenu, Staines and Marshall-Gradisnik contributed to the study conception and design, interpretation of data and critical manuscript revisions.

5.1 ABSTRACT

Background: Abnormal immune function is often an underlying component of illness pathophysiology and symptom presentation. Functional and phenotypic immune-related alterations may play a role in the obscure pathomechanism of CFS/ME. The objective of this study was to investigate the functional ability of innate and adaptive immune cells in moderate and severe CFS/ME patients. The 1994 Fukuda criteria for CFS/ME were used to define CFS/ME patients. CFS/ME participants were grouped based on illness severity with 15 moderately affected (moderate) and 12 severely affected (severe) CFS/ME patients who were age and sex matched with 18 healthy controls. Flow cytometric protocols were used for immunological analysis of dendritic cells, monocytes and neutrophil function as well as measures of lytic proteins and T, NK and B cell receptors. **Results:** CFS/ME patients exhibited alterations in NK receptors and adhesion markers and receptors on CD4⁺T and CD8⁺T cells. Moderate CFS/ME patients had increased CD8⁺ CD45RA effector memory T cells, SLAM expression on NK cells, KIR2DL5⁺ on CD4⁺T cells and BTLA4⁺ on CD4⁺T central memory cells. Moderate CFS/ME patients also had reduced CD8⁺T central memory LFA-1, total CD8⁺T KLRG1, naïve CD4⁺T KLRG1 and CD56^{dim}CD16⁻ NK cell CD2⁺ and CD18⁺CD2⁺. Severe CFS/ME patients had increased CD18⁺CD11c⁻ in the CD56^{dim}CD16⁻ NK cell phenotype and reduced NKp46 in CD56^{bright}CD16^{dim} NK cells. **Conclusions:** This research accentuated the presence of immunological abnormalities in CFS/ME and highlighted the importance of assessing functional parameters of both innate and adaptive immune systems in the illness.

5.2 BACKGROUND

The innate immune system exhibits rapid effector functions and acts as the first line of defence, protecting the host cells while activating the adaptive immune system. NK cells in particular are an important interface for the innate and adaptive immune systems; hence, impaired function potentially leads to immunological disturbances. The presence of immunological dysfunction may impair physiological functioning and may play a role in disease pathogenesis (Maes et al., 2012). NK cell dysregulation has been demonstrated in a number of illnesses, including: HIV, systemic lupus erythematosus (SLE), MS and Major Depressive Disorder (MDD) (Blanca et al., 2001; Schepis et al., 2009; Seide et al., 1996).

Patients with CFS/ME exhibit reduced NK and CD8⁺T cell cytotoxic activity and differences in a number of adaptive immune cell phenotypes (Hardcastle et al., 2014a; Landay et al., 1991). Significant decreases in NK cell cytotoxic activity in CFS/ME patients who were moderately affected by symptoms and characterised using the Fukuda criteria for the illness have been extensively reported (Aoki et al., 1993; Barker et al., 1994; Brenu et al., 2013a; Brenu et al., 2013b; Brenu et al., 2013c; Brenu et al., 2010; Brenu et al., 2012b; Brenu et al., 2012c; Brenu et al., 2011; Caligiuri et al., 1987; Hardcastle et al., 2014a; Klimas et al., 1990; Levine et al., 1998; Maher et al., 2005; Ojo-Amaize et al., 1994; Patarca-Montero et al., 2001; Patarca et al., 1995; Tirelli et al., 1994). The reduced NK cell cytotoxic activity in CFS/ME patients may be associated with abnormalities in NK cell phenotypes, receptors or lytic proteins. Of the previous CFS/ME studies, five have also found significant differences in perforin and granzymes in NK cells of CFS/ME patients (Brenu et al., 2012a; Brenu et al., 2011; Huth et al.,

2014; Maher et al., 2005; Saiki et al., 2008). These changes in functional and phenotypic components of immune cells may be playing a role in the pathomechanism of CFS/ME.

CFS/ME is an enigmatic illness which has no known pathomechanism or cause, with diagnoses based on symptom specific criteria and a range of exclusions. Due to the multifactorial and complex nature of CFS/ME, there are often misconceptions and inconsistencies surrounding diagnosis which highlights the importance of thorough screening of participants in both clinical and research settings. This is particularly important in determining those with other illnesses such as major depression who may satisfy the CFS/ME symptom specific criteria and also demonstrate NK cell abnormalities (Seide et al., 1996). CFS/ME is characterised by persistent fatigue and a combination of symptoms which are often severely debilitating and the illness also tends to vary greatly in the nature of onset and symptom severity (Baraniuk et al., 2013; Brenu et al., 2013a; Carruthers et al., 2011; Fukuda et al., 1994a; Hardcastle et al., 2014a; Jason et al., 2005; Zaturenskaya et al., 2009). Moderate patients are mostly able to maintain normal daily activities but may be hampered by reduced mobility while severe CFS/ME patients experience high levels of daily fatigue and are typically housebound (Wiborg et al., 2010b).

Importantly, severe CFS/ME patients' NK cell cytotoxic activity has only been reported in three previous investigations (Brenu et al., 2013a; Hardcastle et al., 2014a; Ojo-Amaize et al., 1994). Severe CFS/ME patients have demonstrated significant reductions in NK cell cytotoxic activity as well as increased NK cell receptor KIR3DL1 and enhanced plasma IL-4, TNF- α and IFN- γ (Brenu et al., 2013a; Ojo-Amaize et al., 1994).

This research also suggested a correlation between low NK cell cytotoxic activity and severity of CFS/ME, based on clinical status (Ojo-Amaize et al., 1994). As studies have investigated innate and adaptive immune cells in CFS/ME patients, it appears important to further examine functional parameters such as cell activity, receptors and adhesion molecules. Along with NK cell and CD8⁺T cell aberrations (Brenu et al., 2013a; Caligiuri et al., 1987; Hardcastle et al., 2014a; Klimas et al., 1990; Lloyd et al., 1989), DC phenotypes have been previously abnormal in CFS/ME patients (Hardcastle et al., 2014a) although to date no study has assessed DC activity in the illness. Similarly, iNKT cells are seldom examined although one study found differences in iNKT cell phenotypes in CFS/ME patients (Hardcastle et al., 2014a), suggesting the possibility of iNKT cell dysfunction in the illness and the need to assess cytotoxic granules in these cells. Cytotoxic granules have not previously been assessed in $\gamma\delta$ or Tregs in CFS/ME patients, which may be cells of interest according to differences found in these cell types in previous research (Bansal et al., 2012; Brenu et al., 2013b; Brenu et al., 2013c; Brenu et al., 2011).

Previous research has outlined the presence of NK cell dysfunction and immunological abnormalities in CFS/ME patients although most studies only assessed moderately affected CFS/ME patients. Immune dysregulation can also be associated with the clinical features and aetiology of CFS/ME. This is supported as research has found significant clinical improvements in CFS/ME patients' symptoms after B cell depletion (Fluge et al., 2011). It is also possible that further immune cells may be affected by the illness and have yet to be assessed in CFS/ME patients. The current investigation was the first to measure the functional activity of DCs, neutrophils and monocytes, lytic

proteins in iNKT, $\gamma\delta$ and Tregs as well as receptors and adhesion molecules of NK, T and B cells in moderate and severe CFS/ME patients.

5.3 METHODS

5.3.1 Participants

Participants aged between 20 and 65 years old were recruited from Queensland and New South Wales areas of Australia through CFS/ME support groups, email advertisements and social media. In the absence of a diagnostic test for CFS/ME, the 1994 Fukuda criteria were used and patients must have had the illness for a period of at least 6 months prior to the study. All CFS/ME patients had been diagnosed by a primary physician in order to take part in the research. CFS/ME patients were identified as either moderate (mobile) or severe (housebound). These severity groups were then confirmed using an extensive questionnaire which included FSS, Dr Bell's Disability Scale, the FibroFatigue Scale and the KPS as determinants of severity. Participants were then excluded if they were previously diagnosed or had a history of an autoimmune disorder, MS, psychosis, major depression, heart disease or thyroid-related disorders or if they were pregnant, breast feeding, smokers, or experiencing symptoms of CFS/ME that did not conform to the Fukuda criteria for CFS/ME (Hardcastle et al., 2014a). The questionnaire obtained detailed information regarding the onset of illness, presence, frequency and severity of symptoms, comorbidities, overall health and quality of life. This allowed an analysis of all participants on an individual basis to ensure there were no confounding factors influencing CFS/ME patient symptoms. In order to identify psychological exclusions in participants that were not specifically outlined, the

questionnaire (including the FSS, SF-36 and WHO DAS2.0) also included questions and scales regarding emotional stability, social interaction, motivation and wellbeing (Üstün, 2010; Ware Jr & Sherbourne, 1992).

Participants (n=45) included in the study were patients moderately (n=15) or severely (n=12) affected by CFS/ME symptoms as well as a healthy non-fatigued healthy control group (n=18). All participant groups were matched for age and sex. All CFS/ME patients had the illness for at least six months prior to their participation in the research and the average duration of illness of a CFS/ME patient was 6.5 years. It was then ensured that all participating CFS/ME patients fulfilled the Fukuda criteria for CFS/ME at the time of blood collection according to questionnaires which assessed their symptoms in the 30 days prior and at the time of collection. Written informed consent was obtained from all participants and all research protocols were granted ethical clearance after review by the Griffith University Human Research Ethics Committee (GU Ref No: MSC/23/12/HREC).

5.3.2 Sample Preparation and Routine Measures

Blood collection occurred between 8:00am and 11:30am and samples were analysed within 12 hours. A non-fasting blood sample of 50mL was collected into lithium heparinised and EDTA tubes from the antecubital vein of all participants. Initial full blood count results were determined by Pathology Queensland to assess routine levels of white blood cell and red blood cell markers.

5.3.3 Lytic Proteins Analysis

Lytic proteins were assessed as previously described (Brenu et al., 2013a; Brenu et al., 2011) in Tregs, iNKT, $\gamma\delta 1$ and $\gamma\delta 2$ T cells. Ficoll-hypaque (Sigma, St Louis, MO) density gradient centrifugation was used to isolate PBMCs from EDTA whole blood. PBMCs were adjusted to 1×10^7 cells/mL and stained with monoclonal antibodies for iNKT, Tregs, $\gamma\delta 1$ and $\gamma\delta 2$ T cells, see Additional Table 1. Cells were incubated for 30minutes in Cytotfix then perforin, granzyme A and granzyme B monoclonal antibodies were added for 30minutes. Cells were analysed on the flow cytometer where perforin, granzyme A and granzyme B expression was measured in iNKT, Tregs, $\gamma\delta 1$ and $\gamma\delta 2$ T cells, see Appendix 13.

5.3.4 DC Cell Activity Analysis

DC activity was measured from 300uL lithium heparinised whole blood, incubated in Roswell Park Memorial Institute (RPMI)-1640 culture media (Invitrogen, Carlsbad, CA), Phorbol 12-myristate 13-acetate (PMA) and Ionomycin for 5 hours at 37°C, 5% CO₂. Cells were labelled with monoclonal antibodies (see Additional Table 1) and FACS Lyse (BD Biosciences, San Diego, CA) was used to remove red blood cells. Flow cytometric analysis (Becton Dickinson Immunocytometry Systems) was used to measure DC activity based on unstimulated versus stimulated assessments.

5.3.5 Phagocytosis Analysis

The Phagotest kit was used to determine leukocyte phagocytosis in whole blood based on the ability of neutrophils and monocytes to engulf bacteria (Orpegen Pharma, Germany). Manufacturer's instructions were followed for the procedure. Lithium heparinised whole blood (100uL) was aliquot into two 5mL tubes as control and test samples. Control and test samples were incubated with *E.coli* bacteria (Orpegen Pharma, Germany) on ice or at 37°C respectively, before quenching solution were added to stop phagocytosis. Samples were washed and red blood cells were lysed with lysing solution (Orpegen Pharma, Germany). DNA staining solution was used as a measure of neutrophil and monocyte phagocytosis based on differential gating on a flow cytometric analysis (Becton Dickinson Immunocytometry Systems).

5.3.6 Respiratory Burst Analysis

Respiratory burst analysis was measured in granulocytes from whole blood. Intracellular oxidation was performed by incubating 100uL lithium heparinised whole blood in Dihydrohodamine (DHR) for 10minutes at 37°C. PMA was then added, followed by 10minutes incubation at 37°C. FACS Lyse (BD Biosciences, San Diego, CA) was used to remove red blood cells and DHR was used as a measure of neutrophil and monocyte respiratory burst using differential gating on the flow cytometer (Becton Dickinson Immunocytometry Systems).

5.3.7 Isolated Natural Killer Cell Receptor Analysis

As previously described (Hardcastle et al., 2014a), NK cells were isolated from whole blood cells using negative selection with 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 SLAM, integrin and NCRs, see Additional Table 1 (Miltenyi Biotec). Analysis was undertaken on the flow cytometer (Becton Dickinson Immunocytometry Systems), where NK cells were gated using CD56, CD16 and CD3 and SLAM, integrin and NCR receptors were assessed for each NK cell phenotype (CD56^{dim}CD16⁺, CD56^{bright}CD16⁺, CD56^{dim}CD16⁻ and CD56^{bright}CD16^{dim}) see Appendix 13 (Brenu et al., 2013a).

5.3.8 T and B Cell Whole Blood Analysis

Whole blood analysis was undertaken as previously described (Hardcastle et al., 2014a). Monoclonal antibodies were added to lithium heparinised whole blood samples and incubated for 30minutes, see Appendix 13. Cells were then lysed, washed and fixed. CD4⁺T and CD8⁺T cell KIR receptors and phenotypes, B cell receptors and B regulatory cells were assessed using appropriate antibodies (see Appendix 13) and gating strategies on the flow cytometer (Becton Dickinson Immunocytometry Systems) (Montoya et al., 2007).

5.3.9 Statistical Analysis

Data were compared among the three participant groups (control, moderate CFS/ME and severe CFS/ME) with statistical analysis performed based on the distribution. Shapiro-Wilk normality tests were performed and if normally distributed, the ANOVA was used. If data was not normally distributed, the Kruskal Wallis test of independent variables based on rank sums to determine the magnitudes of group differences was used. The Bonferroni Post Hoc or Mann-Whitney U tests determined p values of significance for parametric and non-parametric data respectively, with statistical significance set at an alpha criterion at $p < 0.05$. Spearman's non-parametric correlation was conducted on significant parameters to determine correlates where significance was accepted as $p < 0.01$. Outliers were identified using a boxplot technique and handled by eliminating particular extreme data points from the analysis (Aguinis et al., 2013).

SPSS statistical software version 22.0 was used for all statistical analysis and data represented in this study are reported as plus/minus the standard error of the mean (\pm SEM) or plus/minus the standard deviation (SD) as specified.

5.4 RESULTS

5.4.1 No differences in participant characteristics between groups

Principal participant results are reported in Table 5. We found no statistical difference for age or gender between participant groups ($p = 0.325, 0.607$ respectively) (Table 5) and the Fukuda criteria for CFS/ME were satisfied by all moderate and severe CFS/ME participants.

Table 5: Participant characteristics and comparisons of the age (mean + SEM) and gender distribution of each participant group (control, moderate and severe).

Parameter	Control (n=18)	Moderate (n=15)	Severe (n=12)	<i>p</i> value
Age in years (Mean ± SEM)	40.39 ± 2.65	45.93 ± 2.96	41.25 ± 2.77	0.325
Gender Female, Male	12, 6	11, 4	10, 2	0.607

Age data is represented as Mean±SEM in control (n=18), moderate CFS/ME (n=15) and severe CFS/ME (n=12). *signifies $p < 0.05$ between participant groups. There were no significant differences in age or gender within the research groups.

5.4.2 Severity scale scores differ between participant groups

Severity scales used, included: the Fatigue Severity Scale (FSS), Dr Bell's Disability Scale, the FibroFatigue Scale and the Karnofsky Performance Scale (KPS). For all

scales used, there were significantly different scores between all participant groups, with the exception of ‘sadness’ ($p = 0.064$). Moderate CFS/ME patient scores were significantly worsened compared with healthy controls in all parameters. Severe CFS/ME patients also displayed significantly worsened scores when compared with healthy controls, with the exception of ‘sadness’ ($p = 0.194$) and ‘sleep’ ($p = 0.091$). The KPS and Dr Bell’s Disability Scale scores were significantly lower in the severe CFS/ME patients compared with the moderate CFS/ME patients ($p < 0.0011$ and 0.001).

5.4.3 Differences in routine full blood count parameters between participant groups

Our data have shown a significantly increased monocyte count in the moderate CFS/ME patients compared with the healthy controls and severe CFS/ME patients (Figure 10).

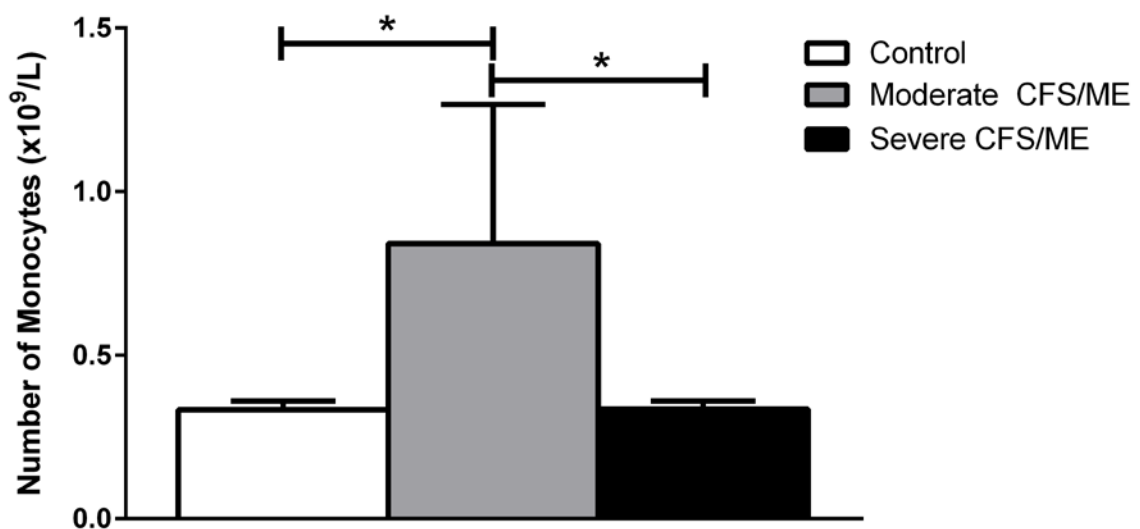


Figure 10: Monocyte full blood count for controls, moderate and severe CFS/ME participants

*Monocyte numbers ($\times 10^9/L$) obtained from routine full blood counts are shown for controls, moderate and severe CFS/ME patients. Data is represented as mean \pm SEM. * represents results that were significantly different where $p < 0.05$.*

There were no other statistically significant differences in the routine full blood count parameters between the participant groups (data not shown).

5.4.4 No differences in flow cytometric analysis of DC, neutrophil and monocyte function or lytic proteins

Previous research has reported differences in DC phenotypes in moderate and severe CFS/ME patients (Hardcastle et al., 2014a), however, this was the first research to assess DC activity in the illness. Our data have found no significant differences in the DC activity markers CD80 and CD86, in unstimulated or stimulated DCs between any of the participant groups, see Appendix 14. Neutrophil and monocyte function were examined as neutrophil respiratory burst has previously been reduced in moderate CFS/ME patients (Brenu et al., 2010). There were no significant alterations between any of the participant groups in the ability of neutrophils or monocytes to phagocytose or undergo respiratory burst, see Appendix 15. iNKT, $\gamma\delta$ T cells and Tregs have previously shown dysfunction in CFS/ME patients (Hardcastle et al., 2014a), however no studies had examined lytic proteins in these cell types. We found no significant differences in iNKT, $\gamma\delta$ T cells or Treg levels of perforin, granzyme A, granzyme B or CD57, see Appendix 16.

5.4.5 NK cell adhesion molecules and natural cytotoxicity receptors differ between moderate and severe CFS/ME patients

Previous investigations have shown significant differences in NK cell receptors in CFS/ME patients, however signaling lymphocytic activation molecule (SLAM) receptors, adhesion molecules and NCRs have not been reported and are critical for NK cell function (Brenu et al., 2011; Hardcastle et al., 2014a). SLAM receptor (CD150) was significantly increased in our data in total NK cells of moderate CFS/ME patients compared with severe CFS/ME patients ($p = 0.046$). $CD56^{\text{bright}}CD16^{\text{dim}}$ NK cells expression of NKp46 was significantly reduced in severe CFS/ME compared with controls and moderate CFS/ME ($p = 0.021$ and 0.021 respectively) (Figure 11). $CD56^{\text{dim}}CD16^{-}$ NK cell CD2 expression was significantly lower in moderate CFS/ME compared with severe CFS/ME patients ($p = 0.033$) while $CD18^{+}CD2^{-}$ was increased in moderate CFS/ME patients compared with controls and severe CFS/ME in $CD56^{\text{dim}}CD16^{-}$ NK cells ($p < 0.0019$ and 0.035 respectively). In the $CD56^{\text{dim}}CD16^{-}$ NK cells phenotype, $CD18^{+}CD11c^{-}$ was significantly increased in the severe CFS/ME compared with controls ($p = 0.036$) (Figure 11) (Table 6).

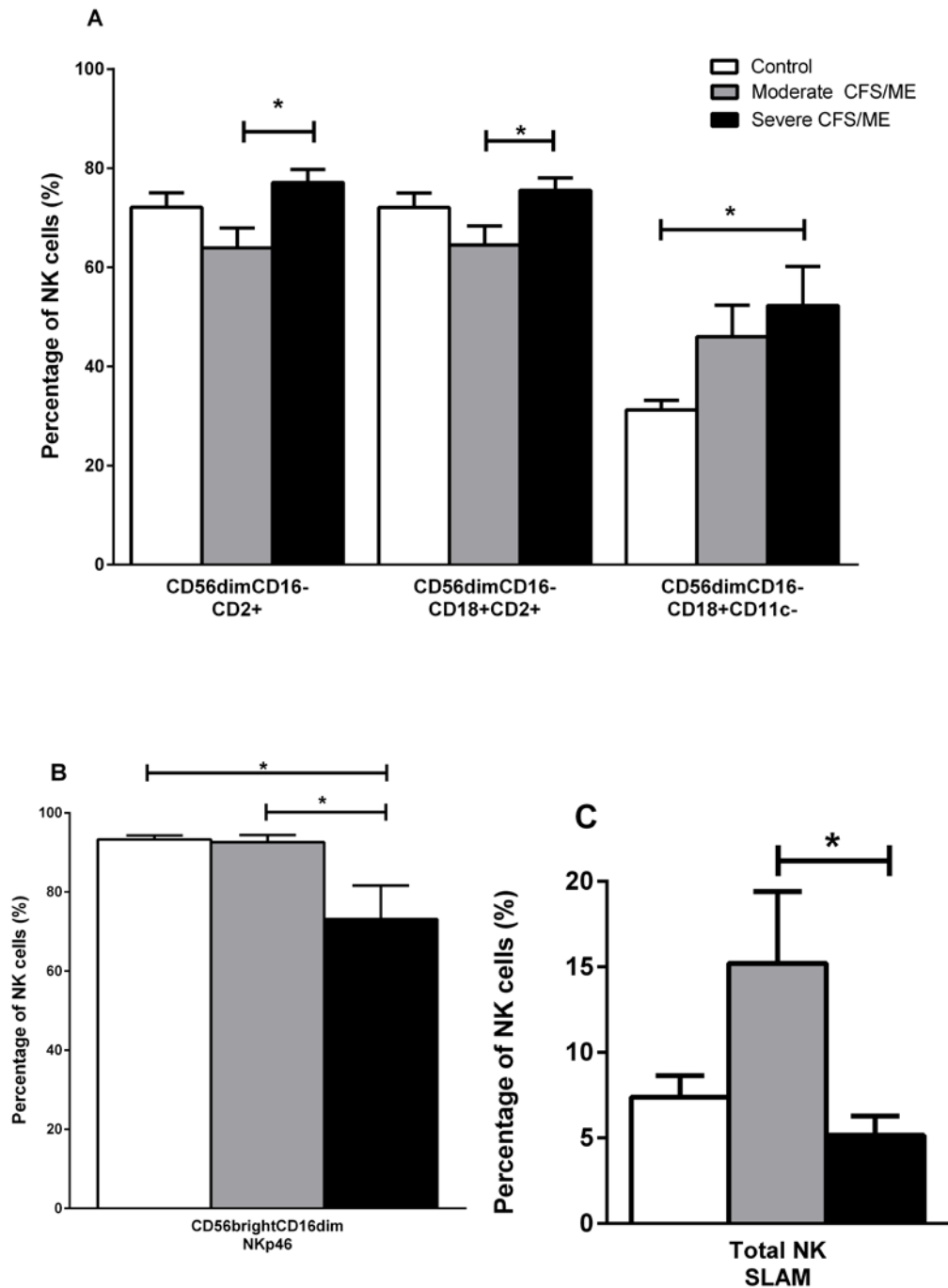


Figure 11: The profile of receptors and adhesion molecules on NK cells in control, moderate and severe CFS/ME participants.

A $CD2^+$, $CD18^+CD2^+$, $CD18^+CD11c^-$ adhesion molecule expression on $CD56^{dim}CD16^-$ NK cells in control, moderate and severe CFS/ME patients. *B* $CD56^{bright}CD16^{dim}$ NK cell expression in control, moderate and severe CFS/ME patients, represented as

percentage of NK cells. C Total NK cells expressing the SLAM receptor in control, moderate CFS/ME and severe CFS/ME patients. All data is represented as percentage of total NK cells (%) and shown as mean±SEM. * represents results that were significantly different where $p < 0.05$.

Table 6: Summary of significant differences in parameters found between groups.

	<u>Significant Parameters</u>	<u>Group</u>	<u>Potential Result of Changes</u>
NK cell	↓ CD56 ^{dim} CD16 ⁻ CD2 ⁺	M v S	Impaired ability to adhere to target cells (Huth et al., 2014).
	↓ CD56 ^{dim} CD16 ⁻ CD18 ⁺ CD2 ⁺	M v S	Reduced NK cells in an active state hence lessened NK cell activation and cytotoxic activity (Huth et al., 2014; Lima et al., 2002).
	↑ CD56 ^{dim} CD16 ⁻ CD18 ⁺ CD11c ⁻	S v C	Decreased adhesive abilities or lower number of activated cells (Lima et al., 2002).
	↓ CD56 ^{bright} CD16 ^{dim} NKp46	S v C, S v M	Reduced recognition and lysis of target cells (Biassoni et al., 2001).
	↓ Total SLAM	S v M	Lessened ability of NK cells to undergo cytotoxic activity (Veillette, 2006).
CD4⁺ T cell	↑ Total KIR2DL5	M v S	Inhibition of T cell functions (Vilches et al., 2000).
	↓ Naïve KLRG1	M v C, M v S	Enhanced T cell activation (Henson & Akbar, 2009).
	↑ Central Memory BTLA4	M v C	Greater inhibitory signalling and modulation of immune responses (Hurchla et al., 2007).
CD8⁺ T cell	↑ CD45RA Effector Memory	M v C	Heightened capacity for cytotoxic activities (Anane et al., 2010).
	↓ Central Memory LFA-1	M v C	Lack of LFA-1 adhesion required for optimal cytotoxic activity (Nielsen et al., 2012).
	↓ Total KLRG1	M v C	Enhanced T cell activation (Henson & Akbar, 2009).

↓ represents significant reductions and ↑ represents significant increases in the group listed first in the ‘Group’ column compared with the group listed second in the ‘Group’ column. S=Severe CFS/ME patients; M= Moderate CFS/ME patients; C=Controls.

5.4.6 No differences in Bregs and BCRs

Significant B cell phenotypes have been reported in both moderate and severe CFS/ME patients (Hardcastle et al., 2014a), however, regulatory B (Breg) cells and B cell receptors (BCRs) in CFS/ME cohorts are yet to be examined (Fluge et al., 2011; Hardcastle et al., 2014a). We found no significant differences in Breg cell phenotypes or BCRs between the participant groups, see Appendix 17.

5.4.4 Increased KIR2DL5 in CD4⁺T cells of moderate CFS/ME patients

Killer immunoglobulin-like receptor (KIR)s have previously shown significant differences in NK cells of CFS/ME patients, although these had not been examined in CD4⁺T or CD8⁺T cells in CFS/ME patients (Brenu et al., 2013a; Hardcastle et al., 2014a). Our data found no significant alterations in the expression of KIRs on CD8⁺T cells between the participant groups. KIR2DL5 expression was significantly higher on CD4⁺T cells in moderate CFS/ME compared with severe CFS/ME patients ($p = 0.011$) (Figure 12) (Table 6).

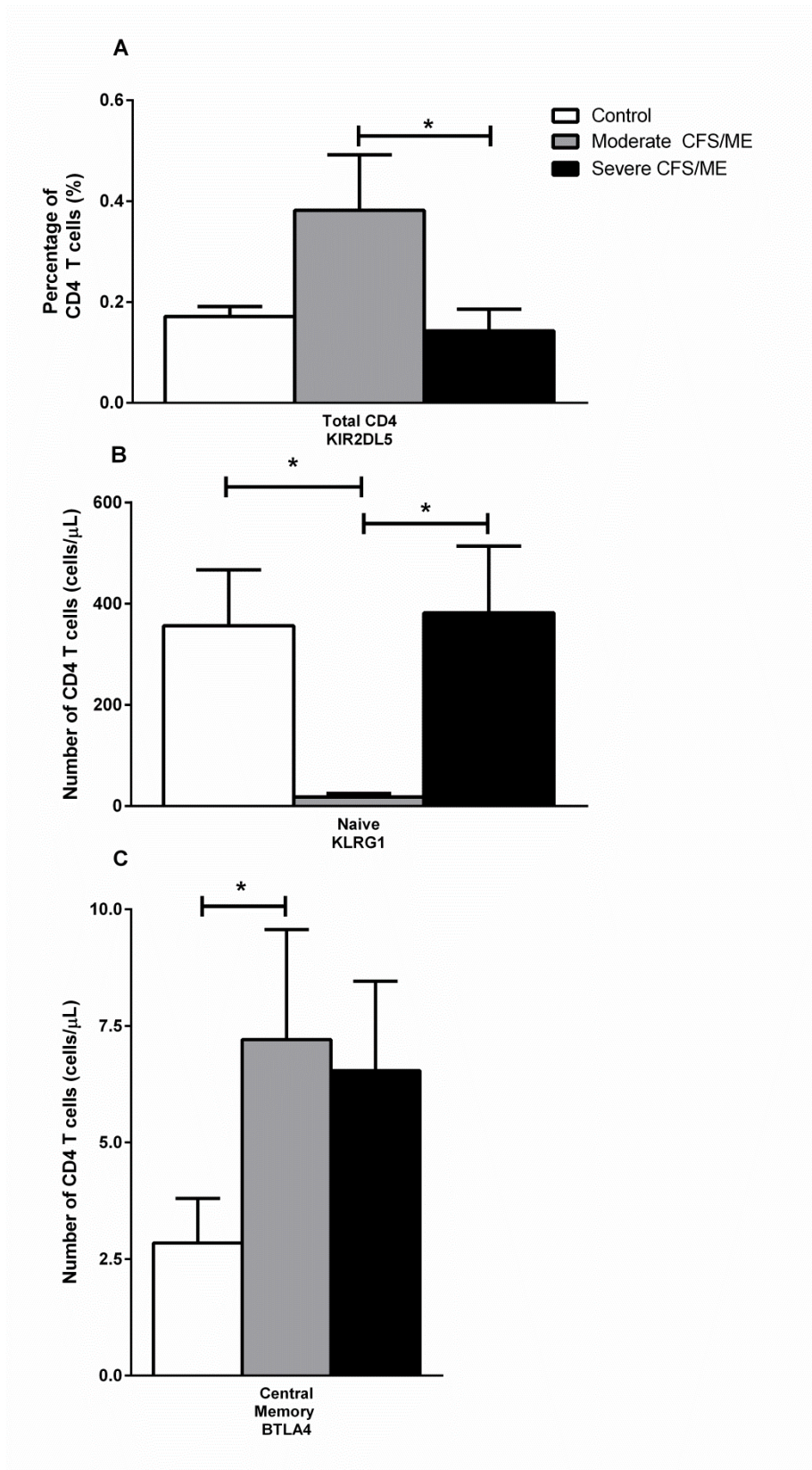


Figure 12: KIR and receptor expression in CD4⁺T cells of control, moderate and severe CFS/ME participants.

*A KIR2DL5 expression in total CD4⁺T cells as represented by a percentage of total CD4⁺T cells (%). B Number of naïve CD4⁺T cells expressing KLRG1 in in control, moderate and severe CFS/ME participants shown as a number of cells per microliter (cells/ μ L). C BTLA4 receptor expression in central memory CD4⁺T cells of control, moderate and severe CFS/ME patients, presented as cells/ μ L. Data is represented as mean \pm SEM. * represents results that were significantly different where $p < 0.05$.*

5.4.5 Differences in CD8⁺T and CD4⁺T cells and phenotypes between CFS/ME patient groups

CD8⁺T cells have been significantly different in CFS/ME patients in previous investigations, however, receptors on CD8⁺T and CD4⁺T cells had not yet been examined (Brenu et al., 2012a; Klimas et al., 1990; Landay et al., 1991). We found that the CD45RA effector memory CD8⁺T cell phenotype formed a significantly higher percentage of total CD8⁺T cells in moderate CFS/ME compared with controls ($p=0.016$) (Figure 13). Central memory CD8⁺T cells had significantly reduced lymphocyte function-associated antigen (LFA) -1 in moderate CFS/ME compared with controls ($p=0.032$). Total CD8⁺T cell expression of killer cell lectin-like receptor subfamily G member (KLRG)1 was also reduced in moderate CFS/ME compared with controls ($p=0.014$) (Figure 13).

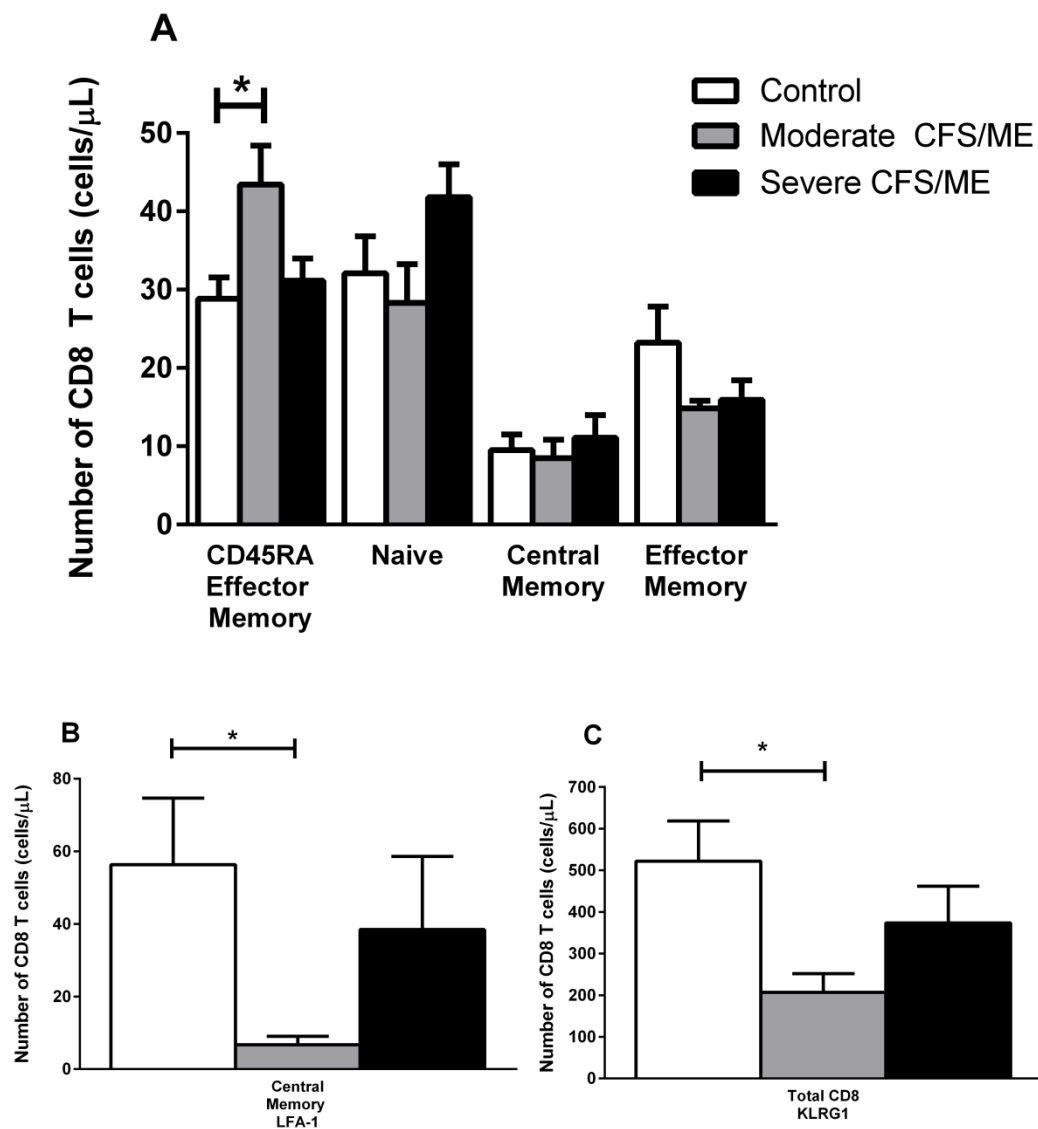


Figure 13: Alterations in phenotypes and receptors in CD8⁺T cells in control, moderate and severe CFS/ME.

A CD45RA effector memory, naïve, central memory and effector memory CD8⁺T cell phenotypes represented as number of CD8⁺T cells (cells/ μ L). **B** LFA-1 expression in central memory CD8⁺T cells for control, moderate CFS/ME and severe CFS/ME participants. **C** KLRG1 expression in total CD8⁺T cells for control, moderate CFS/ME and severe CFS/ME participants. All data is represented as mean \pm SEM. * represents results that were significantly different where $p < 0.05$.

In our data, CD4⁺ central memory T cells, B and T lymphocyte attenuator (BTLA)4 had significantly increased expression in moderate CFS/ME patients compared with controls ($p = 0.038$) (Figure 12). KLRG1 was also significantly reduced in CD4⁺ naïve T cells in moderate CFS/ME compared with controls and severe CFS/ME ($p = 0.013$ and 0.019 respectively) (Table 6). There was no significant difference in CD4⁺T cell phenotypes between any of the participant groups, see Appendix 18.

5.4.6 Correlations across severity and immune parameters

Our data showed a significantly positive correlation between the KPS and Dr Bell's Disability Scale scores. Both the KPS and Dr Bell's Disability scale were negatively correlated with the total $\gamma\delta$ T cell CD45RA effector memory phenotype values and CD56^{dim}CD16⁻ NK cells with CD18⁺CD11c⁻ (Table 7). There were also a number of parameters that were correlated with one another, such as CD4⁺T and CD8⁺T cell markers, NK cell adhesion markers and $\gamma\delta$ and CD8⁺T cell phenotypes (Table 7).

Table 7: Spearman's correlation to identify correlates between significant parameters.

		1	2	3	4	5	6	8	9	10	13
1	CM CD8 ⁺ LFA-1										
2	Naïve CD4 ⁺ KLRG1										
3	Total CD8 ⁺ KLRG1		.50								
4	CM CD4 ⁺ BTLA4	.55									
5	CM CD4 ⁺ LFA-1			.56							
6	CD56 ^{dim} CD16 ⁻ NK CD2 ⁺										
7	CD56 ^{dim} CD16 ⁻ NK CD18 ⁺ CD2 ⁺						-.60				
8	CD56 ^{dim} CD16 ⁻ NK CD18 ⁺ CD11a ⁻		-.47		.49		-.057				
9	CD56 ^{dim} CD16 ⁻ NK CD18 ⁺ CD11c ⁻										
10	Total NK SLAM			.46			-.53		-.50		
11	Total CD8 ⁺ EMRA			-.44		-.75					
12	CD4 ⁺ KIR2DL5							.48		.75	
13	KPS								-.43		
14	Dr Bell's Disability Scale								-.46		.95

Table 2 shows the significant correlation values between bivariate parameters for significant parameters for combined control, moderate CFS/ME and severe CFS/ME participant groups. All correlations shown have $p < 0.01$.

5.5 DISCUSSION

This study is the first to provide an overview of cell function and receptor interactions, including: assessing DC, neutrophil and monocyte function and receptors on T cells, BCRs and Bregs, in moderate and severe CFS/ME patients. This is also the first study to examine lytic proteins in $\gamma\delta$ T cells, iNKT cells and Tregs in CFS/ME patients. Our results suggest that in some cases, functional immunological impairment may be related to differences in severity of CFS/ME patients, highlighting the variation in the illness. Significant alterations shown in moderate CFS/ME patients were often not present in severe CFS/ME patients and controls, revealing the possibility that moderate CFS/ME patients may have a different aetiology compared with the severe subgroup of CFS/ME patients.

Increased SLAM expression on total NK cells in moderate CFS/ME patients in this study may be a mechanistic response to the typically reduced NK cell cytotoxic activity in CFS/ME (Brenu et al., 2011; Klimas et al., 2012; Klimas et al., 1990). SLAM is a receptor expressed on the surface of T, B, NK and DC cells, functioning as an activating adaptor protein to amplify the recruitment of inflammatory cells, such as DCs, by activating IFN- γ (Lanier, 2001; Sidorenko & Clark, 2003; Wang et al., 2001). SLAM receptors regulate NK cell activity via association with SLAM associated protein (SAP) family adapters, where binding receptors are then coupled to the Src kinase FynT to evoke protein tyrosine phosphorylation signals (Veillette, 2006). Heightened expression of SLAM in moderate CFS/ME may enhance the ability of NK cells to undergo cytotoxic activities (Brenu et al., 2013a; Brenu et al., 2010; Brenu et al., 2012c; Brenu et al., 2011; Hardcastle et al., 2014a; Klimas et al., 1990). The activating receptor

NKp46 is also typically involved in the recognition and lysis of target cells and was reduced in CD56^{bright}CD16^{dim} NK cells of severe CFS/ME patients (Biassoni et al., 2001). NKp46 is highly expressed on the CD56^{bright}CD16^{dim} NK cell subset and although it is only weakly involved in cytotoxic activation, this reduction may contribute to the reduced NK cell cytotoxic activity prevalent in severe CFS/ME patients (Biassoni et al., 2001; Hardcastle et al., 2014a; Poli et al., 2009). The expression of SLAM and NKp46 on NK cells significantly differed between moderate and severe CFS/ME patients, suggesting that perhaps these severity subgroups may vary in immunological presentation, as proposed by previous studies (Brenu et al., 2013a; Hardcastle et al., 2014a; Ojo-Amaize et al., 1994).

Effector memory and CD45RA effector memory cells demonstrate NK-like functions as they have the ability to detect abnormal major histocompatibility complex (MHC) expression and have a high capability for cytotoxic activities (Anane et al., 2010). Our findings suggest that the number of CD45RA effector memory CD8⁺T cells of moderate CFS/ME patients may be enhanced due to sub-optimal function. In T cells, CD45RA effector memory cells are upregulated following cytokine directed proliferation, indicating that the subset is generated via homeostasis rather than antigen-dependent pathways (Farber et al., 2014; Sallusto et al., 2004). Moderate CFS/ME patients have an increased number of CD8⁺T CD45RA effector memory cells, potentially as a result of homeostasis where the cells may not be effectively undergoing degranulation and apoptosis (Farber et al., 2014; Sallusto et al., 2004).

This research also found reductions in KLRG1 expression in total CD8⁺T and naïve CD4⁺T cells of moderate CFS/ME patients, which suggests that these cells may have a

reduced ability to inhibit T cell function and activation. KLRG1 ligation inhibits the nuclear factor of activated T cells (NFAT) signalling pathway and downregulates CD95 mediated lysis to inhibit the activation of T cells (Henson & Akbar, 2009). Hence, blockades of inhibitory receptors tend to improve CD8⁺T cell responses by preventing inhibitory pathways (Blackburn et al., 2008). It is therefore possible that reduced KLRG1 may be contributing to the pro-inflammatory response and T cell activation often found in CFS/ME patients (Brenu et al., 2011; Curriu et al., 2013).

Increased KIR2DL5 on CD4⁺T cells in moderate CFS/ME patients may also be associated with alterations in KIR receptors in T cells in the same cohort (Brenu et al., 2013a; Hardcastle et al., 2014a). KIR2DL5 is an inhibitory KIR found in variable proportions of circulating T cells (Cisneros et al., 2012; Estefanía et al., 2007) which is directly linked to a greater number of random combinations of KIR receptors expressed on these cells, which may be influencing optimal T cell functions in the illness (Vilches et al., 2000). Enhanced inhibitory signalling and modulation of immune responses are typical attributes of increased BTLA expression in T cells which may be present in moderate CFS/ME patients who have amplified expression of inhibitory receptor BTLA4 in central memory CD4⁺T cells (Hurchla et al., 2007). Activation and function of CD4⁺T cells by NK cells is dependent on the engagement of the β_2 integrin LFA-1. LFA-1 adhesion is necessary for optimal cytotoxic activity by both NK cells and CD8⁺T cells, also mediating NK cell degranulation via synergy with NKG2D (Nielsen et al., 2012). Decreased expression of LFA-1 on central memory CD8⁺T cells in moderate CFS/ME patients suggests that there may be a lack of LFA-1 adhesion in CFS/ME which is required for ideal cytotoxic activity by NK cells and CD8⁺T cells (Nielsen et al., 2012). Similar to the pattern shown in SLAM and NKp46 receptors on NK cells,

CD45RA effector memory CD8⁺T, CD4⁺T and CD8⁺ TCRs significantly differed between moderate and severe CFS/ME patients.

Cellular adhesion may be important in CFS/ME as it is required for target cell contact and NK cell effector function. Regulation of adhesion molecules is necessary for integrin target cell ligand interactions as the release of adherence results in lymphocyte movement (Bryceson et al., 2006). CD2 expression in CD56^{dim}CD16⁻ NK cells is reduced in moderate CFS/ME patients compared with severe CFS/ME patients, suggesting that these cells may have an impaired ability to adhere to target cells. This confirmed previous findings where CD2/CD18 co-expression was reduced in the same CD56^{dim}CD16⁻ NK cell phenotype in a cohort of moderate CFS/ME patients (Huth et al., 2014). Increased CD2 is often associated with a higher cytotoxic ability (Lima et al., 2002) as CD2 acts as a contributor to induce NK cell activation (Bryceson et al., 2006; Lima et al., 2002). Higher expression of CD2 in severe CFS/ME patients potentially implies that more NK cells in these patients are in an active state and may have a greater ability than the moderate CFS/ME patients to induce NK cell activation and cytotoxic activities (Lima et al., 2002). CD18⁺/CD2⁻ CD56^{dim}CD16⁻ NK cells were also increased in the moderate CFS/ME patients, strengthening the theory that CFS/ME patients may have a weakened ability to activate NK cells as well as having impaired NK cell cytotoxic activity. Adhesion molecules CD18 and CD2 on CD56^{dim}CD16⁻ NK cells were also significantly altered in the moderate CFS/ME patient group compared with the severe CFS/ME patients, who appeared similar to the controls. In the case of CD18⁺CD11c⁻ on the same CD56^{dim}CD16⁻ NK cell subset, however, increases in CD18⁺CD11c⁻ increased in the moderate CFS/ME patients and significantly increased in the severe CFS/ME patients. Expression of the adhesion marker CD11c is

heterogeneous and variable in NK cells although, typically activated NK cells are CD11c⁺ (Lima et al., 2002). Increased CD18⁺CD11c⁻ on CD56^{dim}CD16⁻ NK cells in severe CFS/ME patients indicates that these patients may have a reduced ability to adhere or that they may have a low number of activated NK cells. Therefore, differences in CD18⁺CD11c⁻ adhesion molecules in severe CFS/ME patients may be associated with the reduced NK cell cytotoxic activity found in the illness (Brenu et al., 2011; Hardcastle et al., 2014a; Klimas et al., 1990).

CFS/ME symptom severity and presentation may be related to the immune dysregulation shown as the immune system interacts with physiological functioning via a number of body systems, including: the central nervous system, digestive system and endocrine system (Glaser & Kiecolt-Glaser, 2005). Unrefreshing sleep and sleep disturbances are symptoms of CFS/ME and reports have indicated that NK cells are altered after sleep deprivation, demonstrating interactions between physiological symptoms and the immune system (Bryant et al., 2004), particularly in CFS/ME patients. Similarly, it has previously been suggested that clinical severity status appears to be associated with reduced NK cell activity in CFS/ME patients (Brenu et al., 2013a; Ojo-Amaize et al., 1994). Although there are limited research findings for severe CFS/ME patients, the differences in NK cells, CD4⁺T and CD8⁺T cells between severity groups, found in this research, suggest that immune dysfunction in CFS/ME may be related to clinical symptoms and hence severity.

5.6 CONCLUSIONS

This study was the first to show significant differences in a number of receptors in NK, CD4⁺T and CD8⁺T cells in CFS/ME (Brenu et al., 2013a; Hardcastle et al., 2014a) suggesting dysregulation in NK cell cytotoxic activity, receptor regulation and potentially cell adherence. Consistent with previous literature, our research suggests that CFS/ME patients have immunological dysregulation in the innate and adaptive immune cells. We have also highlighted significant differences in NK, CD4⁺T and CD8⁺T cells between moderate and severe CFS/ME patients, suggesting severity subgroups may have distinct immune perturbations and consequently aetiology. Further studies examining severity subgroups of CFS/ME patients may therefore contribute to the understanding of the pathomechanism associated with the illness.

CHAPTER 6: PROJECT FOUR: LONGITUDINAL ANALYSIS OF IMMUNE ABNORMALITIES IN VARYING DEGREES OF CHRONIC FATIGUE SYNDROME/MYALGIC ENCEPHALOMYELITIS (CFS/ME) PATIENTS

Hardcastle, SL., Brenu, EW., Johnston, S., Nguyen, T., Huth, T., Ramos, S., Staines, D and Marshall-Gradisnik, S. 201-5. Longitudinal analysis of immune abnormalities in varying severities of Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients. *Journal of Immunology Research*, 13: 299.

Author contributions:

Hardcastle was the principle contributor to this manuscript. Hardcastle was responsible for contributions to study design, acquisition of data, analysis and interpretation of data and primary drafting of the manuscript. Johnston, Nguyen, Huth and Ramos contributed to acquisition of data and manuscript revisions. Brenu, Staines and Marshall-Gradisnik contributed to the study conception and design, interpretation of data and critical manuscript revisions.

6.1 ABSTRACT

Research has identified immunological abnormalities in CFS/ME, a heterogeneous illness with an unknown cause. There have been no CFS/ME studies examining innate and adaptive immune cells longitudinally in patients with varying severities. This is the first study to investigate immune cells over six months (week 24) while also examining CFS/ME patients of varying symptom severity. Participants were grouped into 18 non-fatigued healthy controls, 12 moderate and 12 severe CFS/ME patients and flow cytometry was used to examine cell parameters at zero and six months (week 24). Over time, iNKT CD62L expression significantly increased in moderate CFS/ME patients and CD56^{bright} NK cell receptors differed in severe CFS/ME. Naïve CD8⁺T cells, CD8⁻CD4⁻ and CD56⁻CD16⁻ iNKT phenotypes, $\gamma\delta$ 2T cells and effector memory subsets were significantly increased in severe CFS/ME patients at six months (week 24). Severe CFS/ME patients were significantly reduced in CD56^{bright}CD16^{dim} NKG2D, CD56^{dim}CD16⁻ KIR2DL2/DL3, CD94⁻CD11a⁻ $\gamma\delta$ 1T cells and CD62L⁺CD11a⁻ $\gamma\delta$ 1T cells at six months. Severe CFS/ME patients differed from controls and moderate CFS/ME patients over time and expressed significant alterations in iNKT cell phenotypes, CD8⁺T cell markers, NK cell receptors and $\gamma\delta$ T cells at six months (week 24). This highlights the importance of further assessing these potential immune abnormalities longitudinally in both moderate and severe CFS/ME patients.

6.2 INTRODUCTION

In the immune system, lymphocytes are subject to continual checkpoints, signals and regulation to allow successful cell development, homeostasis and to subsequently prevent illness (Rathmell & Thompson, 2002). Immune responses generated as a result of these signals between the innate and adaptive cells can fluctuate and have a critical influence on the maintenance of physiological homeostasis (Brenu et al., 2012c; Rathmell & Thompson, 2002). CFS/ME is a heterogeneous illness, varying in severity and nature of onset although research has consistently established immunological abnormalities (Barker et al., 1994; Brenu et al., 2013a; Brenu et al., 2013b; Brenu et al., 2011; Curriu et al., 2013; Klimas et al., 1990; Patarca, 2001).

Reduced NK cell cytotoxic activity is the most predominant and consistent outcome of immunological studies in CFS/ME. A number of parameters have also been shown to alter in patients, including: Tregs, iNKT cells, CD8⁺T cells and cytokines (Brenu et al., 2011; Hardcastle et al., 2014a; Klimas et al., 1990). Alterations in both innate and adaptive immune cells reflect the extent of immune dysregulation in CFS/ME which may potentially be linked to the illness pathomechanism.

Longitudinal studies of CFS/ME have also demonstrated consistently reduced NK cell cytotoxic activity while there was variation in cytokine levels over time. It appears that longitudinal examination of immune cells in CFS/ME may allow an assessment of consistent immune parameters as potential biomarkers for the illness (Brenu et al., 2012c; ter Wolbeek et al., 2007). This research further investigates immunological

markers of the innate and adaptive immune system at zero and six months (week 24) in moderate and severe CFS/ME patients.

6.3 METHODS

6.3.1 Participants

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

Participants previously recruited from Queensland and New South Wales areas of Australia were again approached for this follow-up study. All assessments were taken at zero and six months (week 24). Participants were between 20 and 65 years old and CFS/ME patients had the illness for a period of at least six months prior to the study. CFS/ME was defined based on the 1994 Fukuda criterion in the absence of a biomarker or diagnostic test for the illness. CFS/ME patients were identified as either moderate or severe and these groups were confirmed using an extensive questionnaire to assess symptomatology, health status, quality of life, severity and mobility in all participants (Hardcastle et al., 2014a). 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, smoking, or experiencing symptoms of CFS/ME that did not conform to the Fukuda criterion for CFS/ME.

Participants (n=42) in the follow up study included moderately (n=12) or severely (n=12) affected CFS/ME patients as well as an age and sex matched non-fatigued healthy control group (n=18). The severe CFS/ME group were housebound and the FSS, Dr Bell's Disability Scale, the FibroFatigue Scale and the KPS were assessed in all participant groups as a determinant of severity (Hardcastle et al., 2014a; Hardcastle et al., 2014b).

6.3.2 Sample Preparation

A non-fasting blood sample of 50mL was collected from the antecubital vein of participants into lithium heparinised and EDTA tubes. Blood was collected between 8:00am and 11:30am and samples were analysed within 12 hours of collection. Initial full blood count assessment was undertaken to determine levels of white blood cell and red blood cell markers.

6.3.3 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 Treg phenotypes, NK cell lytic proteins and CD8 lytic proteins as described (Brenu et al., 2013a; Brenu et al., 2011) (Appendix 14). 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 PBS (Gibco Biocult, Scotland), cells were analysed on the flow cytometer (BD Immunocytometry Systems) where the expression of FOXP3 Tregs

was determined on CD4⁺CD25⁺CD127^{low} T cells (Brenu et al., 2011). NK and CD8 T cell lytic proteins were assessed as previously described (Brenu et al., 2011). Cells were incubated for 30 minutes 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 then washed and analysed on the flow cytometer where perforin, granzyme A and granzyme B expression was measured in NK and CD8 T cells (Brenu et al., 2011).

6.3.4 NK Cell Phenotype and Receptors 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 (Appendix 19) (Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells were analysed on the flow cytometer (BD Immunocytometry Systems) where NK cells were gated using CD56, CD16 and CD3 antibodies (Appendix 19) (Brenu et al., 2013a).

6.3.5 Whole Blood Analysis

Appropriate antibodies (Appendix 19) were added to whole blood samples and incubated for 30 minutes. Following which cells were lysed, washed, fixed and analysed on the flow cytometer. iNKT, DC, B, $\gamma\delta$ T and CD8⁺T cell phenotypes were assessed

using appropriate antibodies (Appendix 19) and gating strategies on the flow cytometer (Montoya et al., 2007).

6.3.6 Data and Statistical Analysis

All statistical analysis was performed using SPSS statistical software version 22.0. Paired *t* tests were used to examine changes in each immune parameter between zero and six months (week 24) for each of the groups. A one-way repeated ANOVA with time as a within-subject factor and group as a between-subject factor was used to assess the interaction of time and group. Results were classified as statistically significant at an alpha criterion of $p < 0.05$ if there were significant differences between groups over time.

The six month (week 24) single time point analysis was assessed among the three participant groups (control, moderate CFS/ME and severe CFS/ME) based on the distribution. If normally distributed, an ANOVA was used. Shapiro-Wilk and Kruskal Wallis test of independent variables based on rank sums to determine the magnitudes of group differences was used if data was not normally distributed. The Bonferroni Post Hoc or Mann-Whitney U tests determined *p* values of significance for parametric and non-parametric data respectively, with statistical significance set at an alpha criterion at $p < 0.05$. Clinical data are presented as mean \pm SD and immunological data are represented using mean \pm SEM. Extreme outliers were identified using an SPSS boxplot and handled by eliminating particular data points from the analysis (Aguinis et al., 2013).

6.4 RESULTS

6.4.1 Patient Characteristics

The participant ages (mean \pm SD) for the control (n=18), moderate CFS/ME (n=12) and severe CFS/ME (n=12) patient groups were 41.94 \pm 10.76, 44.73 \pm 12.90 and 41.27 \pm 10.05 respectively, with no statistically significant differences ($p < 0.05$) in age between the groups (Table 8). Gender distribution was also not significantly different between the groups as they were all predominantly female with control, moderate CFS/ME and severe CFS/ME groups having 72%, 67% and 83% female participants, respectively (Table 8).

Table 8: Participant characteristics, including: age and gender, for control, moderate CFS/ME and severe CFS/ME participant groups.

	Control (n=18)	Moderate (n=12)	Severe (n=12)	<i>p</i> value
Age in years (<i>Mean \pm SD</i>)	41.94 \pm 10.76	44.73 \pm 12.90	41.27 \pm 10.05	0.540
Gender (% Female)	72%	67%	83%	0.566

Age data is represented as Mean \pm SD and gender is represented as percentage of group which is female in control (n=18), moderate CFS/ME (n=12) and severe CFS/ME (n=12) groups. There were no significant differences in age or gender within the research groups.

All CFS/ME patients in the moderate and severe CFS/ME groups satisfied the 1994 Fukuda criterion for CFS/ME, as those who did not were excluded from the study. According to the FibroFatigue Scale, all CFS/ME patients scored significantly worse than the control group except in relation to ‘sadness’ which had no differences in scores between any participant groups. There was no statistically significant difference between moderate and severe CFS/ME patients in the FibroFatigue Scales (data not shown). Dr Bells Disability scale and the KPS were significantly different between all groups, with severe CFS/ME patients scores being further worsened significantly compared with moderate CFS/ME (Figure 14).

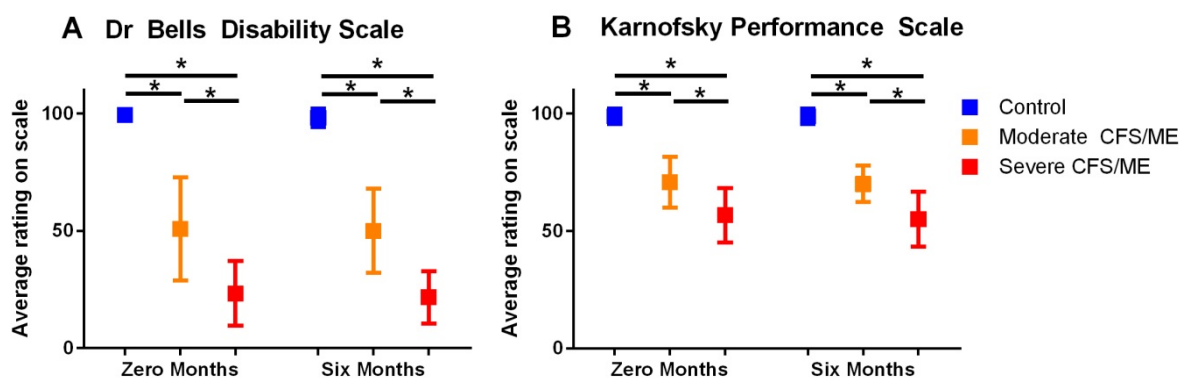


Figure 14: Average severity scale scores at zero and six months (week 24) for control, moderate and severe CFS/ME patients.

A Dr Bells Disability Scale scores for each group, represented as a score between 0 and 100. B KPS scores for each group, represented as a score between 0 and 100. All data is presented as mean±SEM where statistical significance was accepted as $p < 0.05$.

6.4.2 No Change to Intracellular Parameters

There were no significant differences between any of the groups and between zero and six months (week 24) for Tregs, NK or CD8⁺T cell lytic proteins.

6.4.3 No Change to Whole Blood Phenotypes

This research found no significant differences in DC or B cell phenotypes between any of the groups or between zero and six months (week 24).

6.4.4 iNKT Cells

Between zero and six months (week 24), iNKT cells expressing CD62L were significantly increased at six months (week 24) in moderate CFS/ME patients ($p < 0.0014$) (Figure 15).

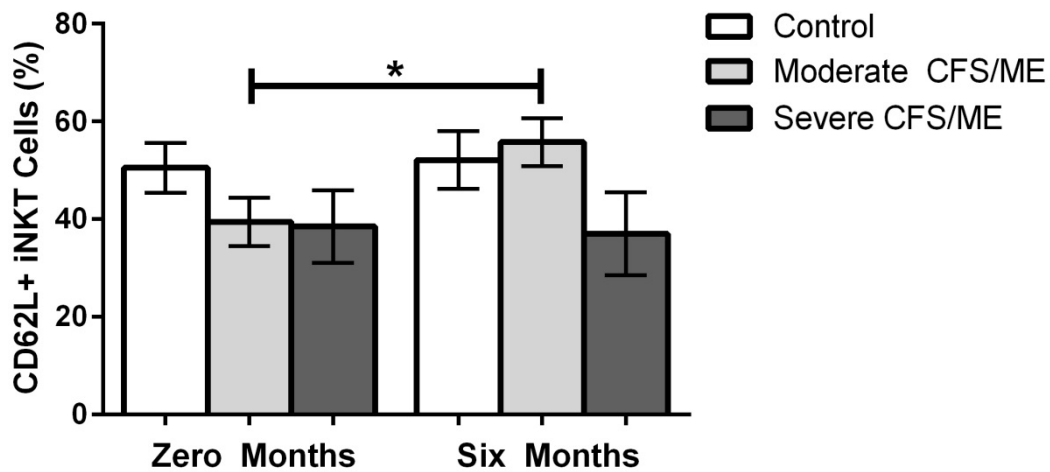


Figure 15: iNKT cell expression of CD62L in control, moderate and severe CFS/ME patients at zero and six months (week 24).

iNKT cells expressing CD62L as a percentage of total iNKT cells. Data is presented as mean±SEM where statistical significance was accepted as $p < 0.05$.

At six months, CD8⁺CD4⁻ and CD56⁺CD16⁻ iNKT cells were significantly increased in severe CFS/ME compared with controls ($p = 0.024$ and 0.030) (Figure 16).

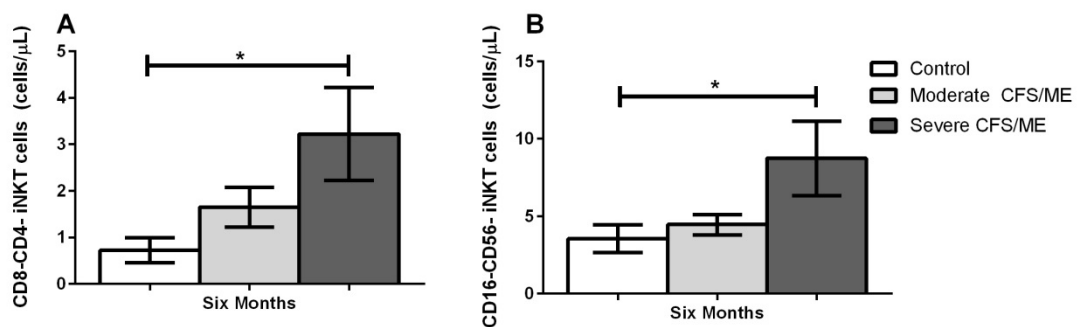


Figure 16: iNKT cell expression profile in control, moderate and severe CFS/ME patients.

A iNKT cells expressing a CD8⁺CD4⁻ phenotype as a number of total iNKT cells (cells/μL). B iNKT cells expressing a CD56⁺CD16⁻ phenotype as a number of total iNKT

cells (cells/ μ L). Data is shown as mean \pm SEM where statistical significance was accepted as $p < 0.05$.

6.4.5 KIRs

CD56^{bright}CD16^{dim} NK cells expressing KIR3DL1/DL2 were significantly increased in controls and moderate CFS/ME patients after six months (week 24) ($p < 0.000$ and 0.004) (Figure 17A). CD56^{bright}CD16⁺ NK cells expressing KIR2DL1 were significantly increased in severe CFS/ME patients after six months (week 24) ($p = 0.011$) (Figure 17B). CD56^{bright}CD16⁺ NK cells expressing KIR2DL2/DL3 were significantly increased in controls and moderate CFS/ME patients after six months (week 24) ($p = 0.018$ and 0.049) (Figure 17C). CD56^{bright}CD16⁺ NK cells expressing KIR2DS4 were also significantly increased in controls and moderate CFS/ME patients after six months (week 24) ($p = 0.038$ and 0.023) (Figure 17D).

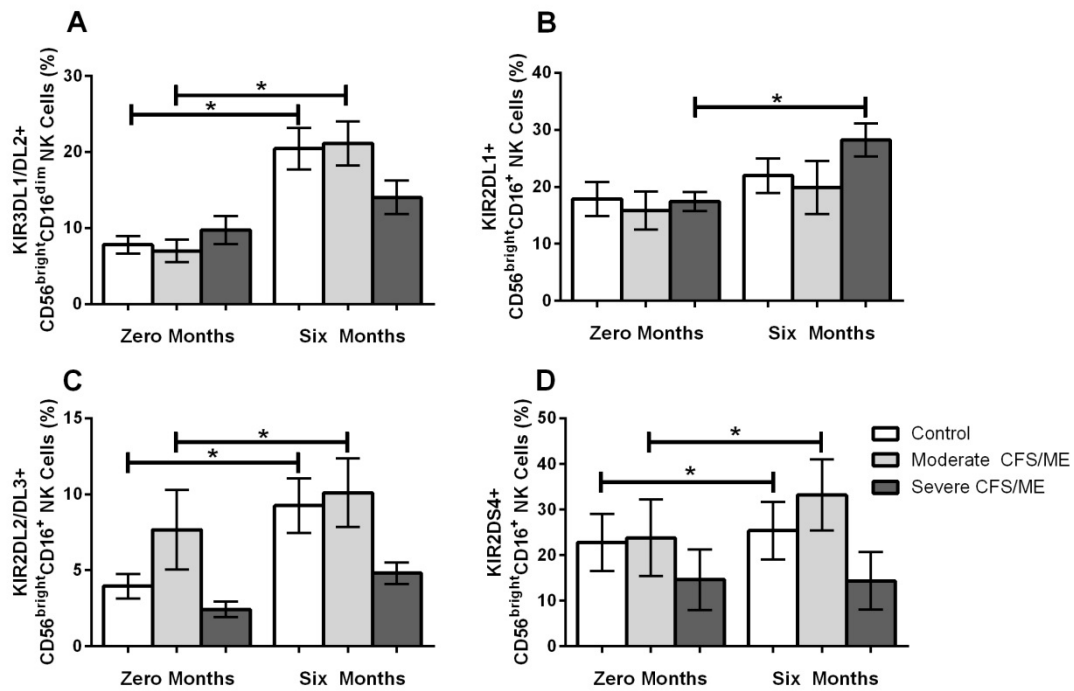


Figure 17: Alterations in CD56^{bright} NK cell receptors between zero and six months (week 24) in control, moderate and severe CFS/ME patients.

A Percentage of CD56^{bright}CD16^{dim} NK cells expressing KIR3DL1/DL2. *B* Percentage of total CD56^{bright}CD16⁺ NK cells expressing KIR2DL1. *C* Percentage of CD56^{bright}CD16⁺ NK cells expressing KIR2DL2/DL3. *D* Percentage of CD56^{bright}CD16⁺ NK cells expressing KIR2DS4. Data is shown as mean±SEM where statistical significance was accepted as $p < 0.05$.

At six months (week 24), CD56^{bright}CD16^{dim} NK cells expressing NKG2D were significantly reduced in severe CFS/ME compared with moderate CFS/ME patients ($p = 0.014$) (Figure 18A). Also at six months (week 24), KIR2DL2/DL3 expression in CD56^{dim}CD16⁻ NK cells was significantly reduced in severe CFS/ME patients compared with controls ($p = 0.045$) (Figure 18B).

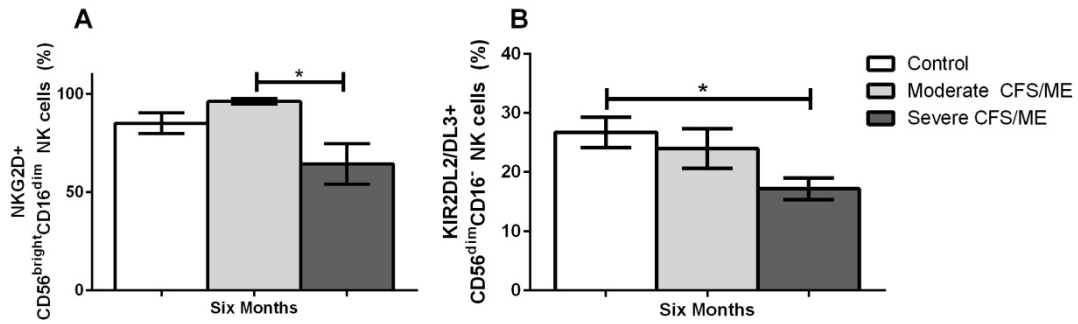


Figure 18: NK cell receptors in control, moderate and severe CFS/ME participant groups.

A Percentage of total $CD56^{bright}CD16^{dim}$ NK cells expressing the receptor *NKG2D*. *B* Percentage of total $CD56^{dim}CD16^{-}$ NK cells expressing the receptor *KIR2DL2/DL3*. Data is shown as $mean \pm SEM$ where statistical significance was accepted as $p < 0.05$.

6.4.6 CD8 T cells

At six months (week 24), naïve CD8 T cells were significantly increased in severe CFS/ME patients compared with moderate CFS/ME patients ($p = 0.041$) (Figure 19).

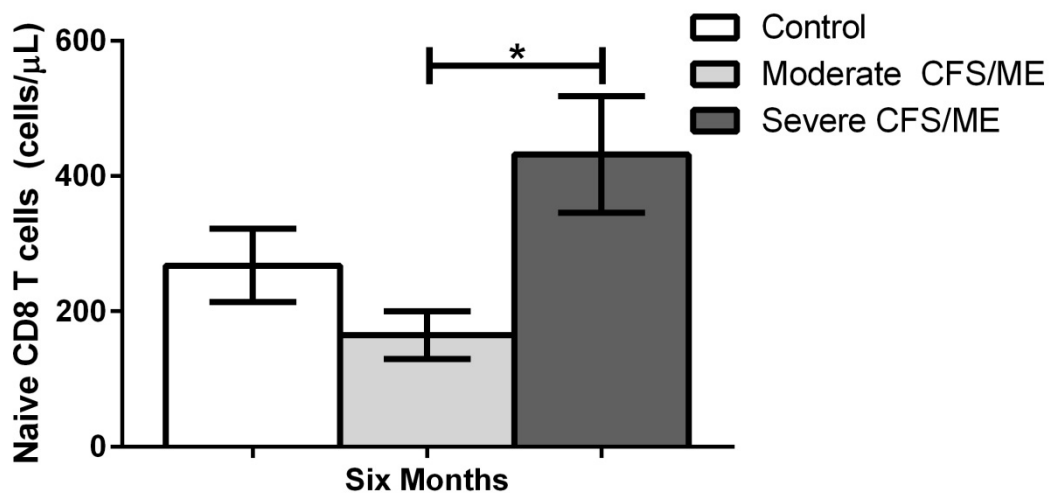


Figure 19: Increased naïve CD8⁺T cells in the severe CFS/ME participant group.

Number of total CD8⁺T cells (cells/ μ L) of the naïve CD8⁺T cell subset in controls, moderate CFS/ME and severe CFS/ME. Data is shown as mean \pm SEM where statistical significance was accepted as $p < 0.05$.

6.4.7 $\gamma\delta$ T cells

At the six months (week 24), total $\gamma\delta$ T cells were significantly increased in severe CFS/ME compared with controls and moderate CFS/ME patients ($p = 0.035$ and 0.034) (Figure 20A). At six months (week 24), $\gamma\delta$ effector memory and CD45RA⁺ effector memory T cells were also significantly increased in the severe CFS/ME patient group compared with controls and moderate CFS/ME patients respectively ($p = 0.003$, 0.013 and 0.017 , 0.032) (Figure 20B and 20C).

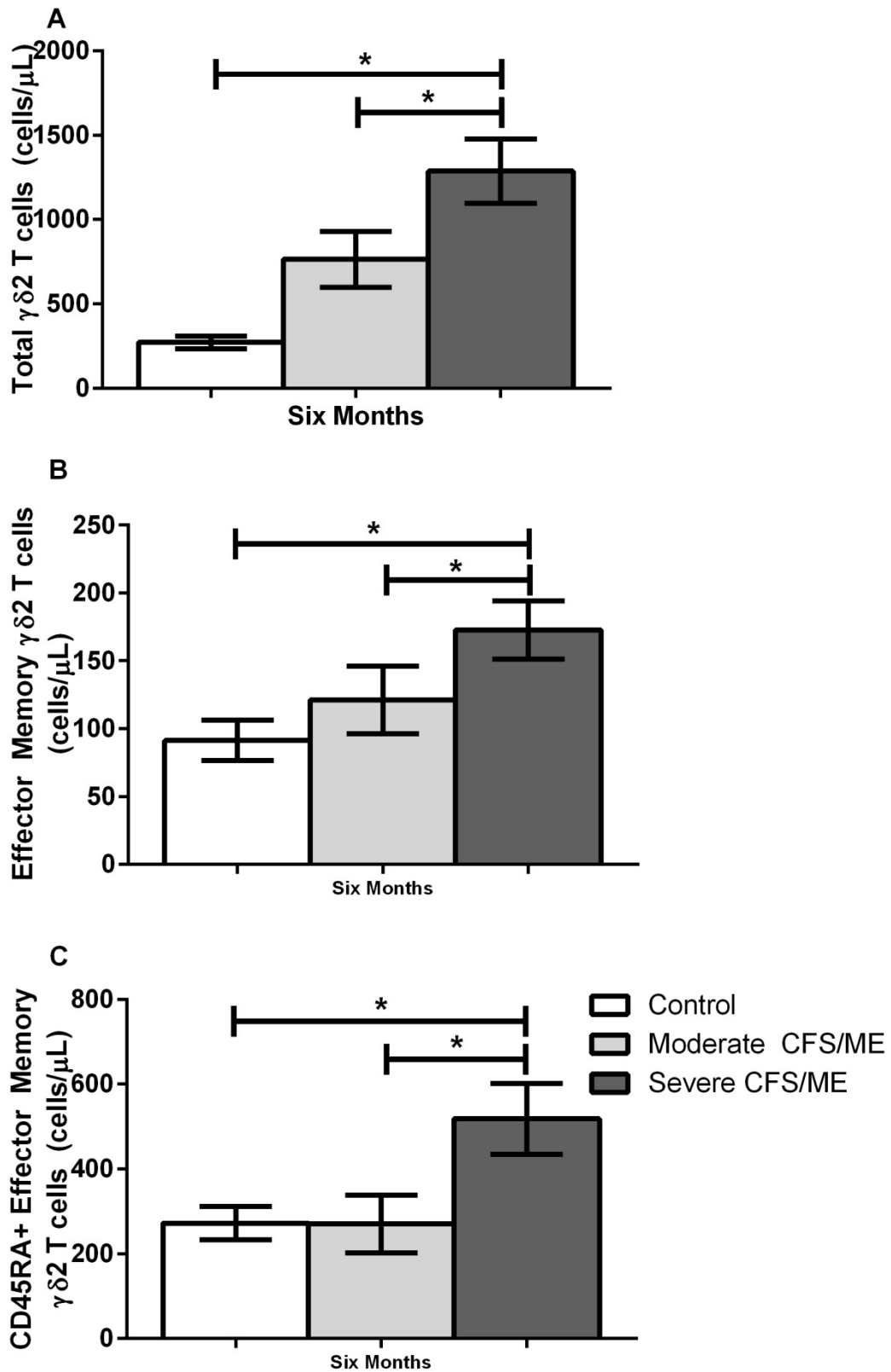


Figure 20: Alterations in $\gamma\delta 2$ T cell phenotypes in control, moderate CFS/ME and severe CFS/ME patients.

A Total number of $\gamma\delta 2$ T cells expressed as total cells/ μL . B Total number of $\gamma\delta 2$ T cells (cells/ μL) expressing the effector memory cell phenotype. C Total number of $\gamma\delta 2$ T cells (cells/ μL) expressing the CD45RA^+ effector memory cell phenotype. Data is shown as mean \pm SEM where statistical significance was accepted as $p < 0.05$.

At six months (week 24), $\gamma\delta 1$ T cells in the severe CFS/ME group displayed significantly lower $\text{CD94}^-\text{CD11a}^-$ expression when compared with the control and moderate CFS/ME group ($p = 0.018$ and 0.047) (Figure 21A). At six months (week 24), $\text{CD94}^-\text{CD11a}^-$ expression in $\gamma\delta 2$ T cells of severe CFS/ME patients was significantly higher than controls and moderate CFS/ME ($p = 0.019$ and 0.005) (Figure 21B). The severe CFS/ME group also had significantly higher $\text{CD94}^-\text{CD11a}^+$ expression on $\gamma\delta 2$ T cells compared with controls ($p = 0.025$) in the six month (week 24) (Figure 21C).

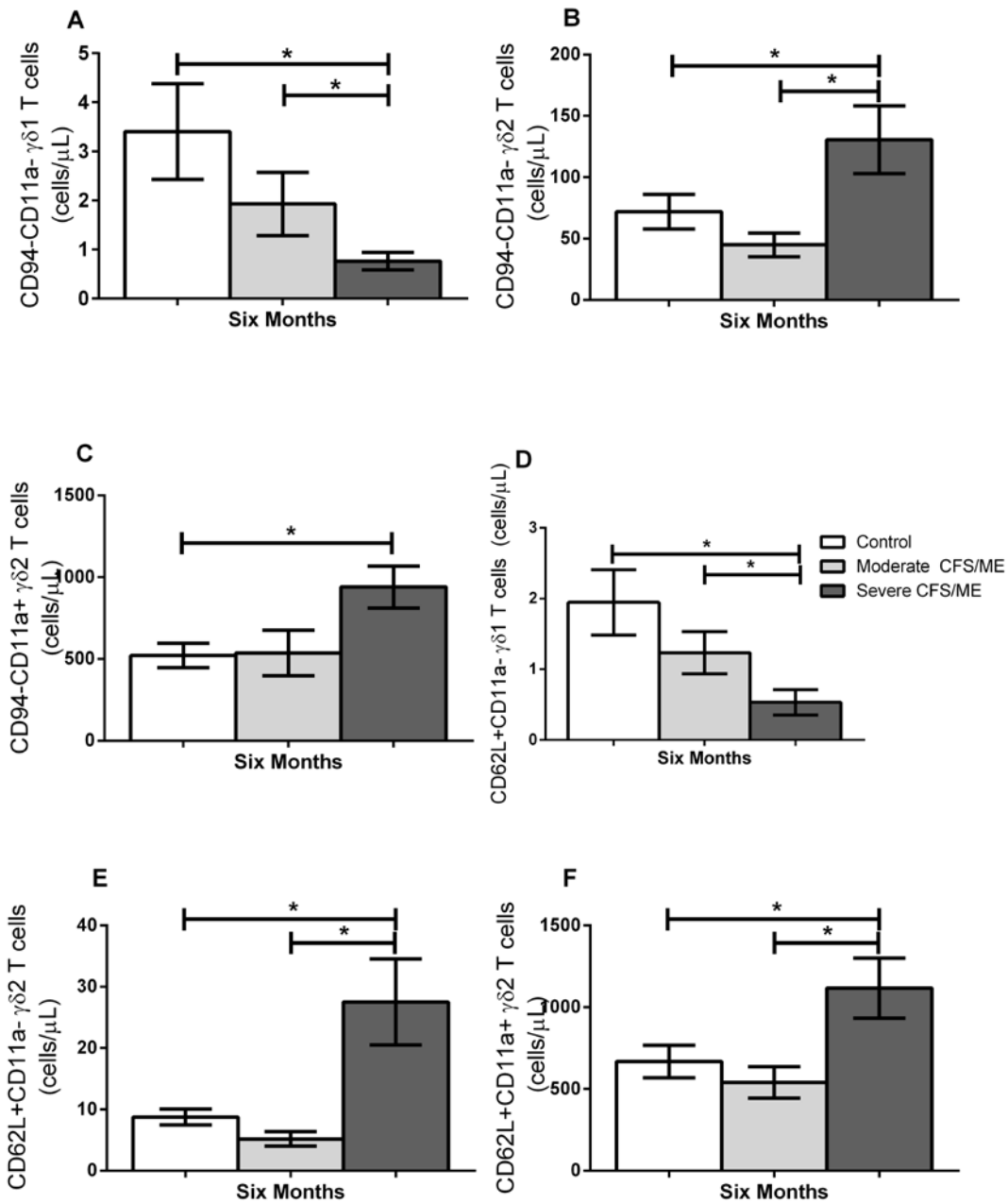


Figure 21: Profile of $\gamma\delta$ 1 and $\gamma\delta$ 2 T cell expression of receptors and adhesion molecules in control, moderate CFS/ME and severe CFS/ME patients.

A $\gamma\delta$ 1 T cells with the CD94⁻CD11a⁻, as a number of total $\gamma\delta$ 1 T cells (cells/ μ L). *B* $\gamma\delta$ 2 T cells with the CD94⁻CD11a⁻, as a total number of $\gamma\delta$ 2 T cells (cells/ μ L). *C* $\gamma\delta$ 2 T cells with the CD94⁻CD11a⁺, as a total number of $\gamma\delta$ 2 T cells (cells/ μ L). *D* $\gamma\delta$ 1 T cells with CD62L⁺CD11a⁻ as a total number of $\gamma\delta$ 1 T cells (cells/ μ L). *E* $\gamma\delta$ 2 T cells with CD62L⁺CD11a⁻ as a total number of $\gamma\delta$ 2 T cells (cells/ μ L). *F* $\gamma\delta$ 2 T cells with the

expression CD62L⁺CD11a⁺, as a total number of $\gamma\delta$ T cells (cells/ μ L). Data is shown as mean \pm SEM where statistical significance was accepted as $p < 0.05$.

At the sixth month (week 24), $\gamma\delta$ 1 T cells expression of CD62L⁺CD11a⁻ was significantly reduced in severe CFS/ME compared with both controls and moderate CFS/ME ($p = 0.013$ and 0.023) (Figure 21D). At six months (week 24), $\gamma\delta$ 2 T cells expression of CD62L⁺CD11a⁻ as well as CD62L⁺CD11a⁺ was significantly increased in the severe CFS/ME group compared with controls and moderate CFS/ME patients ($p = 0.002$, 0.001 and 0.045 , 0.018 respectively) (Figure 21E, 8F).

6.5 DISCUSSION

The present study examined innate and adaptive immune cells at zero and six months (week 24) to investigate longitudinal changes in moderate and severe CFS/ME. Severe CFS/ME patients displayed significant NK cell receptor differences over time when compared with controls and moderate CFS/ME. At the sixth month (week 24), severe CFS/ME patients also demonstrated significant alterations in iNKT cell phenotypes, CD8⁺T cell markers, NK cell receptors and $\gamma\delta$ T cells compared with the control and/or moderate CFS/ME patients.

Our study demonstrated immunological variation over time as there were differences between participant groups between zero and six months (week 24). iNKT cells had not previously been examined in CFS/ME and the current study found expression of CD62L was significantly increased in moderate CFS/ME patients between zero and six months (week 24). The function of CD62L in iNKT cells is not known although this

may suggest variation in iNKT cell markers or adhesion over time in CFS/ME. The CD56^{bright} NK cell subset also varied between participant groups over time, particularly in the severe CFS/ME patients. CD56^{bright}CD16^{dim} NK cells expressing KIR3DL1/DL2 and CD56^{bright}CD16⁺ NK cells expressing KIR2DS4 and KIR2DL2/DL3 were significantly increased after six months (week 24) in controls and moderate CFS/ME patients, while severe CFS/ME patients showed significantly increased CD56^{bright}CD16⁺ NK cells expressing the KIR2DL1 receptor after six months (week 24). This research showed changes in NK cell receptors over time notably in CD56^{bright} NK cells. CD56^{bright} NK cells form around 10% of total peripheral NK cells and are the primary producers of NK cell-derived cytokines, particularly IFN- γ , TNF- β , macrophage colony-stimulating factor (M-CSF), IL-10 and IL-13 during an innate immune response (Cooper et al., 2001a; Cooper et al., 2001b). Previously, peripheral levels of IL-10 and IFN- γ were shown to be significantly increased and longitudinal analysis has shown the CD56^{bright}CD16⁻ NK cell phenotype to be decreased over time in CFS/ME patients (Brenu et al., 2012c). The current study potentially suggests that the alterations in CD56^{bright} NK cell subsets may be influencing cytokine production over time in CFS/ME. Cytokine imbalances between pro-inflammatory cytokines or cytokine inhibitors may play a role in the initiation of a number of diseases, particularly Th1/Th2 cytokine shifts which have been used to explain immunological disease pathogenesis (Müller, 2002).

NK cell receptors are particularly important in CFS/ME as reduced NK cell cytotoxic activity is one of the most consistent markers of the illness (Barker et al., 1994; Brenu et al., 2013a; Brenu et al., 2012c; Brenu et al., 2011; Curriu et al., 2013; Hardcastle et al., 2014a; Huth et al., 2014; Klimas et al., 1990). NK cell cytotoxic activity can be

regulated to by NKG2D, which is an activating receptor that has previously been significantly elevated in ICC-defined CFS/ME patients when compared with CFS/ME patients defined using the 1994 Fukuda definition (Brenu et al., 2013b). CD94 is a NK cell receptor which is dependent on NKG2 protein association and has also been significantly increased in CD56^{dim}CD16⁻ NK cells in CFS/ME patients in previous research (Hardcastle et al., 2014a). Our study found significantly reduced NKG2D expression in CD56^{bright}CD16^{dim} NK cells in severe CFS/ME compared with moderate CFS/ME at the sixth month (week 24). Therefore, reduced NKG2D may be associated with the reduced NK cell cytotoxic activity previously shown in severe CFS/ME patients compared with moderate CFS/ME patients (Brenu et al., 2013a; Hardcastle et al., 2014a). Previous research has also suggested that impairment of NK cell cytolytic function may be derived in part by reduced activating NK cell receptors, such as NKG2D (Epling-Burnette et al., 2007).

KIR2DL2/DL3 is an inhibitory receptor that has been previously reduced in severe CFS/ME compared with moderate CFS/ME patients (Hardcastle et al., 2014a). The current study supports previous findings where KIR2DL2/DL3 expression in CD56^{dim}CD16⁻ NK cells in severe CFS/ME patients was again significantly reduced when compared with controls. This reduction in the inhibitory receptor of CFS/ME patients may be a result of a larger regulatory response to the reduced NK cell cytotoxic activity that is shown in the illness, particularly as CD56^{dim} NK cells are highly cytotoxic (Brenu et al., 2013a; Cooper et al., 2001a).

Significantly raised naïve CD8⁺T cell numbers at six months (week 24) of this research in severe CFS/ME patients may be promoting the ability of these severely affected

patients to develop an immune response against novel antigens and lower the susceptibility of infections (Roederer et al., 1995). CD8⁺T cells are also responsible for cytotoxic activities and have previously shown significantly reduced activity in CFS/ME patients (Brenu et al., 2011). In contrast, CFS/ME patients have also previously been associated with CD8⁺T cell immune activation, a reduced level of CD8⁺ suppressor T cells and an increase in CD8⁺ cytotoxic T cells (Landay et al., 1991). Therefore the current study validates previous research where significantly enhanced CD8⁺T cell activation and CD8⁺T cell numbers were found in CFS/ME patients (Klimas et al., 1990; Landay et al., 1991).

There is little research on iNKT cells in CFS/ME patients, although one study has shown significantly elevated iNKT cell numbers in severe CFS/ME patients, reduced CD8CD4, CD8aCD4 phenotypes in moderate CFS/ME, increased CD56CD16 and CCR7SLAM phenotypes in severe CFS/ME compared with both moderate CFS/ME patients and controls (Hardcastle et al., 2014a). The present study again found significantly increased iNKT cells expressing CD56⁻CD16⁻ in severe CFS/ME patients at the sixth month (week 24). The function of CD56 and CD16 on iNKT cells is unknown (Montoya et al., 2007) however, altered expression of these markers on NK cell phenotypes is often shown in CFS/ME patients (Barker et al., 1994; Brenu et al., 2013b; Brenu et al., 2011; Caligiuri et al., 1987; Hardcastle et al., 2014a; Landay et al., 1991). The CD8⁻ CD4⁻ subset of iNKT cells is primarily responsible for cytotoxic activities and was previously reduced in moderate CFS/ME patients (Hardcastle et al., 2014a). The present study has shown a significant increase in CD8⁻ CD4⁻ iNKT cells in severe CFS/ME patients, suggesting a possible regulatory mechanism where cytotoxic activities may be enhanced in iNKT cells as a regulatory response to the reduced

cytotoxic activity that has been consistently documented in NK cells and CD8⁺T cells of CFS/ME patients (Barker et al., 1994; Brenu et al., 2013a; Brenu et al., 2011; Hardcastle et al., 2014a; Klimas et al., 1990).

The present study found significantly increased overall numbers of $\gamma\delta$ 2 T cells in severe CFS/ME at the sixth month (week 24). As $\gamma\delta$ T cells are sentinel cells with cytotoxic properties, this may suggest an activation as an immune response to bacterial infection, wound repair, antigen presentation or immunoregulation (Anane et al., 2010). Significantly enhanced numbers of effector memory and CD45RA⁺ effector memory $\gamma\delta$ 2 T cells also in severe CFS/ME patients suggests that they have greater potential for cytotoxic activity, tissue homing and target recognition (Anane et al., 2010; Hardcastle et al., 2014a). Effector memory phenotypes of $\gamma\delta$ T cells exhibit NK-like functions, detecting MHC expression and undergoing cytotoxic activities following cytokine directed proliferation and regulatory pathways (Anane et al., 2010; Farber et al., 2014; Sallusto et al., 2004). Interesting, both effector memory and CD45RA⁺ effector memory T cell phenotypes are preferentially mobilized during adrenergic stimulation, suggesting severe CFS/ME patients' immune responses may be enhanced similar to a situation of psychological stress (Anane et al., 2010). There may potentially be a homeostatic mechanism taking place in severe CFS/ME patients, leading to greater immune activation, similarly to that also shown in CD8⁺T cells and Tregs in CFS/ME (Brenu et al., 2012c; Brenu et al., 2011; Curriu et al., 2013; Morris & Maes, 2013b).

CD94⁻CD11a⁻ expression was significantly reduced in severe CFS/ME patients in $\gamma\delta$ 1 T cells and significantly increased in severe CFS/ME patients in $\gamma\delta$ 2 T cells. CD94 is a surface molecule with NK-like abilities, important in MHC expression detection and

high cytotoxic activities while CD11a is an adhesion molecule which aids migration to inflammatory sites (Anane et al., 2010). $\gamma\delta 2$ T cells expressing $CD94^+CD11a^+$ were also significantly increased in severe CFS/ME patients, suggesting that the majority of $\gamma\delta$ T cells in these patients may have improved adhesion and migration to sites of inflammation (Anane et al., 2010). $\gamma\delta 1$ and $\gamma\delta 2$ T cells also showed variation in CD62LCD11a expression, as severe CFS/ME patients demonstrated significantly reduced $CD62L^+CD11a^-$ $\gamma\delta 1$ T cells as well as significantly increased $CD62L^+CD11a^-$ $\gamma\delta 2$ T cells. $CD62L^+CD11a^+$ expression was increased in $\gamma\delta 2$ T cells of severe CFS/ME patients, again potentially suggesting severe patients may have an enhanced immune activation and an increased adhesive and migratory ability compared with moderate CFS/ME and controls (Anane et al., 2010). The alternative expression of these markers in $\gamma\delta 1$ and $\gamma\delta 2$ T cells may be a result of the differing $\gamma\delta$ T cells phenotypes while $\gamma\delta 1$ T cells are mainly present in epithelial tissues and low levels in the bloodstream and $\gamma\delta 2$ T cells represent most of circulating $\gamma\delta$ T cells (Poggi et al., 2004).

6.6 CONCLUSIONS

This research was the first to assess innate and adaptive immune cells over time in moderate and severe CFS/ME patients. Severe CFS/ME patients had significantly altered NK cell receptors over time in comparison with moderate CFS/ME patients and controls. Severe CFS/ME patients also expressed significant changes in iNKT cell phenotypes, $CD8^+$ T cell markers, NK cell receptors and $\gamma\delta$ T cells compared with the control and/or moderate CFS/ME patients at six months (week 24). This research highlighted the importance of longitudinally assessing varying severities of CFS/ME patients to further examine variation in illness severity and consistency of potential

immune abnormalities that have been shown. This research may also contribute to further understanding CFS/ME and potentially assist in leading to a diagnostic test based on distinct immunological markers.

CHAPTER 7: DISCUSSION AND CONCLUSION

This research is the first to assess potential immunological abnormalities in CFS/ME patients with varying symptom severity. This research has also determined longitudinal differences in immune parameters in severe CFS/ME patients that were not found in moderate CFS/ME patients and non-fatigued healthy controls.

Previously, immunological CFS/ME research had been primarily focused on patients who have the physical ability to visit collection sites (Bansal et al., 2012; Brenu et al., 2010; Brenu et al., 2012c; Brenu et al., 2011; Broderick et al., 2010; Chia & Chia, 2008; Curriu et al., 2013; Fukuda et al., 1994a; Gupta & Vayuvegula, 1991b; Huth et al., 2014; Klimas et al., 2012; Klimas et al., 1990; Levy, 1994; Lloyd et al., 1989; Patarca, 2001). Consequently, previous CFS/ME studies are limited as the patients represented in these data were not inclusive of those who had severe CFS/ME (Hooper, 2007; Jason et al., 2009a). The inclusion of severity subgroups to distinguish patients with varying symptom severities is widely recognised in illnesses such as cancer, MS and FM (Meeus et al., 2011; Saa'd et al., 2012). Severity scales may enable measures of subgroups in CFS/ME patients to determine if severity does play a role in the illness and allow for it to be represented in research findings (Brenu et al., 2013a; Ojo-Amaize et al., 1994; Wiborg et al., 2010b). CFS/ME is a heterogeneous illness, therefore assessing distinct moderate and severe CFS/ME patients in this research allows for an identification of immunological parameters that may not have been distinguished in a group of CFS/ME patients with varied and undisclosed severity.

The collective results in this current research investigation included immunological analysis of moderate and severe CFS/ME patients as well as controls at baseline (week 0) and six months (week 24). Initial baseline (week 0) results found significantly reduced NK cell cytotoxic activity, CD56^{dim}CD16⁻ KIR2DL1/DS1 NK cells, CD45RA⁺ effector memory $\gamma\delta$ 1T cells in both moderate and severe CFS/ME patients compared with controls. Moderate CFS/ME patients at baseline (week 0) also had significantly reduced effector memory $\gamma\delta$ 1T cells, CD8⁻CD4⁻, CD8a⁻CD4⁻ and CD8a⁻CD4⁺ iNKT cells compared with controls. Severe CFS/ME patients had reduced CD56^{bright}CD16^{dim} KIR2DL2/DL3, transitional and Bregs compared with moderate CFS/ME patients. Compared with both moderate CFS/ME patients and controls at baseline (week 0), severe CFS/ME patients demonstrated significantly heightened CD14⁻CD16⁺ DCs and CD56⁺CD16⁺, CD56⁻CD16⁻, CD56⁻CD16⁺, CCR7⁻SLAMF⁻ and CCR7⁻SLAMF⁺ iNKT cells. Severe CFS/ME patients also displayed significantly increased CD56^{bright}CD16^{dim} and CD56^{dim}CD16⁺ NK cells, memory and naïve B cells in comparison with moderate CFS/ME patients. CD94⁺ CD56^{dim}CD16⁻ NK cells and iNKT cell numbers were significantly increased in moderate and severe CFS/ME patients compared with controls. Moderate CFS/ME patients then also showed significantly increased CD56^{dim}CD16⁻ NK cells and pDCs compared with controls.

Baseline (week 0) serum samples were also used for an analysis of immunoglobulins and cytokines between controls, moderate and severe CFS/ME patients. In these measures, severe CFS/ME patients displayed significantly increased serum IFN- γ compared with moderate CFS/ME patients and significantly increased IL-7 and IL-8 compared with both controls and moderate CFS/ME patients. Moderate CFS/ME patients also showed significantly enhanced levels of RANTES and IL-1 β compared

with severe CFS/ME patients as well as significantly reduced IL-6 compared with both controls and severe CFS/ME patients. All significant differences found in baseline (week 0) serum cytokines in this current research investigation were between moderate and severe CFS/ME patient subgroups, emphasising the presence of potential severity subgroups in the illness.

The six month (week 24) follow up included a longitudinal analysis of parameters as well as a single time point analysis (week 24). According to Dr Bells Disability Scale and the KPS, there were no significant differences in the severities of any groups between the two time points, suggesting that the severity of the CFS/ME patients over that time was maintained. Only the moderate CFS/ME patients displayed a significant increase between baseline (week 0) and six months (week 24) in CD62L⁺ iNKT cells. Similarly, only severe CFS/ME patients demonstrated a significant increase in CD56^{bright}CD16⁺ KIR2DL1 between baseline (week 0) and the six month (week 24) time point. A significant increase was shown between the two time points of baseline (week 0) and six months (week 24), in both controls and moderate CFS/ME patients in CD56^{bright}CD16^{dim} KIR3DL1/DL2 and CD56^{bright}CD16⁺ KIR2DL2/DL3 and KIR2DS4. Overall, the longitudinal analysis demonstrated significant disparities in the severe CFS/ME patients when compared with the differences found in controls and moderate CFS/ME patients. This particularly highlights consistent immunological dysfunction in CFS/ME and the incidence of distinct immune cell presentation between the symptom severity subgroups in CFS/ME patients, which has not previously been found in other investigations prior to this research.

Analysis at the six month (week 24) time point found that severe CFS/ME patients showed significantly increased CD8⁻CD4⁻ and CD56⁻CD16⁻ iNKT cells when compared with controls. This significant increase in CD8⁻CD4⁻ and CD56⁻CD16⁻ iNKT cells was also shown at baseline (week 0). At six months (week 24), total effector memory and CD45RA⁺ effector memory $\gamma\delta$ 2T cells, CD94⁻CD11a⁻, CD62L⁺CD11a⁻, CD62L⁺CD11a⁺ $\gamma\delta$ 2T cells and CD62L⁺CD11a⁻ $\gamma\delta$ 2T cells were significantly higher in severe CFS/ME patients compared with both controls and moderate CFS/ME patients. Severe CFS/ME patients demonstrated significantly increased CD94⁻CD11a⁺ $\gamma\delta$ 2T cells at six months (week 24) compared with controls. Severe CFS/ME patients had significantly reduced CD56^{dim}CD16⁻ KIR2DL2/DL3 NK cells at six months (week 24) compared with controls and CD62L⁺CD11a⁻ $\gamma\delta$ 1T cells were significantly lower in severe CFS/ME patients compared with both controls and moderate CFS/ME patients. Furthermore, significant differences were also present between severity subgroups at the six month (week 24) time point, as severe CFS/ME patients showed significantly higher naïve CD8⁺T cells and CD56^{bright}CD16^{dim} NKG2D NK cells compared with moderate CFS/ME patients.

The analysis of adhesion markers at the sixth month (week 24) time point found significantly increased SLAM in NK cells in moderate compared with severe CFS/ME patients. Severe CFS/ME patients demonstrated significantly increased CD56^{dim}CD16⁻ CD18⁺CD11c⁻ compared with controls. Moderate CFS/ME patients had significantly reduced CD56^{dim}CD16⁻ NK cell CD2⁺, CD56^{dim}CD16⁻ CD18⁺CD2⁺ compared with severe CFS/ME patients. Adhesion molecules appeared to vary in moderate CFS/ME patients, potentially suggesting differing aetiologies between moderate and severe CFS/ME patients in some cases. Moderate CFS/ME patients also showed significantly

increased central memory CD4⁺T cell BTLA4 compared with controls and significantly enhanced CD4⁺T cell KIR2DL5 compared with severe CFS/ME patients. In comparison with controls, moderate CFS/ME patients showed significantly increased CD45RA⁺ effector memory CD8⁺T cells. Moderate CFS/ME patients then had significantly reduced central memory CD8⁺T cell LFA-1 and CD8⁺T cell KLRG1 compared to controls. Compared with both controls and moderate CFS/ME patients, severe CFS/ME patients also showed significantly reduced CD56^{bright}CD16^{dim} NK p46 receptor expression. Naïve CD4⁺T cell KLRG1 was significantly reduced in moderate CFS/ME patients compared with controls and severe CFS/ME patients. In CD4⁺ and CD8⁺ TCRs, it appears that moderate CFS/ME patients may express a different immunological aetiology to severe CFS/ME patients as differences shown were predominantly in the moderately affected CFS/ME patients.

The results from this research therefore suggest that there are a number of immunological parameters that are significantly different between moderate and severe CFS/ME patient subgroups. Immune dysregulation can be associated with clinical features and presentation; hence this may be reflected in the varying severity of CFS/ME patients (Fluge et al., 2011; Glaser & Kiecolt-Glaser, 2005; Ojo-Amaize et al., 1994). The discussion sections below will highlight and provide further detailed explanation on the results from this research.

7.1 INCREASED CD56^{BRIGHT} NATURAL KILLER CELL PHENOTYPES COUPLED WITH REDUCED NATURAL KILLER CELL CYTOTOXIC ACTIVITY IN SEVERE CFS/ME

Reduced NK cell cytotoxic activity has been previously observed in moderately affected CFS/ME patients (Brenu et al., 2012c; Brenu et al., 2011; Carruthers et al., 2011; Klimas et al., 2012; Klimas et al., 1990; Lloyd et al., 1989; Patarca-Montero et al., 2001; ter Wolbeek et al., 2007). While there were no significant differences between moderate and severe CFS/ME patients in NK cell cytotoxic activity, the severe CFS/ME patients demonstrated consistently lower NK cell cytotoxic activity compared with the moderate CFS/ME patients. These findings have validated our previous research, which was the first to assess NK cell cytotoxic activity in moderate and severe CFS/ME patients (Brenu et al., 2013a).

NK cell cytotoxic activity is crucial to host defence and is achieved following target cell recognition when the balance of activating signals reaches the threshold required for target cell lysis (Grier et al., 2012). Reduced NK cell cytotoxic activity in CFS/ME patients may suggest compromised cytotoxic activity pathways, such as those involving the apoptotic mediators, perforin and granzymes (Bade et al., 2005; Brenu et al., 2011; Trapani & Smyth, 2002). Perforin and granzymes contribute to the granule-mediated cell death pathway by facilitating granzyme entry into target cells and subsequently activating apoptosis respectively (Chowdhury & Lieberman, 2008; Saiki et al., 2008). Previous research has shown inconsistent differences in perforin and granzyme levels in CFS/ME patients (Brenu et al., 2011; Maher et al., 2005), although the present research found no significant difference in perforin or granzymes between the control, moderate

CFS/ME and severe CFS/ME patients. This may highlight the heterogeneity of the illness between research studies, particularly as previous studies did not separate CFS/ME patients based on severity (Brenu et al., 2012a; Brenu et al., 2011; Maher et al., 2005; Saiki et al., 2008). Further reduced NK cell cytotoxic activity in severe CFS/ME patients may also reflect an association between symptom severity and patient physical mobility and altered immune dysfunction (Brenu et al., 2013a; Hardcastle et al., 2014a; Ojo-Amaize et al., 1994).

NK cell cytotoxic activity may be regulated by NK cell phenotypes. CD56^{bright} and CD56^{dim} NK cell phenotypes have specific roles in an immune response, being responsible for cytokine secretion and cytotoxic granule release respectively (Cooper et al., 2001b). Increases in both CD56^{bright} and CD56^{dim} NK cell phenotypes may therefore suggest heightened secretion of cytokines such as IFN- γ and increased release of cytotoxic granules, including perforin and granzymes, in CFS/ME patients (Caligiuri, 2008; Cooper et al., 2001a). This may suggest that while NK cell phenotypes are significantly increased in the illness, they may have impaired functioning as they play a role in NK cell cytotoxic activity, which is significantly decreased in these CFS/ME patients. Severe CFS/ME patients appear to have further reduced NK cell cytotoxic activity and this may be reflected by the significantly increased NK cell phenotypes which also occurs in these patients.

CD56^{bright} NK cell receptors were significantly different in severe CFS/ME patients between baseline (week 0) and six month (week 24) time points when compared with differences in moderate CFS/ME and non-fatigued healthy controls. CD56^{bright}CD16^{dim} KIR3DL/DL2, CD56^{bright}CD16⁺ KIR2DL2/DL3 and CD56^{bright}CD16⁺ KIR2DS4 NK

cells were significantly increased between the two time points in controls and moderate CFS/ME patients while there were no significant changes in the same markers in severe CFS/ME patients. Similarly, only CD56^{bright}CD16⁺ KIR2DL1 NK cells were significantly increased in severe CFS/ME patients between baseline (week 0) and six months (week 24). Therefore, according to these differences, severe CFS/ME patients appear to have significantly different levels of CD56^{bright} NK cell receptors over time when compared with both moderate CFS/ME patients and non-fatigued healthy controls. CD56^{bright} NK cells are primarily responsible for the secretion of immunoregulatory cytokines, including: IFN- γ , TNF- β , IL-8, IL-10 and IL-13 which induce cytotoxic activity and assist in the recruitment of B cells and DCs (Cooper et al., 2001a; Cooper et al., 2001b). Therefore, alterations in CD56^{bright} NK cell receptors in severe CFS/ME patients may suggest potential cytokine imbalances in this cohort which may subsequently be influencing interactions and activation of DCs, B cells, T cells and macrophages (Robertson, 2002; Vivier et al., 2008).

In support of these findings, this research also found significantly increased serum IFN- γ and IL-7 in severe CFS/ME patients at baseline (week 0) and although the source is unknown, it may be associated with the increased CD56^{bright} NK cells in these patients (Michaud et al., 2010). The levels of serum IFN- γ and IL-7 at baseline (week 0) were also significantly correlated. This may be a result of the interaction between IFN- γ and IL-7 in CD56^{bright} NK cells as IL-7 is expressed in high levels in these cells modulates IFN- γ . IL-7 promotes the survival of the CD56^{bright} NK cell phenotype and also inhibiting apoptosis by increasing B cell lymphoma (BCL)2 expression (Michaud et al., 2010). IL-7 also plays a critical role in the regulation of lymphoid homeostasis, which

may also be imbalanced in CFS/ME patients who have previously demonstrated Th1/Th2 cytokine shifts {Ma, 2006 #540;Fletcher, 2009 #342}.

Enhanced numbers of CD56^{bright} NK cells are found in diseases such as HIV, SLE and MS (Alter & Altfeld, 2009; Blanca et al., 2001; Reis et al., 2009; Schepis et al., 2009). In SLE, there was a significantly increased number of CD56^{bright} NK cells, an associated increase in levels of serum IFN- γ and low NK cell cytotoxic activity (Schepis et al., 2009). These immune abnormalities shown in SLE are similar to that shown in CFS/ME, particularly as shown in severe CFS/ME patients in this research (Schepis et al., 2009). In SLE it has been suggested that NK cell alterations are contributing to the autoimmune disease, promoting T cell activation and subsequent B cell responses as well as directly promoting B cell responses via CD40/CD154 interactions (Blanca et al., 2001; Reis et al., 2009; Schepis et al., 2009). A cellular mechanism similar to that in SLE may be present in CFS/ME patients as research has suggested the possibility of increased activation of both T and B cells (Brenu et al., 2011; Fluge et al., 2011).

7.2 ABERRANT NATURAL KILLER CELL RECEPTORS IN SEVERE CFS/ME

KIR3DL1 and KIR2DL2/DL3 specifically bind to MHC-I molecules, providing an explanation for the relationship between NK cell sensitivity to cytotoxic activity and the absence of MHC-I molecules on target cells (Bryceson et al., 2006; Stewart et al., 2003). These KIRs then induce inhibitory actions on NK cell cytotoxic activity (Bryceson et al., 2006). Individual KIR molecule expression is independent of other KIR molecules, often resulting in a NK cell population with a diverse repertoire of receptors and a range of specificities to HLA class I allotypes (Raulet et al., 2001; Stewart et al., 2003). The current findings suggest the inhibitory receptor KIR2DL2/DL3 may be dysregulated in severe CFS/ME patients and that this appears consistent over time. Reduced inhibitory NK cell receptors are typically associated with a corresponding increase in NK cell cytotoxic activity, although in severe CFS/ME patients, it may be a regulatory response where inhibitory receptors are being reduced in an attempt to improve NK cell cytotoxic activity (Barker et al., 1994; Brenu et al., 2010; Brenu et al., 2011; Caligiuri et al., 1987; Gupta & Vayuvegula, 1991a; Klimas et al., 1990; Lanier, 1998; Levine et al., 1998; Lloyd et al., 1989; Ojo-Amaize et al., 1994; Swanink et al., 1996; Tirelli et al., 1994). Furthermore, it is possible that antigenic stimulation from NK cells may be initiating inhibitory receptor expression in response to cytotoxic stimulation (Raulet et al., 2001). This may be particularly important in severe CFS/ME patients as NK cell cytotoxic activity appears to be further reduced in this cohort.

There were significantly increased NK cell KIR2DS4 over time in both controls and moderate CFS/ME patients while the severe CFS/ME patients demonstrated no change in KIR2DS4. This KIR2DS4 interaction has low binding affinity compared with other KIR2D receptors and peptide-specific interactions may enhance the binding of activating KIRs, such as KIR2DS4 to enhance the lysis of abnormal target cells (Graef et al., 2009; Katz et al., 2004; Kulkarni et al., 2008). Differences in KIR2DS4 between moderate and severe CFS/ME patients may suggest potential diversity in the genetics and aetiology between moderate and severe CFS/ME patients.

NKG2D and NKp46 are also NK cell cytotoxic activity activating receptors (Gonzalez et al., 2006; Raulet et al., 2013; Thorén et al., 2012) which were both significantly reduced in CD56^{bright}CD16^{dim} NK cells in severe CFS/ME patients at the sixth month (week 24) time point. Although CD56^{bright} NK cells are not primarily involved in cytotoxic activities, the significant reduction in these activating receptors may be associated with significantly decreased NK cell cytotoxic activity in CFS/ME patients, particularly in the severe CFS/ME patient cohort. The cytokine induced CD94 NK cell receptor, which is dependent on the NKG2 protein associated with NKG2D, was significantly increased at baseline (week 0) in both moderate and severe CFS/ME patients. Furthermore, enhanced CD94 receptors are often associated with upregulated HLA-E expression, which protects target cells from NK cell lysis, reducing overall NK cell cytotoxic activity (Lanier, 2005; Tomasec et al., 2000).

SLAM was significantly increased at the six month (week 24) time point of the current research in moderate CFS/ME patients. Increased SLAM typically enhances NK cell cytotoxic activity (Lanier, 2001; Sidorenko & Clark, 2003; Wang et al., 2001). SLAM

is differentially expressed on NK cells to act as an activating adaptor protein to amplify IFN- γ secretion and the recruitment of inflammatory cells, such as DCs (Bouchon et al., 2001; Lanier, 2001; Sidorenko & Clark, 2003; Wang et al., 2001). Differences in SLAM, in CFS/ME patients may be influencing downstream pathways, including the CD2-like Receptor Activating Cytotoxic Cell (CRACC) pathway, which in turn reduce NK cell cytotoxic activity in these patients (Bouchon et al., 2001; Chan et al., 2003; Cruz-Munoz et al., 2009; Kim & Long, 2012).

Over time, this research found similar trends in NK cell receptor expression in both controls and moderate CFS/ME patients while severe CFS/ME patients appeared to significantly differ. This may be important in validating a significant distinction between CFS/ME patients based on symptom severity.

The consistent significant reduction of NK cell cytotoxic activity in CFS/ME patients may play an important role in the activation of DCs during an immune response (Tomescu et al., 2010).

7.3 INCREASED DC PHENOTYPES IN SEVERE CFS/ME

DC cell activity and phenotypes in moderate and severe CFS/ME patients had not been assessed prior to this research. This research found no significant differences at six months (week 24) between any participant groups in CD80 and CD86 costimulatory molecules and markers of DC activity (Fu et al., 1996). This presupposes that DCs in CFS/ME patients appear to actively respond to stimulus. This may suggest that it may be cell-cell interactions or cytokine secretion may be influencing DC dysfunction in CFS/ME patients.

At baseline (week 0), pDCs were significantly increased in moderate CFS/ME patients compared with controls and also showed a statistically insignificant increase in severe CFS/ME. pDCs secrete high amounts of cytokines, such as IFNs, present antigens and stimulate T cells during an immune response (Barchet et al., 2005). Increases in pDCs are often associated with improved NK cell activation and functioning (Tomescu et al., 2010) however, NK cell cytotoxic activity is often significantly reduced in CFS/ME patients (Brenu et al., 2013a; Brenu et al., 2010; Brenu et al., 2011; Caligiuri et al., 1987; Klimas et al., 2012; Klimas et al., 1990). pDCs enhance NK cell mediated cytotoxic activity by producing type I IFNs, particularly IFN- α (Colonna et al., 2004; Hoene et al., 2006). NK cell and DC interactions result in activation and cytokine production by both cell types, leading to NK cell proliferation, cytotoxic activities and DC maturation (Cooper et al., 2004). This suggests that there may be diminished efficiency in cell-cell cross talk, including inhibitory and activating signals between DCs and NK cells in CFS/ME patients, as the increase in pDCs is not associated with an improvement in NK cell cytotoxic activity. Moreover, at baseline (week 0) serum

cytokine analysis found no significant differences in IFN- α between any participant groups, which is secreted by pDCs (Tomescu et al., 2010). pDCs also have the potential to stimulate T cell immune responses and generate Tregs by upregulating MHC and costimulatory molecules (Barchet et al., 2005; Fu et al., 1996). pDCs act as precursor cells following antigen interaction they may mature into DCs and act as initiators of adaptive immune responses, including the promotion of Treg differentiation (Barchet et al., 2005). This may suggest that the significant increase in pDCs may correspond to the increased Treg phenotypes also found in CFS/ME patients (Brenu et al., 2012a; Brenu et al., 2011; Morris & Maes, 2013a). The relationship between pDCs and Tregs is also thought to play an important role in the pathogenesis and progression of HIV (Strickler et al., 2014). The dual roles of pDCs and Tregs may also play a role in the CFS/ME immune mechanism as both parameters are increased in the illness and may be related to an enhanced immune response.

CD14⁻CD16⁺ DCs have a distinct Fc γ R pattern to other DC phenotypes (Henriques et al., 2012). Increased CD14⁻CD16⁺ DCs in severe CFS/ME patients may also contribute to the priming of T cell responses, implying improved maintenance of the peripheral inflammatory environment during an immune response (Henriques et al., 2012). IL-4, IL-10 and IL-12 cytokines are primarily produced by CD14⁻CD16⁺ DCs to enhance an inflammatory response and activate NK cells (Henriques et al., 2012; Piccioli et al., 2007), although the present research found no differences in these serum cytokines at baseline (week 0) between groups. Serum IL-4, IL-10 and IL-12 cytokines may not have differed in this research because although the number of CD14⁻CD16⁺ DCs were significantly enhanced in severe CFS/ME patients, there may not be an associated increase in DC-derived cytokine secretion which is required to create and regulate an

inflammatory environment. It is also possible that the broad assessment of serum cytokines may not specifically detect any potential alterations in DC-derived cytokines.

The secretion of cytokines by DCs may also be regulated by iNKT cells which promote the release of IL-10, activate DC function by secreting IFN- γ and present CD1d molecules (Cerundolo et al., 2004; Mars et al., 2004). iNKT cells had not been assessed in CFS/ME patients prior to this research.

7.4 ALTERATIONS IN iNKT CELLS IN CFS/ME

iNKT cells interact with a number of innate and adaptive immune cells to regulate disease severity (Godfrey et al., 2000; Mars et al., 2004). This was the first to assess iNKT cells in CFS/ME patients, finding significantly increased total iNKT cell numbers in severe CFS/ME patients compared with both controls and moderate CFS/ME patients at baseline (week 0). Significantly enhanced numbers of iNKT cells may contribute to an improved cell-mediated regulation of immunity and further promotion of iNKT cell proliferation in the bone marrow (Godfrey et al., 2000; Mars et al., 2004).

iNKT cells also promote the release of IL-10 which reduces IL-12 in DCs and causes anergy apoptosis in T and B cells (Mars et al., 2004). There were no significant differences in serum IL-10 and IL-12 levels at baseline (week 0). However, increased numbers of DCs, B cells and serum IFN- γ in correspondence with reduced NK cell ability may still allude to dysfunctional cross-talk between innate and adaptive immune cells in CFS/ME patients, potentially worsened in severe CFS/ME patients (Mars et al., 2004).

At baseline (week 0), severe CFS/ME patients had significantly increased numbers of CD56⁻CD16⁻, CD56⁺CD16⁺ and CD56⁻CD16⁺ iNKT cell phenotypes. The function of CD56 and CD16 in iNKT cells is unknown although alterations in CD56 and CD16 NK cell phenotypes are common in CFS/ME patients (Barker et al., 1994; Caligiuri et al., 1987; Klimas et al., 1990; Landay et al., 1991; Lorusso et al., 2009; Straus et al., 1993). CD56⁻CD16⁻ iNKT cell phenotypes were consistently increased in severe CFS/ME patients at both baseline (week 0) and six months (week 24). It may be possible that the

mechanism contributing to altered NK cell phenotypes in CFS/ME patients may be similar to that influencing differences in CD56 and CD16 iNKT cell phenotypes.

Severe CFS/ME patients had significantly increased numbers of CCR7⁺SLAM⁺ and CCR7⁻SLAM⁻ iNKT cell phenotypes at baseline (week 0) compared with moderate CFS/ME patients and controls. CCR7 is expressed in 20% of peripheral iNKT cells and is important in immunosurveillance, migration and the production of IL-2, IL-10 and TNF- α (Kim et al., 2002). Increased peripheral iNKT cells not expressing CCR7 in severe CFS/ME patients may potentially contribute to the reduced ability of these iNKT cells to interact with other immune cells, such as DCs via cytokines (IL-2, IL-10 and TNF- α) (Kim et al., 2002).

The presence of SLAM on iNKT cells is important in cytokine production, particularly the amplification and induction of IFN- γ and IL-4. SLAM is therefore necessary for the subsequent recruitment of inflammatory cells, including: DCs, NK, B and T cells as well as for cytotoxic activities by iNKT cells (Baev et al., 2008; Bouchon et al., 2001; Lanier, 2001; Sidorenko & Clark, 2003; Wang et al., 2001; Wang et al., 2004). Aberrant levels of CCR7 and SLAM in the severe CFS/ME patients may suggest these severe CFS/ME patients potentially have further immune dysfunctions. This research has highlighted the importance of assessing iNKT cells and their phenotypes in CFS/ME research. This is of particular value as iNKT cells primarily function to interact with cells of the innate and adaptive immune systems.

7.5 B CELL PHENOTYPES DIFFER IN SEVERE CFS/ME

At baseline (week 0), naive and memory B cell phenotypes were significantly increased in severe CFS/ME patients compared with moderate CFS/ME patients. This potentially suggests an amplification of B cell activation in those who are more severely affected by symptoms (McHeyzer-Williams & McHeyzer-Williams, 2005). This is also potentially related to the alterations found in NK cells, iNKT and T cells in CFS/ME patients at baseline (week 0) and six months (week 24) as these cells are responsible for moderating B cell activation (Blanca et al., 2001). The enhanced numbers of naive and memory B cell phenotypes may also be associated with the significant increase in serum IFN- γ found at baseline (week 0) in severe CFS/ME patients as well as alterations demonstrated in NK cells in the illness (Brenu et al., 2013a; Curriu et al., 2013; Klimas et al., 1990). NK cells interact directly with B cells through the CD40-CD154 pathway which is necessary for B cell maturation, Ig secretion and isotype switching. In turn, B cells also activate NK cells, further stimulating their production of IFN- γ (Blanca et al., 2001). IL-8 is another pro-inflammatory mediator produced by activated NK cells which promotes the attraction of T cells and B cells (Robertson, 2002). The importance of this cytokine is in this study, as serum IL-8 was significantly increased in severe CFS/ME patients at baseline (week 0). Although the origin of the IL-8 in serum was unknown, the association of IL-8 with the activation of B cells may lead to increased serum IL-8 being associated with the heightened B cell activation shown previously in CFS/ME patients (Fluge et al., 2011; Robertson, 2002).

Additionally, the severe CFS/ME patient group had significantly reduced transitional B cells and Bregs compared with controls at baseline (week 0). Transitional B cells are

essential in the process of B cell development, particularly in negative selection checkpoints of B cell autoreactivity (Carsetti et al., 1995; Palanichamy et al., 2009). Research has previously suggested that reduced transitional B cells in autoimmune diseases appears to correspond with increased autoreactive B cells in both mature and naïve B cell phenotypes (Palanichamy et al., 2009).

Reduced Bregs may suggest decreased B cell maturation and progression in severe CFS/ME patients. This may be a result of ineffective T cell-mediated extrinsic signals or CD40-CD40 cell interactions and further contribute to immune dysfunction in CFS/ME patients, including NK cell cytotoxic activity (Blanca et al., 2001; Chung et al., 2003; Palanichamy et al., 2009).

Significant changes in B cell phenotypes, particularly in the severe CFS/ME patient group may play a significant role in the clinical features and aetiology of CFS/ME, as previous investigations have reported clinical improvements in CFS/ME patients following treatment using B cell depletion (Fluge et al., 2011). It was speculated that this may be explained by an elimination of disease-associated autoantibodies or interaction of B cells with T cell antigen presentation, as implied in SLE (Fluge et al., 2011; Mackay et al., 2006; Sanz & Lee, 2010). Potential B cell activation in CFS/ME is similar to SLE, with the B cell depleting agent Rituximab showing improvement in SLE patients as well as in CFS/ME patients (Fluge et al., 2011; Sanz & Lee, 2010). Interestingly, SLE also presents with enhanced CD56^{bright} NK cells and reduced NK cell cytotoxic activity, similarly to CFS/ME patients, demonstrating potential immune parallels between the illnesses (Fluge et al., 2011; Mackay et al., 2006; Sanz & Lee, 2010; Urbańska-Krawiec & Hrycek, 2010). B cell antibody production and activation is

regulated by a number of cells, including $\gamma\delta$ T cells. It has been proposed that in autoimmune diseases, $\gamma\delta$ T cell self-reactivity may be directed against B cells (Häcker et al., 1995). This relationship between $\gamma\delta$ T cells and B cells may suggest that immune abnormalities in B cells in CFS/ME patients may subsequently be associated with abnormal numbers of $\gamma\delta$ T cells, as found in this research.

7.6 $\gamma\delta$ 2T INCREASES IN SEVERE CFS/ME

This research was the first to assess $\gamma\delta$ T cells in CFS/ME patients over six months (week 24) and the first to examine $\gamma\delta$ T cells in moderate and severe CFS/ME patients. At baseline (week 0), EMRA $\gamma\delta$ 1T cells were significantly lower in moderate and severe CFS/ME patients compared with controls. Effector memory $\gamma\delta$ 1T cell phenotypes were also significantly reduced in moderate CFS/ME patients compared with controls. At the sixth month (week 24), $\gamma\delta$ 2T cell total numbers, along with $\gamma\delta$ 2T cell EMRA and effector memory phenotypes were significantly increased in severe CFS/ME patients compared with both controls and moderate CFS/ME.

Effector memory $\gamma\delta$ T cells demonstrate NK-like functions including: detection of non-self MHC expression on target cells, cytotoxic activities, tissue homing and rapid innate-like target cell recognition (Anane et al., 2010). $\gamma\delta$ 1T and $\gamma\delta$ 2T cells have differential roles during an immune response (Hahn et al., 2004; Poggi et al., 2004). $\gamma\delta$ 1T cells are present in epithelial tissues and low levels in the blood stream, responsible for epithelial tumour cell lysis, tissue and wound repair, airway inflammation and Th2 cytokine levels (Hahn et al., 2004; Poggi et al., 2004). In this research, it appears that $\gamma\delta$ 1 effector memory phenotypes are reduced in the bloodstream of CFS/ME patients and subsequently, they may potentially have reduced target cell recognition in the endothelium, tissues and airways (Anane et al., 2010; Hahn et al., 2004).

$\gamma\delta$ 2T cells play a role in B cell proliferation, Th1 cytokine levels, immunosuppression and the activation of neutrophils, monocytes and NK cells during an immune response

(Hahn et al., 2004; Poggi et al., 2004). $\gamma\delta$ 2T cells are responsible for activating a number of innate and adaptive immune cells. At six months (week 24), $\gamma\delta$ 2T cells were significantly enhanced in severe CFS/ME patients (Anane et al., 2010; Hahn et al., 2004). Serum IFN- γ was increased at baseline (week 0) in the severe CFS/ME patient group and although the origin was unknown, it may be associated with the increased $\gamma\delta$ 2T cells, which secrete IFN- γ to activate NK cell cytotoxic activity (Carding & Egan, 2002; Cerwenka & Lanier, 2001).

It is possible that the heightened number of $\gamma\delta$ 2T cells in CFS/ME patients may also be associated with increased Tregs previously reported in the illness (Aspler et al., 2008; Brenu et al., 2011). Similarly, serum IL-7 was increased in CFS/ME patients and is an important cytokine required for the expansion of naive CD4⁺ and CD8⁺ T cells as well as overall T cell homeostasis. The potential relationship between IL-7 and T cell aberrations shown in CFS/ME patients may be important in the illness pathogenesis and understanding the apparent immune dysregulation (Schluns et al., 2000; Tan et al., 2001; Tan et al., 2002).

Overall, this research has contributed to CFS/ME research by confirming the presence of immunological dysfunction in the illness, such as reduced NK cell cytotoxic activity as well as identifying cellular manifestations present in the illness that had not previously been examined, including increased iNKT cell numbers and phenotypes. The research also observed immune abnormalities that are specific to either severe CFS/ME or moderate CFS/ME patients. Importantly the severe CFS/ME patients presented with abnormalities in NK cell phenotypes, receptors and adhesion molecules, B cell phenotypes, iNKT cell markers, DC phenotypes, cytokines, $\gamma\delta$ T cells compared with

moderate CFS/ME patients. The heterogeneous nature of CFS/ME may be contributing to the varied immunological abnormalities and clinical presentation attributed to the illness. This research was the first of its kind, particularly assessing immunological parameters in moderate and severe CFS/ME patients, creating a platform in immune research of subgroups in CFS/ME. This has highlighted the potential for severity subgroups in the illness and contributed to future research into the illness mechanism or potential diagnostic tools.

7.5 LIMITATIONS AND FUTURE RESEARCH

Most importantly, this research has advanced progress towards understanding the pathomechanism of CFS/ME and/or a diagnostic panel. CFS/ME is a heterogeneous illness exemplified by patients having varied course and age of onset, illness duration, co-morbidities, symptom severity and activity levels. This research categorised symptom severity subgroups for analysis although further studies may also categorise CFS/ME patients based on age and course of onset, illness duration and activity levels. This research has highlighted the potential for CFS/ME subgroups in clinical and research settings which may be beneficial for the implementation of specific management or treatment strategies in future.

Often, due to the severity of their illness, severe CFS/ME patients are difficult to recruit and liaise with, to determine home visit logistics for research. For this reason the number of participants in the research was limited by the number of severe CFS/ME patients included. It is necessary to examine these immunological cell markers in a larger cohort of severe CFS/ME patients in a longitudinal study to further assess the consistency in immunological markers and reproducibility of results that may assist in developing a diagnostic panel for the illness. In order to further analyse the results from this thesis in future, longitudinal examination of significant immune parameters over three or more time points may be important to evaluate consistencies.

Individual CFS/ME patients also experience varying symptom severity and can fluctuate between ‘moderate’ and ‘severe’ states over long periods of time. It may also be beneficial to examine individual patients longitudinally to identify potential

variations between moderate and severe symptoms. This may assist in understanding the direct role of immunological dysfunction on the physiological state at different stages of the illness. This may be important in future if treatment for CFS/ME becomes available because it may be tailored based on these symptom severities.

Serum cytokine analysis in this research found significant alterations, although the origin of the cytokines was unknown. This analysis was not specific to cell types as it represents total cytokines secreted by all cells in peripheral blood circulation at the time of collection. Further studies may assess specific cytokine levels by isolating cell types for cytokine analysis. Future research may also analyse key cytokines, including IL-7, IL-8 and IFN- γ , in participants serum on a longitudinal scale to assess whether cytokine alterations are consistent over time.

iNKT cells had not previously been assessed in CFS/ME patients and this current research demonstrated significant differences in iNKT cell parameters between moderate and severe CFS/ME patients. Therefore, assessing iNKT cell receptors and function may be of interest for further studies. Similarly, $\gamma\delta$ 1T and $\gamma\delta$ 2T cell phenotypes have shown significant differences in severe and moderate CFS/ME patients, highlighting the potential to further examine $\gamma\delta$ T cell function, such as cytotoxic activities in the illness. B and T cell phenotype differences were found in CFS/ME patients at baseline (week 0) and six months (week 24), potentially suggesting dysregulation of these cells. Proliferative profile of B cells (Mond & Brunswick, 2003) along with B and T cell stimulatory and activation investigations may further elaborate on the mechanism or consequences of these differences in CFS/ME patients.

Medication and supplement intake varies greatly in CFS/ME patients and this can influence the immune system. It may benefit immunological research to include CFS/ME patient groups who are not taking medication for the duration of the research to ensure medication intake does not confound the results.

Overall, this research has provided a preliminary investigation into immunological dysfunction in different symptom severities of CFS/ME patients. This highlights the benefit of future research directing at subgroups of CFS/ME patients as it may contribute to increased sensitivity and identification of immune biomarkers. Distinctive symptom severity subgroups may also allow more specific management strategies for CFS/ME patients in clinical settings.

7.6 CONCLUSION

Presently, CFS/ME appears to be a heterogeneous and idiopathic illness with no known biomarker for a diagnostic test. This research has significantly contributed to the knowledge of CFS/ME by highlighting a number of immunological abnormalities in CFS/ME patients which may be important in understanding the illness mechanism, severity subgroups and also contribute to a panel of unique biomarkers for identifying CFS/ME.

The international knowledge of CFS/ME has been significantly enhanced by this research as it has contributed unique assessments that had not previously been undertaken in the illness. This original research found that NK cell, DC, iNKT, $\gamma\delta$ T cell and B cell phenotypes significantly differed in severe CFS/ME patients when compared with moderate CFS/ME patients. These results have demonstrated the importance of assessing symptom severity subgroups among patient cohorts in both clinical and research settings. These results were also the first to examine iNKT and $\gamma\delta$ T cells in CFS/ME patients. This research found significantly increased iNKT cell numbers, increased iNKT cell markers and enhanced $\gamma\delta$ T cell phenotypes in severe CFS/ME patients. Longitudinal assessments had not been previously undertaken in severity subgroups of CFS/ME patients and in this research, severe CFS/ME patients had a significantly different number of CD56^{bright} NK cell receptors when compared with moderate CFS/ME patients and controls over time.

The results generated from this research are valuable as they have identified novel immunological abnormalities in CFS/ME as well as the presence of potential patient

subgroups, which appear important in understanding the pathomechanism of the illness. The implementation of severity subgroups may enhance research specificity as well as clinical understanding of CFS/ME by recognising variation between patients. This research has also demonstrated potential relationships between changes in both innate and adaptive immune cells and proteins, NK cells, DCs, iNKT, B cells, T cells and cytokines that should be assessed in future. Further investigation is now necessary to ascertain the mechanism(s) and potential biomarker(s) for the illness. Future investigations may also take advantage of CFS/ME subgroups to reinforce understanding of the heterogeneous illness.

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9.0 APPENDICES

APPENDIX 1: INFORMATION SHEET

Analysis of Innate and Adaptive Immune Cell Functions within patients with varying severities of Chronic Fatigue Syndrome

PARTICIPANT INFORMATION SHEET

**Griffith University Ethics Reference Number: MSC / 23 / 12 /
HREC**

Who is conducting the Research

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Participant Coordinators

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Why is the research being conducted?

Chronic Fatigue Syndrome (CFS) is an illness diagnosed based on symptom-specific criteria. There are substantial costs associated with CFS worldwide and currently there is no known cure, successful treatments or a useful method for

diagnosing the disorder. This research examines the pathology of the disorder with a specific focus on white blood cells. Two distinct groups of CFS patients will be examined, the first being those with mild symptoms, mobility and able to go about their daily activities. The second group of CFS patients will be those with 'severe' symptoms who are homebound.

The expected benefits of the research

A review of the available research to date demonstrates a significant lack of research focused on 'severely affected' CFS patients. Alterations in immune function have been identified in a number of CFS cohorts however, majority of these individuals often demonstrate moderate CFS symptoms as they are mobile and unlikely to be homebound at the time of the experiment. Only one study has investigated and shown changes in particular white blood cells known as Natural Killer cells in patients with severe CFS. These Natural Killer cells are important in the immune system as they can quickly detect and kill infected cells. This research forms part of Sharni Hardcastle's higher degree PhD research. Hence, the focus of this research is to determine the extent or difference in white blood cells in CFS patients with moderate and severe symptoms in relation to a non-fatigued healthy control group.

The purpose of this study is to:

1. Provide an extensive analysis of immune cell function, gene expression and protein expression in NK cells, neutrophils, monocytes, dendritic cells, B cells and T cells in CFS subjects with a particular focus on subjects with moderate and severe CFS symptoms in comparison to a non-CFS group.

2. Assess the role of immune cell phenotypes in severe and moderate CFS compared to non-CFS controls.
3. Determine potential biomarkers for the disease.
4. Analyse heart rate, blood pressure and temperature of CFS patients in comparison to non-CFS controls.

The basics by which you will be selected or screened

Prior to participation in the study, all participants will be required to complete a questionnaire which will be provided at the initial assessment phase to determine if a participant is suitable for the above mentioned study/research. The questionnaires will consist of the CDC and International Consensus Criteria for CFS, Fatigue Severity Scale, Karnofsky Performance Status and the SF-36 Health Questionnaire. Participants will be excluded if they are smokers, have a diagnosed autoimmune disease, are pregnant, are currently breast feeding, have diabetes or have heart disease. The purpose of the exclusion criteria is to ensure that other confounding factors are not in conflict with the results generated from the study. Hence it is highly recommended that participants inform the researchers in the event that they identify with certain aspects of the exclusion criteria.

What will you be asked to do?

Following completion of the questionnaires all participants will be notified by researchers to determine if they are in the inclusion or exclusion criteria.

Appointments will be made with individuals in the inclusion criteria for blood and clinical measure collections. Therefore, as a volunteer participant for this study, we will pre-arrange a date, time and place for blood and clinical measure collection. A volume of 80ml of blood will be collected as well as temperature, heart rate and blood pressure from all participants by a qualified General Practitioner (GP) or a qualified and trained phlebotomist on the appointed date. Participants who are severely affected by CFS and unable to leave their homes will be visited by a qualified medical practitioner and/or phlebotomist and a member of the research team. Participants, who are able, will visit the facility at Griffith University for collections.

A strict protocol will be adhered to for the collection of samples. Firstly, researchers will ensure that the patient is seated for at least 15 minutes prior to blood collection. During this time, the researcher will observe the patient and if they appear distressed they will be asked if they would consider another day for blood collection or do they wish to withdraw from the study. The patient may withdraw at their own free will. The qualified professional will then take a temperature reading from the outer ear of the patient and an arm blood pressure reading. After 15 minutes of being seated, the patient will then have 80mL of blood collected from the antecubital vein of their arm.

Risks to you

Some participants may feel dizzy after blood collection hence following collection; each patient will remain seated for 10 minutes and be provided a biscuit, water and/or orange juice by the researcher. After the post 10 minutes

from blood draw, the patient will be relocated to an adjacent room where a family member, carer or a friend will accompany them to their home.

Participants will be contacted by a research team member post blood collection to confirm your wellbeing. Researchers will be available to address any issues or questions relating to the research or your wellbeing. You may contact the Chief Investigator, Professor Sonya Marshall-Gradisnik or Student Researcher, Sharni Hardcastle on the contact details above.

Your confidentiality

The identity, with respect to your name and personal details will be disclosed to the researchers and remain at all times confidential. You will be given an alpha numerical code to avoid identification. Your contact details will also be stored in a private and confidential file. You will not be informed of your participant code and researchers will only identify you and your code for the purpose of sending you results if requested and once they have been stringently quality assured.

Following data collection and after completion of the study, you will be contacted and provided with explanatory and summary information pertaining to the results from this study. You will also be sent Full Blood Count (FBC) results from their own blood a basic explanation of the results in lay-mans. You will only be given your own personal FBC result alongside a 'reference' range of values.

Your participation is voluntary

Participation in this study is entirely voluntary and with no obligation. You are free to withdraw from the study at any time.

Questions / further information

If you have any further questions regarding this research, feel free to contact a member of the research team on the contact details above. Contact details provided are for:

Senior Investigator: Professor Sonya Marshall-Gradisnik,
PhD Candidate conducting this research: Sharni Hardcastle

Ethical conduct of this research

This research is conducted in accordance with the National Statement on Ethical Conduct in Human Research.

As a participant of this research, you may withdraw at any time. Should you have any complaints or concerns regarding the ethical conduct of this research, you may contact the Manager of Research Ethics at Griffith University at research-ethics@griffith.edu.au or on (07) 37555585.

Privacy Statement

This research involves the collection and use of your identified personal information. The information collected is confidential and will not be disclosed to third parties without your consent, except to meet government, legal or other regulatory authority requirements. A de-identified copy of this data may be used for other research purposes. However, your anonymity will at all times be safeguarded. For further information consult Griffith University's Privacy Plan at: <http://www.griffith.edu.au/privacy-plan>, or telephone Griffith University on (07) 37354375.

Again, thank you for your participation in this research, it is much appreciated and you are invited to contact us on the numbers above should you have any questions or if you would like to withdraw.

APPENDIX 2: CONSENT FORM

Analysis of Innate and Adaptive Immune Cell Functions within patients with varying severities of Chronic Fatigue Syndrome

CONSENT FORM

**Griffith University Ethics Reference Number: MSC / 23 / 12 /
HREC**

Research Team

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By signing below, I confirm that I have read and understood the information package and in particular have noted that:

- I understand my involvement in this research;
- I have had any questions answered to my satisfaction;
- I understand the risks involved;
- I understand that there will be no direct benefit to me from my participation in this research;
- I understand that my participation in this research is voluntary;
- I understand that if I have any additional questions I can contact the research team;
- I understand that I am free to withdraw at any time, without comment or penalty;
- I understand that any information about me is confidential, and that no information that could lead to the identification of any individual will be disclosed in any reports on the project, or to any other party;
- I have read the attached explanatory information and I am willing to have my blood used in this study, however data will only be used in any publication or presentation and my identity not stated;
- I understand that I can contact the Manager, Research Ethics, at Griffith University Human Research Ethics Committee on 37355585 (or research-ethics@griffith.edu.au) if I have any concerns about the ethical conduct of the research project; and
- I agree to participate in this project;

Name: **(please print)**

Signature:

Date:..... **Date of Birth:**.....

APPENDIX 3: IN PERSON QUESTIONNAIRE

Analysis of Innate and Adaptive Immune Cell Functions within patients with varying severities of Chronic Fatigue Syndrome

Examination date: ___/___/___ Time: _____ Patient ID: _____

Activity during the past week

(100% same as healthy to 10% completely bedridden)

Best day:

10%

100% 90%

Worst day:

10%

100% 90%

Today:

10%

400% 300% 200% 70% 60%

Fibro Fatigue Scale

Fatigue:

0 Ordinary recovery

1 2 3 4 5 6 Debilitating

Short term memory:

0 Memory as usual 1 2 3 4 5 6 Complete inability to remember

Concentration:

0 No difficulties 1 2 3 4 5 6 Complete inability to concentrate

Sleep:

0 Sleeps as usual 1 2 3 4 5 6 Severe sleep disturbances

Headache:

0 Absent or transient 1 2 3 4 5 6 Severely interfering or crippling

Pain:

0 Absent or transient

1 2 3 4 5 6

Muscle tension:

0 No increase 1 2 3 4 5 6 Painful; Completely incapable of relaxing physically.

Infection: 0 No symptoms of infection

1 2 3 4 5

crippling

Irritable bowel:

0 No irritable bowel

1 2 3 4 5 6 Incapacitating

Autonomic:

0 No autonomic disturbances 1 2 3 4 5 6 Incapacitating

Irritability:

0 Not easily irritated 1 2 3 4 5 6 Persistent or anger which is difficult or impossible to control

Sadness:

0 Occasional sadness may occur in the circumstances 1 2 3 4 5 6 Continuous experience of misery or extreme despondency

Kanorfsky's Performance Scale

- 100%** Normal, no complaints, no evidence of disease
- 90%** Able to carry on normal activity. Minor signs or symptoms of disease
- 80%** Normal activity with efforts; some signs or symptoms of disease
- 70%** Cares for self, unable to carry on normal activity or to do active work
- 60%** Requires occasional assistance, but is able to care for most of his/her personal needs
- 50%** Requires considerable assistance and frequent medical care
- 40%** Disabled; requires special care and assistance
- 30%** Severely disabled; hospital admission is indicated although death not imminent
- 20%** Very sick; hospital admission is necessary. Active supportive treatment necessary.
- 10%** Moribund; fatal processes progressing rapidly
- 0%** Dead

Dr Bell's CFS Disability Scale

- 100** No symptoms at rest. No symptoms with exercise; normal overall activity level; able to work full-time without difficulty.
- 90** No symptoms at rest; mild symptoms with activity; normal overall activity level; able to work full-time without difficulty.
- 80** Mild symptoms at rest, symptoms worsened by exertion; minimal activity restriction noted for activities requiring exertion only; able to work full-time with difficulty in jobs requiring exertion.
- 70** Mild symptoms at rest; some daily activity limitation clearly noted. Overall functioning close to 90% of expected except for activities requiring exertion. Able to work full-time with difficulty.
- 60** Mild to moderate symptoms at rest; daily activity limitation clearly noted. Overall functioning 70%-90%. Unable to work full-time in jobs requiring physical about, but able to work full-time in light activities if hours flexible.
- 50** Moderate symptoms at rest; moderate to severe symptoms with exercise or activity; overall activity level reduced to 70% of expected. Unable to perform strenuous duties, but able to perform light duty or desk work 4-5 hours a day, but requires rest periods.
- 40** Moderate symptoms at rest. Moderate to severe symptoms with exercise or activity; overall activity level reduced to 50%-70% of expected. Not confined to house. Unable to perform strenuous duties; able to perform light duty or desk work 3-4 hours a day, but requires rest periods.
- 30** Moderate to severe symptoms at rest. Severe symptoms with any exercise; overall activity level reduced to 50% of expected. Usually confined to house. Unable to perform any strenuous tasks. Able to perform desk work 2-3 hours a day, but requires rest periods.
- 20** Moderate to severe symptoms at rest. Severe symptoms with any exercise; overall activity level reduced to 30%-50% of expected. Unable to leave house except rarely; confined to bed most of day; unable to concentrate for more than 1 hour a day.
- 10** Severe symptoms at rest; bedridden the majority of the time. No travel outside of the house. Marked cognitive symptoms preventing concentration.
- 0** Severe symptoms on a continuous basis; bedridden constantly; unable to care for self.

APPENDIX 4: ONLINE QUESTIONNAIRE



Griffith
UNIVERSITY

CFS/ME Questionnaire

The National Centre for Neuroimmunology and Emerging Diseases

SECTION A: BACKGROUND INFORMATION

1. Date of birth _____
2. Your Sex Female Male
3. Please indicate which of the following **ethnic** groups you identify with?
 - Non indigenous Australian
 - Indigenous Australian
 - Oceanian (New Zealand, Melanesian, Papuan, Micronesian, Polynesian)
 - North-West European (British, Irish, Western European, Northern European)
 - Southern or Eastern European
 - North African or Middle Eastern
 - South East Asian
 - North East Asian
 - South or Central Asian
 - North, South or Central American
4. Your **highest** level of education obtained?
 - Primary school
 - Secondary (high) school
 - Professional training (not university)
 - Undergraduate
 - Post graduate/Doctoral
5. Your **current** employment status?
 - Work full time
 - Work part time/casual
 - On disability pension
 - Retired
 - Currently not working
 - Study full time
 - Study part time
6. Where in Australia are you currently living?

Queensland

- Brisbane
- Gold Coast
- Beaudesert
- Caboolture
- Ipswich
- Logan
- Pine Rivers
- Redcliffe
- Sunshine Coast
- Moreton
- Wide Bay Burnett
- Darling Downs
- South West
- Redlands

New South Wales

- Richmond Tweed
- Mid north Coast
- Northern
- North Western
- Central West
- South Eastern
- Murrumbidgee
- Murray
- Far West

7. How old were you when you first noticed signs of CFS/ME? ____ years

8. How old were you when a clinician diagnosed you with CFS/ME? ____ years

9. Do you have a family member/relative that has or has had ME/CFS?
Yes No

If yes, how are you related? _____

10. How did your symptoms begin?

- Suddenly (within 24 hours)
- Gradually
- After an infection
- Other

11. How severe were your symptoms when they began? (**please circle**)

(mild) **1** **2** **3** **4** **5** **6** **7** **8** **9** **10** (severe)

12. Just before your symptoms began did you experience any of the following?

- A minor infection
- Immunization
- Upper respiratory infection
- Sinusitis
- Pneumonia
- Dental infection
- Urinary tract infection
- Blood transfusion

13. Just before your symptoms began were you exposed to any of the following?

- Sick people
- An unfamiliar infection while travelling
- Contaminated water
- Poor quality recycled air
- Chemical toxins
- Heavy metals
- Moulds
- Severe physical trauma eg. whiplash/spinal injury/surgery
- Anaesthetics
- Undue stress
- Steroids

14. Where did your symptoms start?

- Australia: City _____ State _____
- Overseas: Country _____

15. How did your symptoms begin?

- Suddenly (within 24 hours)
 - Gradually
 - After an infection
 - Other
- _____

16. Please estimate the number of visits to the following health care providers you have **made during the past 12 months** (indicate 0 if no visits)

General practitioner	_____	visits
Neurologist	_____	visits
Physiotherapist	_____	visits
Occupational therapist	_____	visits
Chiropractor	_____	visits
Psychologist/psychiatrist	_____	visits
Social worker	_____	visits
Surgeon	_____	visits
Acupuncturist	_____	visits
Podiatrist	_____	visits
Osteopath	_____	visits
Urologist	_____	visits
Massage therapist	_____	visits
Other	_____	visits

17. Do you have any other chronic conditions (eg. lung disease, high blood pressure)? If yes, please list below:

18. Do you have a history of any of the following?

- Heart disease
- Diabetes
- Psychiatric disorder

19. Are you pregnant or breastfeeding?

- Yes No

20. Are you a smoker, or have been in the past 2 years?

- Yes No

PLEASE CONTINUE TO PART B

PART B: SYMPTOMS

Have you experienced any of the following **during the past 4 weeks?**

21. Any of the following cognitive symptoms?

- Confusion
- Disorientation
- Cognitive overload
- Difficulty making decisions
- Slowed speech
- Dyslexia
- Short term memory loss

22. Any of the following difficulties with pain?

- Headache
- Muscle pain
- Joint pain
- Abdomen pain
- Chest pain
- Pain worsens the more physical/mental activity I do

23. Any of the following sleep disturbances?

- Insomnia
- Prolonged sleep including naps
- Sleeping most of day and being awake at night
- Frequent awakenings
- Awakening earlier than before illness started
- Vivid dreams/nightmares
- Unrefreshing sleep

24. Any of the following sensory and perceptual disturbances?

- Inability to focus vision
- Sensitivity to light, noise, vibration, odour, taste and touch
- Impaired depth perception
- Muscle weakness
- Twitching
- Poor coordination
- Feeling unsteady on feet

25. Any of the following immune symptoms?

- Recurrent or chronic sore throat
- Recurrent or chronic sinus issues
- Recurrent or persistent infections with prolonged recovery periods
- Flu-like symptoms activate or worsen with activity

26. Any of the following gastro issues?

- Nausea
- Abdominal pain
- Bloating
- Irritable bowel syndrome
- Sensitivities to food, medications, odours or chemicals
- Urinary urgency or frequency, or need to wake up at night to urinate

27. Any of the following problems?

- Heart palpitations
- Light-headedness or dizziness
- Air hunger
- Laboured breathing
- Fatigue of chest walls or muscles
- Abnormal body temperature
- Marked fluctuations in body temperature
- Sweating episodes
- Recurrent feelings of feverishness
- Cold hands and feet
- Intolerance of extreme temperature

28. Have you been diagnosed with any of the following conditions?

- Orthostatic intolerance
- Neurally mediated hypotension
- Postural orthostatic tachycardia syndrome
- Ataxia

29. After minimal daily activity do you experience the following?

- A marked, rapid physical or mental fatigue
- Worsening of your symptoms
- A prolonged recovery period of usually 24 hours or longer?
- Immediate exhaustion
- Delayed exhaustion (hours or days after)
- Exhaustion not relieved by rest

30. How would you rate your activity levels the past four weeks, compared to before your illness began?

- 100% (my activity is the same as before any signs of illness)
- 90%
- 80%
- 70%
- 60%
- 50% (my activity has been reduced by half because of my illness)
- 40%
- 30%
- 20%
- 10%
- 0% (I am completely bedridden by my illness)

31. During the past 4 weeks, how many average hours per night did you usually sleep? ____ hours

32. How would you rate the quality of your sleep the past 4 weeks?

10 (excellent) 9 8 7 6 5 4 3 2 1 (extremely poor)

33. How frequently did you experience the following symptoms the past four weeks?

- | | |
|-----------------------------------|--|
| Difficulty processing information | <input type="checkbox"/> Never <input type="checkbox"/> Rarely <input type="checkbox"/> Often <input type="checkbox"/> Quite often |
| Pain | <input type="checkbox"/> Never <input type="checkbox"/> Rarely <input type="checkbox"/> Often <input type="checkbox"/> Quite often |
| Sleep disturbances | <input type="checkbox"/> Never <input type="checkbox"/> Rarely <input type="checkbox"/> Often <input type="checkbox"/> Quite often |
| Sensitivity to light, sound etc. | <input type="checkbox"/> Never <input type="checkbox"/> Rarely <input type="checkbox"/> Often <input type="checkbox"/> Quite often |
| Flu-like symptoms | <input type="checkbox"/> Never <input type="checkbox"/> Rarely <input type="checkbox"/> Often <input type="checkbox"/> Quite often |
| Bowel or urinary problems | <input type="checkbox"/> Never <input type="checkbox"/> Rarely <input type="checkbox"/> Often <input type="checkbox"/> Quite often |

Heart conditions Never Rarely Often Quite often

Breathing problems Never Rarely Often Quite often

Problems with body temperature Never Rarely Often Quite often

34. How severe were the following symptoms the past four weeks? (please circle): 1 (no problem) 2 3 4 5 6 7 8 9 10 (severe)

Difficulty processing information 1 2 3 4 5 6 7 8 9 10

Pain 1 2 3 4 5 6 7 8 9 10

Sleep disturbances 1 2 3 4 5 6 7 8 9 10

Sensitivity to light, sound etc. 1 2 3 4 5 6 7 8 9 10

Flu-like symptoms 1 2 3 4 5 6 7 8 9 10

Bowel or urinary problems 1 2 3 4 5 6 7 8 9 10

Heart conditions 1 2 3 4 5 6 7 8 9 10

Breathing problems 1 2 3 4 5 6 7 8 9 10

Problems with body temperature 1 2 3 4 5 6 7 8 9 10

PLEASE CONTINUE TO PART C

PART C: SF-36 SCALE

35. In general, would you say your health is:

- Excellent
- Very good
- Good
- Fair
- Poor

36. Compared to one year ago, how would you rate your health in general now?

- Much better now than a year ago
- Somewhat better now than a year ago
- About the same as one year ago
- Somewhat worse now than one year ago
- Much worse now than one year ago

37. The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No not limited at all
a. Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Lifting or carrying groceries.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
d. Climbing several flights of stairs.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
e. Climbing one flight of stairs.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
f. Bending, kneeling or stooping.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
g. Walking more than one kilometre.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
h. Walking several blocks	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
i. Walking one block	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
j. Bathing or dressing yourself	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

38. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

- | | Yes | No |
|--|--------------------------|--------------------------|
| a. Cut down the amount of time you spent on work or other activities? | <input type="checkbox"/> | <input type="checkbox"/> |
| b. Accomplished less than you would like? | <input type="checkbox"/> | <input type="checkbox"/> |
| c. Were limited in the kind of work or other activities? | <input type="checkbox"/> | <input type="checkbox"/> |
| d. Had difficulty performing the work or other activities (eg. needed extra time)? | <input type="checkbox"/> | <input type="checkbox"/> |

39. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?

- | | Yes | No |
|---|--------------------------|--------------------------|
| a. Cut down the amount of time you spent on work or other activities? | <input type="checkbox"/> | <input type="checkbox"/> |
| b. Accomplished less than you would like? | <input type="checkbox"/> | <input type="checkbox"/> |
| c. Didn't do work or other activities as carefully as usual | <input type="checkbox"/> | <input type="checkbox"/> |

40. During the past 4 weeks...

- | | Not at all | Slightly | Moderately | Quite a bit | Extremely |
|---|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| a. To what extent has your physical health, or emotional problems interfered with your normal social activities with family, friends, neighbours or groups? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| b. How much bodily pain have you had? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| c. How much did pain interfere with your normal work (including both work outside the home and housework)? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

41. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks:

- | All the time | Most of the time | A good bit of the time | Some of the time | A little of the time | None of the time |
|--------------|------------------|------------------------|------------------|----------------------|------------------|
|--------------|------------------|------------------------|------------------|----------------------|------------------|

time

- | | | | | | | |
|---|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| a. Did you feel full of pep? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| b. Have you been a very nervous person? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| c. Have you felt so down in the dumps nothing could cheer you up? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| d. Have you felt calm and peaceful? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| e. Did you have a lot of energy? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| f. Have you felt downhearted and blue? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| g. Did you feel worn out? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| h. Have you been a happy person? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| i. Did you feel tired? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| j. How much of the time has your physical health or emotional problems interfered with your social activities (like visiting friends, relatives, etc.)? | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

42. How TRUE or FALSE is each of the following statements for you.

- | | Definitely true | Mostly true | Don't know | Mostly false | Definitely false |
|---|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| a. I seem to get sick a little easier than other people | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| b. I am as healthy as anybody I know | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| c. I expect my health to get worse | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| d. My health is excellent | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

PART D: WHO DAS 2.0

Think back over the past 30 days and answer these questions, thinking about how much difficulty you had doing the following activities. For each question, please indicate only one response.

In the past 30 days, how much difficulty did you have in:

	None	Mild	Moderate	Severe	Extreme or cannot do
43 a. Concentrating on doing something for ten minutes?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Remembering to do important things?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Analysing and finding solutions to problems in day to day life?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
d. Learning a new task, for example, learning how to get to a new place?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
e. Generally understanding what people say?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
f. Starting and maintaining a conversation	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
44 a. Standing for long periods such as 30 minutes?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Standing up from sitting down?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Moving around inside your home?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
d. Getting out of your home?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
e. Walking a long distance such as a kilometre (or equivalent)?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
45 a. Washing your whole body	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Getting dressed?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Eating?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

d. Staying by yourself for a few days?

46 a. Dealing with people you do not know

None Mild Moderate Severe Extreme or cannot do

b. Maintaining a friendship

c. Getting along with people who are close to you?

d. Making new friends

e. Sexual activities

47a. Taking care of your household responsibilities

b. Doing most important household tasks well?

c. Getting all the household work done that you needed to do?

d. Getting your household work done as quickly as needed?

e. Your day-to-day work/school?

f. Doing your most important work/school tasks well?

g. Getting all the work done that you need to do?

h. Getting your work done as quickly as needed?

48 a. How much of a problem did you have in joining in community activities (for example, festivities, religious or other activities) in the same way as anyone else can?

b. How much of a problem did you have because of barriers or hindrances in the world around you?

None Mild Moderate Severe Extreme or cannot do

c. How much of a problem did you have living with dignity because of the attitudes and actions of others?

d. How much time did you spend on your health condition, or its consequences?

e. How much have you been emotionally affected by your health condition?

f. How much has your health been a drain on the financial resources of you or your family?

g. How much of a problem did your

family have because of your health problems?

h. How much of a problem did you have in doing things by yourself for relaxation or pleasure?

49. Overall, in the past 30 days, how many days were these difficulties present Number of days _____

50. In the past 30 days, for how many days were you totally unable to carry out your usual activities or work because of any health condition? Number of days _____

51. In the past 30 days, not counting the days that you were totally unable, for how many days did you cut back or reduce your usual activities or work because of any health condition? Number of days _____

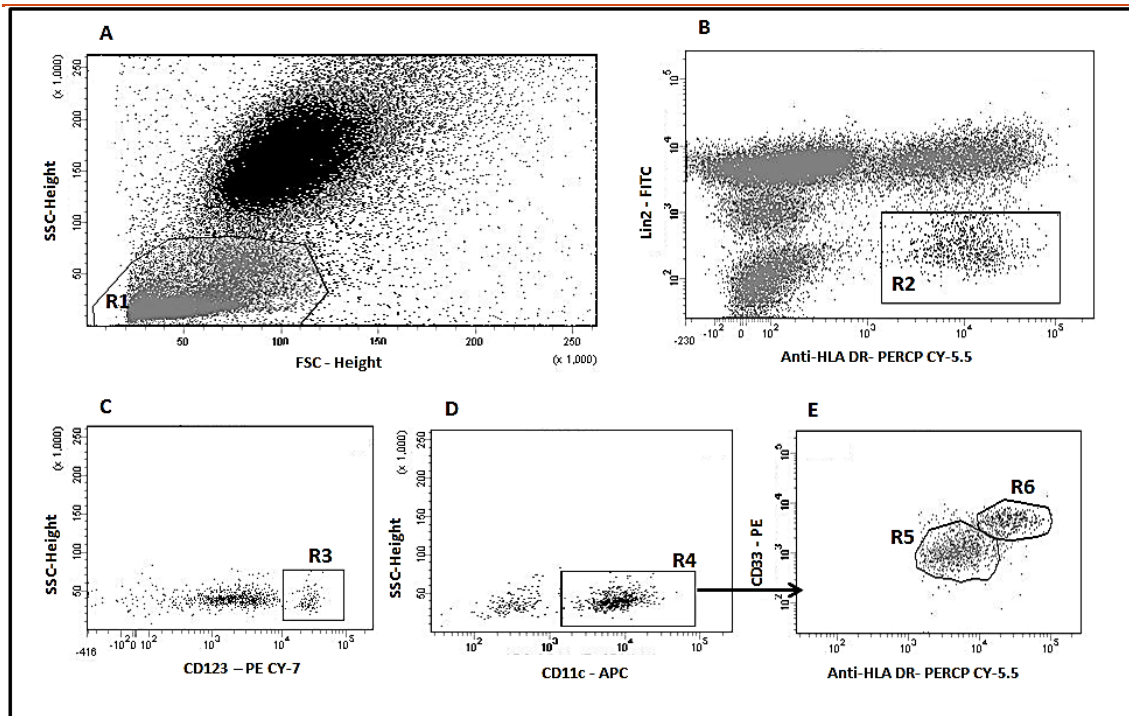
THIS CONCLUDES THE SURVEY. THANK YOU VERY MUCH

APPENDIX 5: PROJECT ONE MONOCLONAL ANTIBODY COMBINATIONS

Cell	Measured Phenotype	Monoclonal Antibody Marker Combinations
NK Cells	Phenotypes: KIRs: Lytic Proteins:	CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} KIR2DL1 (CD158a), KIR3DL1 (CD158e), KIR2DL2/DL3 (CD158b), KIR2DS4 (CD158i), KIR2DL1/DS1 (CD158a/h), KIR3DL1/DL2 (CD158e/k), KIR2DL5 (CD158f), NKG2D (CD314), NKG2 (CD94) CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} Perforin ⁺ , CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} GranzymeA ⁺ , CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} GranzymeB ⁺
iNKT Cells	Phenotypes:	6B11 ⁺ CD3 ⁺ CD8 ^{+/-} CD4 ^{+/-} , 6B11 ⁺ CD3 ⁺ CD8a ^{+/-} CD4 ^{+/-} 6B11 ⁺ CD3 ⁺ CD45RO ^{+/-} CD28 ^{+/-} , 6B11 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} , 6B11 ⁺ CD3 ⁺ CCR7 ^{+/-} SLAMF ^{+/-} , 6B11 ⁺ CD3 ⁺ CD56 ^{+/-} CD16 ^{+/-}
CD8 T cells	Phenotypes: Lytic Proteins:	CD8 ⁺ CD3 ⁺ CD45RO ^{+/-} CD27 ^{+/-} , CD8 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} , CD8 ⁺ CD3 ⁺ CCR7 ^{+/-} CCR5 ^{+/-} , CD8 ⁺ CD3 ⁺ CCR5 ^{+/-} CD28 ^{+/-} CD8 ⁺ CD3 ⁺ Perforin ⁺ , CD8 ⁺ CD3 ⁺ GranzymeA ⁺ , CD8 ⁺ CD3 ⁺ GranzymeB ⁺
Tregs	Treg FOXP3⁺:	CD127 ^{low} CD25 ⁺ CD4 ⁺ FOXP3 ⁺
γδ T cells	γδ 1 T cells: γδ 2 T cells: Phenotypes:	γδ 1 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} γδ 2 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} Naïve: γδ ⁺ CD3 ⁺ CD45RA ⁺ CD27 ⁺ Central Memory: γδ ⁺ CD3 ⁺ CD45RA ⁻ CD27 ⁺ Effector Memory: γδ ⁺ CD3 ⁺ CD45RA ⁻ CD27 ⁻ CD45RA ⁺ Effector Memory: γδ ⁺ CD3 ⁺ CD45RA ⁺ CD27 ⁻
DCs	Phenotypes:	CD14 ⁻ CD16 ⁺ DCs: Lin2 ⁻ HLA-DR ⁺ CD16 ⁺ pDCs: Lin2 ⁻ HLA-DR ⁺ CD123 ⁺ mDCs: Lin2 ⁻ HLA-DR ⁺ CD33 ⁺
Monocytes	Phenotypes:	Proinflammatory: HLA-DR ⁺ CD11a ⁺ CD16 ⁺ CD14 ⁻ Intermediate: HLA-DR ⁺ CD11a ⁺ CD16 ⁺ CD14 ⁺ Classical: HLA-DR ⁺ CD16 ^{+/-} CD11a ⁺ CD16 ⁻ CD14 ⁺
Neutrophils	Phenotypes:	CD16 ⁺ CD62L ⁺ CD66 ⁺ CD177 ^{bright} , CD16 ⁺ CD62L ⁺ CD66 ⁺ CD177 ^{dim}
B Cells	Phenotypes:	Naïve: CD19 ⁺ CD20 ⁺ CD21 ⁺ CD38 ⁺ CD27 ⁻ Memory: CD19 ⁺ CD20 ⁺ CD27 ⁺ CD38 ⁺ Plasma: CD19 ⁺ CD138 ⁺ CD27 ⁺ CD38 ⁺ Transitional: CD19 ⁺ CD10 ⁺ CD27 ⁺ CD38 ⁺ Breg: CD19 ⁺ CD1d ⁺ CD24 ⁺ CD38 ⁺

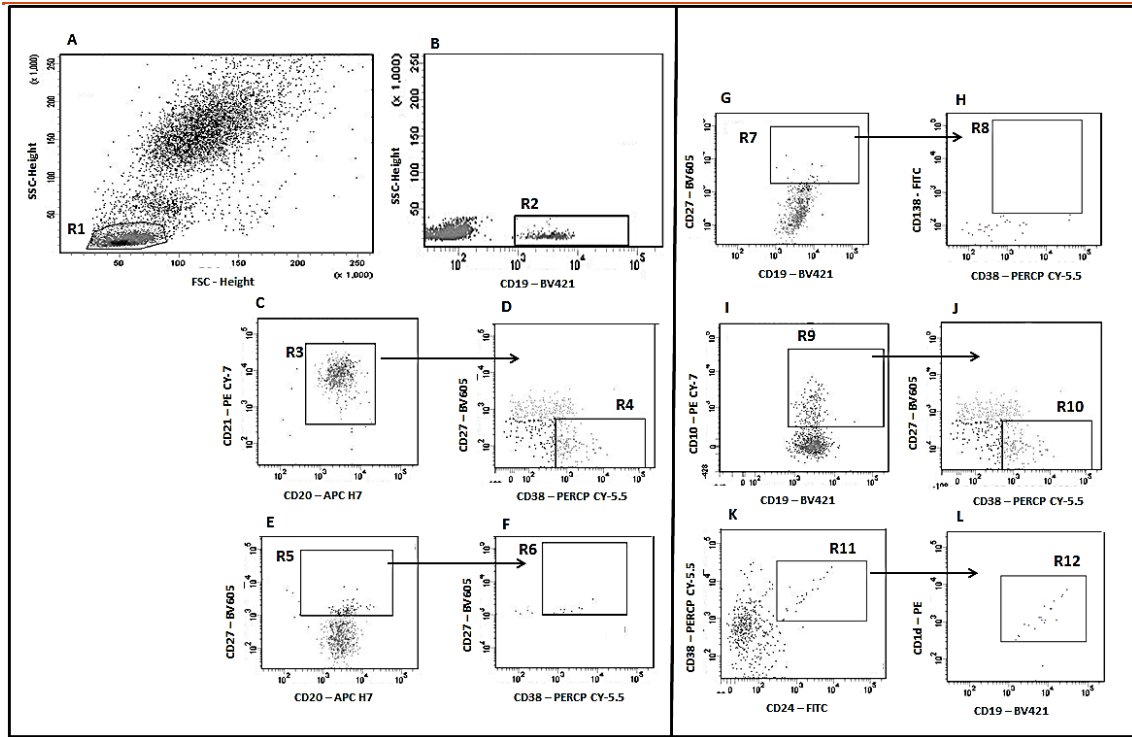
Appendix 5 shows the monoclonal antibody combinations used in project one to identify each of the cells and parameters for gating on the flow cytometer.

APPENDIX 6: PROJECT ONE FLOW CYTOMETRIC GATING STRATEGIES FOR IDENTIFICATION OF DENDRITIC CELL PHENOTYPES IN CFS/ME AND CONTROL PARTICIPANTS.



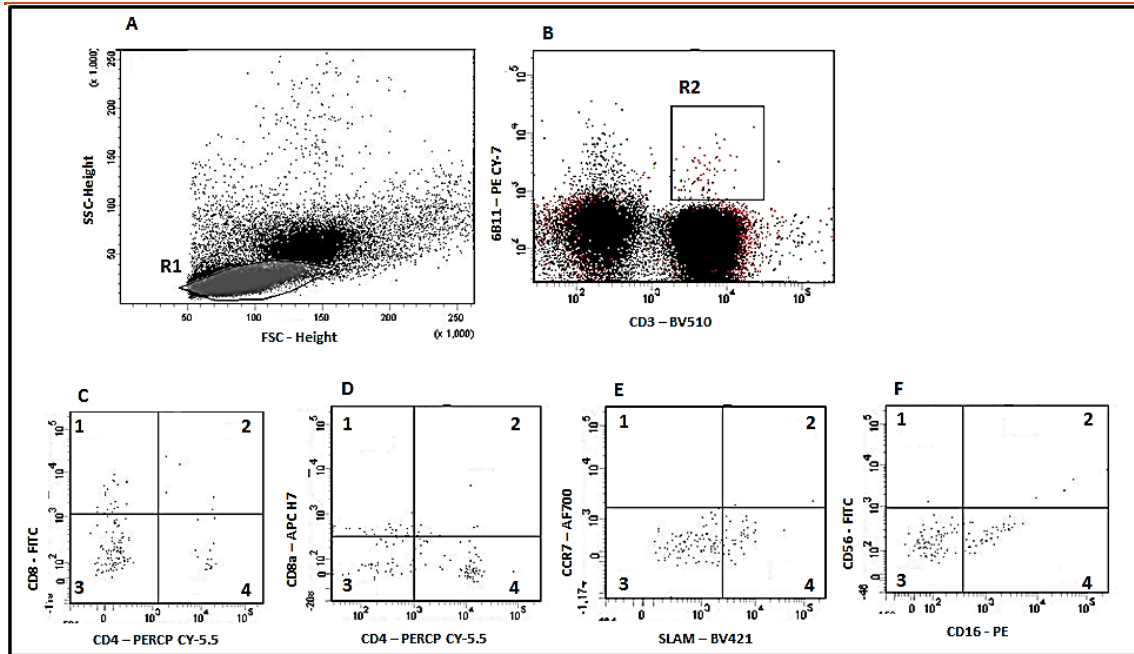
The DC phenotypes assessed in project one were pDCs, mDCs and CD14⁻CD16⁺ DCs. R1 represents the leukocyte gate. From the leukocyte gate (R1), cells expressing only HLA DR and no lineage markers were identified as R2, total DCs. R2 was then portrayed along with the markers CD123, CD11c and CD33 to identify the different DC phenotypes. The population expressing CD123 were identified as **pDCs (R3)**. DCs expressing CD123 were identified as mDCs and CD14⁻CD16⁺ DCs (R4). Plot E was used to distinguish mDCs and CD14⁻CD16⁺ DCs (R4) using CD33 and HLA DR (E). Populations of **mDCs (R6)** and **CD14⁻CD16⁺ DCs (R5)** were then recognised (Henriques et al., 2012).

APPENDIX 7: PROJECT ONE FLOW CYTOMETRIC GATING STRATEGIES FOR IDENTIFICATION OF B CELL PHENOTYPES IN CFS/ME AND CONTROL PARTICIPANTS.



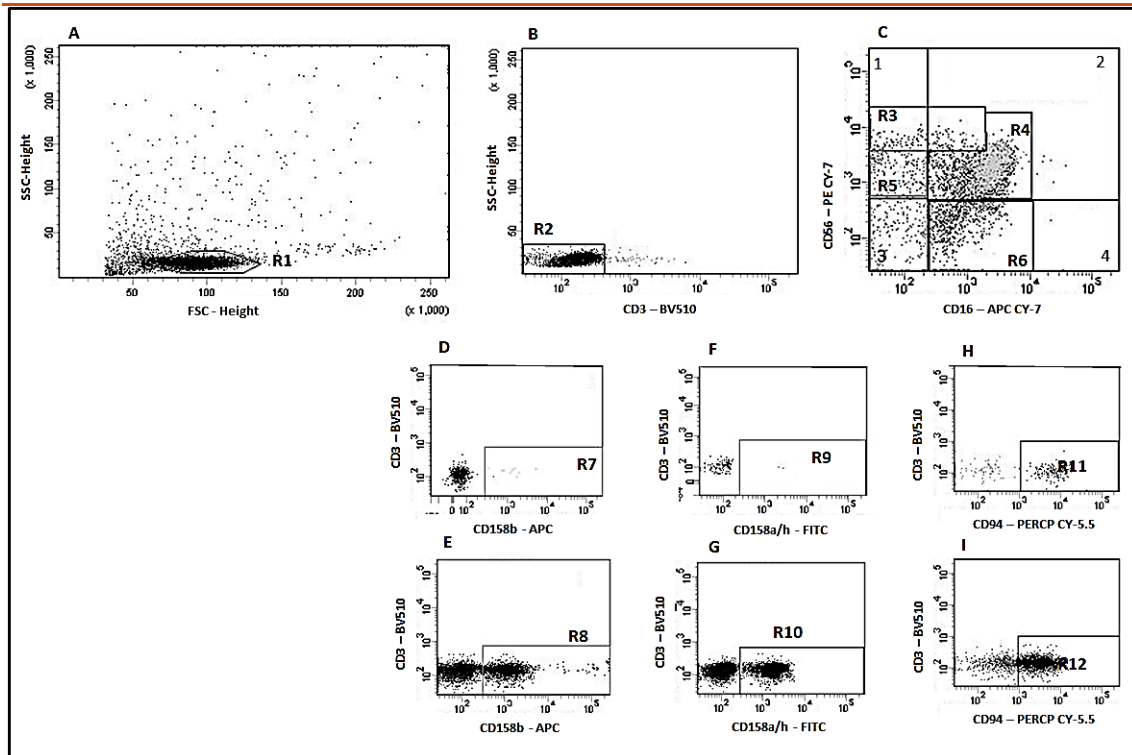
B cell phenotypes assessed in project one were naïve, memory, plasma, transitional and Bregs. R1 represents the gated lymphocytes. CD19 was then used to identify total B cells (R2) from the total lymphocytes. R2 was represented on plot C and subsequently R3 was represented on plot D to identify CD19⁺ CD21⁺ CD20⁺ CD27⁻ CD38⁺ B cells as **naïve B cells (R4)**. Plots E and F were used to identify CD19⁺ CD27⁺ CD20⁺ CD38⁺ B cells as **memory B cells (R6)**. Plots G and H were used to determine CD19⁺CD27⁺CD138⁺CD38⁺ **plasma B cells (R8)**. **Transitional B cells (R10)** were identified from total B cells (R2) and plots I and J for the phenotype CD19⁺CD10⁺CD27⁻CD38⁺. Lastly, **Bregs (R12)** were identified from total B cells (R2) in plots K and L for the phenotype CD19⁺CD24⁺CD38⁺CD1d⁺.

APPENDIX 8: PROJECT ONE FLOW CYTOMETRIC GATING STRATEGIES FOR IDENTIFICATION OF INKT CELL PHENOTYPES IN CFS/ME AND CONTROL PARTICIPANTS.



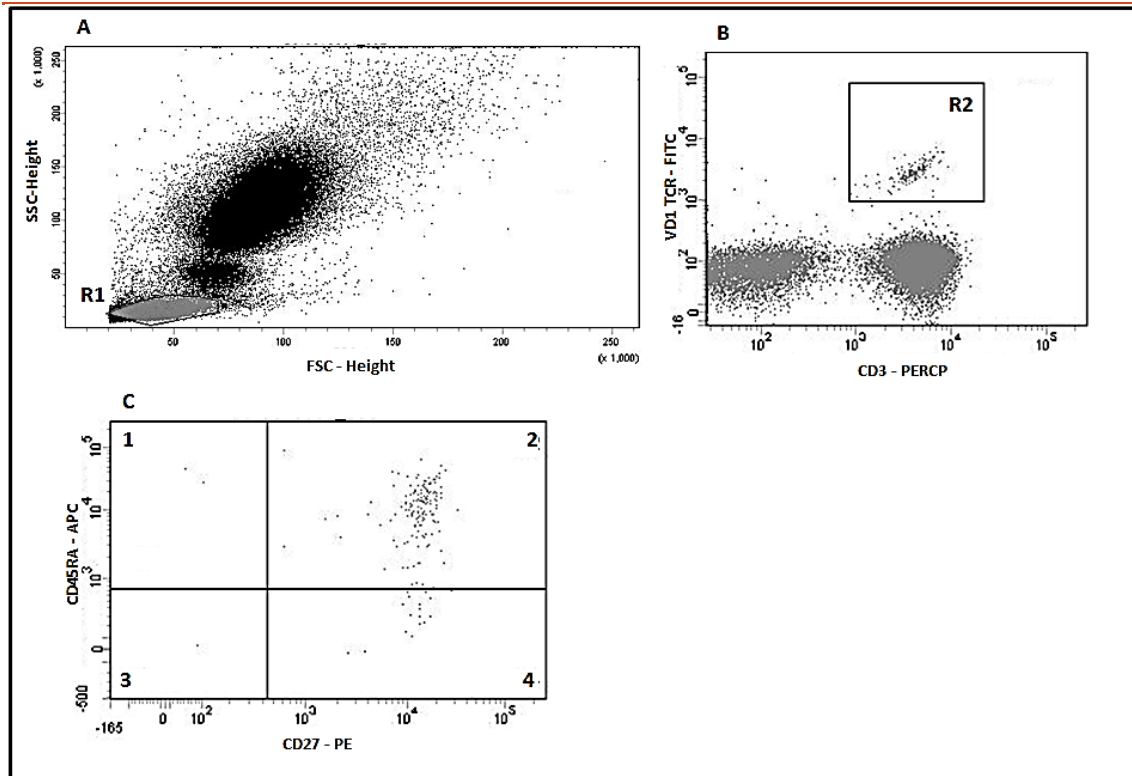
iNKT cells in project one were assessed for phenotypes using the markers CD8, CD4, CD8a, CCR7, SLAM, CD56 and CD16. Lymphocytes were gated (R1) and represented with the markers 6B11 and CD3 to identify total iNKT cells (R2). iNKT cell phenotypes $6B11^+CD3^+CD8^{+/-}CD4^{+/-}$ and $6B11^+CD3^+CD8^{+/-}CD4^{+/-}$ were identified in plots C and D. The expression of $CCR7^{+/-}$, $SLAM^{+/-}$, $CD56^{+/-}$ and $CD16^{+/-}$ were also identified on total iNKT cells in plots E and F.

APPENDIX 9: PROJECT ONE FLOW CYTOMETRIC GATING STRATEGIES FOR IDENTIFICATION OF NATURAL KILLER CELL PHENOTYPES IN CFS/ME AND CONTROL PARTICIPANTS.



NK cells were identified in project one by the four phenotypes, $CD56^{\dim}CD16^+$, $CD56^{\dim}CD16^+$, $CD56^{\dim}CD16^-$ and $CD56^{\text{bright}}CD16^{-/\dim}$. Total lymphocytes were gated as R1. Lymphocytes were assessed for CD3 expression (B) and those negative for CD3 were labelled R2. Plot C shows isolated lymphocytes negative for CD3 in relation to CD56 and CD16 expression. NK cell phenotypes were plotted in gate C, $CD56^{\text{bright}}CD16^{-/\dim}$ (R3), $CD56^{\dim}CD16^+$ (R4), $CD56^{\dim}CD16^-$ (R5) and $CD56^-CD16^+$ (R6). NK cell phenotypes, were assessed in relation to the KIR receptors, with $CD56^{\text{bright}}CD16^{-/\dim}$ (R3 shown in D, F and H) and $CD56^{\dim}CD16^-$ (R5 shown in E, G and I). KIR receptors CD158b (D, E), CD158a/h (F, G) and CD94 (H, I) were then assessed for the NK cell phenotypes (Brenu et al., 2011).

APPENDIX 10: PROJECT ONE FLOW CYTOMETRIC GATING STRATEGIES FOR IDENTIFICATION OF $\gamma\delta$ T CELL PHENOTYPES IN CFS/ME AND CONTROL PARTICIPANTS.



Total lymphocytes were gated in project one as R1 and $\gamma\delta$ 1 T cells were identified using $\gamma\delta$ 1 TCR and CD3 markers (R2). CD27 and CD45RA were used to identify the four main $\gamma\delta$ 1 T cell phenotypes based on the quadrants 1, 2, 3 and 4 in plot C; **1 was CD45RA⁺ effector memory ($\gamma\delta$ 1⁺CD3⁺CD45RA⁺CD27⁻)**, **2 was naïve ($\gamma\delta$ 1⁺CD3⁺CD45RA⁺CD27⁺)**, **3 was effector memory ($\gamma\delta$ 1⁺CD3⁺CD45RA⁻CD27⁻)** and **4 was central memory ($\gamma\delta$ 1⁺CD3⁺CD45RA⁻CD27⁺)**.

APPENDIX 11: PEARSON CORRELATION USED TO IDENTIFY CORRELATES BETWEEN B CELLS, NK CELL PHENOTYPES AND NK KIR RECEPTORS IN PROJECT ONE.

Pearson Correlation	Memory B Cells	Plasma B Cells	Naïve B Cells
Naïve B cells	-	0.427	-
NK Cells CD56^{dim}CD16⁻ CD158a/h⁺ (KIR2DL1/DS1)	-	0.374	0.393
NK Cells CD56^{bright}CD16^{-/dim}	0.470	-	-

Appendix 11 shows value of Pearson Correlation between bivariate parameters including: B cell, NK cell phenotypes and NK KIR receptors for combined control, moderate CFS/ME and severe CFS/ME participant groups in project one. All correlations listed have $p < 0.01$. ‘-‘ is shown where Pearson correlation value was not significant ($p > 0.01$).

APPENDIX 12: PEARSON CORRELATION USED TO IDENTIFY CORRELATES BETWEEN NK CELL CYTOTOXIC ACTIVITY AND INKT MARKERS IN PROJECT ONE.

Pearson Correlation	NK Cell Activity 12.5 : 1	NK Cell Activity 25 : 1	iNKT Cells CD8 ⁻ CD4 ⁻	iNKT Cells CD8a ⁻ CD4 ⁺	iNKT Cells CCR7 ⁻ SLAMF ⁻	iNKT Cells CD56 ⁻ CD16 ⁺	iNKT Cells CD56 ⁻ CD16 ⁻	iNKT Cells CD56 ⁺ CD16 ⁺
NK Cell Activity 25 : 1	0.921		-	-	-	-	-	-
NK Cell Activity 50 : 1	0.660	0.660	-	-	-	-	-	-
iNKT Cells CD8a ⁻ CD4 ⁻	-	-	0.823	0.909	-	-	-	-
iNKT Cells CD8a ⁻ CD4 ⁺	-	-	0.891		-	-	-	-
iNKT Cells CCR7 ⁻ SLAMF ⁻	-	-	0.551	0.534		-	-	-
iNKT Cells CCR7 ⁻ SLAMF ⁺	-	-	-	-	0.695	0.420	0.701	0.603
iNKT Cells CD56 ⁺ CD16 ⁺	-	-	-	-	0.633	0.880	0.766	
iNKT Cells CD56 ⁻ CD16 ⁻	-	-	0.552	0.537	0.947	0.636		-
iNKT Cells CD56 ⁻ CD16 ⁺	-	-	0.404	0.351	0.506		-	-

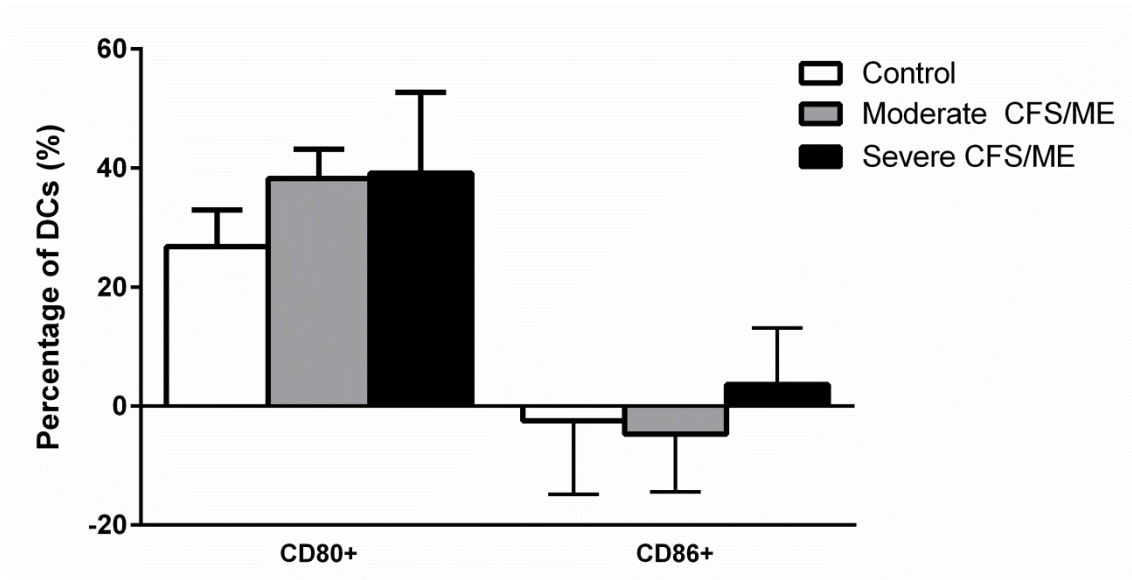
Appendix 12 shows value of Pearson Correlation between bivariate parameters for NK cell cytotoxic activity and iNKT markers for combined control, moderate CFS/ME and severe CFS/ME participant groups in project one. All correlations listed have $p < 0.01$. ‘-’ is shown where Pearson correlation value was not significant ($p > 0.01$). Cells in table with strike-through represent parameters shown in both rows and columns.

APPENDIX 13: PROJECT THREE MONOCLONAL ANTIBODIES

Cell	Phenotype	Monoclonal Antibody Marker Combinations
NK Cells	Phenotypes: Integrins: SLAM: NCRs:	CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} CD2, CD18, CD11a, CD11b, CD11c CD150 NKp30, NKp44, NKp46, NKp80
CD8⁺ and CD4⁺ T cells	CD8 CD4 Phenotypes KIRs Receptors and Markers:	CD8 ⁺ CD3 ⁺ CD4 ⁺ CD3 ⁺ Naïve: CD45RA ⁺ CD27 ⁺ Central Memory: CD45RA ⁻ CD27 ⁺ Effector Memory: CD45RA ⁻ CD27 ⁻ CD45RA ⁺ Effector Memory: CD45RA ⁺ CD27 ⁻ KIR2DL1 (CD158a), KIR3DL1 (CD158e), KIR2DL2/DL3 (CD158b), KIR2DS4 (CD158i), KIR2DL1/DS1 (CD158a/h), KIR3DL1/DL2 (CD158e/k), KIR2DL5 (CD158f), NKG2D (CD314), NKG2 (CD94) PD1, CD160, TIM3, 2B4, CD44, PSGL, CD62L, CXCR3, CD49d/CD29, LFA-1, KLRG1, CD127, BLTA4, CTLA4, Tregs (CD25 ⁺ CD28 ⁺ CD56 ⁺), CCR5, CD28, CCR7
iNKT Cells	Lytic Proteins:	6B11 ⁺ CD3 ⁺ Perforin, GranzymeA, GranzymeB
Tregs	Lytic Proteins:	CD127 ^{low} CD25 ⁺ CD3 ⁺ CD4 ⁺ Perforin, GranzymeA, GranzymeB
γδ T cells	γδ 1 T cells: γδ 2 T cells: Phenotypes: Lytic Proteins:	γδ 1 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} γδ 2 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} Naïve: γδ ⁺ CD3 ⁺ CD45RA ⁺ CD27 ⁺ Central Memory: γδ ⁺ CD3 ⁺ CD45RA ⁻ CD27 ⁺ Effector Memory: γδ ⁺ CD3 ⁺ CD45RA ⁻ CD27 ⁻ CD45RA ⁺ Effector Memory: γδ ⁺ CD3 ⁺ CD45RA ⁺ CD27 ⁻ Perforin, GranzymeA, GranzymeB
DCs	Phenotypes:	CD14 ⁻ CD16 ⁺ DCs: Lin2 ⁻ HLA-DR ⁺ CD16 ⁺ pDCs: Lin2 ⁻ HLA-DR ⁺ CD123 ⁺ mDCs: Lin2 ⁻ HLA-DR ⁺ CD33 ⁺
B Cells	BCRs: Breg:	CD19 ⁺ CD79a, CD79b, IgA, IgE, IgD, IgM, CD154, CD27 ⁻ CD19 ⁺ CD5 ⁺ CD1d ⁺ CD81 ^{+/-} , CD21 ^{+/-}

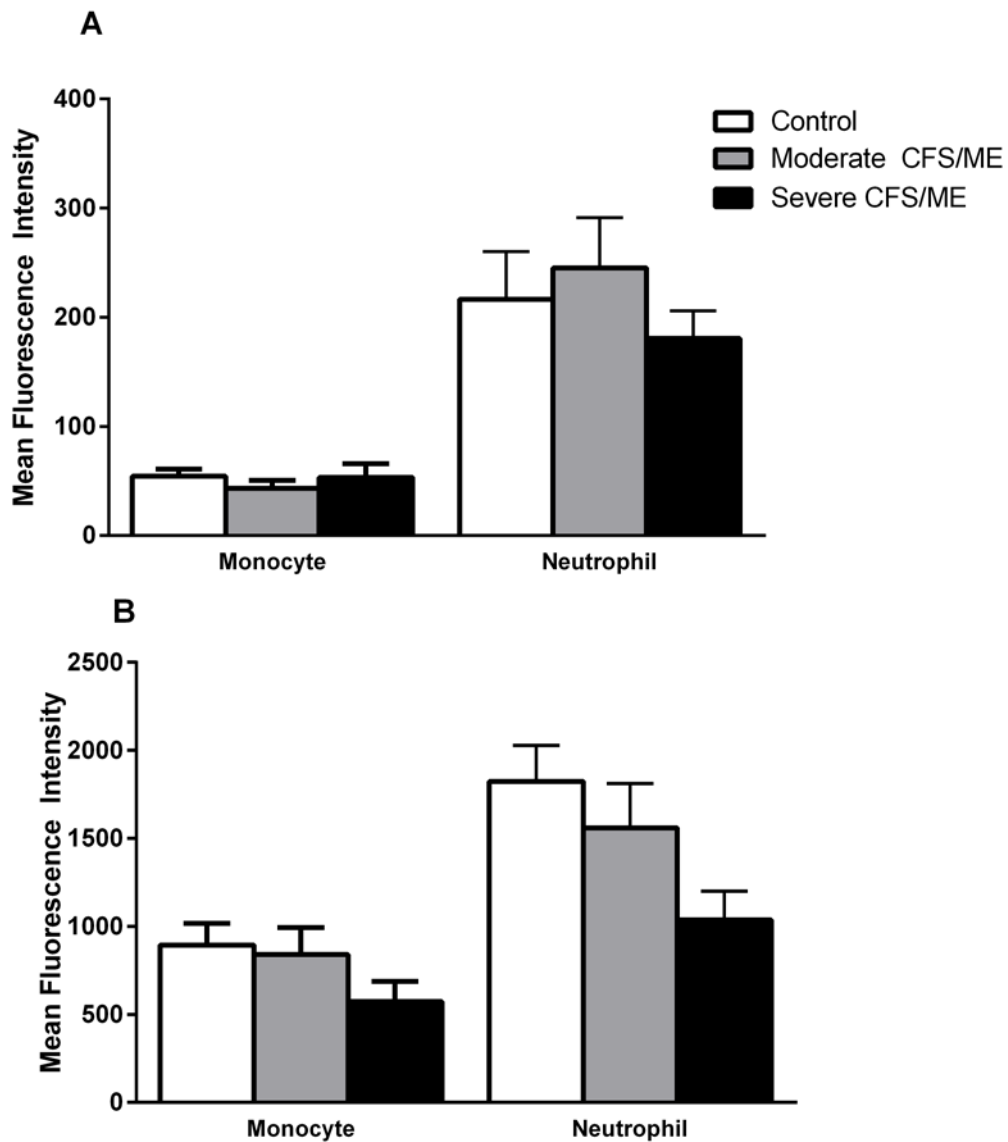
Appendix 13 shows the monoclonal antibody combinations used in project three identify each of the cells and parameters for gating on the flow cytometer.

APPENDIX 14: PROJECT THREE FLOW CYTOMETRIC ANALYSIS OF DC ACTIVITY



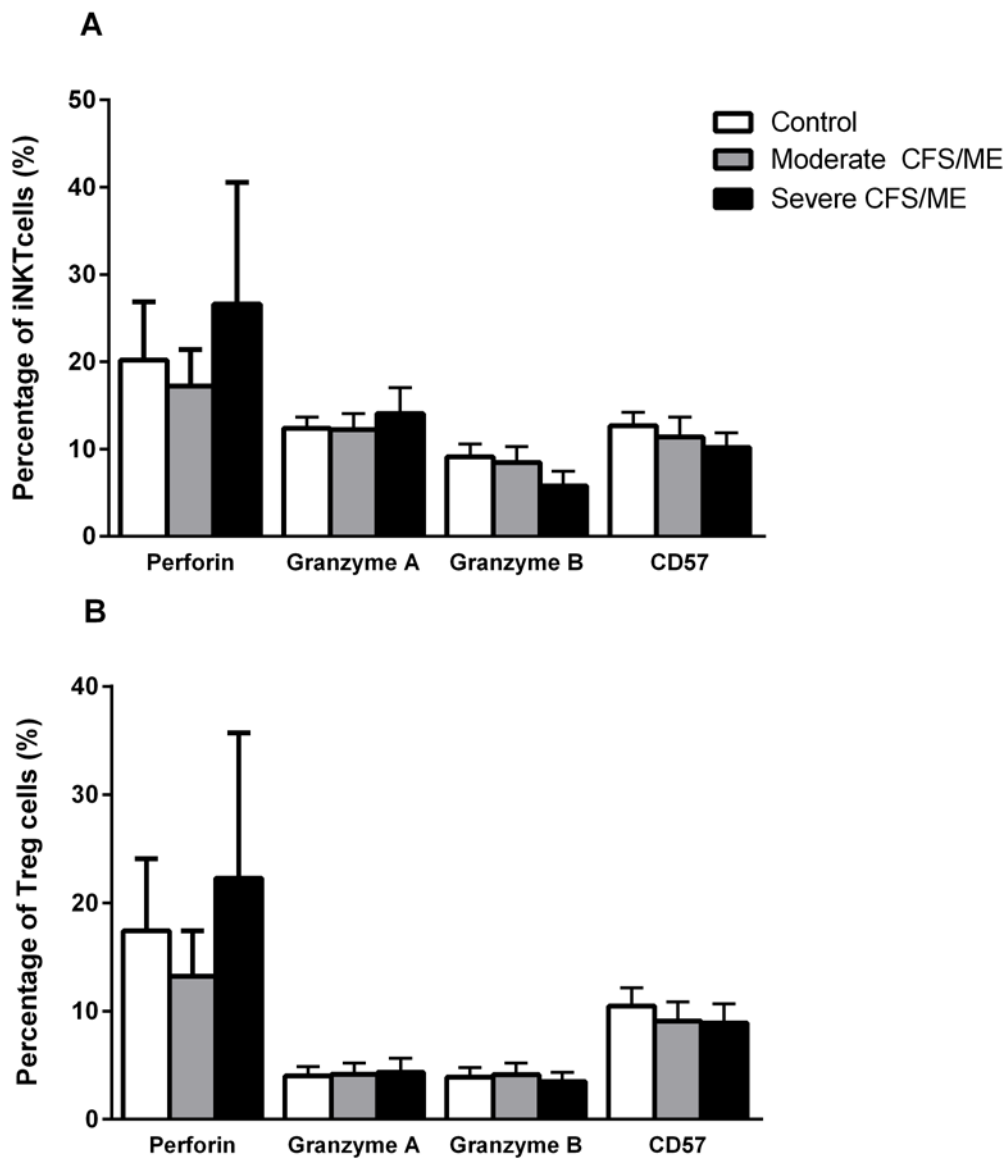
DC activity is shown based on the difference in expression of activation markers (CD80 and CD86) following stimulation. Measures are represented as percentage of DCs expressing markers (%) in controls, moderate CFS/ME and severe CFS/ME. All data are represented as mean \pm SEM.

APPENDIX 15: PROJECT THREE FLOW CYTOMETRIC ANALYSIS OF MONOCYTE AND NEUTROPHIL FUNCTION



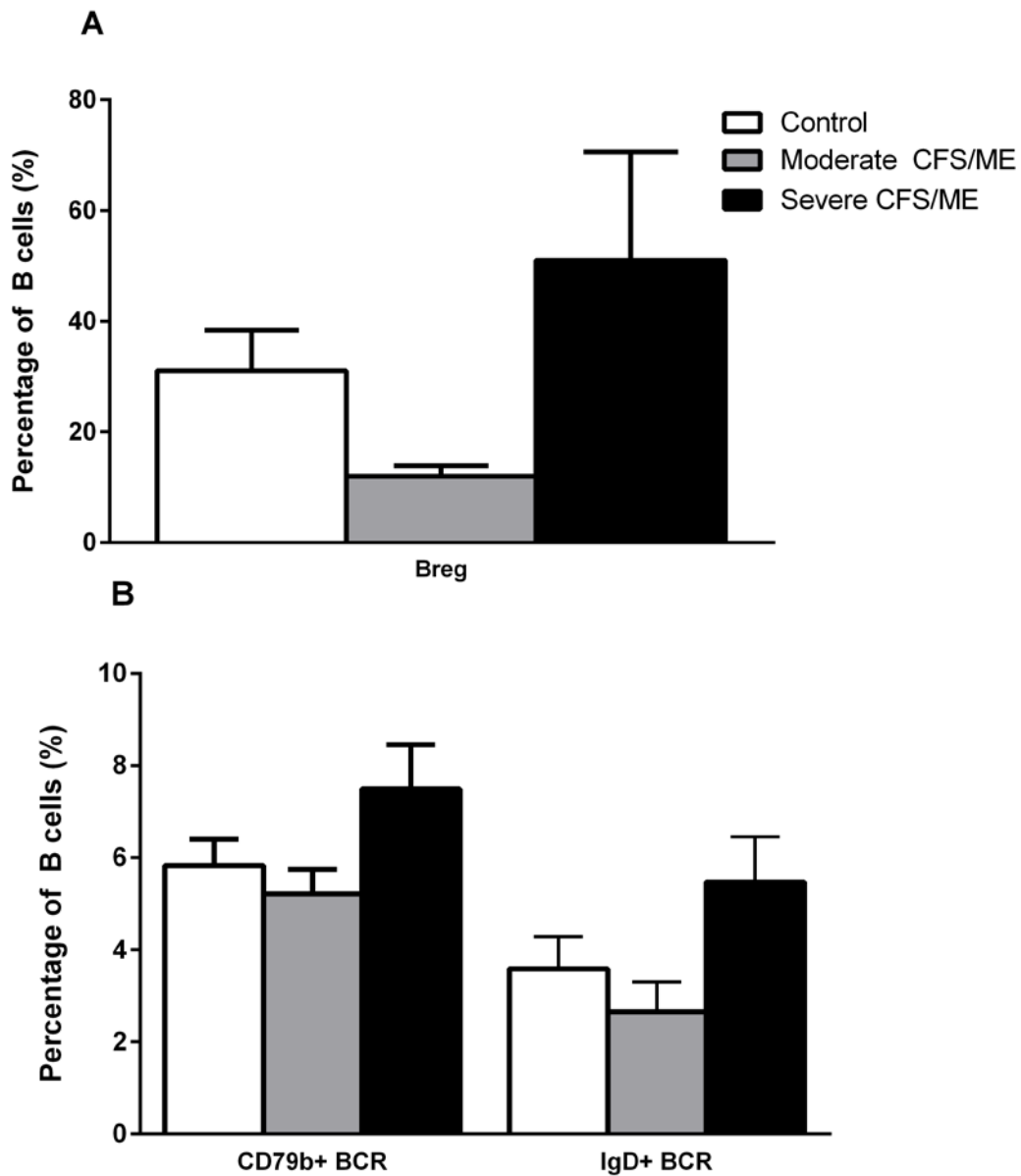
A Monocyte and Neutrophil function respiratory burst, shown as difference in mean fluorescence intensity following stimulation. Measures are shown for controls, moderate CFS/ME and severe CFS/ME. **B** Monocyte and Neutrophil function phagocytosis, shown as difference in mean fluorescence intensity following stimulation. Measures are shown for controls, moderate CFS/ME and severe CFS/ME.

APPENDIX 16: PROJECT THREE INTRACELLULAR ANALYSIS OF LYTIC PROTEINS



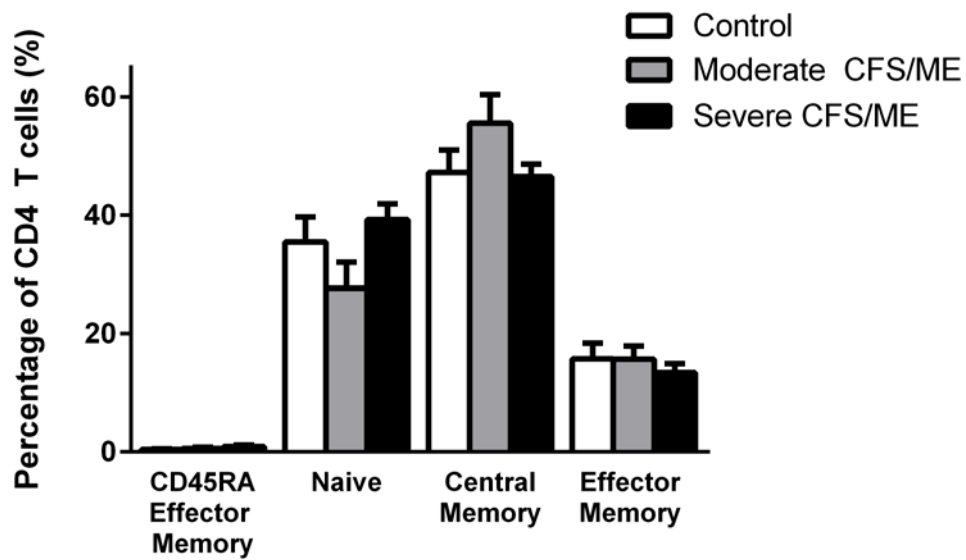
A Lytic proteins in iNKT cells are shown as percentage of iNKT cells (%) expressing perforin, granzyme A, granzyme B and CD57 for controls, moderate CFS/ME and severe CFS/ME patients. **B** Lytic proteins in Tregs are shown as percentage of Tregs (%) expressing the lytic proteins perforin, granzyme A, granzyme B and CD57 in controls, moderate CFS/ME and severe CFS/ME patients. All data are represented as mean \pm SEM.

APPENDIX 17: PROJECT THREE PROFILE OF BREGS AND BCRS IN CONTROLS, MODERATE AND SEVERE CFS/ME PATIENTS



A Bregs are as percentage of B cells (%) in controls, moderate CFS/ME and severe CFS/ME patients. **B** CD79b and IgD expression to represent BCRs and shown as a percentage of B. BCRs shown in controls, moderate CFS/ME and severe CFS/ME patients. All data are represented as mean \pm SEM.

APPENDIX 18: PROJECT THREE PERIPHERAL BLOOD CD4⁺T CELL PHENOTYPES



CD45RA effector memory, naïve, central memory and effector memory CD4⁺T cell phenotypes represented as percentage of total CD4⁺T cells (%). All data are represented as mean_±SEM.

APPENDIX 19: PROJECT FOUR MONOCLONAL ANTIBODIES

Cell	Measured Phenotype	Monoclonal Antibody Marker Combinations
NK Cells	Phenotypes: KIRs: Lytic Proteins:	CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} KIR2DL1 (CD158a), KIR3DL1 (CD158e), KIR2DL2/DL3 (CD158b), KIR2DS4 (CD158i), KIR2DL1/DS1 (CD158a/h), KIR3DL1/DL2 (CD158e/k), KIR2DL5 (CD158f), NKG2D (CD314), NKG2 (CD94) CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} Perforin ⁺ , CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} GranzymeA ⁺ , CD3 ⁻ CD56 ^{+/-} CD16 ^{+/-} GranzymeB ⁺
iNKT Cells	Phenotypes:	6B11 ⁺ CD3 ⁺ CD8 ^{+/-} CD4 ^{+/-} , 6B11 ⁺ CD3 ⁺ CD8a ^{+/-} CD4 ^{+/-} 6B11 ⁺ CD3 ⁺ CD45RO ^{+/-} CD28 ^{+/-} , 6B11 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} , 6B11 ⁺ CD3 ⁺ CCR7 ^{+/-} SLAMF ^{+/-} , 6B11 ⁺ CD3 ⁺ CD56 ^{-/+} CD16 ^{-/+} , 6B11 ⁺ CD3 ⁺ CD62L ^{-/+} CD11a ^{-/+} , 6B11 ⁺ CD3 ⁺ CD94 ^{-/+} CD11a ^{-/+}
CD8 T cells	Phenotypes: Lytic Proteins:	CD8 ⁺ CD3 ⁺ CD45RO ^{+/-} CD27 ^{+/-} , CD8 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} , CD8 ⁺ CD3 ⁺ CCR7 ^{+/-} CCR5 ^{+/-} , CD8 ⁺ CD3 ⁺ CCR5 ^{+/-} CD28 ^{+/-} CD8 ⁺ CD3 ⁺ Perforin ⁺ , CD8 ⁺ CD3 ⁺ GranzymeA ⁺ , CD8 ⁺ CD3 ⁺ GranzymeB ⁺
Tregs	Treg FOXP3⁺:	CD127 ^{low} CD25 ⁺ CD4 ⁺ FOXP3 ⁺
γδ T cells	γδ 1 T cells: γδ 2 T cells: Phenotypes:	γδ 1 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} γδ 2 ⁺ CD3 ⁺ CD45RA ^{+/-} CD27 ^{+/-} Naïve: γδ ⁺ CD3 ⁺ CD45RA ⁺ CD27 ⁺ Central Memory: γδ ⁺ CD3 ⁺ CD45RA ⁻ CD27 ⁺ Effector Memory: γδ ⁺ CD3 ⁺ CD45RA ⁻ CD27 ⁻ CD45RA ⁺ Effector Memory: γδ ⁺ CD3 ⁺ CD45RA ⁺ CD27 ⁻
DCs	Phenotypes:	CD14 ⁻ CD16 ⁺ DCs: Lin2 ⁻ HLA-DR ⁺ CD16 ⁺ pDCs: Lin2 ⁻ HLA-DR ⁺ CD123 ⁺ mDCs: Lin2 ⁻ HLA-DR ⁺ CD33 ⁺
B Cells	Phenotypes:	Total: CD19 ⁺ Memory: CD19 ⁺ CD27 ⁺ CD38 ⁻ Plasma: CD19 ⁺ CD138 ⁺ CD27 ⁺ CD38 ⁺ Plasmablast: CD19 ⁺ CD138 ⁻ CD27 ⁺ CD38 ⁺ Immature: CD19 ⁺ CD27 ⁻ CD38 ⁺

Appendix 14 shows the monoclonal antibody combinations used in project four to identify each of the cells and parameters for gating on the flow cytometer.