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Assessment of Immune and Molecular Changes in T Lymphocytes, Natural Killer Cells and Vasoactive Neuropeptides in Chronic Fatigue Syndrome

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Assessment of Immune and Molecular Changes in T Lymphocytes, Natural Killer Cells and Vasoactive Neuropeptides in Chronic Fatigue Syndrome

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A thesis submitted in fulfilment of the degree of Doctor of Philosophy to Bond University

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Professor Mieke van Driel

Abstract

Immune dysregulation due to infection, inflammation or compromises to immune and molecular mechanisms can have detrimental effects on normal physiological functions. Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a disorder of unknown mechanism where diagnosis is often delayed and evidence-based effective treatments are lacking. CFS/ME is characterised by severe fatigue, flu-like symptoms, pain and cognitive disturbances. To date a mechanism explaining the relationship between these symptoms and the physiological irregularities presented by patients remains obscure. As such, CFS/ME patients spend a considerable amount of money on health service cost in pursuit of the most effective treatment to alleviate their symptoms. An important initiative towards better management of CFS/ME is the development of accurate diagnosis. The principal aim of this research is to identify feasible biomarkers that can be used in effective diagnosis of CFS/ME. This research examines the status of lymphocytes in CFS/ME with a specific focus on their cellular function including apoptosis, protein secretion, receptor expression and gene expression. It is hypothesized that irregularities in these cellular processes contribute to the mechanism of CFS/ME and thus may form a suite of diagnostic markers for assessing CFS/ME patients.

Participants for the study comprised 95 CFS/ME patients and 50 non-fatigued controls at baseline. Data for 50 CFS/ME and 27 non-fatigued controls was available for the follow up study at 6 and 12 months. Participants were aged between 25-65 years. The criteria for inclusion were based on the Centre for Disease Prevention and Control (CDC) 1994 clinical diagnostic criteria. Whole blood was collected from each participant at baseline, at 6 months and at 12 months. Flow cytometric protocols were

employed in examining measures of cytotoxic activity in CD8^{+}T and Natural Killer (NK) cells, levels of CD56^{bright}CD16[negative] NK cells CD56^{dim}CD16[positive] NK phenotypes, CD4^{+}T helper cytokine secretion and levels of Foxp3 and vasoactive neuropeptide receptor (VPACR2). The expression pattern of cytotoxic related genes including granzyme A (*GZMA*), granzyme K (*GZMK*), perforin (*PRF1*) and interferon-gamma (*IFN-G*) and the expression of microRNA (miRNA) molecules in NK and CD8^{+}T cells were investigated.

At baseline, compared to the non-fatigued controls, CFS/ME patients exhibited significant decreases in cytotoxic activity of NK and CD8^{+}T cells, levels of CD56^{bright}CD16[negative] NK cells and repression of *IFN-G, GZMA* and *GZMK* gene expression. At the same time, significant increases in cytokines, IL-10, IFN-γ and TNF-α, FOXP3, VPACR2 and *PRF1*expression were noticed in the CFS/ME patients compared to controls. At 6 months follow up, significant reductions in NK activity, CD56^{bright}CD16[negative] NK cells, IL-10 and IL-17A were observed in the CFS/ME patients compared to the non-fatigued controls. At 12 months, in contrast to the non-fatigued controls, CFS/ME patients continued to demonstrate significant decreases in NK activity and significant increases in only IL-2. Assessment of miRNA expression revealed a significant down-regulation of a number of miRNAs in CFS/ME patients in comparison to the non-fatigued controls, specifically, *miR-21, miR-146a, miR-223, miR-17-5p, miR-103, miR-106, miR-10a, miR-191* and *miR-152* were significantly down-regulated mainly in the CFS/ME patients in comparison to the controls.

In conclusion, the results from this study have elucidated the extent of decreases in cytotoxic activity in CFS/ME. In addition, this study has identified unique immune

related processes and molecules that are compromised in CFS/ME patients. These novel parameters may have important implications in the development of biomarkers for CFS/ME. Moreover the consistent decrease in cytotoxic activity and NK phenotypes over the 12 month period strongly supports their usefulness as biomarkers for diagnosing CFS/ME. These biomarkers if implemented in the clinical setting could potentially assist in improving diagnosis and demonstrating a clear pathomechanism for CFS/ME. Eventually, this may result in the development of better therapeutic and treatment strategies for managing CFS/ME.

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Abbreviations

AB	Antibody
APC	Antigen presenting cell
AIDS	Acquired Immune Deficiency Syndrome
AU	Australia
cAMP	Cyclic adenosine monophosphate
CCR	Chemokine receptor
CD	Cluster of differentiation
CDC	Centre for disease prevention and control
CFS/ME	Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis
CNS	Central Nervous System
CB4	Coxsackie's B4 virus
CD2BP2	CD2 (cytoplasmic tail) binding protein 2
CTSC	Cathepsin C
CXC	Chemokine
DEFB1	Defensin beta 1
EBV	Epstein Barr Virus
EIF2B4	Eukaryotic translation initiation factor 2B, subunit 4
EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1
FasL	Fas ligand
EGR3	Early growth response 3
FCγR	Fc gamma receptor
FOXP3	Forkhead/winged helix transcription
GSN	Gelsolin (amyloidosis, Finnish type)

GZMA	Granzyme A
GZMB	Granzyme B
GZMK	Granzyme K
HHPV	Human Herpes Virus
HIF1A	Hypoxia inducible factor 1 alpha
HLA-DR	Human Leuckocyte Antigen
HTLV-1	Human T-lymphotropic Virus Type I
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-10RA	Interlukin 10 receptor alpha
IL-6R	Interleukin 6 receptor
IL-6ST	Interleukin 6 signalling transducer
JAK	Janus kinase
LPS	Lipopolysaccharide
LTα	Lymphotoxin alpha
LU	Lytic units
MAPK9	Mitogen-activated protein kinase 9
miRNA	Micro ribonucleic acid
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor zeta
NK	Natural Killer
PACAP	Pituitary adenylate cyclase activating polypeptide
Prf1	Perforin

RISC	RNA induced silencing complex
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SOCS	Suppressor of cytokine signalling
STAT	Signal transduction and activators of transcription
Th	T helper cell
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
TRAF	TNF receptor associated factor family of proteins
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T lymphocytes
US	United States of America
VN(s)	Vasoactive neuropeptide(s)
VIP	Vasoactive intestinal peptide
VPACR	VIP and PACAP receptor

Statement of Novelty

To the best of my knowledge I, Ekua Weba Brenu, declare that this work is an original documentation of the present research. All components of the written work were produced by the candidate unless otherwise specified where due acknowledgement has been given in the form of references.

Ekua Weba Brenu

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1. Introduction

1.1. Overview of Chronic Fatigue Syndrome

Chronic Fatigue Syndrome (CFS) also known as Myalgic Encephalomyelitis (ME) is an unexplained multi-symptom debilitating disorder with multi-factorial heterogeneous characteristics that undermine efficient development of a comprehensive mechanism to explain this disorder (Masuda *et al.*, 1994). Currently, the method of diagnosis is based on individuals demonstrating prevailing symptoms of severe fatigue, neurological disturbances and physical impairments enduring for more than six months (Fukuda *et al.* 1994). The prevalence rate of CFS/ME is between 0.2-2.6% (Grinde, 2008) with a higher prevalence in females compared to males at a ratio of 6:1 (Devanur & Kerr, 2006). In 2002, the Royal Australian College of Physicians reported that 25% of Australians who visit the general practitioner with fatigue are diagnosed with CFS/ME, hence, the prevalence rate of CFS/ME in Australia is 0.4% (Jason, 1999; Jason *et al.*, 2008; Reynolds *et al.*, 2004).

Significant economic and social losses incurred by patients in pursuit of the best therapy to manage their disorder can include direct and indirect costs. Direct costs refer to medical, ambulatory and hospital expenses, prescription medications, over-the-counter medications, and medical laboratory testing and indirect costs include transportation costs, work productivity losses, disability reimbursements, lower quality of life, social isolation, loss of leisure, services provided by family members, friends, or other informal care providers (Jason *et al.,*2008). At an estimated prevalence rate of 0.4% in Australia, the economic and social cost to society would be exceptionally high, emphasizing substantial decreases in overall economic contribution from this group due to this disorder. Using data extrapolated from indirect and direct costs incurred by a United States (US) CFS/ME population, the

annual cost associated with treatment and management of CFS/ME in Australia is estimated to be approximately AU$379 million per annum (assuming a 0.4% prevalence rate in Australia and a direct cost of US$ 5,509 (AU$5,176) per patient) (Jason *et al.,* 2008).

The diagnostic criteria for CFS/ME are not uniform and there currently exists four separate guidelines/definitions, the Australian, British, Canadian and the Centre for Disease Prevention and Control (CDC 1994) criteria (Carruthers *et al*., 2003, Fukuda *et al*., 1994, Lloyd *et al*., 1990, Sharpe *et al*., 1991). The most widely used guidelines for research purposes are the CDC 1994 criteria which describes CFS/ME as a novel onset of lingering relentless fatigue for over a period of at least 6 months, where four of the following symptoms (i) impaired short term memory or concentration, (ii) sore throat, (iii) tender cervical or axillary lymph nodes, (iv) muscle pain, (v) multijoint pain with no indication of swelling or redness, (vi) severe headaches, (vii) unrefreshing sleep and (viii) postexertional malaise must be experienced by the patient (Fukuda *et al.* 1994). Exclusion criteria for this definition consist of indications of psychiatric disorders such as melancholic depression, substance abuse, bipolar disorder, psychosis and eating disorders (Fukuda *et al.* 1994). In contrast, mental fatigue, psychosis, organic brain disorder and neuropsychiatric symptoms are incorporated in the other three definitions (Wyller 2007). Psychosis and organic brain diseases are indicative of a psychiatric disorder and are therefore not included in the CDC 1994 definition (Wyller 2007). Although, these definitions assist health professionals in diagnosing CFS/ME, they are not definitive as assessment is based on self-report from patients. Therefore, discrepancies can occur during diagnosis increasing the possibility of misdiagnosing CFS/ME as purely psychological. The

CDC 1994 case definition minimises these errors as it is more robust and is constantly being evaluated to ensure that the definition is appropriate and effective in isolating CFS/ME cases from other patients (Reeves *et al.* 2003; Jones *et al.* 2007). However, recently, the Canadian definition has been found to be a better assessment tool when compared with the CDC1994 case definition as it provides a more structured format of assessing patient illness and symptoms with focus specificity in physiological systems known to be compromised in CFS/ME (Caruthers *et al.*, 2003).

CFS/ME is a multi-system disorder that may result in impaired physiological homeostasis associated with imbalances in endocrine, immune, muscular, central and autonomic nervous system (Klimas and Koneru, 2007, Maes et al., 2006, Fulle et al., 2003, Di Giorgio et al., 2005, Cleare, 2003, Goertzel et al., 2006). Previously, CFS/ME was thought to have a psychological origin where depression was the prevailing cause for the symptoms patients described (Wessely and Powell, 1989). To date despite the vast research in CFS/ME, a singular causal agent for CFS/ME has not yet been identified, and infections originating from viral antigens may be a component of the disease mechanism. Additionally, currently there are no definitive biomarkers established for assessing CFS/ME as diagnosis is based on the previously described definitions. Whether CFS/ME occurs as a consequence of either post or pre-infectious episodes, toxins and other related factors remains to be determined although post or pre-infectious episodes may occur in some patients with CFS/ME. Immune deterioration appears to be a hallmark of CFS/ME and there is now compelling evidence to support this proposition. In particular, cell numbers, function and proteins implicated in the immune response to pathogens and inflammation may be defective in these patients. Immune cells that have been investigated in association with

CFS/ME include neutrophils, T (Cluster of Differentiation (CD) 4$^+$ and CD8$^+$T) cells, B cells and Natural Killer (NK) cells. Results on the total numbers of these lymphocytes in CFS/ME are most often inconsistent, nonetheless, NK cell activity is consistently noted to be severely decreased in CFS/ME patients while expression levels of genes related to immune function are also abnormally expressed in some CFS/ME individuals, suggesting that the changes in immune response may develop from cellular and molecular changes in immune cells and proteins. Deficiencies in these immune factors prompt inflammatory symptoms (Dantzer *et al*., 2008) and this may present in the form of fatigue, pain, severe headaches and swollen lymph nodes as occurs in CFS/ME.

As CFS/ME is a multi-system disorder, neuroendocrine and neuroimmune interactions and molecular patterns may be compromised. These changes may involve novel immune regulators including vasoactive neuropeptide (VNs) and the recently discovered small non-protein coding molecules known as microRNAs (miRNAs). These VNs are important neuroimmune regulators with expressions and receptors on immune cells in the periphery and central nervous system (CNS). They have neuroprotective effects as they prevent CNS insults, restore immune balance and act as anti-inflammatory mediators. Deficiencies in these peptides have been associated with a number of autoimmune conditions. Similarly an association between miRNAs and disease pathology and immune function exists (Liston et al., 2010b). As these parameters have not been investigated in CFS/ME, it is important to examine the link between these markers and CFS/ME as it further expands and may have the potential to assist in understanding the immunomodulation profile in CFS/ME. Additionally,

changes in gene expression in CFS/ME can affect immune, neurological, metabolic and important transcriptional processes.

This research attempts to fill the ambiguities in the current knowledge on CFS/ME such as (i) to determine the specific role of immune cells that have not being previously measured in CFS/ME, (ii) to investigate the relationship between immune function and disease progression overtime, (iii) to assess the contribution of novel molecules VNs, FOXP3 and miRNAs to the immune profile of CFS/ME individuals and (iv) to identify potential biomarkers for CFS/ME.

1.2. Immunological Parameters

The following sections review the immune system with specific focus on T and Natural Killer (NK) lymphocytes. A review of the extent of research on these cells in CFS/ME is also provided.

The immune system comprises a wide array of cells and proteins widely distributed throughout the human body and involve many physiological functions including neurological, endocrine, cardiovascular and gastrointestinal systems. Generally, the immune system is subdivided into the innate and adaptive systems. Immunological molecules are widespread throughout the human body. Hence, these components are intertwined with other systems to sustain optimal physiological homeostasis. Therefore, perturbations in any part of the immune process affect the outcome of other physiological systems and alter the homeostatic control.

The innate immune system is the primary defence mechanism that reacts to initial stages of infection, inflammation or other inflammatory insults. The adaptive immune system comes into play where the actions of the innate immune system are insufficient to effectively remove the infection or inflammatory insult and a more specific response is required. Cells of the innate immune system include neutrophils, macrophages, dendritic cells, NK cells, eosinophils and basophils, while those in the adaptive immune system are largely comprised of T and B cells, the latter of which produce antibodies. These cells are important during inflammatory episodes as they produce either anti- or pro-inflammatory mediators as well as antibodies to thwart pathogenesis and excessive inflammation. Dendritic cells, macrophages, neutrophils and NK cells eliminate pathogens either through phagocytosis (macrophages and

neutrophils) or cytotoxic activity. They also secrete cytokines and chemokines that induce inflammation, recruit, and activate adaptive immune cells (B and T lymphocytes) and present peptide fragments of the pathogen to T lymphocytes. Once activated the adaptive immune cells secrete antibodies (B cells) that help eradicate pathogens and retain memory of the pathogen, repair tissues, induce apoptosis of the infected cells and release cytokines that either enhance or dampen the inflammatory response.

During infection or inflammation, innate immune cells are firstly recruited to sites of pathogen infiltration or inflammation where they either phagocytose (macrophages and neutrophils) or induce apoptosis (NK lymphocytes) of cells infected by the pathogen and release proteins known as cytokines or chemokines that induce inflammation. To promote effective pathogen clearance, the cells of the innate immune system activate T and B cells via presentation of pathogen peptide fragments to the T lymphocytes or secrete cytokines that activate these cells (Figure 1). Activated T lymphocytes induce further pathogen lysis, secrete additional cytokines that further stimulate B and T lymphocytes to create memory and enhance further inflammation. T lymphocytes are thus important regulators of these immune reactions and restore immunological stability (Hoebe *et al.*, 2004). Failure to effectively eliminate pathogens or restore immune balance can affect physiological homeostasis and potentially present a symptom profile similar to CFS/ME.

The main cells of interest to this study are the NK and T lymphocytes and their cytokines. The exact role of these cells in the immune system is discussed in the following sections. Several factors such as neuropeptides and gene expression

enhance the ability of immune cells, such as T lymphocytes to successfully regulate immune response and homeostasis. Compromises to their functions have been shown to significantly alter anti- and pro-inflammatory actions of T lymphocytes in various diseases which share similar symptoms to CFS/ME (Moldofsky, 1995, Whistler *et al.*, 2009). The activities of VNs and small non-coding RNA molecules in the immune function are also discussed.
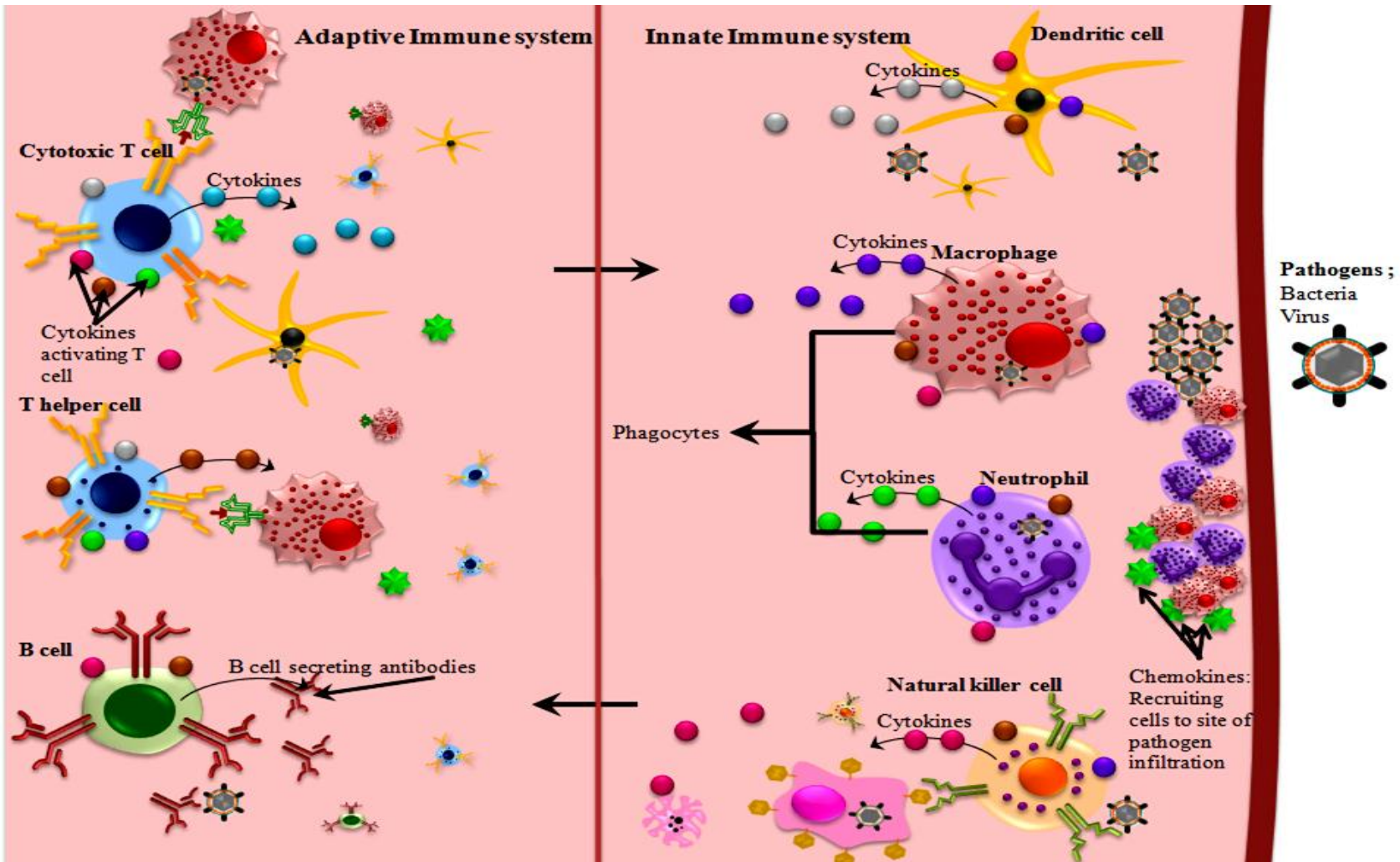
Figure 1: The components of the immune system. The immune system is made up of the innate and adaptive components, bidirectional communication between these components via the release of cytokines, chemokines and antibodies activate immune cells to respond to pathogens, maintain tolerance and regulate inflammatory reactions. *Copyright Ekua Weba Brenu*

1.2.1. T Lymphocytes

T lymphocytes are important components of immunological function and are most often explored in immune related disorders such as CFS/ME. T lymphocytes are generally involved in adaptive immune responses against antigens released during pathogen invasion or inflammation and are recruited via the release of soluble proteins from cells of the innate immune system, dendritic cells, macrophages and neutrophils. They characteristically express surface protein CD3. There are two major types of T lymphocytes described, $CD3^+CD4^+$ and $CD3^+CD8^+$. $CD3^+CD4^+$ and $CD3^+CD8^+$ have receptors that recognise and selectively bind to peptides known as major histocompatibility complex (MHC), specifically MHC class II and MHC class I respectively (Mazza and Malissen, 2007).

$CD3^+CD4^+$ T lymphocytes are helper cells and are further classified into T helper (Th) cells 1, Th2, Th17 and regulatory T lymphocytes (Treg). $CD3^+CD8^+$ T lymphocytes can be further classified in to $CD8^+CD11b^+$ (suppressor activity) and $CD8^+CD11b^-$ (cytotoxic activity) (Reddy and Grieco, 1991). Immature and mature T lymphocytes express a variety of co-stimulatory receptors at different stages of immune defence against infection. A number of these receptors have been shown to be differentially expressed in CFS/ME patients (Table 1). For example CD28 is required for the propagation of T lymphocytes and cytokines during an immune response to infections (Boise *et al*., 1995). In the occurrence of an infection, antigen presenting cells (APC) such as dendritic cells and cytokines bind with high affinity to CD28 on the naïve T lymphocytes, activating the T lymphocyte. The T lymphocyte encounters the antigen and retains a representation of its receptor thus becoming memory T lymphocyte. $CD45RO^+$ and $CD45RA^+$ represent the naïve or memory component of the T

lymphocytes respectively (de Jong *et al*., 1991, Hamann *et al*., 1996, Sohen *et al*., 1990). Memory T lymphocytes, CD45RA, and other T lymphocyte surface markers, tend to be significantly low in some cases of CFS/ME. This can have severe consequences on overall immune function as the vast majority of the T lymphocyte repertoire is imperative for physiological homeostasis.

1.2.1.1. CD4$^+$ T helper Lymphocytes

Naïve CD4$^+$T lymphocytes differentiation into subtypes is dependent on signals received during initial antigen interactions. Figure 2 describes the immune factors released by these cells and also molecules implicated in their differentiation from naïve CD4$^+$T lymphocytes into the different subtypes. CD4$^+$ T cells can be categorised into Th1, Th2, Th17 and Tregs (Figure 3) where Th1 and Th17 predominantly promote inflammation while Th2 inhibit inflammation. Tregs are suppressors of autoimmune reaction and autoreactive T lymphocytes that alter immune homeostasis. The mechanism of action of these cells occurs via the secretion and expression of soluble proteins and receptors for these proteins and include cytokines, chemokines and their receptors (Mosmann and Coffman, 1989). Immune perturbations affecting physiological functions similar to those exhibited by CFS/ME patients may occur where there is either an excessive or inadequate production of these cytokines. Importantly, shifts in cytokine profiles have been noticed in CFS/ME where there is a preferential shift towards either a Th1 or Th2 cytokine profile. A governing Th1 and Th17 immune response is thought to be associated with autoimmune diseases such as Rheumatoid Arthritis and Multiple Sclerosis (Singh *et al*., 2007, Drulovic *et al*., 2009). An overwhelming Th2 mediated immune response triggers most systemic and allergic disorders (Nevala *et al*., 2009).
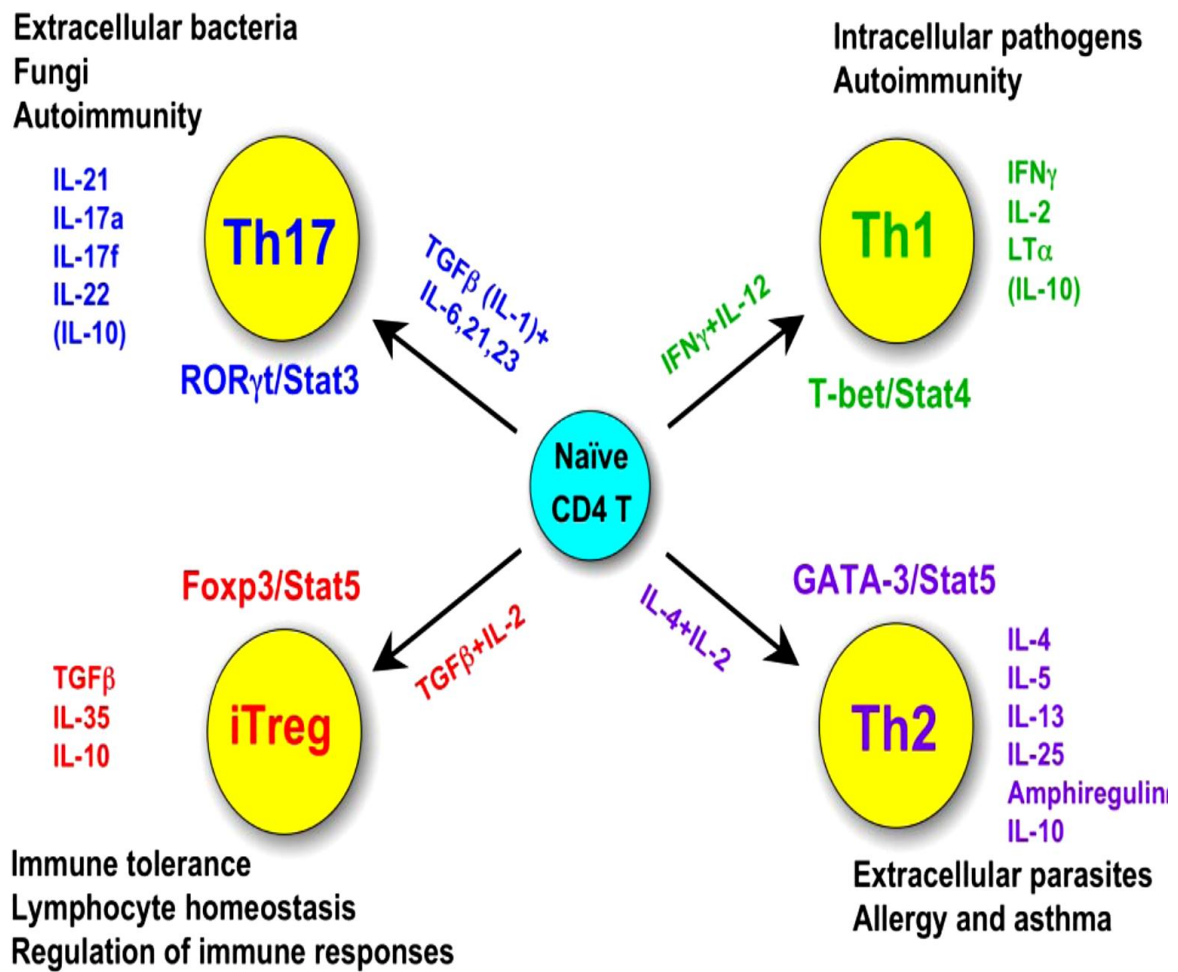
Figure 2: Differentiation of CD4$^+$ T helper cells. CD4$^+$T lymphocytes expansion into various subsets generates four distinct populations of CD4$^+$T lymphocytes in the presence of different transcription factors and molecules necessary for their differentiation. These cells secrete cytokines that assist in maintaining immune homeostasis (Zhu and Paul, 2008).

Among the different subsets of T cells, Th1 cells are the main producers of IFN-γ, IL-2 and lymphotoxin α (LTα) (Rook *et al.* 1997; Hanson *et al.* 2001). These cells are important in ensuring efficient anti-inflammatory activity during infiltration of the intracellular environment by mycobacteria particles. IFN-γ stimulates macrophages to phagocytise microbial pathogens while the production of IL-2 is paramount for the

formation of memory $CD4^+$ and $CD8^+$ T lymphocytes. This assists in successful detection and elimination of mycobacterial, viral and tumour antigens should they reappear (Williams *et al*., 2006, Yamane *et al*., 2005). Equivocal levels of IL-2 and IFN-γ have been reported in CFS/ME (Patarca *et al*., 1994, Linde *et al*., 1992, Lloyd *et al*., 1992, Peakman *et al*., 1997, Gold *et al*., 1990) and this may explain the occurrence of persistent infections and heightened inflammation. Where pathogens are not adequately cleared due to a lack of $CD4^+$ and $CD8^+$ T lymphocytes memory formation against pathogenic antigens or an increase in IFN-γ pro-inflammatory reactions tissue damage occurs and may encourage autoimmune related immune response in these patients.

Conversely Th2 is an anti-inflammatory mediator secreting cytokines IL-4, IL-9, IL-10, IL-13 and IL-25 (Swain et al., 1990, Longphre et al., 1999, Wynn, 2003, Urban et al., 1998, Fort et al., 2001). Induction of these cytokines is necessary for protecting against extracellular pathogenesis which includes parasites (Mosmann and Coffman, 1989, Paul and Seder, 1994). Immune responses to allergic reactions involves IL-5, IL-9, while IL-4 and IL-10 modulate inflammatory reactions (Le Gros et al., 1990, Longphre et al., 1999, Wynn, 2003, Urban et al., 1998, Fort et al., 2001). Hence, discrepancies in Th2 immune related responses in CFS/ME patients may affect the differentiation of both Th2 and Th1 cells, as cytokines, IL-2 and IL-4, produced by these cells are necessary for their survival (Zhu *et al*., 2006, Jankovic *et al*., 2000, Min *et al*., 2004, Finkelman *et al*., 2000, Zhu *et al*., 2002). This can have severe consequences on the ability of the body to respond to extracellular pathogens and restore balance after an inflammatory episode.

Th17 cells are secretors of pro-inflammatory IL-17 (IL-17A and IL-17F), IL-21, IL-22 and TNF-α and direct the production of pro-inflammatory chemokines CCL2, CCL3 and CCL20 (Paust *et al.*, 2009, Pene *et al.*, 2008). These chemokines stimulate the migration of monocytes and T lymphocytes (Paust *et al.*, 2009, Pene *et al.*, 2008). Th17 induces the generation of antimicrobial peptides, facilitates neutrophil migration to sites of infection and secretes an array of pro-inflammatory cytokines and chemokines (Weaver *et al.*, 2007, Fossiez *et al.*, 1996, Liang *et al.*, 2006, Ouyang *et al.*, 2008). Similarities between Th1 and Th17 can be attributed to the finding that both Th17 cells and Th1 cells increase the production of pro-inflammatory cytokine IFN-γ (Bending *et al.*, 2009). Currently, data regarding Th17 cells in CFS/ME are insufficient, however, elevated levels of cytokines, TGF-β and IL-6, required for Th17 differentiation (Bettelli *et al.* 2006; Zhou *et al.* 2007) have been reported in CFS/ME patients (Chao *et al.*, 1991, Chao *et al.*, 1990, Bennett *et al.*, 1997). An increase in TGF-β and IL-6 may favour Th17 production. Antigenic modifications of the Th17 cells may cause neuropathic pain, systemic intestinal inflammation and the production of anti-apoptotic molecules (Kleinschek *et al.*, 2009, Hou *et al.*, 2009, Koenders *et al.*, 2005, Lubberts *et al.*, 2005). Interestingly, in CFS/ME patients many viral antigens have been observed. These antigens may modify the activities of Th17 cells (Hofstetter *et al.*, 2009) and promote severe fatigue and other CFS/ME like episodes.

The fourth types of T cells are the Tregs. Tregs are classified as either natural or adaptive. Natural Tregs make up 5-10% of all $CD4^+T$ lymphocytes (Sakaguchi, 2005). An important attribute of Tregs is the expression of the forkhead/winged helix transcription factor (FOXP3), a member of the FOXP subfamily of transcriptional

repressors and activators (Fontenot *et al*., 2003, Hori *et al*., 2003). FOXP3 suppresses IL-2 and IFN-γ thus modulating the amount of pro-inflammatory factors in circulation (Bettelli *et al*., 2005, Bettelli and Kuchroo, 2005, Wu *et al*., 2006b). This protects against elevated production of autoreactive T lymphocytes, Tregs and inflammation through the joint action of enzymes, adenosine diphosphate hydrolysing enzyme CD39 and ecto-5'-nucleotidase CD73 (Deaglio *et al*., 2007) and cyclic adenosine monophosphate (cAMP) (Bopp *et al*., 2007). Natural Tregs secrete IL-10 and TGF-β (Tang and Bluestone, 2006). TGF-β has a role in the delineation of Th17 cells whereas IL-10 reduces Th1 directed pro-inflammatory responses (Bettelli *et al*., 2006). Dysfunctional CD4$^+$CD25$^+$Treg is a distinct contributory factor to many autoimmune diseases (Shevach, 2002, Sakaguchi *et al*., 2001) and a similar presentation may occur in CFS/ME. The other types of Tregs are the adaptive Tregs which secrete anti-inflammatory cytokines IL-5, IL-10, TGF-β and IFN-γ (Roncarolo *et al*., 2001, Roncarolo *et al*., 2006, Weiner, 2001, Faria and Weiner, 2006). Interestingly, these cytokines are required for the generation of some CD4$^+$T cell subsets, regulation of naïve T lymphocytes and the prevention of autoimmune reactions (Sundstedt *et al*., 2003, Oida *et al*., 2003, O'Garra and Barrat, 2003). Collectively, these immune related processes assist in preserving homeostasis.

Currently, a clear profile of CD4$^+$T cell subtypes and their cytokine secretion in CFS/ME has not yet been demonstrated. The tremendous contribution of these cells to inflammatory pathways, suggest the need for further investigations to clarify their role in the mechanism of CFS/ME pathogenesis as this can assist in both diagnosing and treating this disorder.

1.2.1.2. CD8$^+$ T Lymphocytes

CD8$^+$T lymphocytes are the second subset of T lymphocytes that are necessary for the control of both chronic and intracellular infections. They express MHCI and are derived from multi-potent T lymphocytes and are able to either remain in stasis or differentiate in to cytotoxic, suppressor and memory CD8$^+$T lymphocytes. As such CD8+T cells can be sub grouped into four major subsets (Figure 4), including Tc1, Tc2, Tc17 and CD8$^+$Treg cells (Huber *et al*., 2009, Xystrakis *et al*., 2004). Antigen specific memory CD8$^+$T lymphocytes are more prominent in the periphery and constantly survey the peripheral environment for secondary infection (Kaech and Wherry, 2007) thus guarding against re-infection. CD8$^+$T lymphocytes are highly cytotoxic and secrete antiviral proteins (Grayson *et al*., 2001, Luckey *et al*., 2006, Bachmann *et al*., 2006). Cytotoxic CD8$^+$T lymphocytes are the focus of this project. These lymphocytes are more differentiated in comparison to the other CD8$^+$T cells thus they express high quantities of granzymes, perforin, cytokines and chemokine receptors (Peixoto *et al*., 2007).

CD8$^+$T lymphocytes differentiate into CD8$^+$CD25$^+$T lymphocytes (CD8$^+$Tregs) in the presence of exclusive APCs, tumour and viral antigens that alter co-stimulatory molecules (Alatrakchi *et al*., 2007, Jarnicki *et al*., 2006, Myers *et al*., 2005). They are important in preserving sites of immune privilege and homeostatic balance in immune response. They can either initiate suppression through direct killing (cytotoxic) or stimulate the production of immunosuppressive cytokines such as TGF-β and IL-10 (Niederkorn, 2008). However, their actions are largely effective in the presence of IFN-γ, thus in most cases, the CD8$^+$Tregs are only functionally effective after IFN-γ activation (Niederkorn, 2008). The idea of lowered or deteriorating immune function in CFS/ME that allows recurrent infections and an inability to clear old and/or new

infections may be due to a lowered number of memory and suppressive CD8^{+} T lymphocytes. Thus cell phenotyping may be a better method of determining the type of cells that are compromised in CFS/ME patients if any. Additionally, as NK cytotoxic activity is reduced in CFS/ME, CD8^{+}T cell activity may also be reduced and this requires more confirmatory studies.
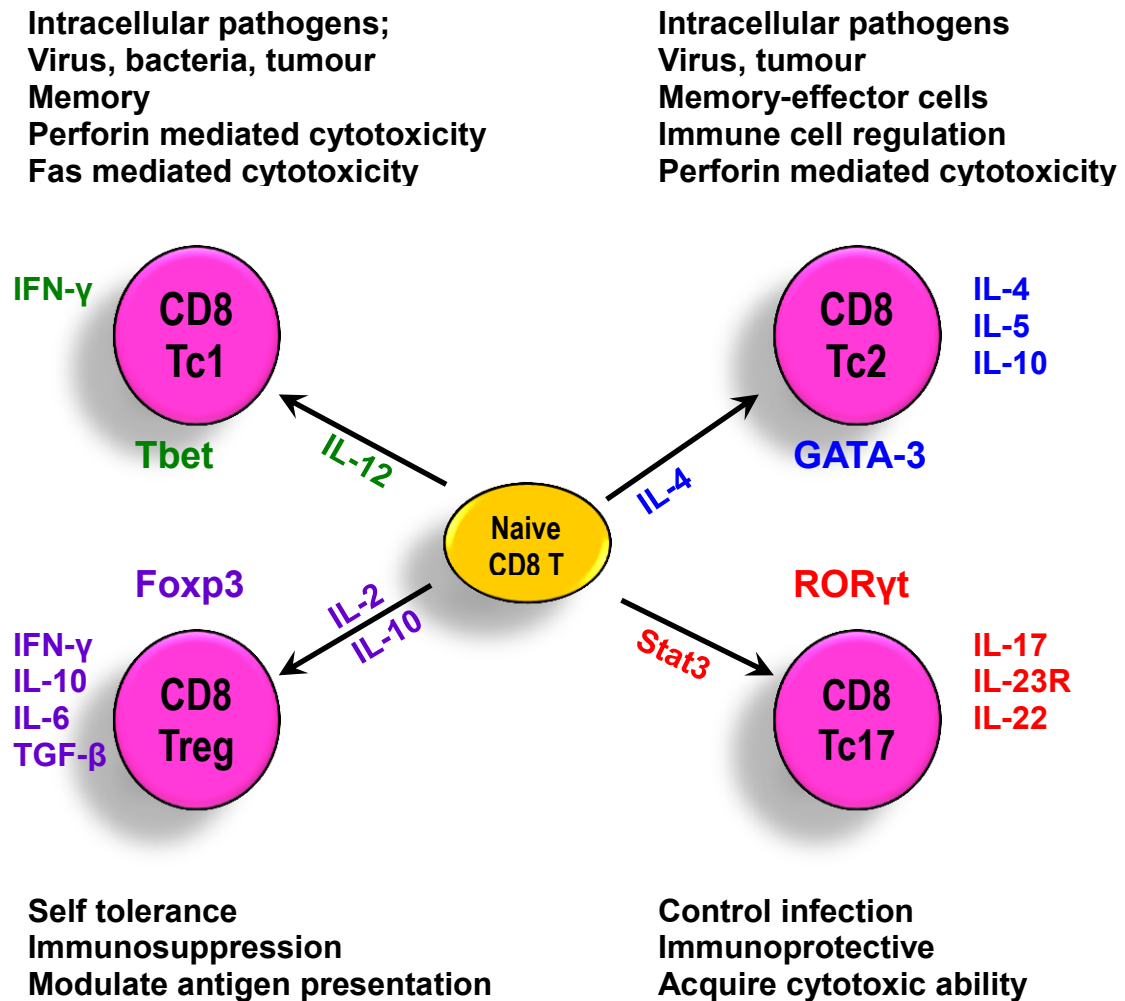
Figure 3: Differentiation of CD8^{+} T cell. CD8^{+}T cells can be subdivided into these phenotypes depending on the cytokines and other co-stimulatory molecules present. These cells interact with viral infected and transformed cells using the peptide MHCI peptide recognition pathways. This induces various cytotoxic and immunosuppressive pathways ensuring complete clearance of pathogens and maintenance of immune homeostasis.

1.2.1.3.   T Lymphocyte in Chronic Fatigue Syndrome

To date over 20 studies have investigated immune function in CFS/ME, the parameters most often examined are viral antigens, inflammatory signals, cytokine levels, NK function and lymphocyte numbers (Table 1). The results from these studies with respect to T lymphocytes are however inconsistent, as variations in the number of these cells and their surface markers in peripheral blood circulation occur in the CFS/ME population in comparison to the controls where participants have either identical or equivocal numbers in the overall quantity of T lymphocytes and their subsets.

Immunological assessments of antibody positive and negative Epstein Barr Virus (EBV) CFS/ME patients have identified similarities in the levels of T lymphocytes in both CFS/ME patients and controls (Jones, 1988, Jones *et al*., 1988). However, only one patient demonstrated a significant change in T lymphocytes (Jones, 1988). CFS/ME patients demonstrate similarities in levels of activated, naïve and memory CD4$^+$T and CD8$^+$T lymphocytes to that of healthy individuals (Visser *et al*., 1998). T lymphocyte counts remain constant despite symptom severity and disease persistence (Landay *et al*., 1991, Tirelli *et al*., 1994).

Contrary to these studies, CFS/ME patients may have reduced T lymphocytes particularly the CD4$^+$T and CD8$^+$T lymphocytes with diminished response of T lymphocytes during delayed hypersensitivity (Lloyd *et al*., 1989). In some cases the levels of CD4$^+$T lymphocytes were similar to controls while CD8$^+$T lymphocytes had a propensity to increase among the CFS/ME patients. However, CFS/ME patients with an incidence of EBV infections may demonstrate high levels of CD4$^+$T lymphocytes with unaltered CD8$^+$T lymphocytes and an increase in overall T cell

suppression (Straus *et al*., 1985). Hence, it is likely that although the CD8$^+$T lymphocytes remained similar to that of healthy controls the prevalence of suppressor CD8$^+$T lymphocytes was much higher in the CFS/ME group. These findings could suggest a relationship between CD8$^+$T lymphocytes suppressor function and CFS/ME, however, the activities of subtypes of CD8$^+$T and CD4$^+$T lymphocytes were not indicated in this study. Additionally, lymphocytes subsets may differ between CFS/ME patients and healthy individuals according to the mode of onset of illness, that is, either sudden or gradual (Hanson *et al*., 2001). Both patients with gradual and sudden onset of CFS/ME exhibit decreases in the percentage of total T lymphocytes and CD8$^+$T lymphocytes nevertheless CD4$^+$T lymphocytes are elevated (Hanson *et al*., 2001). Significantly higher levels of antiviral antibodies such as human herpes virus-6 (HHPV-6), EBV-EA and Coxsackie's B4 virus (CB4) are prevalent in some cases of CFS/ME regardless of the symptom severity (Landay *et al*., 1991). This may indicate an inability of the CD8$^+$T and NK lymphocytes to effectively clear viral antigens permitting increases in viral loads.

These studies have highlighted the profile of T lymphocytes in CFS/ME, however, the constraint with these studies relates to the lack of measurements for T cell functions. Changes in T cell number are not necessarily a reflection of the function of these cells especially in a disorder such as CFS/ME where the exact cause is not known. Additionally, it is important to know the precise subsets of CD4$^+$T and CD8$^+$T lymphocytes that are reduced. Similarities may exist between the overall counts of these cells in both healthy and CFS/ME patients. It is likely that in CFS/ME patients some subsets of these cells may be more or less prevalent than others. This project provides an insightful view of the function and subsets of T lymphocytes in CFS/ME,

with specific focus on $CD4^+T$ lymphocytes and $CD8^+T$ lymphocytes. In this study, $CD4^+T$ cell studies are concerned with their cytokine profiles while $CD8^+T$ cell investigations are centred on cytotoxic activity. Although, NK cell studies have indicated significant decreases in NK function in CFS/ME patients, as CFS/ME is heterogeneous and multifactorial with inconsistencies in immune parameters, further investigative studies are required to assess the function of $CD8^+T$ lymphocytes in a novel CFS/ME cohort as both NK and $CD8^+T$ cells perform some parallel immune functions.

1.2.2. Natural Killer Lymphocytes

NK lymphocytes are the cytotoxic cells of the innate immune system. These cells mature in the bone marrow from $CD34^+$ hematopoietic progenitor cells (Farag *et al*. 2002). They represent 10-18% of the total lymphocyte population in both lymphoid and non-lymphoid tissues (Caligiuri 2008). Their primary functions are to eliminate foreign pathogens and tumours, activate other immune cells and preferentially provide an adequate supply of cytokines to the immune system (Caligiuri 2008; Vivier *et al*. 2008). These cells express CD56 and CD16 receptor molecules on their cell surfaces (Fan *et al*. 2008). CD16 is also known as the fragment crystallisation gamma receptor III (FcγRIII) (Farag *et al*. 2002). FcγRIII is required for the initiation of antibody dependent cytotoxic activity in NK lymphocytes (Vivier *et al*. 2008). The two main subtypes of NK lymphocytes are $CD56^{dim}CD16^{positive}$ and $CD56^{bright}CD16^{negative}$ NK lymphocytes (Farag *et al*. 2002). The name $CD56^{bright}CD16^{negative}$ NK depicts high levels of CD56 (neural cell adhesion molecule (NCAM)) molecules and low levels of CD16 (Fc γ receptor III for lysis) receptors on the cell surface of these cells (Fan *et al*. 2008). $CD56^{bright}CD16^{negative}$ NK lymphocytes are primarily responsible for producing cytokines, TNF-β, IFN-γ, IL-10, TNF-α and granulocyte macrophage colony-

stimulating factor (GM-CSF) (Caligiuri 2008). CD56^{dim}CD16^{positive} NK lymphocytes can be distinguished from CD56^{bright}CD16^{negative} NK lymphocytes by the presence of low levels of the adhesion molecule, CD56, but high levels of CD16 (Caligiuri 2008). Increased expression of FcγRIII/CD16 enhances target killing via ADCC when the FcγRIII/CD16 recognises and binds targets activating death receptor pathways (Vivier *et al*. 2008). These particular NK lymphocytes with a high density of CD16 contain increasing amounts of lytic granules perforin and granzymes, necessary for ADCC and natural cytotoxicity (Bryceson *et al*. 2006).

A
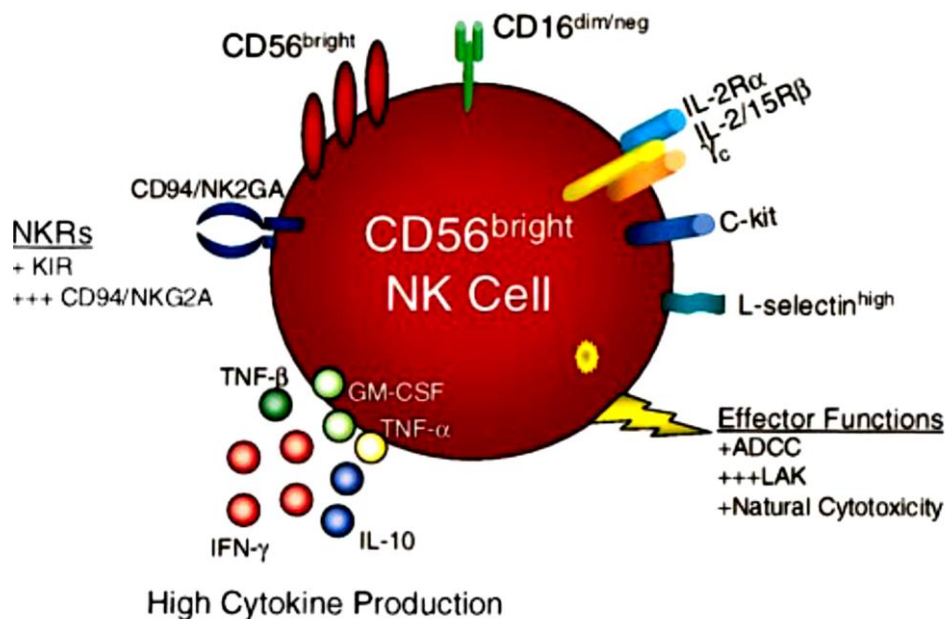
B
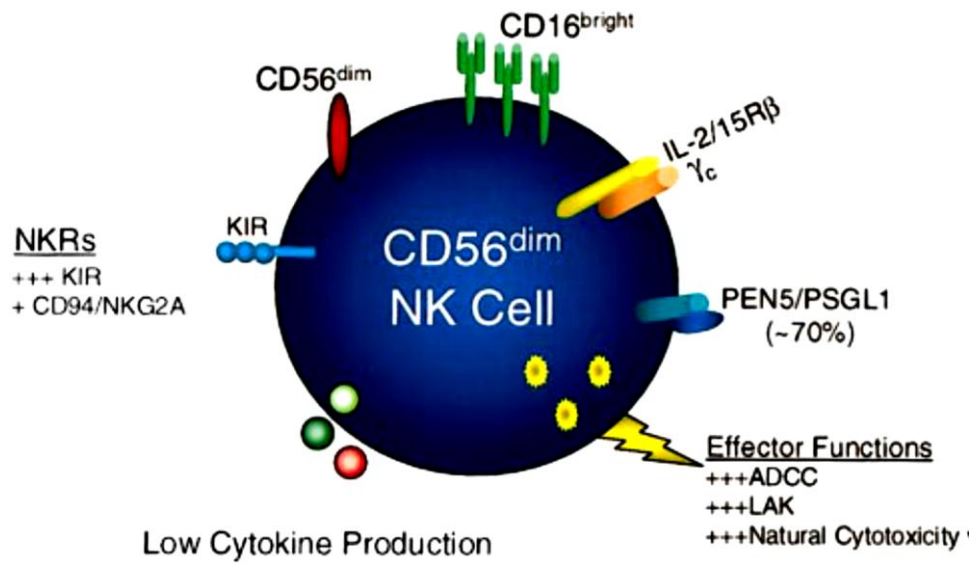
Figure 4: The profile of NK lymphocyte subsets. The two main types of NK cells are morphologically and functionally distinct cells. The CD56^{bright}CD16[negative] (A) NK lymphocyte secretes less granule containing lytic molecules, are less cytotoxic and express different NK receptors. They are high cytokine producers. CD56^{dim}CD16[positive] NK lymphocyte (B) secretes less cytokines. However, they are more cytotoxic express more NK receptors and granules containing lytic molecules (Cooper *et al*., 2001).

1.2.3. Cytotoxic Activity

NK lymphocytes and CD8^{+}T lymphocytes exert their effector function via granule-dependent cytotoxic activity (Chowdhury and Lieberman, 2008, Russell and Ley, 2002). In the granule-dependent pathway, granules containing perforin, proteases and granzymes (include Granzyme (GZM) A, B, H, K and M) are released in to the target cell (Figure 5) where they induce death or apoptosis (Ashton-Rickardt, 2005, Bots and Medema, 2006, Lieberman, 2003). During viral infection or tumour growth receptors on the surfaces of cytotoxic cells CD8^{+}T and NK lymphocytes are activated when cytokines and adhesion molecules attach to these receptors (Makrigiannis & Anderson

2003; Cerwenka & Lanier 2001). Activation of these receptors stimulates signal transduction molecules, intracellular signalling, tyrosine phosphorylation and the release of cytokines and granules containing granzymes and perforin into the plasma membrane of the target cell (Cerwenka & Lanier 2001). The exact role of perforin in the mechanism of cytolysis remains unknown however there are suggestions that perforin sabotages the plasma membrane to allow endocytosis and release of the contents of the cytotoxic granules into the cytoplasm (Catalfamo and Henkart, 2003, Pipkin and Lieberman, 2007). They may also direct the granzymes into the endosomes and subvert the endosome, thus facilitating the movement of granzymes to appropriate organelles and the nucleus of target cells where they induce apoptosis (Voskoboinik et al., 2006, Voskoboinik and Trapani, 2006, Scott et al., 2008).

Granzymes act via a number of mechanisms to induce cell death in the target cell. Granzyme K (GZMK) has been observed to activate the release of reactive oxygen species (MacDonald et al., 1999), degrade mitochondria, induce single stranded nicks in the DNA, caspase-independent nuclear fragmentation and nuclear condensation (Zhao et al., 2007). GZMB, a serine protease, acts via a caspase-dependent apoptosis pathway (Sutton et al., 2003) while GZMA, GZMH, and GZMM act independently of caspase activation (Johnson et al., 2003, Kelly et al., 2004, MacDonald et al., 1999). GZMA activates slow apoptosis by inducing single stranded DNA nicks in the nucleus (Warren & Smyth 1999; Cullen & Martin 2008). Gram B activates the caspase pathway by acting on the mitochondria to release cytochrome C and BCL-2 (Warren & Smyth 1999; Cullen & Martin 2008). BCL-2 suppresses the activation BAX and BAK, thus preventing apoptosis, while cytochrome C induces an apoptosome formation (Warren & Smyth 1999; Cullen & Martin 2008). The death promoters and apoptosome stimulate caspase resulting in eventual death of the target

cells. Other death receptor pathways exist involving CD95 (Fas), TNF or TRAIL. NK and CD8^{+} T lymphocytes are highly equipped to maintain homeostasis in the immune systems and a deficiency in their function can have severe consequences on immune response to pathogens. These mechanisms are illustrated in Figure 5. These important attributes of NK cell function suggests that they are a necessary component of immune function and breaches in this mechanism can affect viral clearance following infection or memory creation that facilitates rapid anti-viral activity upon subsequent exposure.
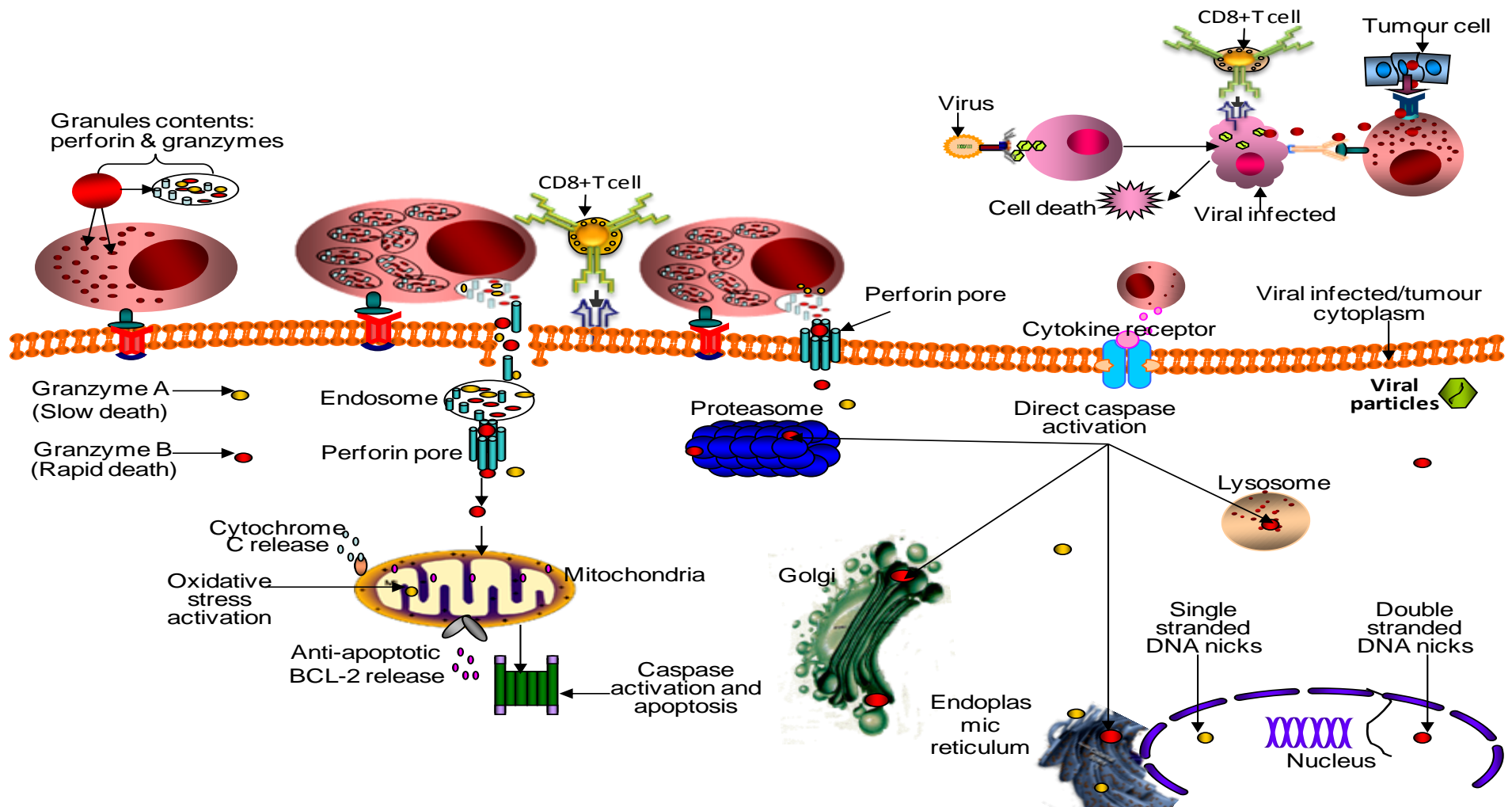
Figure 5: The mechanism of cytotoxic activity of NK and CD8^{+}T lymphocytes. Recruitment of NK and CD8^{+}T lymphocytes to sight of infection or tumour growth results in the release of granules containing granzymes and perforin and induction of apoptosis. Apoptosis is stimulated by the binding of granzymes to organelles of the viral infected or tumour cell (Lieberman, 2003).

1.2.3.1. Natural Killer Lymphocytes in CFS/ME

NK lymphocytes are the primary lytic agents that eliminate viral infected cells, hence, their deterioration in terms of function may evoke immune compromised situations. NK lytic activity, also referred to as cytotoxic activity, has been examined in CFS/ME where most often decreases in NK activity have been observed to be a characteristic of CFS/ME (Klimas *et al*. 1990; Ojo-Amaise *et al* 1994; Barker *et al*. 1994; Brenu *et al*., 2010).

CFS/ME patients with abnormal serological levels of EBV demonstrate a 65% reduction in NK cell cytotoxicity compared to the controls (Klimas *et al*. 1990, Ojo-Amaise *et al* 1994) suggesting a possible association between NK lysis and viral infections. However, decreases in NK cell activity in CFS/ME patients may occur without viral antigens compared to healthy controls (Barker *et al*. 1994) although, in some cases the presence of viral antigens was not measured (Brenu *et al*., 2010; Maher *et al.* 2005; Fletcher *et al.* 2010). Hence, it is difficult to draw conclusions on the role of viral antigens in NK activity. When NK cell function in CFS/ME is compared to patients with Acquired Immune Deficiency Syndrome (AIDS) and healthy subjects, CFS/ME patients had a higher killing rate of the target cells compared to the AIDS patients though they were considerably reduced when evaluated against the healthy controls (See *et al*. 1997). In contrast, CFS/ME patients demonstrate lower NK activity in comparison to fatigue-non-CFS/ME and healthy individuals (Masuda *et al*. 1994).

These studies indicate substantial deficiencies in the cytotoxic activity of NK lymphocytes in CFS/ME, however, the prevalence of incompetent NK lymphocytes are not as severe as in diseases such as AIDS. AIDS is an established disorder where a

mechanism for the disease pathogenesis is known while CFS/ME remains an unexplained phenomenon. Nonetheless, NK cell lytic deficiencies possibly occur from paucities in lytic proteins, perforin and granzymes, in CFS/ME patients (Maher *et al*., 2005; Saiki *et al*., 2008). For example, a two-fold decrease in perforin and granzymes in CFS/ME patients compared to healthy subjects (Saiki *et al*., 2008) can reduce lysis capabilities of NK lymphocytes. Moreover changes in NK cell phenotypes, $CD56^{dim}CD16^{positive}$ NK lymphocytes and $CD56^{bright}CD16^{negative}$ NK have been associated with some cases of CFS/ME (Brenu *et al*., 2010, Maher *et al*., 2005, Robertson *et al*., 2005, Tirelli *et al*., 1994). Abnormalities in NK cell phenotype can affect soluble protein production required to ensure successful pathogen lysis. For instance, a decline in $CD56^{dim}CD16^{positive}$ NK lymphocytes suggests ineffective cytotoxic activity while an increase or decrease in $CD56^{bright}CD16^{negative}$ NK lymphocytes causes an increase in inflammation or a decrease in lymphocyte activation and effector function. Further studies are therefore required to confirm these inept NK lymphocytes in CFS/ME patients. Remarkably, longitudinal assessment of NK phenotypes and NK activity has not been performed, this study therefore assesses these attributes of NK lymphocytes in CFS/ME patients over a long period of time.

Table 1: A summary of studies investigating immune function in CFS/ME and their findings

T lymphocytes	NK lymphocytes	CD molecules	Authors
↓CD4$^+$ T lymphocytes ↓ CD8$^+$ T			Lloyd *et al.* 1989
↑CD4$^+$T ↕CD8$^+$T			Straus *et al.* 1985
↕Total T cell			Jones *et al.*, 1988
↕CD4$^+$T ↓Suppressor CD4$^+$ ↓CD45RA$^+$ T lymphocytes	↑NK lymphocytes ↓NK activity	↑CD20$^+$ ↑CD21$^+$ ↑CD2$^+$	Klimas *et al.*, 1990
↓CD8$^+$T		↓CD38$^+$	Landay *et al.* 1991
↑T suppressors			(Kato *et al.*, 1992)
	↓NK ↓CD56^{dim}CD16^{positive} ↑CD56^{bright}CD16^{negative}		Tirelli *et al.*, 1994
	↓NK activity		Ojo-Amaize *et al.*, 1994
Pathological T lymphocytes			Hilgers and Frank, 1994
↕Total T cell ↓CD8$^+$CD11b$^+$	↕CD16$^+$ ↕CD56$^+$NK ↓NK cell activity	↑CD38$^+$ ↑CD28$^+$	Barker *et al.*, 1994
	↓NK ↓CD56^{dim}CD16^{positive} ↓CD56^{bright}CD16^{negative} ↓NK activity		Masuda *et al.*, 1994
	↕ NK activity		Mawle *et al.*, 1997
↓Total T cell			Vedhara *et al.* 1997
↓CD8$^+$ T-cell	↓NK activity	↓CD5$^+$ ↓CD11a$^+$	See *et al.*, 1998
↕T proliferation			Visser 1998
↓Total T cell			Kavelaars *et al.*, 2000
↓CD8$^+$ T ↑CD4$^+$T		↓CD34$^+$	Hanson *et al.*, 2001
	↓CD56$^+$ NK		Racciatti *et al.*, 2004
↑CD8$^+$ T ↓Naïve T cells			Nijs *et al.*, 2003
	↓CD56$^+$		Stewart *et al.*, 2003

↓CD4^{+} and CD8^{+}T ↑CD4^{+}/CD8^{+} ratio	↓CD56^{+}NK		Racciatti *et al*., 2004
	↓NK activity		Maher *et al*. 2005
	↓NK activity		Robertson *et al*., 2005
↓Early T cell activation		↓CD69	Maes *et al*., 2005
		↓CD69^{+}	Mihaylova *et al*., 2007
	↓NK activity		Siegel *et al*., 2006
	↓CD56^{bright}CD16[negative] ↓NK activity		(Brenu *et al*., 2010)

↑ refers to significant increase while ↓ refers to a significant decrease and ↕ refers to no changes when CFS/ME patients are compared with healthy individuals.

1.2.4. Soluble Proteins

Immune cells express CD molecules on their cell surfaces which are important during differentiation, priming of T lymphocytes during antigen presenting cells (APC) presentation and elimination of pathogens. T lymphocytes express receptors for surface markers, cytokines and chemokines and also secrete some of these markers. Deficiencies or defects in these markers may have severe consequences on physiological function.

Cytokines are important determinants of all immune related responses including cytotoxic function, phagocytosis, allergies, immunosuppression, antigen presentation, humoral and cellular mediated immunity. They are responsible for growth, differentiation and activation of immune cells. The classification of these markers can be confined to the ability to induce anti-inflammatory or pro-inflammatory immune responses. Additionally, they can also be grouped according to their functions. Cytokines that are necessary for antigen presentation cell (APCs) are produced following binding of ligand to the pattern recognition receptors on the surface of

APCs. This interaction generates the production of TNF, IL-1, IL-6, IL-8, IL-12, IL-15, IL-18 and IL-23. Importantly, cytokines also contribute to effective cytotoxic activity and this is necessary to eliminate viral infections. Cytokines such as IFN-α inhibit viral growth and therefore have antiviral qualities. Other cytokines that may be useful for cytotoxic activity also include IL-1. The proliferation and differentiation of NK and cytotoxic T cells is in part modulated by IL-7 while INF-γ, IL-17, IL-21 and IL-2 are responsible for cell mediated immunity involving T cells (Commins *et al.* 2010). Chemokines are chemotactic molecules that primarily induce the migration of immune cells to sites of immune compromise or immune insults. In the adaptive immune system they are responsible for the generation of haematopoiesis, lymphocyte trafficking and immune homeostasis (Borish *et al*., 2003).

Paucities in cytokine quantity are likely to have negative consequences on cytotoxic function, humoral immunity and cellular mediated immunity. Additionally, skewness in the cytokine distribution either towards a dominant anti-inflammatory or pro-inflammatory state may activate autoimmune or systemic inflammation respectively (Elenkov *et al*., 2005).

1.2.4.1.   CD Molecules and Cytokines in CFS/ME

In CFS/ME levels of soluble proteins are inconsistent across studies. High levels of inter-cellular adhesion molecule (ICAM)-1 and CD4$^+$T lymphocyte function-associated antigen (LFA)-1 on the surfaces of CD4$^+$T lymphocytes may be observed in CFS/ME individuals (Gupta and Vayuvegula, 1991). In CFS/ME, patients with less severe symptoms exhibit higher increases in surface markers such as CD11b$^-$, CD38$^+$ and HLA-DR on CD8$^+$T lymphocytes (Landay *et al*., 1991). However, compared to

healthy individuals, some CFS/ME patients regardless of their symptom severity may display significantly higher levels of CD38$^+$ and HLA-DR on their CD8$^+$T lymphocytes (Landay *et al*., 1991). Alterations in these molecules may occur as a consequence of significantly high levels of antiviral antibodies such as HHPV-6, EBV-EA and CB4 virus in the circulation of CFS/ME patients. This is indicative of potential decrease in pathogen lysis and recurring viral infection. Similarly, reductions in CD11b$^+$ (Barker *et al*., 1994, Swanink *et al*., 1996) with increasing levels of CD38$^+$ and CD28$^+$ markers have been noticed on CD8$^+$T lymphocytes in some CFS/ME patients (Barker *et al*., 1994). Equivalent expression of other surface molecules CD38$^+$, CD45RO, HLA-DR on CD8$^+$ T lymphocytes and CD4$^+$ T lymphocytes are present in some CFS/ME patients and healthy participants (Hassan *et al*., 1998). CD28$^+$ is heterogeneously expressed on CD8$^+$T lymphocytes in CFS/ME, it may be reduced (Hassan *et al*., 1998) or elevated (Barker *et al*., 1994) in comparison to healthy individuals. This highlights the heterogeneity associated with CFS/ME and implicates an imbalance in inflammatory markers in CFS/ME, however more studies are required to investigate if these changes depict CFS/ME as an autoimmune (high levels of Th1) or systemic (predominant Th2) disorder or whether patients express attributes of both.

Similarly, grouping of CFS/ME patients into two populations using both 1988 and the 1994 CDC definitions yields comparable results with respect to levels of surface molecules (CD8$^+$CD38$^+$, CD8$^+$HLA-DR$^+$, CD8$^+$CD11b, CD8$^+$CD28$^+$, CD4$^+$CD45RO$^+$, CD4$^+$CD45RA$^+$) on both CD4$^+$T and CD8$^+$T lymphocytes (Natelson *et al*., 1998). Surprisingly, the levels of surface markers were akin to patients with Multiple Sclerosis and depression (Natelson *et al*., 1998). Conversely,

CFS/ME patients meeting the British criteria may show decreases in CD11b$^+$ and CD56$^+$ (Swanink *et al*., 1996) and increases in CD38$^+$ (Peakman *et al*., 1997) on CD8$^+$T lymphocytes, while levels of CD25$^+$ and HLA-DR on CD4$^+$ T and CD8$^+$ T lymphocytes remain unchanged (Peakman *et al*., 1997).

Memory (CD45RA$^+$) CD4$^+$T lymphocytes may also diminish in CFS/ME with an increase in CD8$^+$HLA-DR cells (Klimas *et al*., 1990). A deterioration in CD4$^+$CD45RA$^+$T lymphocytes in CFS/ME may be problematic as these cells are important in creating memory for the CD4$^+$T lymphocytes, allowing immediate action in response to insults and pathogens and also activating suppressor or cytotoxic CD8$^+$T lymphocytes (Klimas *et al*., 1990). Accordingly, decreases in the production of CD4$^+$CD45RA$^+$T cells reduce B lymphocyte regulation. Hence, inefficient suppression by B lymphocytes and cytotoxic T lymphocytes may likely promote high levels of viral reactivation. This study however did not provide a detailed analysis of the B lymphocyte activity. Declining levels of CD45RA$^+$T lymphocytes may be accompanied by an increase CD45RO$^+$T lymphocytes (Hanson *et al*., 2001). Although, the specificity of T lymphocytes was not indicated in the Hanson *et al*., study it is likely that CD45RA$^+$ and CD45RO$^+$ measurements were inclusive of both CD4$^+$T and CD8$^+$T lymphocytes. Increases in naïve T lymphocytes without an increase in memory creation may incite an increase in viral load as there will be a limited amount of cells able to recognise and effectively lyse a recurring pathogen or create memory for a novel pathogen (Picker *et al*., 2004).

Thymocytes secrete numerous amounts of soluble proteins or cytokines and chemokines and these can either have a pro (type 1/Th1) or anti-inflammatory (type 2/

Th2) immune response (Lucey *et al*., 1996). Anti-inflammatory cytokines, IL-4, IL-6, IL-10 and IL-13, avert unwarranted inflammatory reactions of pro-inflammatory cytokines, and other immune modulators (Keane and Strieter, 2002). Contrarily, pro-inflammatory cytokines, IL-1, IL-2, IL-12, TNF-α and IFN-γ arbitrate allergic reactions and mobilise other cells during pathogen infiltration (Opal and DePalo, 2000). Disequilibrium in cytokine production favouring either anti-inflammatory or pro-inflammatory cytokine profile may be prevalent in some CFS/ME individuals (Skowera *et al*., 2004, Swanink *et al*., 1996), causing changes in inflammatory homeostasis. Only one study has identified defective IL-17F in some cases of CFS/ME, and this was characterised by significantly lowered expression of Histidine-161-Arginine in the protein composition of this cytokine. This allele is mainly responsible for dampening the pro-inflammatory action of this cytokine (Metzger *et al*., 2008). CFS/ME patients lacking this protein may therefore experience heightened levels of pro-inflammatory markers in the presence or absence of Th2 deficiencies. This ultimately overwhelms anti-inflammatory modulations and increases inflammation in the joints and other areas. Nevertheless, whether an anti- or pro-inflammatory immune modulation is a hallmark of CFS/ME has not yet been determined and therefore requires additional research. Table 2 summarizes studies that have investigated cytokine distribution in CFS/ME. The differences in results can be attributed to the heterogeneous nature of the disorder.

Although, the results are not consistent, they do indicate defects in the cellular networks of T lymphocytes in CFS/ME. Hyper or hypo expression of these markers coupled with shifts in Th1/Th2/Th17 cytokines affect the ability of the innate and adaptive immune components to collectively lyse and restore order during or after

pathogenesis and subsequent recurring infections. Particularly, these studies have not highlighted the relationship between Tregs and CFS/ME, this may be compromised in CFS/ME where changes in cytokines such as IL-6, IL-10 and TGF-β are either elevated or decreased. However, lack of Treg activities may explain the increase in pro-inflammatory cytokines and chemokines in some of these CFS/ME patients. Therefore, it is imperative to explore the role of subtypes of CD4^{+}T lymphocytes with respect to their cytokines and transcription factors, which this study proposes to investigate.

Table 2: Studies investigating cytokines in CFS/ME compared to non-CFS/ME participants.

Cytokines	Significant Increase in expression	Significant Decrease in Expression	No Change	
IL-1α	+++		+++	Patarca *et al.*, 1994, Linde *et al.*, 1992, Lloyd *et al.*, 1992, Peakman *et al.*, 1997, Rasmussen *et al.*, 1994, Swanink *et al.*, 1996
IL-1β	+	+	+++++	Linde *et al.*, 1992, Morte *et al.*, 1989, Patarca *et al.*, 1994, Peakman *et al.*, 1997, Rasmussen *et al.*, 1994, Straus *et al.*, 1989
sIL-1R	+		+	Cannon *et al.*, 1997, Swanink *et al.*, 1996
IL-2	++	++	+++	Cheney *et al.*, 1989, Gold *et al.*, 1990, Kibler *et al.*, 1985, Linde *et al.*, 1992, Patarca *et al.*, 1994, Rasmussen *et al.*, 1994, Straus *et al.*, 1989
sIL-2	+		+	Linde *et al.*, 1992, Patarca *et al.*, 1994
IL-4	+		+	Visser *et al.*, 1998
IL-5	+			Fletcher *et al.*, 2009
IL-6	+++		+++++	Gupta *et al.*, 1997, Buchwald *et al.*, 1997, Chao *et al.*, 1990, Chao *et al.*, 1991, Linde *et al.*,

				1992, Lloyd *et al*., 1992, Peakman *et al*., 1997, See *et al*., 1997
sIL-6R	+			Patarca, 2001
IL-8		+		Fletcher *et al*., 2009
IL-10		+	+	Gupta *et al*., 1997, Fletcher *et al*., 2009
IL-12	+			Fletcher *et al*., 2009
IL-13		+		Fletcher *et al*., 2009
IL-15		+		Fletcher *et al*., 2009
IL-23			+	Fletcher *et al*., 2009
TNF-α	++	+	++	Gupta *et al*., 1997, Patarca *et al*., 1994, Kriegler *et al*., 1988, Lloyd *et al*., 1992, Peakman *et al*., 1997
TNF-β	+		++	Lloyd *et al*., 1992, Patarca *et al*., 1994, Peakman *et al*., 1997
IFN-α	+		++	Linde *et al*., 1992, Straus *et al*., 1989, Vojdani *et al*., 1997
IFN-γ	+	++	++++	Klimas *et al*., 1990, Linde *et al*., 1992, Lloyd *et al*., 1992, Peakman *et al*., 1997, Straus *et al*., 1989, Visser *et al*., 1998
TGF-β	+			Bennett *et al*., 1997

The symbol + represents the number of studies that have examined these cytokines and their corresponding result.

1.3. Gene Expression Profile in CFS/ME

A number of studies have investigated the molecular processes in CFS/ME. This section examines the findings from these gene expression studies in CFS/ME. Table 3 provides a summary of these studies and the genes that were identified to be differentially expressed in CFS/ME patients in contrast with non-fatigued controls. The majority of these genes are involved in immune, metabolic, cellular and neurological functions. The following is a review of the genes that have been identified in relation to immune function in CFS/ME. A systematic review of all the

genes investigated in CFS/ME patients is beyond the scope of this research. As such the information provided herein is only limited to genes identified and related to immune function since this is the focus of this project.

1.3.1. Cytokine and Chemokine Genes

Cytokine and chemokine genes are critical for sustaining, inducing and mediating immune reactions including cell proliferation and activation. A number of these genes have been observed to be differentially expressed in CFS/ME. Among these are IL-8 gene (*IL-8*), (Vernon *et al.* 2002) a chemokine expressed in endothelial cells that serves as a chemoattractant molecule for neutrophils during pathogen invasion and other immunological insults (Huber *et al.*, 1991). Although neutrophil recruitment has not been reported to be defective in CFS/ME, deficiencies in *IL-8* mRNA expression do occur and this has adverse consequences on inflammation (Xie, 2001, Mukaida, 2003, Nozell *et al.*, 2006). Secretion of end products of phagocytosis during neutrophil pathogen lysis, increases IL-8 chemotactics thereby causing an increase in the recruitment of more neutrophils to sites of infection (Sparkman and Boggaram, 2004, Ito *et al.*, 2004). An up regulation of *IL-8* as noted in CFS/ME may be associated with potential declines in oxidative stress owing to the regulatory effects of nitric oxide and oxidative stress on *IL-8* mRNA during inflammation (Sparkman and Boggaram, 2004, Ito *et al.*, 2004). Neutrophil oxidative burst in CFS/ME has been shown to be reduced (Brenu *et al.*, 2010). Another contributory factor to this alteration in expression may be related to declining NF-κB regulation (Huang *et al.*, 2001). NF-κB is an essential transcription factor necessary for leukocyte cytokine production (Artis *et al.*, 2003, Bohuslav *et al.*, 1998). Deficiencies in NF-κB may increase susceptibility to infectious agents (Artis *et al.*, 2003, Sha *et al.*, 1995) and

alter inflammatory reactions (Campbell *et al*., 2000, Yang *et al*., 1998) as observed in most CFS/ME cases (Fletcher *et al*., 2010, Broderick *et al*., 2010).

Most cytokines are regulated and have an involvement in the Janus Kinase signal transducers and activators of transcription (JAK/STAT) pathways. Processes in this pathway involve the transcription and phosphorylation of factors including *STAT5A*. The *STAT5A* transcription factor is induced by cytokines IL-2, IL-4 and IL-7, and is a critical element in the proliferation and survival of Th2 cells (Lin and Leonard, 2000). Changes in this transcription factor in CFS/ME (Saiki *et al*., 2008) are a probable cause for decline in anti-inflammatory reaction/Th1 type immune response and an increase in pro-inflammatory mediators in the immune pathogenesis of this disorder (Skowera *et al*., 2004). *STAT5* together with *JAK1* mediate cytokine signalling in particular IFN-α, IL-2, IL-7, IL-9, IL-10, IL-13 and IL-22 signalling (Schindler *et al*., 2007). An up regulation in *STAT5A* and *JAK1* (Kerr *et al*., 2008b, Saiki *et al*., 2008) can significantly hinder the rate or activity of the above mentioned cytokines and their receptors. This has been observed in some CFS/ME patients where receptors for IL-7, IL-10 and IFN-α are differentially expressed (Kerr, 2008) while these same cytokines for example, IFN-α and IL-10, maybe either increased and decreased respectively in some CFS/ME cases (Gupta *et al*., 1997, Vojdani *et al*., 1997).

The JAK/STAT pathways are also regulated by cytokines such as IL-6 (Kamimura *et al*., 2003, Kristiansen and Mandrup-Poulsen, 2005). Interactions between IL-6 and IL-6R are important for modulating downstream signalling events involving JAKs and STATs which promote the growth of progenitor cells. Intense signalling of suppressors of cytokine signalling (SOCS) proteins result in exacerbation of the

inflammatory response and heightened inflammation (Rawlings *et al*., 2004, Kristiansen and Mandrup-Poulsen, 2005). Decreased expression of IL-6 (Light *et al*., 2009), coupled with a decline in its receptor and signal transducer (*IL6R* and *IL6ST* genes) (Kerr, 2008) may have unfavorable outcomes on the role of IL-6 in the immune response and in regulating *JAK1* in CFS/ME (Guschin *et al*., 1995). An immune profile where IL-6, its receptor and signal transducer are compromised, affects JAK1 and STAT5 signalling and alters cytokine related trans-signalization and inflammatory homeostasis permitting either an autoimmune or defective physiological function in CFS/ME.

The tumour necrosis factor (TNF)-α receptor is encoded by the gene *TNFRSF1A*. It increases pro-inflammatory events via NF-κB activation that results in elevations in gene expression and proliferation of cytokines (Nowlan *et al*., 2006). *TNFRSF1A* is also involved in cellular apoptosis signalling related to the TNFR-associated factor (TRAF) domains (Baud and Karin, 2001). In CFS/ME, heightened cellular apoptosis of neutrophils has been reported (Kennedy *et al*., 2004, Vojdani *et al*., 1997, See *et al*., 1998), this possibly occurs as a consequence of changes in *TNFRSF1A* (Kerr *et al*. 2008). Decreases in the NF-κB gene, *NFKB1*, correlated with a proportional decrease in *TNFRSF1A* in CFS/ME (Kerr *et al*., 2008) may potentially affect proliferation of IL-8. *NFKBIZ* which dampens NF-κB, has been shown to be decreased in expression in CFS/ME (Kerr *et al*. 2008). NF-κB activation involves pattern recognition receptors such as Toll-like receptors (TLR) (Kitamura *et al*., 2000, Yamazaki *et al*., 2001) and other factors including *TRAF3* (He *et al*., 2007, He *et al*., 2006, Hauer *et al*., 2005). It is possible to hypothesise that variations in the expression of these genes may have significant disadvantages on cytokine secretion and regulation in CFS/ME.

HIF1A represents hypoxia induced transcription factor 1 alpha (HIF1α) gene, which has a role in the regulation of apoptosis (Yu *et al*., 2004, Carmeliet and Tessier-Lavigne, 2005, Akakura *et al*., 2001). It regulates phagocytosis induced by neutrophils and macrophages in response to the presence of microbial antigens (Nizet & Johnson 2009) and incidentally phagocytosis is downregulated in CFS/ME (Brenu *et al*., 2010), perchance resulting from differential expression of *HIF1A*.

The degree of *CXCR4* expression by neutrophils, monocytes and T lymphocytes is regulated by cAMP, IL-4, IL-6, IL-10 and reactive oxygen species, and its expression is essential for hematopoietic cell trafficking, differentiation, endothelial migration and immune proliferation in the CNS and immune systems (Moepps *et al*., 1997, Zou *et al*., 1998, Jazin *et al*., 1997). The observation of an upregulation in *CXCR4* in CFS/ME (Kerr *et al*. 2008; Gow *et al*. 2009) relates to a change in cytokine profile present in some CFS/ME patients. Interestingly, most of these CXCR4 regulatory factors are controlled by NF-κB and VNs. VNs will be discussed in later sections. VNs regulate the production of cAMP and some of the anti-inflammatory cytokines, listed above. Perturbations in these VNs may therefore affect CXCR4 and contribute to altered immune profiles in some CFS/ME patients.

The *CD47* gene, is extensively expressed in immune and nervous system and is important for migration of neutrophils and chemotaxis of other cells, stimulation of T cells and cognitive function (Lindberg *et al*., 1993, Brown *et al*., 1990, Parkos *et al*., 1996, Chang *et al*., 1999, Gao *et al*., 1996, Ticchioni *et al*., 1997, Waclavicek *et al*., 1997). Although, differences in lymphocyte numbers remain controversial in CFS/ME, where some studies have reported decreases, increases or no change in

immune cell numbers, migration of these cells to sites of infection may be impaired given that *IL-8*, *CXCR4* and *CD47* are differentially expressed in CFS/ME patients (Kerr *et al*. 2008; Gow *et al*., 2009).

The gene, *TGF-β1,* is an essential component of the cytokine genes, it has an involvement in immune response to injury, cell development and cell proliferation (Marie *et al*., 2005). *TGF-β1* is a transcription factor that regulates Treg differentiation pathways and is also a necessary factor in the survival of Tregs (Marie *et al*., 2005). It induces apoptosis through the Fas apoptotic pathways or oxidative stress that inevitably cause death of the targeted cell (Sanchez-Capelo, 2005). An upregulation in *TGF-β1* may cause an elevation in neutrophil apoptosis (Kennedy *et al*., 2005), consequently decreasing effective oxidative stress in CFS/ME (Brenu *et al*., 2010). Although, Treg function in CFS/ME patients has not being directly measured, it is likely that these cells may be deficient in their expression of FOXP3 and therefore need to be studied in detail to determine if these effects at the transcriptome level can be translated into protein function in these cells.

1.3.2. Genes Involved in Pathogen Lysis

Cytotoxic activity of NK and CD8^{+}T cells as previously mentioned occurs via a number of pathways including the granule dependent cytotoxic pathways. NK and CD8^{+}T cells secrete granzymes that effectively bind to various organelles within the cell systematically inducing apoptosis of the target cell. Granzyme and perforin genes, *GZMA*, *GZMB* and *PRF1,* are important genes associated with activity during natural cytotoxic activity and antibody dependent cytotoxic activity of CD8^{+}T and NK lymphocytes via FCγRII (CD16) receptor (Lahmers *et al.* 2006; Madueno *et al.* 1993).

GZMA induces slow apoptosis while GZMB stimulates rapid apoptosis of the target cell. Diminishing mRNA levels of *GZMA* and *GZMB* in CFS/ME (Saiki *et al.,* 2008) may affect efficient NK and CD8^{+}T cell cytotoxicity. Not surprisingly, this is consistent with studies investigating NK cell activity in CFS/ME patients where most patients demonstrate significant declines in cytotoxic activity (Brenu *et al*., 2010, Klimas *et al*., 1990, Maher *et al*., 2005).

The cathepsin gene (*CTSC*) is prominent in myeloid cells, polymorphonuclear leukocytes, alveolar macrophages and osteoclasts (Hakeda and Kumegawa, 1991, McGuire *et al*., 1997, Rao *et al*., 1997b). Paucity in the expression of *CTSC* is related to poor functioning in lytic proteins in particular GZMA and GZMB in cytotoxic lymphocytes (Pham and Ley, 1999). Thus in patients with CFS/ME uncharacteristic expression of *CTSC* may translate into low levels of GZMB and GZMA proteins (Maher *et al*. 2005; Saiki *et al*., 2008) and consequently resulting in suboptimal cytotoxic activity during infections in both the innate and adaptive immune systems. A decrease in these parameters may contribute to the abnormally low levels in cytotoxic activity of NK cells in CFS/ME patients. High rates of microbial antigens may persist in these patients as the immune system is not able to effectively eliminate these pathogens.

1.3.3. Transcription factors

Transcription factors are essential molecules for initiating the expression of genes and are especially important for development (Farnham, 2009). In CFS/ME, a few of these genes display disparities when compared to healthy individuals. A typical example is the early growth response protein 3 gene (*EGR3*), changes in this transcription factor

alters lymphocyte proliferation, apoptosis and inflammatory response (Yamamoto *et al.* 2005; Beinke & Ley 2004; Inoue *et al.* 2004). Cytotoxic pathways such as the Fas L and ADCC are induced by *EGR3* (Matsuoka and Jeang, 2005). Altered expression of *EGR3* reduces T cell related production of IL-2 (Safford *et al.*, 2005). Changes in this gene in some CFS/ME patients (Kerr *et al.*, 2008) may be linked to reduced cytotoxic activity and high levels of anti-inflammatory factors increasing pathogenesis and pro-inflammatory immune responses. The resultant effects include symptoms of fatigue, loss in memory, concentration and impaired immune function in CFS/ME as observed in mice deficient in this gene (Li *et al.*, 2007a).

In the innate and adaptive immune system *TRAIL* plays a substantial role in the regulation of cytotoxic activity. The presence of TRAIL on NK and CD8^{+}T lymphocytes serves as an alternative pathway for effective cytotoxic activity against viral antigens (Kayagaki *et al.*, 1999, Janssen *et al.*, 2005). Interestingly, TRAIL has positive and negative effects on Th1/Th2 immune responses. TRAIL induces apoptosis in Th1 cells thereby inhibiting pro-inflammatory reactions while increasing anti-inflammatory effectors by activating Th2 differentiation (Zhang *et al.*, 2003). Hence in CFS/ME, deviations in the expression of *TRAIL* may affect cytotoxic activity and cause abnormal shifts in Th1/Th2 inflammatory responses.

The gene *NFATC1* is the gene for nuclear factor of activated T lymphocytes. It is part of the NFAT family of transcription factors responsible for the regulation of genes encoding cytokines and cytokine receptors (Crabtree and Clipstone, 1994, Rao *et al.*, 1997a). *NFATC1* is important for T cell proliferation, Th2 differentiation and cytokine production (Yoshida *et al.*, 1998; Ranger *et al.*, 1998). Similarly, irregularities in Th2

cytokine profiles in CFS/ME may ensue from perturbed expression in *DEFB1* or the human β defensin 1 may contribute to skewed Th1 profile (Wehkamp *et al*., 2005). The involvement of *DEFB1* in immunomodulation at both the innate and adaptive immune response, is important for dendritic cells and CD4^{+}T lymphocytes recruitment via the CCR6 receptor (Yang *et al*., 1999) during infection and inflammation (Dommisch *et al*., 2005, Wehkamp *et al*., 2005, Sun *et al*., 2005). Animal models have revealed that dysregulation in *DEFB1*, increases the susceptibility to bacterial and viral infection (Moser *et al*., 2002, Morrison *et al*., 2002). *DEFB1* differential expression may be correlated with the observation of different viral antigens in serological samples from CFS/ME patients.

1.3.4. Immune Regulators

GNAS and cyclic AMP-responsive element-binding protein (*CREBBP*) are transcription factors important in the activation of cAMP in cells such as CNS related cells (Goodman and Smolik, 2000). Importantly, mutation in *GNAS* generates sporadic tumours (Lania *et al*., 2003). *GNAS* and *CREBBP* have been shown to be increased in CFS/ME patients (Zhang *et al*., 2009). As will be discussed later *CREBBP* is vital for VN conversion of ATP to cAMP via adenylate cyclase (Delgado and Ganea, 2001). Differential expression of *CREBBP* and *GNAS* may be detrimental to VN related activities that regulate immune responses in both the CNS and periphery, thus increasing susceptibility to infection and other pathologies (Petrij *et al*., 1995, Stevens and Bhakta, 1995). Similarly mitogen activated protein kinase pathways involving genes such as *MAPK9* encode important signalling molecules known as the JNK2 protein kinase. Differential expression in this gene affects downstream signalling pathways that have been shown to be associated with the

pathogenesis of destructive insulitis (Jaeschke *et al*., 2005). In CFS/ME perturbations in these genes likely affects certain metabolic pathways contributing to severe fatigue.

Similarly, *CMRF35* is the gene that encodes the CD300c leukocyte surface protein present on macrophages (Turnbull and Colonna, 2007) and are important regulators of dendritic cell secretion of TNF-α and IFN-α (Ju *et al*., 2008). Irregularities in CFS/ME cytokine profiles possibly occur as a result of defects in *CMRF35* which inadvertently disrupts anti-viral IFN-α and inflammatory TNF-α activities required for immune homeostasis (Sen, 2001).

In summary, the perceived ramifications of these gene expression changes in immune related genes may contribute substantially to the disease profile of CFS/ME. Modifications in genes affect protein conformation during translation and consequently alter function. The genes discussed above have vital roles in most immune related activities such as inflammatory modulation, lymphocyte and cytokine activation, lymphocyte differentiation and proliferation and are also implicated in the signalling pathways of other genes especially transcription factors. A breach in the optimal capacity of these genes whether towards an upregulation or a down regulation potentially interferes with VN function, prompting either a Th1 or Th2 cytokine shift, decrease in cytotoxic activity, lymphocyte migration and proliferation and perturbations in important physiological networks.

Interestingly, the consistent observation of impaired NK cytolysis in CFS/ME is partly due to the reduced expression of activating cytokines, perforin and granzymes. As previously discussed these granzymes induce apoptosis of antigens within the cell.

Variations in inflammatory reactions, that is, pro- and anti-inflammatory, can be explained by the altered levels of their corresponding cytokine genes. Most of these cytokines are engaged in other physiological functions hence, defects in their production can severely hinder other important processes. Although, most studies in CFS/ME have to some extent provided information on the expression of genes in CFS/ME patients, it is not known whether CFS/ME elicits these changes in gene expression pattern or *vice versa*. Similarly, most of the genes observed in these studies have not been replicated in other CFS/ME patients, and most of the time specific lymphocytes were not studied, it is very difficult to ascertain which specific cells are compromised among the CFS/ME population. It is therefore imperative for further studies to be carried out to determine how changes in gene expression can be related to the mechanism of CFS/ME and the specific cells that may be severely compromised in this disorder and how this relates to the disease profile.

Table 3: A Summary of Gene expression studies in CFS/ME

Authors	Total number of transcripts found in study	Important Genes Relevant to immune function
Vernon *et al*., 2002	7	CMRF35, TRAIL, DEFB1, CTSC
Powell *et al*., 2003	7	Protein kinase R Tumor necrosis factor alpha, Cathepsin C
Kaushik *et al*., 2005	16	**IL10RA,** CD2BP2, GSN, EIF2B4, EIF4G1, ANAPC11
Carmel *et al*., 2006	17	Solute carrier family 1 membrane 6 F-box protein 7 Zinc finger protein 350 T cell leukemia 1A
Goertzel *et al*., 2006a	9	NR3C1, TPH2, COMT, CRHR2, CRHR1, NRC1, TH, POMC, 5-HTT
Kerr *et al*. 2008	88	CD2BP2,CD47,CITED2,CREBBP,CRK,CTBP1,**CXCR4**,EBI2,EIF2B4,EIF3S10,EIF4G1,EIF4G3,GNAS,GSN,IFNAR1,**IL10RA,IL6R,IL6ST**,JAK1,MAPK9,PGM2,PKN1,,PRKARA1A,SNAP23,SOS1,TAF11,TCF3,TNFRSF1A,NFKB1,NHLH1,GABAPA,ETS1,EGR1,EGR3,IL7R, PIK3R1
Saiki *et al*. 2008	9	GZMA, ATP5J2, DBI, COX5B, STAT5A
Presson *et al*., 2008	299	FOXN1, PRDX3, SUCLA2, DCTN2, PGK1, SNURF, PRKCH, RYK, PPP1R14C, VAMP5, PRO0641, TMEM50A, CRNKL1, NPAL2, TFB2M, PBLD, LTV1, MED8, CD302(XM13557)
Light *et al*., 2009	13	**IL-6, IL-10**, TNF-α, TLR4, CD14, TGF-β
Gow *et al*., 2009	366	DEFA1, HBG1, CEACAM8, ITGA2B, HBG2, DEFA4, ITGB3, CD61, **CXCR4**, RPS26, ZNF294, HLA-DQA1

Genes in bold have been examined in other studies with similar pattern of expression however, IL-10RA was inconsistent.

1.4. Immune Regulators as possible markers in CFS/ME

Immune function is regulated by proteins and other molecular products including novel substances which continue to be discovered. The following sections describe novel regulators of immune function and their potential contribution to CFS/ME immune mechanism in cases where they are deficient.

1.4.1. MicroRNAs

Thymocytes and other cells of the immune system are regulated by the change in gene expression, specifically, transcription, translation and protein formation. In relation to the immune systems these proteins may include cytokines, chemokines, lytic proteins and receptors. The synthesis of these proteins from mRNA molecules is regulated by many factors including microRNA (miRNA) molecules. This study examines the expression profiles of small non-coding molecules, miRNAs in CFS/ME patients. This section explores the relevance of these molecules in immune function.

MicroRNAs are recently described highly conserved molecules with regulatory activities in multi-cellular organisms such as mammals. They are small components of the ribonucleoprotein particles belonging to a family of small ribonucleic acid molecules which have effects on diverse physiological function. MicroRNAs are suppressors of gene expression and affect either translational processes or the stability of mRNAs through the encouragement of cellular decay processes, deadenylation and decapping (Mishima *et al*., 2006, Wu *et al*., 2006a) process termed RNA interference (outlined in Figure 6). The generation of mature miRNAs from a miRNA gene occurs when a primary transcript (pri-miRNA) produced following transcriptional processing in the nucleus by RNA polymerase II. This pri-miRNA contains a hairpin stem-loop

structure which is served by the enzyme Drosha (RNA III enzyme) and DGCR8 (DiGeorge critical region 8), resulting in the creation of a structure comprised of a 22 basepair stem, 2-nucleotide 3' overhang and a loop, collectively this is known as the pre-miRNA transcript (Lee *et al.*, 2003). The pre-miRNA transcript is transported into the cytoplasm where RNase III enzyme, Dicer, cleaves the double stranded pre-miRNA transcript into a 18- to -24 base pair product (Lee *et al.*, 2002). The less stable transcript formed from the cleavage by Dicer is integrated into an RNA induced silencing complex (RISC) with argonaute proteins where it is further processed (Khvorova *et al.*, 2003, Lee *et al.*, 2003, Mourelatos *et al.*, 2002, Lingel *et al.*, 2003). The final product formed from this sequence of events is a miRNA-RISC complex (Figure 6). Suppressive effects of miRNA on gene mRNA molecules occur via the RISC complex which contains proteins that have the ability to cleave mRNA molecules and interfere with translation (Hutvagner & Zamore, 2002). The mature miRNA can bind complete and incomplete complementary strands of mRNA molecules and degrade the mRNA or inhibit translation respectively (Hutvagner and Zamore, 2002, Behm-Ansmant *et al.*, 2006, Lim *et al.*, 2005). Interactions between the miRNA and mRNA molecules may be potentially important for physiological processes.
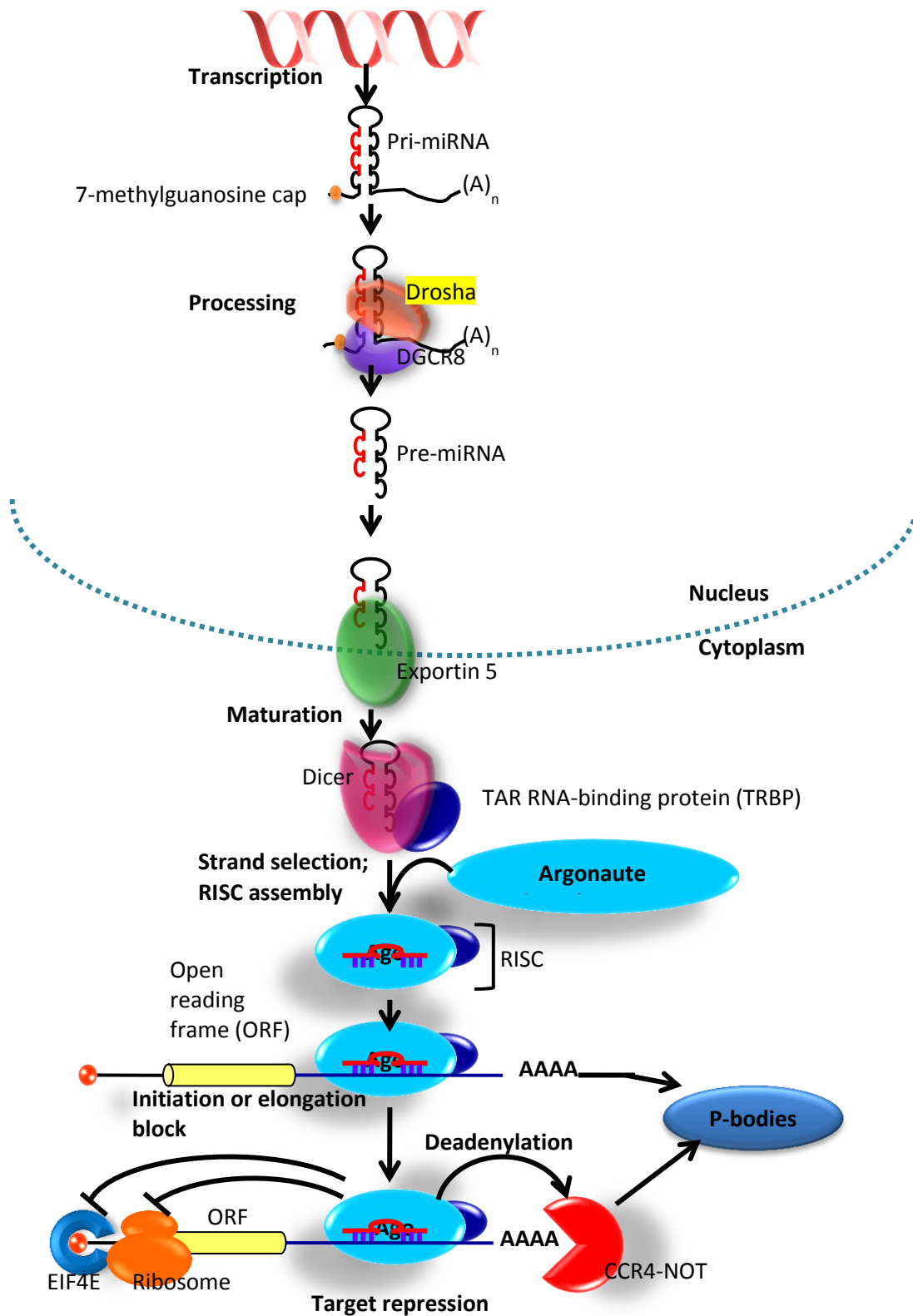
Figure 6: MicroRNA biogenesis and action. A pri-miRNA is converted into a mature miRNA in the presence of various enzymatic processes. Mature miRNA are therefore able to target 3'regions of specific genes (Inui *et al*., 2010).

Similar to mRNAs, miRNAs are regulated by transcription factors (TFs) and vice versa, thus it has been suggested that miRNAs together with TFs regulate the expression of genes (Zhou *et al*., 2007). Recent data have indicated their involvement in immune homeostasis in the adaptive and innate components of immunity and other related cellular processes. In the innate immune system, miRNAs such as *miR-155* enhance the maturation of macrophages and dendritic cells via the TLR receptor pathway, this causes heightened sensitivity in these cells to antigen presentation in circulation (Tili *et al*., 2007, O'Connell *et al*., 2007). M*icroRNA-146* is also associated with the TLR pathways, which are necessary for regulating antigen recognition, innate-adaptive immune interactions stimulating the production of cytokines and they also assist in the generation of CD4$^+$T helper cells thus contributing to inflammatory regulation (Taganov *et al*., 2006). CD4$^+$T and CD8$^+$T cell maturation into various subsets in the periphery are regulated by miRNAs (Wu *et al*., 2007a).

Importantly, deficiencies in components of the miRNA such as Dicer promote a predominant Th1 response governed by IFN-γ with a reduction in the effects of Th2 cells and Treg cells (Cobb *et al*., 2006). In contrast a predominant Th2 CD4$^+$ T cell profile prompting systemic inflammation emanates from deficiencies in the *miR-155* (Rodriguez *et al*., 2007, Thai *et al*., 2007) while in the absence of miR-101 autoreactive T cell mediated autoimmunity occurs (Yu *et al*., 2007). In CFS/ME, there are inconsistent data with regards to the Th1/Th2 profiles. It is likely that in the event that immune related miRNAs are deficient, shifts in Th1 and Th2 immune regulatory effectors and defects in TLR signalling may occur, and this may be related to the pathomechanism of CFS/ME.

The generation of Tregs that express FOXP3 is to some extent dependent on miRNAs (Kohlhaas *et al*., 2009). Any perturbed effects in miRNAs can influence thymic and peripheral derived Tregs especially in response to TGF-β stimulation on naive CD4$^+$ T lymphocytes (Ha, 2011). Modulation of the effects of these molecules is essential for appropriate immune response to bacterial and viral invasion. MicroRNA molecules such as *miR-181* limit the expression of phosphatases required for signal transduction processes that increase the ability of the T cell receptor (TCR) to recognise peptides released from pathogens and initiate a cascade of events that ensures lysis of pathogens (Li *et al*., 2007b). Evidently alterations in these miRNAs significantly promote inflammatory reactions that may be associated with CFS/ME.

Additionally, *miR-155* expressions are regulated by FOXP3 which is important for Treg responsive and the induction of IL-2 (Zheng *et al*., 2007, Marson *et al*., 2007, Lu *et al*., 2009a). A list of potential miRNA molecules that are important for immune function is provided in Table 4. Although, the contribution of miRNAs in the mechanism of CFS/ME has not been clearly outlined to date, further studies are now required to demonstrate or verify the role of miRNAs in CFS/ME.

Table 4: MicroRNAs involved in immune related activities

miRNA	Function	
miR-146	Innate and adaptive immune interactions	Williams *et al*. 2008
miR-150	Lymphocyte development	Zanovello 2011
miR-17-92	Regulation of apoptosis	Nagel *et al*., 2009
miR-223	Regulation and production of granulocytes	Johnnids *et al*., 2008
miR-101	Autoreactive T lymphocytes	Iliopoulos *et al*., 2011
miR-181	TCR signalling	Xiao *et al*. 2009
miR-155	Maturation of macrophages, dendritic cells, FOXP3	Kohlhaas *et al*. 2009
miR-142	Increase production of lymphocytes	Merkerova et al., 2008

1.4.2. Vasoactive Neuropeptides

Vasoactive neuropeptides (VNs) are a class of peptides with diverse regulatory roles in almost all physiological systems. The two most important VNs, associated with a number of neuro-immune disorders, are vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP). These VNs are widespread throughout the mammalian body including areas such as CNS and lymphoid organs and have receptors on immune cells specifically mast cells, T lymphocytes and granulocytes (Bellinger *et al.*, 1996; Ganea & Delgado 2002). VIP and PACAP are highly expressed in the CNS and lymphoid tissues specifically in the cortex, thymus, spleen, lymph nodes, hypothalamus, colon, pituitary gland, neurosecretory fibres, gonads, adrenal, endocrine, germ cells, gastrointestinal tract, ganglia, neurons and muscle fibres (Arimura and Shioda, 1995, Kimura et al., 1994, Shioda et al., 1994, Hannibal et al., 1998, Koves et al., 1993, Bellinger et al., 1996). These peptides are necessary for the regulation of important physiological processes such as maintaining the blood brain/blood spinal barrier (BBB/BSB) (Benagiano *et al*., 1996), cerebellar development (Allais *et al*., 2007) and co-neurotransmitter functioning of cholinergic and catecholamine transmitters (Hamelink *et al*., 2002), inflammatory modulation (Delgado *et al*., 2003), hypoxia and nitric oxide (NO) regulation (Cohen *et al*., 2002, Larocca *et al*., 2007) and inhibition of apoptosis (Delgado and Ganea, 2000b).

Vasoactive intestinal peptide and PACAP act through G-protein coupled receptors (GPCRs), VPAC1, VPAC2 and PAC1. VIP binds with high affinity to VN receptors, VPAC1 and VPAC2 belonging to the G-protein coupled receptor family whilst PACAP binds to all three receptors VPAC1, VPAC2 and PAC1(Harmar *et al*., 1998). In the periphery monocytes, macrophages, T lymphocytes and mast cells secrete VIP

and PACAP and express receptors VPAC1, VPAC2 and PAC1 on their cell surfaces (Gomariz *et al*., 1994). VIP and PACAP adhering to their receptors (VPAC1, VPAC2 and PAC1) activate stimulatory Gα subunit of the GPCR protein. When GDP is replaced by the active form GTP, the βγ subunit dissociates from the complex (Figure 7). The GTPα complex excites adenylate cyclase (AC) to catalyse ATP to produce cAMP. Cyclic AMP once created binds to the regulatory protein kinase A (PKA) resulting in the phosphorylation of cAMP-regulatory element and binding proteins (CREB) phosphorylation (Ganea and Delgado, 2002, Leceta *et al*., 2000) and other signalling pathways. Considering immune function, phosphorylation of CREB initiates a sequence of events that either inhibit the secretion of pro-inflammatory molecules or activates anti-inflammatory factors (Christophe, 1993, Vaudry *et al*., 2000). Thus VIP and PACAP acting through their receptors can inhibit pro-inflammatory cytokines specifically IL-6, IL-12, TNF-α and nitric oxide (NO) production in macrophages and T lymphocytes (Delgado *et al*., 1999d, Martinez *et al*., 1998, Delgado *et al*., 1999c).

Vasoactive intestinal peptide and PACAP also control immune processes involving chemokine (CCL2, CCL5, CCL9, CXCL1, CXCL2, CXCL3, CXCL8, and CX3CL1) release, necessary for the attraction and migration of monocyte and neutrophils to sites of infections (Delgado *et al*., 2004a), activation of anti-inflammatory mechanisms that suppresses macrophage-related activities such as phagocytosis, respiratory burst, and chemotaxis, and limits lymphocyte recruitment and prevents the secretion of pro-inflammatory factors (Gomariz *et al*., 2001, Abad *et al*., 2005, Ganea and Delgado, 2002). In Th1 cells PACAP and VIP prevent the differentiation and proliferation of Th1 type cytokine by suppressing IL-12, a necessary component in

Th1 cell survival and prevalence (Murphy and Reiner, 2002). Antigen induced cell death (AICD) of CD4$^+$ T lymphocytes occurring via Fas ligand activation is also prevented by VIP and PACAP (Delgado and Ganea, 2000a).

An important characteristic of PACAP and VIP is their role as anti-inflammatory effectors. They are able to induce the generation of Th2 type cytokines and chemokines thereby regulating inflammation (Martinez *et al*., 1996, Wang *et al*., 1999, Delgado *et al*., 1999d). This preferential selection and enhancing of Th2 type cytokines is a protective mechanism employed by the physiological system to prevent autoimmune related episodes. In this regard, PACAP and VIP interaction with CD4$^+$T lymphocytes confers an antagonistic effect on Th1 cells through suppression of chemoattractant molecules CXCL10, while enhancing Th2 migration to sites of infection by up-regulating the release of CCL22 from innate immune cells (Jiang *et al*., 2002). CD4$^+$ Th2 cells express predominantly VPAC1 and VPAC2 (Delgado *et al*., 1996) receptors, VPAC1 inhibits excessive production of pro-inflammatory markers from macrophages and microglia cells while VPAC2 sustains Th2 survival and endorses anti-inflammatory effectors (Feldmann *et al*., 1996). These anti-inflammatory effectors include IL-10, IL-4, IL-5 and IL-1Ra (Delgado *et al*., 2004b, Delgado *et al*., 1999a, Feldmann *et al*., 1996). Anti-inflammatory responses are highly necessary to restore immune balance after an infection or inflammatory episode has been resolved. Inflammation due to injury or invasion by pathogens and antigens initiates a sequence of events that initiate the activation of TLR ligands, the release of pro-inflammatory molecules (chemokines and cytokines), the recognition and effective elimination of the pathogens. VIP and PACAP therefore act through their receptors to prevent persistent inflammation in the absence of injury or pathogen. VIP

and PACAP also contribute to Treg expansion and suppressive activities in an attempt to maintain homeostasis (Chorny *et al*., 2006).

Vasoactive intestinal peptide and PACAP deficits have been recognised in autoimmune diseases such as Rheumatoid Arthritis (Gomariz *et al*., 2006). Their direct role in CFS/ME has not yet been determined although there are suggestions for compromises in their function leading to disequilibrium in the Th1/Th2 effector responses (Staines, 2004). As cytokine production in CFS/ME may be defective where either a prevailing Th1 or Th2 response may occur (Swanink *et al*., 1996, Skowera *et al*., 2004), it is perhaps reasonable to propose dysregulation in VNs as a plausible pathomechanism in CFS/ME, since they have a crucial role in the inhibition, release and activation of cytokines. Incidentally, CFS/ME cytokine levels are unclear and the heterogeneity of this disorder and techniques used in measuring may be responsible for these differences in results. Results supporting a Th1 immune dominance in CFS/ME (Rasmussen *et al*., 1994, Gupta *et al*., 1997, Patarca *et al*., 1994, Lloyd *et al*., 1992, Swanink *et al*., 1996), may have a relationship with compromise to VIP and PACAP. This possibly affects the ability of VIP and PACAP to exert anti-inflammatory activities, permitting heightened inflammation in CFS/ME. Consequently, this may result in elevated amounts of IL-2, IL-6, IFN-γ and TNF-α and decreases in IL-10. A prevailing Th2 immune profile in CFS/ME (Skowera *et al*., 2004, Gold *et al*., 1990, Visser *et al*., 1998, Klimas *et al*., 1990, Klimas *et al*., 2009) can also be attributed to changes in VN expression favouring an altered production of cAMP and cytokines that over-compensate the anti-inflammatory reactions and thus exacerbate immune reactions causing systemic insults. These severe anti-inflammatory events may be marked by elevations in IL-4, IL-1α, IL-5 and IL-6 with

decreases in IL-2, IFN-γ and IL-8 (Gold *et al*., 1990, Visser *et al*., 1998, Klimas *et al*., 1990, Klimas *et al*., 2009).

In summary, positive or negative interference of inflammatory events by PACAP and VIP is an effective way of enforcing immune reactions of lymphocytes, cytokines, chemokines and their receptors and an important component for restoring balance after an inflammatory experience. This may be impaired in a disease like CFS/ME and therefore requires further investigations. These studies will further highlight whether CFS/ME is an autoimmune or systemic disorder and perhaps clarify the underlying mechanism associated with this disorder.
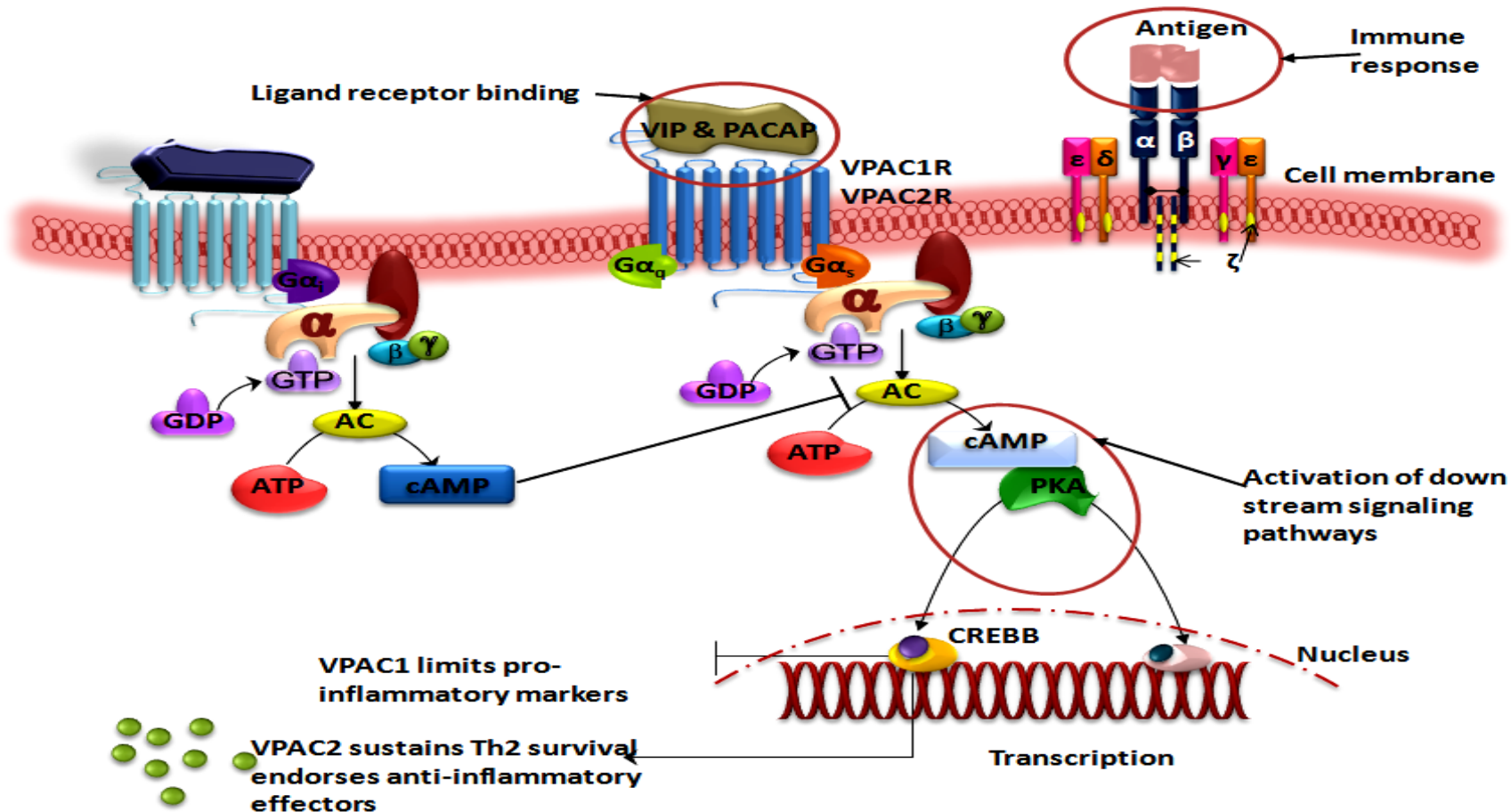
Figure 7: VIP and PACAP signalling. Binding of the VIP and PACAP to their receptors (VPAC1 and VPAC2) stimulate the binding of GTP to alpha and beta gamma subunits, release of the beta and gamma subunits. These evens activate adenylate cyclase to convert ATP to cAMP and activate the cAMP/PKA activation pathways causing either the inhibition of pro-inflammatory cytokines or the activation of anti-inflammatory cytokines.

Although, an adequate mechanism for CFS/ME has not yet been explained, CFS/ME possibly suggests itself in the occurrence of either environmental or physiological stressors. These stressors may be comorbid with e.g. infection (viral or bacterial), pesticide toxicity and other inflammatory instigators which alter the bidirectional communication that exists between the immune system and CNS. Conversely susceptibility to certain stressors may predispose an individual to immune compromise. This may then trigger changes in peptide function and cytokine secretion from the CNS and the peripheral immune system, either at the molecular or cellular level, in favour of either a predominant pro-inflammatory or an anti-inflammatory cytokine profile, and consequently have detrimental effects on immune cell activation and function. Physiological processes would be inadvertently compromised, encouraging persistent infections, increase in viral or bacterial load, severe fatigue, cognitive deficits and a pattern of symptoms observed in CFS/ME. Presently, direct assessment of CNS immune function is not available hence this study is primarily focused on peripheral immune function.

Despite the number of studies examining immune activities in CFS/ME, the explicit function of particularly $CD4^+$ and $CD8^+$ T lymphocytes is not fully known. Changes in T cell numbers which have been widely examined in CFS/ME patients do not provide adequate information on the T cell profile in these participants. Additionally peripheral blood investigations of cytokines in CFS/ME are not specific to cell type, hence, cytokine secretion by Th cells is not currently known. Studies on the cytotoxic activity and suppression by $CD8^+$T lymphocytes and Tregs, respectively, in CFS/ME patients

are currently not available. To facilitate the development of a plausible pathomechanism that will potentially assist in the comprehension of CFS/ME, all aspects of the immune system must be assessed. Here, we present a study that examines cytokine secretion by CD4$^+$T lymphocytes, cytotoxic activity and Treg expression. Notably substantial evidence exists to support the critical role of these VNs in inflammatory reactions in both CNS and peripheral blood. Defects in VNs, VIP and PACAP have adverse effects on Th activities. Alterations in gene expression profiles in immune markers are also suggestive of homeostatic imbalances in CFS/ME. However, in a disorder such as CFS/ME this is very speculative as other factors may be involved. This project assessed VIP and PACAP expression, in addition to gene expression to evaluate their contribution to immune cell function in CFS/ME patients.

The current ambiguities in CFS/ME research and lack of definitive markers for diagnosing this disorder suggest the need for further research. The following section provides the framework for a comprehensive project investigating immune function in CFS/ME.

1.5. Hypotheses

The null hypotheses for the project are:

1. Quantitative assessments of cytokines released by Th1, Th2, Th17 and Tregs in CFS/ME patients are not statistically significantly different from the non-fatigued control population with no preference to either a shift in Th1 or Th2 cytokine profile.

2. Treg FOXP3 expressions in CFS/ME patients are not significantly different from the non-fatigued control population.

3. Assessment of CD8$^+$T lymphocytes cytotoxic activity against tumour cells in CFS/ME patients will not significantly differ from non-fatigued control participants.

4. There is no significant difference in the expression pattern of VIP and PACAP receptor, VPAC2, on T lymphocytes between CFS/ME and non-fatigued participants.

5. The gene expression profile of CFS/ME patients with regards to NK and CD8$^+$T lymphocytes is not statistically significantly different to the non-fatigued control participants.

6. There are no significant differences in miRNA expression between CFS/ME and non-fatigued controls

1.6. Aims

The primary aims of this study were to:

1. Examine NK activity and phenotype expression in CFS/ME patients.

2. Evaluate the protein profile of CD4$^+$T lymphocytes in CFS/ME with specific focus on Th1, Th2, Th17 and Treg related proteins.

3. Directly measure and explore cytotoxic activity of CD8$^+$T cell in CFS/ME.

4. Determine the role of VIP and PACAP in the immune mechanism of CFS/ME by examining their receptor expression on T lymphocytes.

5. Verify the longitudinal profile of these immune parameters.

6. Assess the expression of immune related miRNAs in the optimal function of NK and CD8$^+$T lymphocytes in patients with CFS/ME relative to non-fatigued controls.

7. Develop and establish prospective diagnostic markers for CFS/ME based on the results from this research that could be tested in clinical practice.

1.7.Significance

This study performed a comprehensive analysis of the immune function in CFS/ME at the functional, molecular and protein level.

This is the first research to:

1. Investigate the expression of VN receptors VPAC1 and VPAC2 on lymphocytes in CFS/ME. This investigation illuminates the role of the neuroimmune interactions in CFS/ME.

2. Determine the status of $CD8^+T$ cell cytotoxic activity in CFS/ME.

3. Assess genes of specific lymphocytes such as $CD8^+T$ lymphocytes and NK lymphocytes. Presently only two studies have replicated some of the genes found to be differentially expressed in CFS/ME, this study decisively reproduces a number of these finding in these specific lymphocytes as this can potentially assist in the development of a mechanism for this disorder.

4. Examine miRNA molecules related to immune function specifically NK and $CD8^+T$ cells in CFS/ME.

5. Assess adaptive immune function with respect to T lymphocytes including FOXP3 expression on Tregs in CFS/ME.

The results from this project have potentially served as a basis for developing a disease mechanism for CFS/ME. This can be translated into the generation of prospective diagnostic markers for detecting CFS/ME. Additionally, this may assist in reducing the current cost incurred by patients on management strategies to help relieve their symptoms. These results are likely to increase knowledge on the pathomechanism of CFS/ME which may serve as a basis for future investigations.

2. Project

This study was designed to thoroughly assess aspects of the immune function including innate and adaptive immune system in patients with CFS/ME. It was hypothesised that severe deterioration in multiple components of the immune system are responsible for the symptomatology of this disorder. Hence, this study assessed lymphocyte function, protein levels and genes. The project was divided into three different sections as described below in the following sections. The first part provides an extensive investigation of immune parameters at baseline, this initial study was necessary to establish whether the immune markers are compromised in CFS/ME. In the second part, the immune markers were examined over a time period to determine their stability while the last project was focused on miRNAs. Details of these studies are further discussed in the sections that follow. The specific methods used in each individual study are outlined in a method section within each study.

2.1. Methods

2.1.1. Participant Recruitment and Follow Up

Recruitment for the study was achieved through the CFS/ME support groups in South East Queensland and Northern New South Wales. Additional recruiting strategies such as the media including newspaper and radio advertisement were used to recruit additional participants as most of the prospective participants from the support groups were outside the specified age range (25-65 years). Recruitment of controls was done in close vicinity to the collections sites and this most often included relatives and friends of the participants. Participants were assessed using a CFS/ME questionnaire outlined in the appendix. Using this method the targeted participant population was matched.Not all

immune parameters in the project were measured consistently over the 12 month period. Due to circumstances that were beyond the control of this project, not all parameters were examined at the baseline, 6 or 12 months. The baseline investigations included all parameters. However, at the 6 and 12 months only NK cytotoxic activity, NK phenotypes and cytokines are reported.

The project was divided into three with patients assessed every six months: 1) baseline, 2) 6 month and 3) 12 month. Prior to the baseline collection phase an information session was held for all interested participants. At this information session the study was described to qualified participants and all necessary procedures pertaining to the participants including consent forms, appointment scheduling and blood collection were outlined. This was an important component of the study as it provided an avenue for the patients to ask questions and convey their concerns regarding the study. This first initial contact with the participants was followed up with phone calls for blood collection appointments. After all participants had their blood collected and data analysed, a second follow up feedback session was held to update the patients on the progress of the study and also to highlight some of the findings that were stated in the ethics and consent forms. The mean of the overall results were presented to the patients in power point format. These results included full blood counts, NK phenotypes and NK cytotoxic activity. Similar procedures were applied to both the 6 and 12 months collection times. Since most of the patients and non-fatigued controls were not able to attend these information sessions, a weekly newsletter system was implemented and this contained various updates regarding scheduling times, results and progress of the study.

To better understand the symptoms, clinical presentation and quality of life of participants, a number of questionnaires were formulated. Some of the questions on the questionnaires were based on patient suggestions. These questionnaires also include a Four Dimensional Symptoms assessment criteria. Data construed from these surveys are currently being compiled for publication. Additionally, analysis of the medications administered by the CFS/ME cohort from this study has also being examined and submitted for publication. This study has therefore reviewed certain essential clinical components of CFS/ME that may be useful to health care professionals.

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┌─────────────────────────────────────────────────────────────────────────────┐
│                              Total participants                              │
│                                   N=168                                       │
└─────────────────────────────────────────────────────────────────────────────┘
        │                              │                              │
        ▼                              ▼                              ▼
┌────────────────────┐   ┌────────────────────┐   ┌────────────────────┐
│ 1. Symptoms of     │   │ 1. No fatigue or   │   │ 1. Aged <25 or >65 │
│ fatigue>6 months   │   │ 4<fatigue <6 months│   │ years              │
│ and 4 or more of   │   │ 2. Aged between    │   │ 2. Autoimmune      │
│ the following:     │   │ 25-65 years        │   │ disorder           │
│ • Unrefreshing     │   │ 3. No incidence of │   │ 3. Smoking         │
│ sleep              │   │ anaemia            │   │ 4. Pregnant/breast │
│ • Muscle pain      │   │ 4. No CFS/ME       │   │ feeding            │
│ • Sore throat      │   │ symptoms           │   │ 5. Immobile        │
│ • Pain in the      │   │ 5. Australian      │   │ 6. Cardiovascular  │
│ joints without     │   │ resident           │   │ disease            │
│ swelling or        │   │ 6. Yes/No          │   │ 7. Outside SEQ or  │
│ redness            │   │ medication         │   │ NNSW               │
│ • Headaches        │   │                    │   │                    │
│ • Tender lymph     │   │                    │   │                    │
│ nodes              │   │                    │   │                    │
│ • Impaired         │   │                    │   │                    │
│ concentration      │   │                    │   │                    │
│ or memory          │   │                    │   │                    │
│ 2. Age 25-65 years │   │                    │   │                    │
│ 3. Professionally  │   │                    │   │                    │
│ diagnosed or       │   │                    │   │                    │
│ undiagnosed        │   │                    │   │                    │
│ 4. Australian      │   │                    │   │                    │
│ resident           │   │                    │   │                    │
│ 5. Yes/ no         │   │                    │   │                    │
│ medication         │   │                    │   │                    │
└────────────────────┘   └────────────────────┘   └────────────────────┘
        │                              │                              │
        ▼                              ▼                              ▼
┌────────────────────┐   ┌────────────────────┐   ┌────────────────────┐
│ CFS/ME participants│   │ Non-fatigued       │   │ Excluded           │
│ N=95               │   │ Controls  N=50     │   │ participants  N=53 │
└────────────────────┘   └────────────────────┘   └────────────────────┘
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Figure 8: Criteria for Defining the Various Participant Groups. Using the CDC 1994 case definition an inclusion and exclusion criteria was generated. The inclusion criteria consisted of the CFS/ME participants and non-fatigued controls. The CFS/ME participants were recruited via the media and the CFS/ME support groups, while the controls were mainly selected from the general public and spouses of the CFS/ME patients. The flow chart illustrates the identifiers used for each group. Those participants that were excluded did not meet the CFS/ME or patient criterion.

2.1.2. Cell Culture

Two cell types were used in this project these were the mouse lymphoblast-like mastocytoma (P815) and the human erythromyeloblastoid leukemia (K562) cell lines. These cells were required for the assessment of NK and CD8$^+$T cell cytotoxic activity. These cells were grown in separate cell media supplemented with different substances. However fetal bovin serum (FBS) and peniciliin-streptomycin was added to each media prior to cell culturing. The P815 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine and 4.5g/L of glucose while K562 cells were grown in Roswell Park Memorial Institute (PRMI-1640) medium supplemented with sodium pyruvate and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPPEs) buffer. Each cell type was cultured in an incubator at 37℃ containing 5% carbon dioxide. The cells were passaged 3-4 times following which they were kept frozen in liquid nitrogen in a solution made up of 5% dimethyl sulfoxide (DMSO) and 95% FBS at a concentration of $1x10^6$cells/mL. Prior to cytotoxic activity the cells were gently thawed in a 37℃ water bath and resuspended in a solution containing 50% complete culter media and 50% FBS. Cells were centrifuged for 5 mins at 150 relative centrifugation force (rcf). The cells were then suspended in 5-10ml of complete media in a T25 flask and placed in the incubator until they were required.

2.1.3. Participant Recruitment and Follow Up

A volume of 10 -20mL was used for each assasy as per the procedure. The different isolation steps are outlines below.

Isolation of peripheral blood monobuclear cells PBMCs) from whole blood required 10 mL of whole blood. This was diluted in 20mL of PBS and layered over 10mL of ficol Hypaque and centrifuged for 30 mins at 400rcf. Following centrifugation the solution was contructed into four layers. These were from top to bottoms, plasma plus PBS, PBMC, ficoll and packed red bloode cells. The PBMC layer was transferred into another tube and washsed twice first in 20mL of PBS and then in 10mL of PBS. Cells were counted on a hemacytometer prioto any further secondary assessment.

Gene expression studies required further isolation of the PBMCs into $CD8^+T$ or NK cells using a negative isolation system. Enrichment of NK or $CD8^+T$ cells was performed using NK and $CD8^+T$ Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In these isolations biotinylated antibody cocktails were used to magnetically select non-NK or non-$CD8^+T$ cells and incubated at 8°C for 10 minutes. Anti-biotin micro-beads were added to bind to the biotinylated positively selected cells during a 15 minute incubation period. The cells were washed in PBS and resupended in PBS. The samples were then passed through MACS columns where unwanted cells bind to the magnet while the unlabelled NK or $CD8^+T$ cells pass through the columns and are collected for further analysis. The other remaining cells labelled with the microbeads were attracted to a magnetic apparatus surrounding the column. NK cells and CD8+T cells were snap frozen in liquid nitrogen and then stored in minus 80°C freezer.

3. Project One: Immunological Abnormalities as Potential Biomarkers in Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis

Brenu, E.W., Ashton K.J., van Driel M, Staines, D.R, Keane, J., Ramos, S.B., Klimas, N.G., Marshall-Gradisnik, S.M. 2011. Potential Biomarkers for the Diagnosis of Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis. Journal of Translational Medicine. 9, 81

3.1. Abstract

Background: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is characterised by severe prolonged fatigue, and decreases in cognition and other physiological functions, resulting in severe loss of quality of life, difficult clinical management and high costs to the health care system. To date there is no proven pathomechanism to satisfactorily explain this disorder. Studies have identified abnormalities in immune function but these data are inconsistent. We investigated the profile of markers of immune function (including novel markers) in CFS/ME patients.

Methods: We included 95 CFS/ME patients and 50 healthy controls. All participants were assessed on natural killer (NK) and $CD8^+$T cell cytotoxic activities, Th1 and Th2 cytokine profile of $CD4^+$T cells, expression of vasoactive intestinal peptide receptor 2 (VPACR2), levels of NK phenotypes ($CD56^{bright}$ and $CD56^{dim}$) and regulatory T cells expressing FoxP3 transcription factor.

Results: Compared to healthy individuals, CFS/ME patients displayed significant increases in IL-10, IFN-γ, TNF-α, $CD4^+CD25^+$ T cells, FoxP3 and VPACR2 expression. Cytotoxic activity of NK and $CD8^+$T cells and NK phenotypes, in particular the $CD56^{bright}$ NK cells were significantly decreased in CFS/ME patients. Additionally granzyme A and granzyme K expression were reduced while expression levels of perforin were significantly increased in the CFS/ME population relative to the control population. These data suggest significant dysregulation of the immune system in CFS/ME patients.

Conclusions: Our study found immunological abnormalities which may serve as biomarkers in CFS/ME patients with potential for an application as a diagnostic tool.

3.2. Introduction

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) remains a medically unexplained disorder despite numerous scientific investigations undertaken worldwide. The current worldwide prevalence rate of CFS/ME is estimated to be about 0.5% (Grinde, 2008) with a higher prevalence in females compared to males at a ratio of up to 6:1 (Devanur and Kerr, 2006). The annual cost for treatment and management of CFS/ME in the USA is estimated to be US$319 million with a direct cost of US$7,406 per patient (Jason *et al*., 2008).

Generally, patients with CFS/ME experience severe fatigue, neuropsychological impairments, and other associated flu-like symptoms before a firm diagnosis of CFS/ME is made (Fukuda *et al*., 1994). CFS/ME has been observed to persist for more than six months where symptoms may decrease, remain stable or worsen six months or more(Jason *et al*., 2010). The current diagnostic strategy for health professionals is based on case definition, although this is not the most ideal method as it permits misdiagnosis. CFS/ME may share homology with certain disorders classified as fatigue related disorders where individuals experience fatigue and one or more of CFS/ME related symptoms. Further, there are no biomarkers available to affirm diagnosis thus complicating treatment.

Population based studies have suggested a link between infections, neurological and neuroimmune dysfunctions and clinical manifestations of CFS/ME(Biswal *et al*., 2010, Lakhan and Kirchgessner, 2010, Lorusso *et al*., 2009, Natelson *et al*., 2005, Schutzer *et*

al., 2011). Immunity has been widely investigated in patients with CFS/ME but the results of these studies are inconsistent, reporting different lymphocyte cell numbers and cytokine distributions in patients with CFS/ME. Nonetheless, findings on immunoglobulins, complement markers and activation molecules in CFS/ME, may demonstrate an underlying infringement in immune function (Lorusso *et al*., 2009, Sorensen *et al*., 2009, Nijs *et al*., 2009). Decreased function of lymphocytes, in particular Natural Killer (NK) cell cytotoxic activity in CFS/ME patients compared to healthy controls, seems to be a consistent finding (Brenu *et al*., 2010, Fletcher *et al*., 2010, Klimas *et al*., 1990, Maher *et al*., 2005). The functional capacity of other immune cells, such as T cells, and the contribution of other molecules in the pathophysiological mechanism of CFS/ME, remains to be determined. In particular, the role of subsets of $CD4^+T$ and the $CD8^+T$ cell populations has not been fully studied in CFS/ME. Importantly, recent data on cytokine distribution in CFS/ME patients point towards an increase in pro-inflammatory cytokines suggesting the presence of an underlying viral prevalence in these patients (Broderick *et al*., 2010, Kuratsune, 2007) and this can be detrimental to the immune inflammatory processes.

It is widely known that neuropeptides regulate immunity. Relevant among these are vasoactive neuropeptides (VNs), specifically vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP). They regulate and suppress pro-inflammatory immune processes via the PKA/cAMP pathway (Gomariz *et al*., 2006). Their role in CFS/ME remains unknown although there are suggestions that

they may be implicated in observed CD4$^+$T cell related activities such as cytokine secretion and FOXP3 expression (Staines, 2004).

Immune cell numbers may not necessarily be indicative of diseased states, as stated previously these have been shown to be inconsistent in CFS/ME. However, the functional capacity of these cells during disease progression may provide a better understanding of the mechanism associated with unexplained disorders such as CFS/ME. Alternatively, this may help in identifying specific immune parameters that can be used as diagnostic markers for CFS/ME. The present study thus explores immunological abnormalities that may serve as biomarkers for diagnosing CFS/ME. Additionally, this is the first study to examine the role of the VNs, VIP and PACAP, and FOXP3 expression in CFS/ME.

3.3. Method

The project having been reviewed under an Expedited Review Procedure was granted approval to proceed by the Bond University Human Research Ethics Committee (BUHREC). All participants in this present study signed an informed consent approved by the Bond University Human Research Ethics Committee (BUHREC).

3.3.1. Participants

All participants, both CFS/ME and non-fatigued controls were recruited from Queensland and New South Wales states in Australia through the CFS/ME support groups, newspaper and email advertisements into a prospective study as cases (CFS/ME patients) or non-fatigued controls (healthy volunteers). Participants were eligible if they were between 25 and 65 years old. Prior to inclusion all participants completed a consent form and a Chronic Fatigue Syndrome questionnaire based on the Centre for Disease Prevention and Control case definition (CDC 1994) (Fukuda *et al*., 1994). Participants previously diagnosed with autoimmune disorders, psychosis, epilepsy, heart disease, or who were pregnant or breastfeeding were excluded from the study (Figure 8).

3.3.2. Sample Preparation and Routine Measurements

A volume of 25ml of blood was collected from the antecubital vein of participants into lithium heparinised and EDTA collection tubes between 9am and 11am. Blood samples were analysed within 12 hours of collection. Routine blood cell counts for red blood

cells, lymphocytes, granulocytes and monocytes were performed using an automated cell counter (ACT Differential Analyzer, Beckman Coulter, Miami, FL).

3.3.3. Assessment of NK Cytotoxic Activity

The NK cells were isolated from whole blood samples using Ficoll-Hypaque (GE Healthcare Life Sciences; Milan, Italy) density gradient centrifugation. NK lymphocyte cytotoxic activity was performed as previously described (Aubry *et al*., 1999). Briefly, isolated cells were labelled with 0.4% PKH-26 (Sigma, St Louis, MO). NK cells were incubated with K562 at an effector to target ratio of 25: 1, for 4 hours at 37°C in 95% air, 5% CO_2. Apoptosis of the tumour cells was measured via FACS-Calibur flow cytometry using the Cell Quest Software (Becton Dickinson (BD), San Diego, CA), using Annexin V-FITC and 7-AAD reagent (BD Pharmingen, San Diego, CA). Percent lysis of K562 cells was calculated as previously described (Aubry *et al*., 1999).

3.3.4. Assessment of CD8^{+}T Lymphocyte Cytotoxic Activity

Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples using Ficoll-Hypaque (GE Healthcare Life Sciences; Milan, Italy) density gradient centrifugation. CD8^{+}T lymphocytes were preferentially isolated from PBMCs using CD8^{+}T cell isolation kit (Miltenyi Biotec GmbH; Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Briefly, cells were stained with a CD8^{+}T cell biotin-antibody cocktail, incubated for 10 minutes and then stained with CD8^{+}T cell microbead cocktail for 15 minutes. Cells were then passed through separation columns were cells of interest were collected for further analysis. Cytolysis was performed as

previously described using P815 cells as the target cells (White *et al*., 2007). In brief, P815 cells were stained with 0.4% PKH-26 and activated using anti-CD3 (BD Bioscience, San Diego, CA). The target cells were then incubated with $CD8^+T$ cells at an effector to target ratio of 25: 1, for 4 hours at 37°C in 95% air, 5% CO_2. Annexin V-FITC flow cytometry apoptosis detection was used in assessing cell death of the tumour cells. Percent lysis of K562 P815 cells was calculated as previously described (White *et al*., 2007).

3.3.5. Gene expression in NK and $CD8^+T$ Cells

Isolation of NK and $CD8^+T$ cells was done via MACS separation (Miltenyi Biotec GmbH; Bergisch-Gladbach, Germany) as specified by the manufacturer. Purity was determined on the flow cytometer using the Cell Quest software. Isolated NK cells were coated with PE-CD56CD16 and FITC-CD3 (BD Pharningen, San Diego, CA) monoclonal antibodies to determine the purity of NK cells. To establish the purity of $CD8^+T$ cells, isolated $CD8^+T$ cells were stained and incubated with PE-CD8 and FITC-CD3 monoclonal antibodies (BD Pharmingen, San Diego, CA). Cells were fast frozen in liquid nitrogen and kept in negative 80 degrees freezing conditions for further assessment. Total RNA extractions were performed using the RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified on the NanoDrop 3300 (Thermo Scientific, Wilmington, DE). RNA was synthesised in to cDNA using the SuperScript^{TM} III First-Strand synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) as specified by the manufacturer and stored at negative 20 ^{o}C for later analysis. RT-qPCR was performed using IQ SYBR Green Super Mix (Bio-Rad, Hercules, CA) with *GAPDH* as the

housekeeping gene. Expression levels of granzyme A, granzyme K, perforin and INF-γ (*GZMA, GZMK, PRF1* and *IFN*-G) were collected and quantified using the iQCycler (Bio-Rad, Hercules, CA).

3.3.6. Quantification of NK Phenotypes

Distribution of NK cell phenotypes was assessed as previously described (Cooper *et al*., 2001). NK lymphocytes were isolated from whole blood via negative selection using RosetteSep Human Natural Killer Cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC) and, were labelled with CD56-FITC and CD16-PE monoclonal antibodies (BD Bioscience, San Jose, CA).

3.3.7. VPACR2 Stimulation

Whole blood samples (10mL) diluted with 1x PBS were layered over Ficoll-Hypaque for isolation of peripheral blood mononuclear cells. Cells were stimulated with or without 1µg of Lipopolysaccharide (Invitrogen, Carlsbad, CA) and cultured for 48 hours. Cells were stained with vasoactive intestinal peptide receptor 2 (Sigma, St Louis, MO), FITC-IgG (Sigma, St Louis, MO) and CD4-PE anti-mouse monoclonal antibodies and analysed on the flow cytometer with settings for detecting monocytes and lymphocytes expressing the VPACR2. Percentage of cells expressing both CD4-PE and VIP2-FITC were recorded from these populations to determine the levels of VPACR2 expressed on these cells (Sun *et al*., 2006). In the lymphocyte gate specific reference was made to CD4^{+}T cells.

3.3.8. Cytokine Determination

Isolated CD4$^+$T cells were mitogenically stimulated with 1μg of phytohemagluttinin and cultured at a concentration of 1x10^6 cells/mL for 72 hours. Following incubation, supernatants were removed and stored at -80^{0}C for later assessment. T helper (Th)1, Th2 and Th17 cytokine expressions were investigated using the cytometric bead array kit (BD Pharmingen, San Diego, CA) (Collins *et al*., 1998) for determining levels of interleukin (IL)-2, IL-4, IL-6, IL-10, tumour necrosis factor (TNF)-α, interferon (INF)-γ and IL-17A. The cytokines selected for this study although not conclusive, enough were selected to ascertain the Th1/Th2/Th17 mechanisms in CFS patients.

3.3.9. Regulatory T Cell Assessment

Expression of FOXP3 Tregs was determined on CD4$^+$CD25$^+$ cells. PBMC Cells were stained with monoclonal antibodies FITC-CD4 and APC-CD25 (BD Pharmingen, San Diego, CA) following which cells were permeabilised and stained with anti-FOXP3 and PE-FOXP3 respectively and analysed via flow cytometery (Harmar *et al*., 1998).

3.3.10. Statistical Analysis

Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc, Chicago, USA). A sample size of 59 participants per group was required to obtain statistically significant results with an effect size of 0.5 and a power of 85%. All data represented in this study are reported as means plus or minus standard error of the mean (±SEM). Comparative assessments among participants (that is, the CFS/ME and control

subjects) were performed with the analysis of variance test (ANOVA) and independent sample t-test. All statistically significant results had p-values less than or equal to 0.05.

3.3.11. Ethical Clearance and Participant Selection

Approval for this study was granted after review by the Bond University Human Research Ethics Committee (RO852A).

3.4. Results

Of the 168 participants recruited 95 met the CDC criteria for CFS/ME and 50 qualified as healthy controls. Twenty-three participants were rejected because they did not meet the inclusion criteria for CFS/ME (Figure 10) 58.2% of CFS/ME patients indicated that they experienced 6 or more of the symptoms listed in the CDC criteria list and 21.4% experienced only 4 symptoms. The baseline characteristics of the participants are illustrated in table 5.
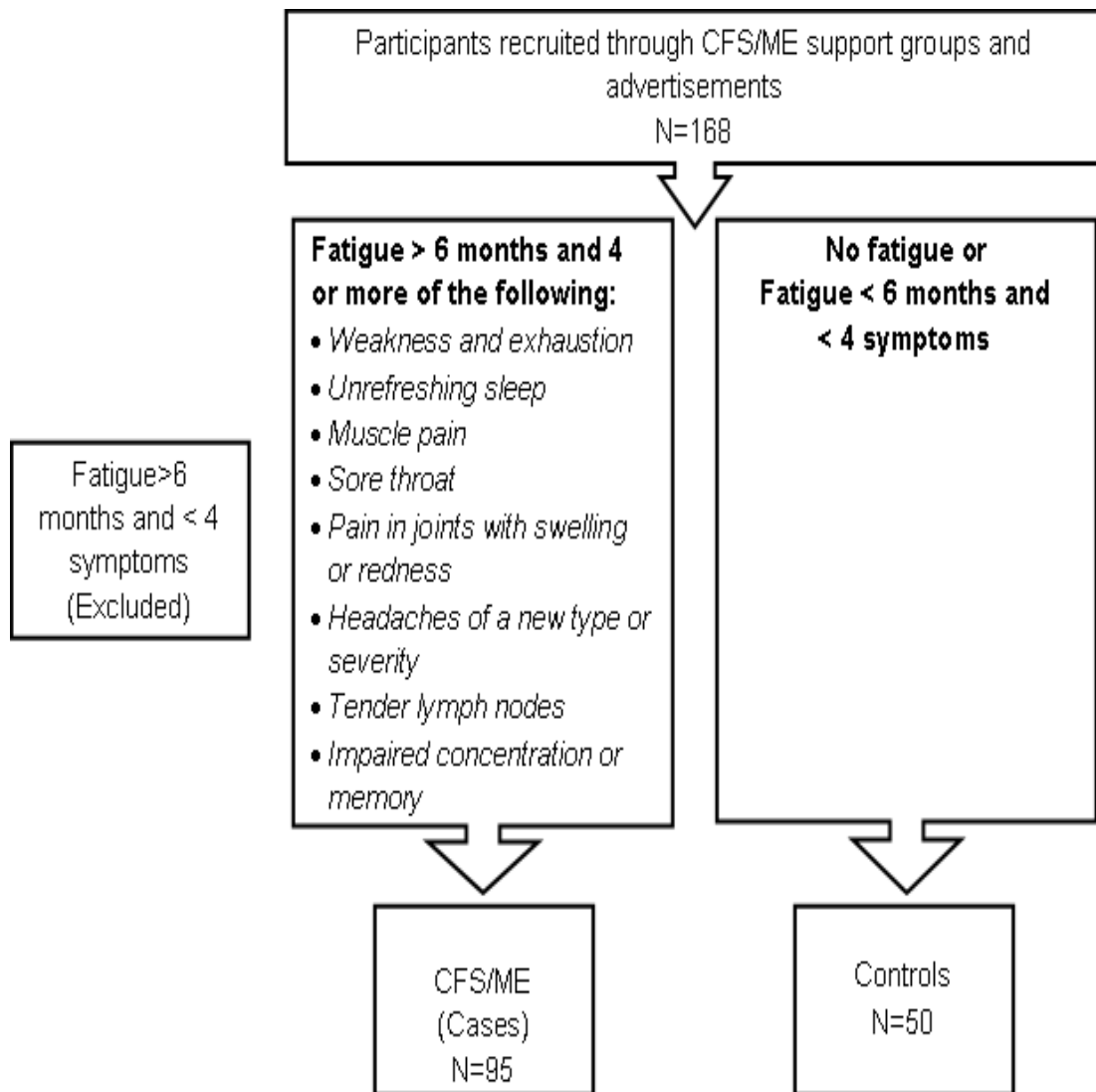
Figure 9: Selection Process for Experimental Groups. Participants for the present project were grouped into CFS/ME, or non-fatigued control groups based on the CDC 1994 case definition symptom criteria. Participants, that is, both CFS/ME and non-fatigued controls, were comprised of both male and females selected using advertisements and through the CFS/ME support groups. Non-fatigued controls were randomly selected from the general population using newspaper and email advertisements. The above flow chart illustrates the process used to generate the final research population.

Table 5: Characteristics of participants in the present study

Parameters Measured	CFS/ME (n=95)	Controls (n=50)	*p*-values
Sex	Female 70.5%	Female 57.7%	
	Male 29.5%	Male 42.3%	
Mean Age	46.47±11.7	41.9±9.6	0.11
Height (cm)	167.47±13.2	167.9±8.9	0.16
Weight (kg)	77.02±18.46	72.48±21.09	0.11
White Blood Cells	5.8±1.4	6.3±1.8	0.68
Lymphocyte (%)	38±5.7	33.6±7.9	0.03
Monocytes (%)	5.90±1.4	5.6±2.2	0.30
Granulocyte (%)	56.3±7.1	60.8±8.2	0.06
Lymphocyte ($x10^3/\mu L$)	2.3±0.8	2.03±0.6	0.24
Monocytes ($x10^3/\mu L$)	0.8±4.2	0.34±0.2	0.51
Granulocyte ($x10^3/\mu L$)	3.3±1.0	3.87±1.5	0.21
Red Blood Cells ($x10^6/\mu L$)	4.3±0.5	4.56±0.4	0.08
Haemoglobin (g/L)	131.6±12.4	137.0±11.7	0.15
Haematocrit (%)	43.8±3.3	43.68±13.0	0.89

3.4.1. Lymphocyte Cytotoxic Activity

Natural Killer and CD8$^+$T (n=71) cytotoxic activity, measured as the ability of NK and CD8$^+$T cells to effectively lyse K562 and P815 cells respectively was significantly decreased ($p< 0.05$) among the CFS/ME patients compared to the control subjects (Figure 10). Similarly granzyme A expression was significantly decreased in both the NK and CD8$^+$T cells in the CFS/ME population. However, IFN-γ and granzyme K were decreased only in the NK cells of the CFS/ME group compared to the healthy controls as shown in Figure 11A and B.
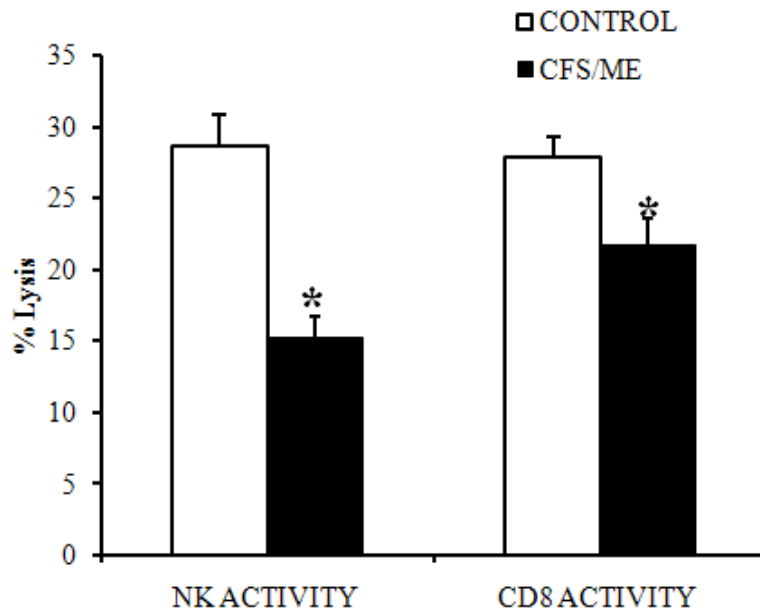
Figure 10: Reduced lytic function of cytotoxic cells in CFS/ME. *In vivo* assessment of NK and CD8$^+$T cell lysis (cytotoxic activity) of tumour cell lines K562 and P815 respectively where, lytic activity is represented as percent (%) lysis on the y-axis. The white bars represent the non-fatigued control population while the black bars represent the CFS/ME cohort. *Denotes statistical significant results. Data presented as mean of % lysis ± SEM.
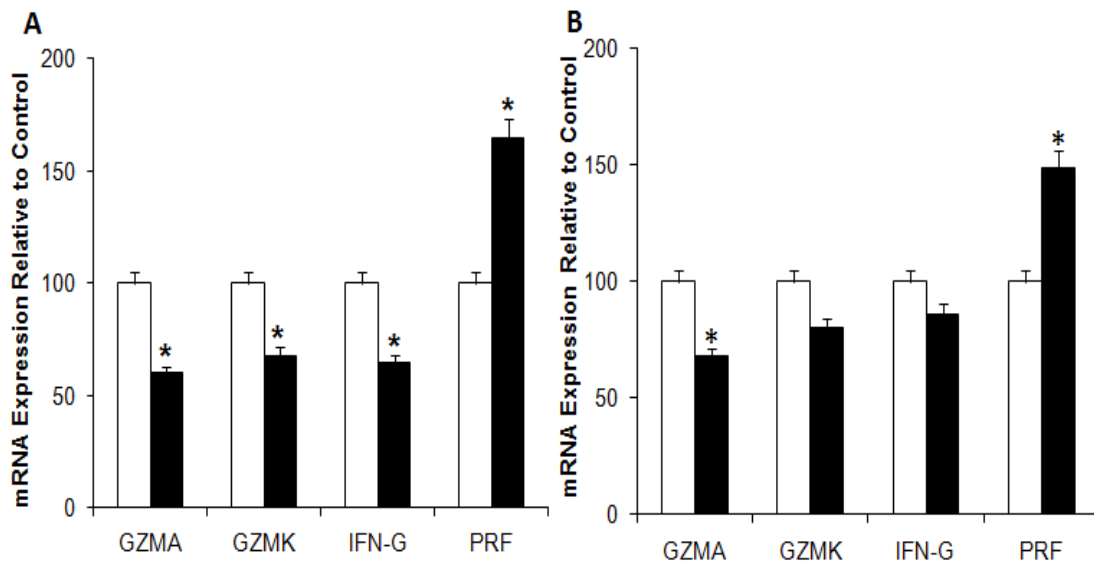
Figure 11: mRNA Expression of Cytotoxic Molecules in NK and CD8+T Cells. Quantitative reverse transcriptase (RT)-PCR demonstrated the relative expression of granzyme A, granzyme K, perforin and IFN-g in NK (A) an dCD8+T cells (B). In NK and CD8+T cells expression levels of GZMA, GZMK and IFN-G were decreased in CFS/ME (black bars) compared to the controls (white bars). PRF1 was however increased in the CFS/ME group. *Denotes statistical significant results (P≤ 0.05). Data presented as means ± SEM.

3.4.2. Altered NK Profiles in CFS/ME

In this study NK phenotypes were classified into two, those expressing $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$NK cells. The number of NK cells expressing $CD56^{bright}CD16^-$ was significantly lower ($p< 0.001$) in the CFS/ME patients compared to the control subjects. However, $CD56^{dim}CD16^+$NK cells remained unchanged across all groups (Figure 12C). The raw data was converted into absolute counts (Figure 12A and B).
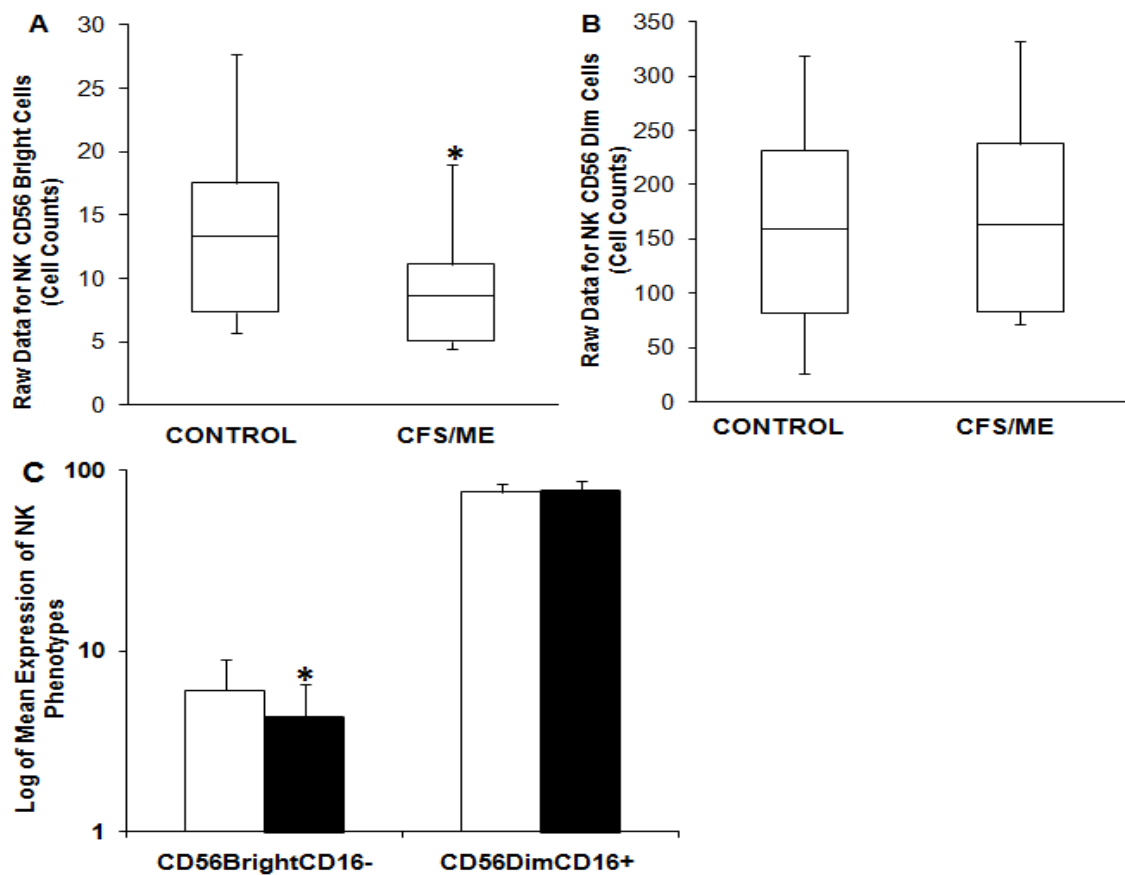
Figure 12: Distribution of NK phenotypes. NK phenotypes that were examined are denoted as either NK Bright (CD56^{bright}CD16$^-$) or NK Dim (CD56^{dim}CD16$^+$). (A) The box plots represent the raw data of the NK Bright cells in the two groups. CFS/ME patients were decreased in their cell numbers for this particular NK phenotype. (B) However, raw data of CD56^{dim}CD16$^+$ NK cells were examined in the control and CFS/ME groups, these were found to be similar. (C) Using the raw data from the flow cytometry results, total cell counts of NK cells were deduced. These measurements are plotted using bar graphs, CD56^{bright}CD16$^-$NK cells are more reduced in the CFS/ME (black bars) group in comparison to the controls (white bars). *Denotes statistical significant results (P≤ 0.05). Data presented as means ± SEM.

3.4.3. Profile of CD4$^+$T cells Subsets and Protein Expressions

After 72 hours of culture Th1 and Th2 cytokine secretions were considerably different between groups, however, Th17 cytokines remained unchanged. In particular IL-10, IFN-γ and TNF-α production was significantly elevated in the CFS/ME group compared to the control group (Figure 13). Other cytokines IL-2 and IL-6 although increased in the CFS/ME population were not statistically different between groups (Figure 13). IL-17A was similarly not significantly different between the two groups. FOXP3 secretion by Tregs was significantly higher in the CFS/ME group compared to healthy participants (Figure 14). Incidentally, Treg cell counts were also higher in the CFS/ME group compared to the healthy population (0.77 ± 0.10 vs. 0.24 ± 0.02). Lymphocyte expression of VPACR2 was significantly higher in the CFS/ME patients compared to the control group (Figure 15).
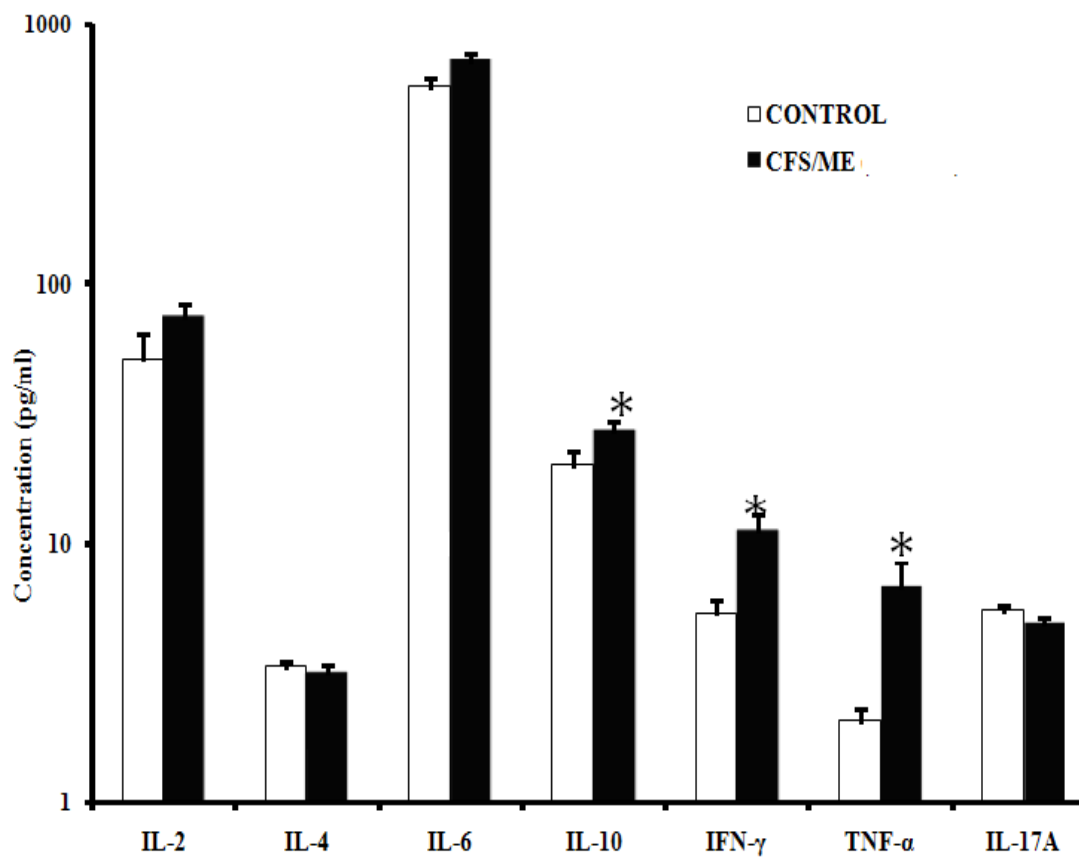
Figure 13: Examination of the expression levels of CD4^{+}T cell Related Cytokines in CFS/ME following mitogenic stimulation. CD4^{+}T cells that is, Th1, Th2 and Th17, cytokine levels in CFS/ME (black bars) and control participants (white bars) measured after stimulation with PHA. The concentrations of cytokines were measured in pg/mL. Both anti-inflammatory (IL-10) and pro-inflammatory (IFN-g, TNF-a) cytokines were increased in the CFS/ME group following mitogenic stimulation. *Statistically significant results at p<0.05. Data presented as log of mean concentration ± SEM.
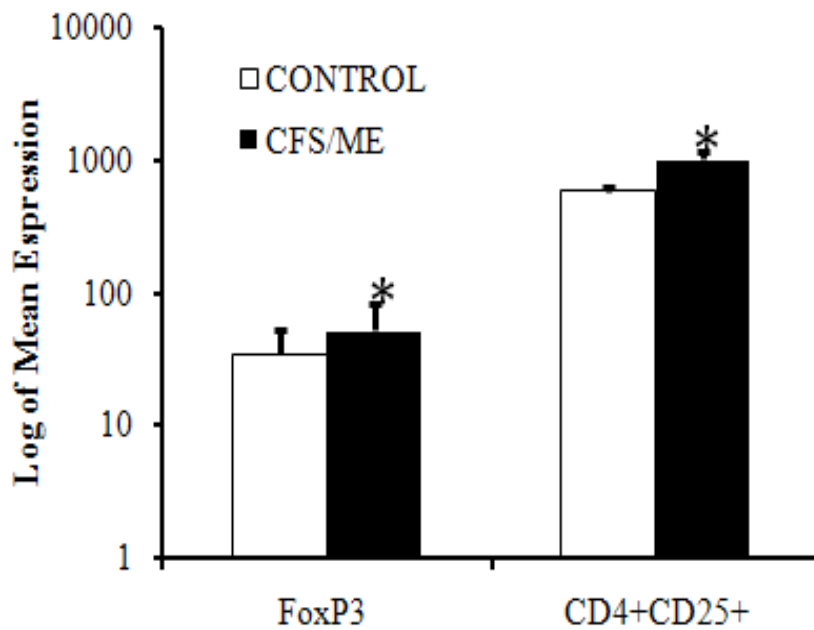
Figure 14: FOXP3 expression and $CD4^+CD25^+T$ cells in CFS/ME. The percentage of CD4+T cells expressing $CD4^+CD25^+FOXP3^+$ markers are represented in the bar graph. Tregs of interest in this study were those positive for FOXP3 and $CD4^+CD25^+$ in CFS/ME (black bars) and control (white bars) participants. *Represents statistically significant results at $p < 0.05$. Data presented as log of mean concentration ± SEM.
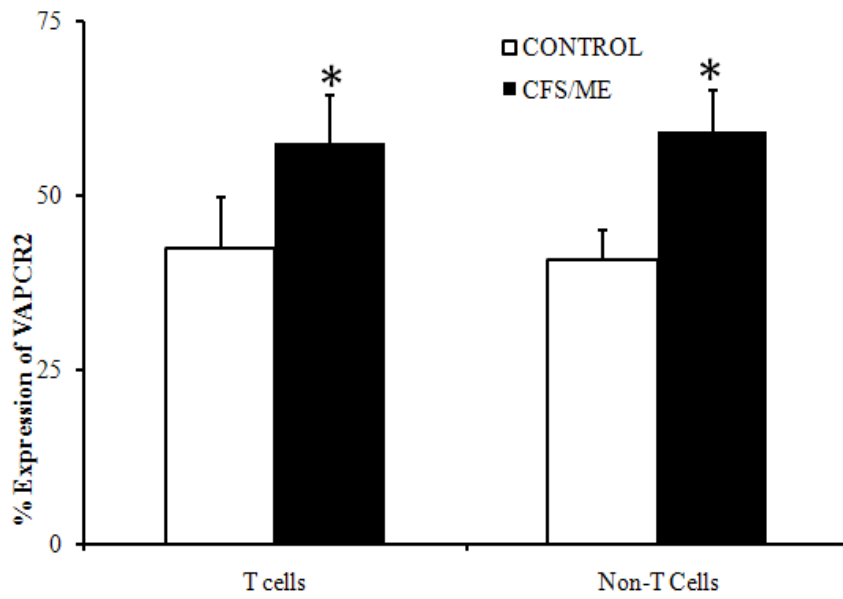
Figure 15: VPAC2R immune cells in CFS/ME. VPAC2R expression on the CD4$^+$T cells was assessed in CFS/ME (black bars) and controls (white bars). The data presented here are based on percentage of cells positive for CD4 and VPAC2R. *Represents statistically significant results at $p<0.05$. Data presented as mean $\pm$ SEM.

3.5. Discussion

This is the first study to show significantly higher levels of VPACR2 receptors, $CD4^+CD25^+Tregs$ and $FoxP3^+Treg$ expression in CFS/ME patients compared to healthy controls. In addition, CFS/ME patients had significantly higher levels of anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines IFN-γ and TNF-α. This profile reflects significant and important immunological dysregulation that could explain some of the clinical symptoms, for example the ongoing sickness that patients experience with CFS/ME.

This is the first study to provide a thorough investigation of the $CD4^+T$ cell profile in CFS/ME patients through the assessment of cytokine secretion and regulatory protein levels in particular VPACR2 receptors and FoxP3 expression. Cytokines are soluble proteins with either anti-inflammatory or pro-inflammatory effects. Equivocal cytokine expression patterns in CFS/ME patients have been reported without a definite identification as to which cytokines may be specifically linked to CFS/ME. Possible explanations for the inconsistencies in cytokine distribution across studies are the heterogeneous nature of the disorder and differences in analytical methods used. However, newer and more sensitive assays have been developed since the conflicting results were reported (Broderick *et al*., 2010). It has been suggested that the mechanism underlying CFS/ME may involve a shift in cytokine production leading to either a predominant Th1 or Th2 cytokine profile (Skowera et al., 2004, Swanink et al., 1996, Nakamura et al., 2010). In the adaptive immune system, $CD4^+T$ cells subsets, Th1, Th2, Th17 and regulatory T cells (Tregs) are the main regulators of cytokine secretion and

the inflammatory immune response. A bimodal Th1/Th2 response was observed in the present study. A predominant Th1 and Th17 immune response has been linked to the development or presence of an autoimmune disease whereas increases in Th2 cytokines suggest the presence of other systemic disorders (Drulovic *et al.*, 2009, Nevala *et al.*, 2009). Th1 cells secrete cytokines IFN-γ and IL-2 while Th2 cells secrete cytokines IL-4 and IL-10 (Zhu and Paul, 2008) and Th17 secrete pro-inflammatory IL-17a, IL-17f and IL-22 (Paust *et al.*, 2009, Pene *et al.*, 2008). Recent data on cytokine networks in CFS/ME show a predominant Th2/anti-inflammatory profile in CFS/ME with a weakened Th1 profile (Broderick *et al.*, 2010).

This study supports the presence of a possible imbalance in Th1/Th2 response in CFS/ME characterised by a significant increase in IL-10 together with significant increases in IFN-γ and TNF-α. Such increases in IL-10 are suggestive of a persistent chronic infectious state and may be associated with a dampening of the NK and CD8^{+}T cell immune response (White *et al.*, 2007). Others have shown that IL-10RA is differentially expressed in CFS/ME patients, highlighting a potential compromise in IL-10 function or its receptor in CFS/ME patients (Kaushik *et al.*, 2005, Kerr, 2008). Nonetheless, increased levels of IL-10, IFN-γ and TNF-α indicate the presence of fungal, bacterial or viral infection (Couper *et al.*, 2008). Incidentally in HIV elevation in IL-10, IFN-γ and TNF-α denote the presence of a chronic infection and this correlated with viral load (Norris *et al.*, 2006). Similarly in CFS/ME such alterations in these cytokines may also suggest an increase in viral load and the occurrence of flu-like symptoms. An increase in IL-10 also may contribute to decreased cytotoxic activity observed in the

NK and CD8^{+}T cells (den Haan et al., 2007, Szkaradkiewicz et al., 2010). The increase in pro-inflammatory cytokines such as TNF-α, may also depict the presence of an inflamed gut or irritable bowel syndrome in some CFS/ME patients (Scully et al., 2010). Inflammation in the gut can alter the central nervous system (Goehler et al., 2007, Lakhan and Kirchgessner, 2010) and affects various physiological mechanisms including neuropeptides.

The changes in both the Th1 and Th2 responses may suggest changes in the function of VN receptor VPACR2 which is a key promoter and stimulator of anti-inflammatory cytokines such as IL-10 (Delgado *et al*., 1999b). It is important to note that VNs, VIP and PACAP have never been assessed in CFS/ME previously. These important neuropeptides increase IL-10 gene expression via the cAMP response element DNA binding complex pathway, therefore changes in VNs such as elevations in VPACR2 may suggest an increase in IL-10 (Pozo *et al*., 2000). Further, an increase in TNF-α and IFN-γ suggests an inability of the heightened VPACR2 to suppress TNF-α and IFN-γ secretion as these neuropeptides are noted to suppress pro-inflammatory cytokines while favouring anti-inflammatory cytokine secretions (Pozo and Delgado, 2004). Additionally, increases in VPACR2 potentially suggest changes in cAMP associated with the inflammatory immune response in CFS/ME. Although our study did not assess the levels of cAMP present in CFS/ME patients, VIP binding to its receptor, in this case VPACR2, is known to stimulate the presence of FoxP3^{+} which assists in regulating the T cell response. Thus it is consistent that heightened levels of VPACR2 will translate into heightened FoxP3 expression. FoxP3^{+} Tregs also secrete IL-10 which maintains the

expression of FoxP3 in Tregs (Gonzalez-Rey and Delgado, 2007). The increased expression of IL-10 and the relatively higher expression of FoxP3 together with significant increases in $CD4^+CD25^+$Tregs suppressive activity suggest a requirement to counter a significant pro-inflammatory response in these patients. While levels of viral antigens were not measured in this study, these observations may suggest a plausible prevalence in viral antigens, adjuvants or autoantibodies in the peripheral circulation of CFS/ME patients (Nancy and Shoenfeld, 2008, Buskila et al., 2008).

Natural Killer cytotoxic activity in CFS/ME has received much attention (Klimas et al., 1990; Masuda et al., 1994; Maher et al., 2005; Roberson et al., 2005; Siegel et al., 2006; Brenu et al., 2010) while only one study has examined $CD8^+$T cell cytotoxic activity. Most studies found significant decreases in NK activity and one study found decreased $CD8^+$T cell cytotoxic activity in a CFS/ME population compared with a control group. These findings are confirmed in our study. In a previous study (Brenu et al., 2010) as well as this study in a larger population, we have found that NK cytotoxic activity and $CD56^{bright}$ NK phenotypes are decreased in CFS/ME patients. These atypical cytotoxic responses may be linked to compromised granule-mediated cell death pathways involving apoptotic mediators, perforin and granzymes. Perforin forms pore-like structures to facilitate the entry of granzymes into the target cell (Scott et al., 2008), and granzymes activate several apoptosis pathways that ensure effective killing of the target cell (Chowdhury and Lieberman, 2008). Perforin and granzymes have been shown to be decreased in both NK and $CD8^+$T cells in CFS/ME (Maher et al., 2005, Saiki et al., 2008). In contrast both granzyme A and granzyme K were significantly

reduced while perforin levels were elevated in both the NK and CD8$^+$T cells of CFS/ME patients. Reduced cytotoxic activity may therefore be an important component of the immune dysregulation seen in CFS/ME.

3.6. Conclusions

These results illustrate a severely compromised immunomodulation mechanism in CFS/ME where attempts to regulate or restore immune homeostasis appear to be impaired. These findings suggest that certain immunological biomarkers as demonstrated in this study may be unique to CFS/ME. To date no routinely available clinical immunological markers have been identified that characterise CFS/ME, resulting in poor recognition and management of patients. The immunological abnormalities identified in our study can potentially fill this void as potential biomarkers and assist clinicians and patients in diagnosis and management of this severely debilitating condition. These biomarkers may include NK phenotypes, NK activity, CD8$^+$T cell activity, IL-10, IFN-γ TNFα, FoxP3 and VPACR2. These markers that seem to be unique to CFS/ME patients could assist in identifying them as a distinct population, enabling more appropriate clinical management and better targeted scientific investigations into the underlying pathomechanisms of the disease.

4. Project Two: Longitudinal Investigation of Natural Killer Cells and Cytokines in Chronic Fatigue Syndrome

Brenu E.W., van Driel M., Staines D.R., Ashton K.J., Keane J., Peterson D., Hardcastle S.L., Tajouri L., Ramos S.B., Marshall-Gradisnik S. 2011. Longitudinal Investigation of Natural Killer Cells and Cytokines in Chronic Fatigue Syndrome. Journal of Affective Disorders. 10 (1), 88.

4.1. Abstract

Objective: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is an etiologically unexplained disorder characterised by irregularities in various aspects of the immunological function. Presently, it is unknown whether these immunological changes remain consistent over time. This study investigates Natural Killer (NK) cell cytotoxic activity, NK cell subsets ($CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$) and cytokines, over the course of a 12 month period in patients with CFS/ME.

Methods: Participants for the study included CFS/ME patients (n=50) assessed using the 1994 Centre for Disease Prevention and Control (CDC) definition and non-fatigued control (n=27). Participants were assessed at baseline (T1), 6 (T2) and 12 months (T3). Flow cytometric protocols were used to assess NK subsets and NK cytotoxic activity. Cytokine secretions were measured following stimulation of peripheral blood mononuclear cells.

Results: NK cytotoxic activity was significantly decreased in the CFS/ME patients at T1, T2 and T3 compared to the non-fatigued group. Additionally, in comparison to the non-fatigued controls, the CFS/ME group had significantly lower numbers of $CD56^{bright}CD16^-$ NK cells at both T1 and T2. Interestingly, following mitogenic stimulation, cytokine secretion revealed significant increases in IL-10, IFN-γ and TNF-α at T1 in the CFS/ME group. A significant decrease was observed at T2 in the CFS/ME group for IL-10 and IL-17A while at T3, IL-2 was increased in the CFS/ME group in comparison to the non- fatigued controls. Overall cytotoxic activity was significantly decreased at T3 compared to T1 and T2. $CD56^{bright}CD16^-$ NK cells were

much lower at T2 compared to the T1 and T3. IL-10 and IL-17A secretion was elevated at T2 in comparison to the T1 and T3.

Conclusion: These results confirm decreases in immune function in CFS/ME patients, suggesting an increased susceptibility to viral and other infections. Furthermore NK cytotoxic activity may be a suitable biomarker for diagnosing CFS/ME as it was consistently decreased during the course of the 12 month study.

4.2. Introduction

Immune responses to infection and inflammation are important aspects of physiological homeostasis. This involves constant co-ordinated responses from and between the innate and adaptive immune systems (Wasowska, Clark and Kupper, 2005). Cells of the innate immune system in particular Natural Killer (NK) cells are important mediators of targeted cell killing of tumor, transformed and virus infected cells (Marcenaro et al., 2011b). NK cells are recruited by interferons and chemoattractive chemokines including CCL22, CX3CL1 and CXCL8 (Gong and Clark-Lewis, 1995, Loetscher et al., 1996, Bochner et al., 1999, Imai et al., 1998). At sites of infection, stochastic expression of NK receptors with the release of granzymes and perforin via mitogenic pathways ensures efficient elimination of unwanted cells (Delgado et al., 2010, Marcenaro et al., 2011a, Marras et al., 2011, Vivier et al., 2004).

As a diseases with unknown pathomechanism and lacking specific diagnostic markers, Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) has been associated with diminished immune function (Lorusso et al., 2009). CFS/ME is characterised by severe fatigue with apparent flu-like symptoms that either fluctuate or deteriorate and persist for many months to years (Fukuda et al., 1994, Wallman et al., 2005). To date cytokines, lymphocyte subsets and cytotoxic activity, have been examined in patients with CFS/ME using serum, plasma or blood samples. The findings from these investigations demonstrate equivocal quantities in lymphocyte numbers and cytokines but interestingly consistent decrease in NK cytotoxic activity (Tirelli et al., 1994, Barker

et al., 1994, Masuda et al., 1994, Racciatti et al., 2004, Stewart et al., 2003, Brenu et al., 2010).

A decrease in NK cytotoxic activity is thus a recurring finding in CFS/ME research (Fletcher et al., 2010, Brenu et al., 2010, Ojo-Amaize et al., 1994, Barker et al., 1994, Maher et al., 2005, Masuda et al., 1994) and remains a hallmark of the disease. Additionally, equivocal changes in NK cell subsets, $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ NK cells have been shown to occur (Brenu et al., 2010, Brenu et al., 2011). Abnormalities in cellular levels of either $CD56^{dim}CD16^+$ NK or $CD56^{bright}CD16^-$ NK cells can affect cytokine production and subsequent clearance of pathogens. Despite these striking alterations there are no follow up studies reporting on the profiling of both NK cells subsets and activity during an extended course of CFS/ME. Perturbations in cytokine production favouring either an anti-inflammatory or pro-inflammatory cytokine profile may be prevalent in some CFS/ME patients (Skowera et al., 2004, Swanink et al., 1996, Metzger et al., 2008). CFS/ME is a chronic disease with a relatively long duration, persisting for more than 6 months, however, it is not reported yet whether alterations in cytokines occur and persist over time in adults with CFS/ME.

At this stage the diagnosis of CFS/ME is based on self-reported clinical symptoms. The difficulty in establishing a stringent medical diagnosis for CFS/ME may be related to the lack of data in monitoring the stability of immune markers during the course of the disease. Almost all studies to date investigating immune function in CFS/ME including cell activity, lymphocyte subsets and cytokines in CFS/ME adults were only restricted

to single time point examination, providing insufficient information on the changes in these makers as the disease progresses. Additionally, there are many factors that can affect the differences in data reported. As CFS/ME is heterogeneous and multi-factorial, CFS/ME patients may experience periods of high, medium or low severity in symptoms which may be related to the levels of cytokines and immune function.

Therefore, the purpose of this study is to examine the validity and stability of immune parameters previously known to be compromised in CFS/ME and to determine most importantly whether these observations are consistent during the course of the disease. Assessment and evaluation of these markers potentially enables both the determination and knowledge of their stability profile over the course of the disease. This is the first longitudinal study assessing NK cytotoxic activity, NK subsets and $CD4^{+}T$ cell cytokine distribution over a period of 12 months in adults suffering from CFS/ME.

4.3. Method

4.3.1. Recruitment

Participants for this study were comprised of CFS/ME patients and non-fatigued controls recruited from an existing cohort in Queensland and New South Wales, Australia (Brenu et al., 2011). Prior to inclusion all participants completed a consent form and a questionnaire. The description of these participants is provided in Table 1. The CFS/ME group met the 1994 CDC criteria for CFS/ME and the control group consisted of non-fatigued volunteers. The non-fatigued controls were recruited from similar locations as the CFS/ME cohort. In both groups, individuals with known autoimmune disorders, psychosis, epilepsy, diabetes and cardiac related disorders prior to the onset of CFS/ME like symptoms were excluded from the study. These exclusions were also applied to the control group.

4.3.2. Data Collection

Samples were collected at baseline (T1), after 6 months (T2) and after 12 months (T3). Blood collections were performed at three testing sites as it was not always feasible for participants to travel to the main testing site. Participants were recruited in 2009 and enlisted for the study in December 2009. The first collection was performed in December 2009. The second sampling point took place in June 2010 and the third in December 2010. Clinical data were collected through self-administered questionnaires at baseline (T1) and T3.

4.3.3. Sample Preparation and Routine Measurements

Non-fasting morning blood samples were collected from the antecubital vein of all participants into lithium heparin (12mL) and EDTA (25mL) tubes. Regular full blood count (Coulter Counter, Beckman Coulter) assessments were performed ahead of immunological assessments. All analyses and experiments were performed immediately following blood collection. On a given day blood samples were collected from a maximum of 10 participants each day. These participants comprised of a mixture of CFS/ME patients and healthy controls, however, as there were more CFS/ME patients compared to healthy controls, in some cases the samples comprised only CFS/ME patients.

4.3.4. NK cytotoxic activity

Natural Killer cytotoxic activity was examined as previously described (Aubry et al., 1999, Brenu et al., 2010, Brenu et al., 2011). In brief, NK lymphocytes were isolated from blood samples using density gradient centrifugation and labelled with 0.4% PKH-2T2 (Sigma, St Louis, MO). Following which NK cells were incubated with K562 cells, for 4 hours at 37°C in 95% air, 5% CO_2 at an effector to target ratio of 25 (NK cells):1 (K562). An E:T ratio of 25:1 was chosen as we have previously found this ratio to be the most optimal condition for assessing cytotoxic activity. In previous studies this ratio has been used (Brenu et al., 2010, Brenu et al., 2011). After four hours of incubation NK lysis of K562 cells was calculated as previously described (Aubry et al., 1999) to determine the ability of NK cells to induce tumor cell death or apoptosis via FACS-Calibur flow cytometry (BD Bioscience, San Jose, CA), using Annexin V-FITC and 7-

AAD reagents (BD Pharmingen, San Diego, CA). The NK assay was performed within 2-4 hours upon receipt of all blood samples for that particular day hence each sample was treated the same. Each sample was performed in duplicates and a control sample was included in each run.

4.3.5. NK subsets

The frequency of NK cell subsets was evaluated as previously described (Brenu *et al.*, 2010, Brenu *et al.*, 2011). Briefly, a negative selection system using RosetteSep Human Natural Killer Cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC) was used to segregate NK lymphocytes from whole blood. Preferentially isolated NK cells were then labelled with CD56-FITC and CD16-PE monoclonal antibodies (BD Pharmingen, San Jose, CA). In a forward scatter and side scatter plot the lymphocyte gate was set on NK cells. Subset profile was measured in a PE versus FITC plot.

4.3.6. T cell specific cytokine distribution

Isolated PBMCs were cultured at $1x10^6$ cells/mL with or without 1μg of phytohemagluttinin mitogenic stimulation for 72 hours. Cellular supernatants were collected following incubation and stored at -80^0C for later assessment. Th1, Th2 and Th17 cytokine concentrations were determined using the cytometric bead array (CBA) kit (BD Pharmingen, San Jose, CA) (Brenu et al., 2011, Dickson and Finlayson, 2009). The cytokines that were measured include IL-2, IL-4, IL-6, IL-10, tumour necrosis factor (TNF)-α, interferon (INF)-γ and IL-17A. The CBA kit includes a standard that is diluted at different concentrations as per manufacturer's instructions to produce a

standard curve for each cytokine to be measured. This was included in the cytokine assessment. During each run of the CBA analysis control samples were included and each set of samples analysed on a given day consisted of both CFS/ME patients and non-fatigued control cell supernatants.

4.3.7. Statistical Analysis

Statistical analysis was performed using SPSS software (18.0; SPSS Inc, Chicago). . The experimental data represented in this study are reported as means plus/minus standard error of the mean (±SEM) while all the clinical date are reported as means plus/minus standard deviation (±SD). Comparative assessments among participants (CFS/ME and control subjects) were performed with the analysis of variance test (ANOVA) and repeated measures. For the repeated measures assessment time was the within-subjects factor and group was the between subject factor. Instances where Mauchly's test indicated that sphericity assumption was breached, the Huynh-Feldt correction was applied. Bonferroni method was used as post-hoc analysis to assess changes in the data. Time effects and group effects on the variables measured were assessed using eta squared, where eta squared (η^2) is the ratio of the sum of squares of the variance to the sum of squares of the total variance ($\eta^2 = SS_{variance\ effect}/SS_{total}$). To determine within subject stability. Pearson and Spearman's rank correlations were determined between the three time points. P-values less than or equal to 0.05 were considered statistically significant.

4.3.8. Ethical Clearance and Participant Selection

Approval for this study was granted after review by the Bond University Human Research Ethics Committee (R0852A).

4.4. Results

4.4.1. Participants

Data for baseline, 6 months and 12 months were available for 86 participants (65 CFS/ME patients and 25 non-fatigued controls). The mean age for the CFS/ME patients was 47.2±11.5 years and 45.2±9.3 years for the non-fatigued controls. 75.4% of CFS/ME patients and 66.7% of the non-fatigued controls were female and the mean BMI in both groups was 24.5±5.0 for the CFS/ME patients and 24.5±4.3 for the non-fatigued controls. The CFS/ME patients had been suffering from fatigue for a mean of 16.4±12.5 years and their mean score on the Fatigue Severity Scale (FSS) was 55.5±9.1 (the lowest possible score of the FSS was 7 and highest is 63) (Krupp et al., 1989). Only 19.4% of patients indicated that they were still able to carry out normal activities. Clinical baseline characteristics are reported in Table 6.

Table 6: Baseline clinical characteristics of chronic fatigue syndrome patients (cases) and non-fatigued controls.

	Cases (n=65)	Controls (n=21)
Age, mean in years (SD)	47.2 (11.5)	45.2 (9.3)
Female (%)	75.4	66.7
BMI kg/m^2 (SD)	24.4 (4.9)	25.3 (5.5)
Smoked in the past 2 years (%)	4.7	4.8
Symptom (%)		
Weakness >24 hours after exercise	93.8	9.5
Unrefreshing sleep	93.8	19.0
Impaired concentration	90.8	4.8
Muscle pain	81.5	14.3
Joint pain	70.8	9.5
Headaches	67.7	9.5
Lymph glands	43.1	0
Sore throat	46.2	8.0
Diagnosed with depression or anxiety	64.6	19.0

4.4.2. Longitudinal assessment of NK cytotoxic activity

Natural Killer cytotoxic activity, the ability of the NK cells to effectively cause apoptosis of K562 cells was significantly reduced ($p< 0.05$) in CFS/ME patients compared to the control group. Significant changes between the groups were noticed at the different time points, i.e. at T1 ($p<0.001$), T2 ($p<0.001$) and T3 ($p<0.001$) (Figure 16A). Regardless of the time point cytotoxic activity remained significantly decreased ($p<0.001$). Time effects had no interaction with the group ($p=0.495$). There were

significant time effects in cytotoxic activity from T1 to T3 (p=0.023) and from T2 to T3 (p=0.016) (Figure 16B).
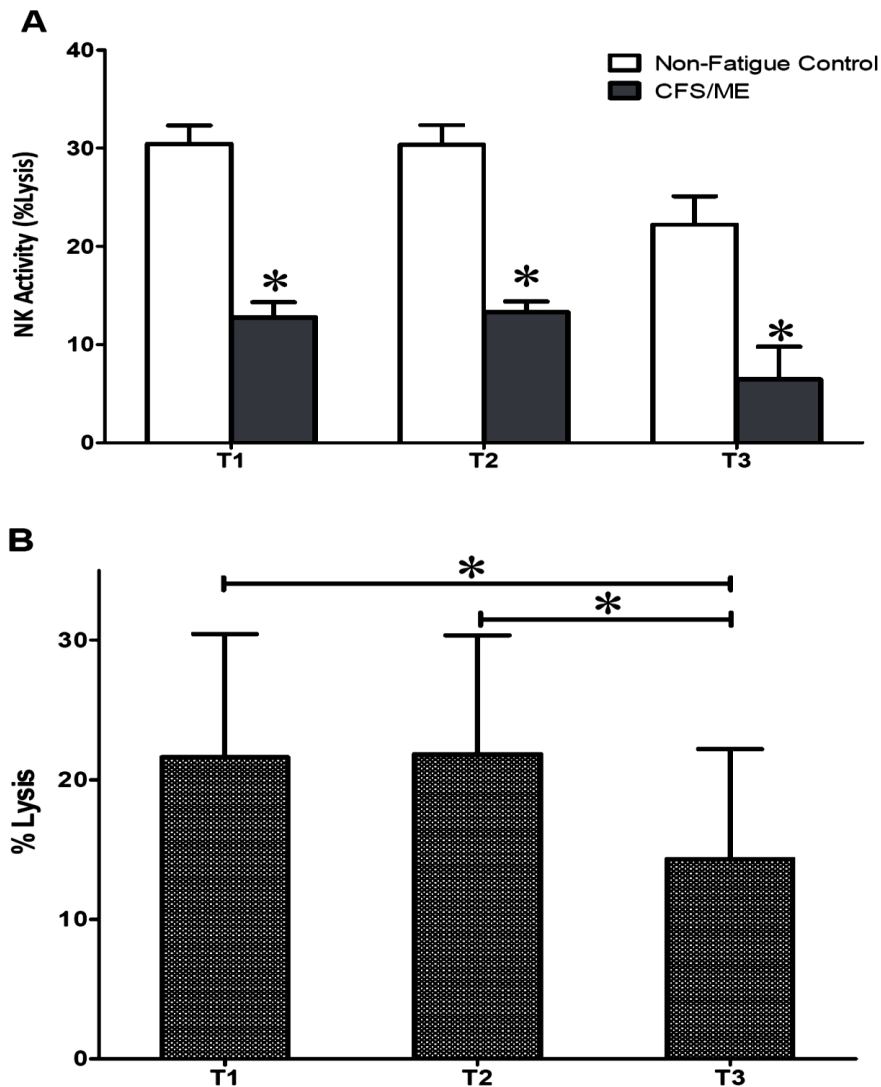
Figure 16: NK cytotoxic activity was decreased at all time points in the CFS/ME patients. (A) Cytotoxic activity presented as % lysis of K562 cells by NK cells assessed overtime at T1, T2 and T3 in the CFS/ME patients (black bars) and control (white bars) participants. (B) Cluster analysis showing the overall cytotoxic activity in the whole participant group. *Indicates statistical significant results relative to controls. Statistics are presented as mean ± SEM.

4.4.3. Differential Distribution of NK Cells Between Groups

The NK subsets were classified as $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ NK cells. $CD56^{bright}CD16^-$ NK cells were significantly lower at T1 ($p=0.020$) and T2 ($p<0.001$) in CFS/ME patients compared to non-fatigued controls (Figure 17A). Significant time effects were observed for $CD56^{bright}CD16^-$ NK cell levels ($p=0.003$) in the overall group. Additionally, there was a significant interaction between time and group ($p=0.015$). Pairwise comparison revealed significant changes in the overall group from the T1 to T2 period ($p=0.037$) and from T1 to T3 ($p=0.014$) (Figure 17B). $CD56^{dim}CD16^+$ NK cells remained unchanged throughout the study for both groups.
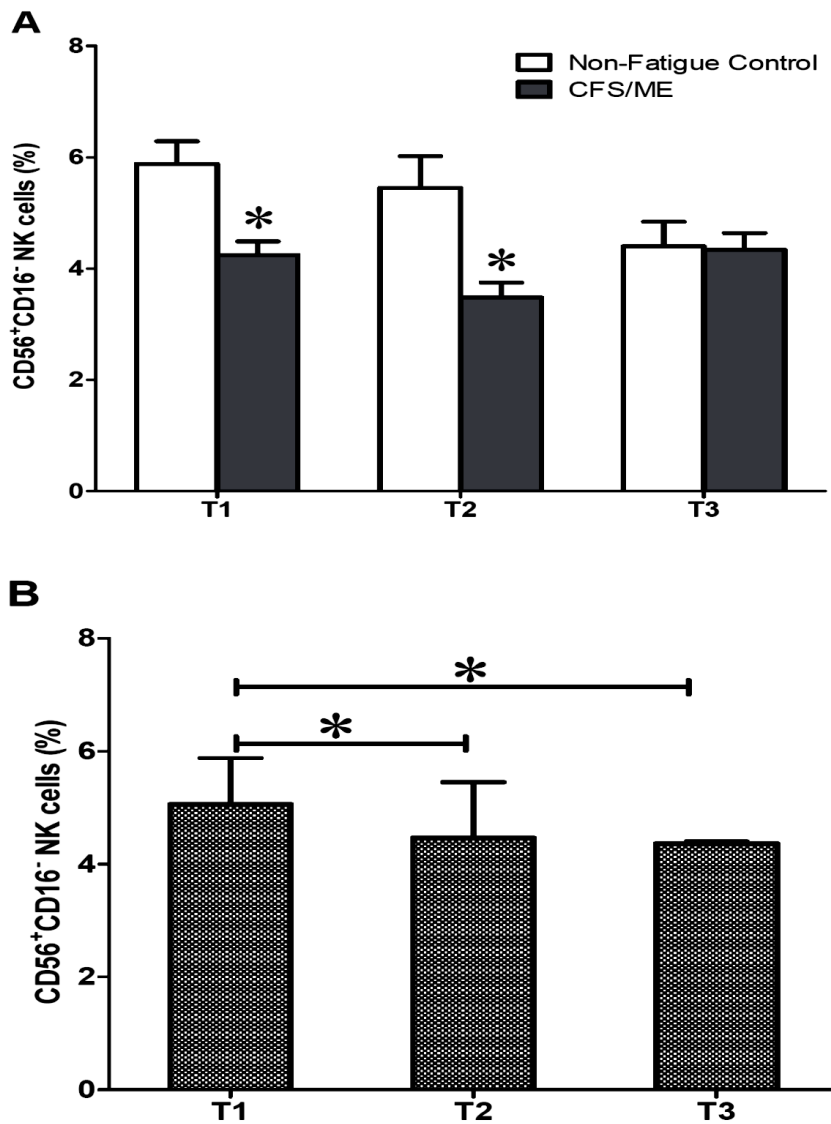
Figure 17: CD56^{bright}CD16$^-$NK Subset distribution in the CFS/ME group. Percentage of NK cells stained positive for CD56^{bright}CD16$^-$ was significantly low in the CFS/ME group at the T1 and at T2. The white bars represent control data while the black bars represent CFS/ME data. (B) Cluster analysis showing the overall CD56^{bright}CD16$^-$ NK cells in the whole participant group. Data are presented in the form of log of the total events collected via flow cytometry ± SEM. *Signifies statistical significant results relative to controls.

4.4.4. T Cell Related Cytokine Distribution

Following mitogenic stimulation, production significant differences in cytokine distribution were observed at T1, T2 and T3. At T1, IL-10 (p=0.051), IFN-γ (p=0.003) and TNF-α (p=0.002) were significantly increased in the CFS/ME patients compared to the non-fatigued controls (Figure 18B, D, E). IL-10 (p=0.026) and IL-17A (p=0.002) were significantly decreased at T2 (Figure 18B, C) and only IL-2 (p=0.005) was significantly increased at T3 (Figure 18A). There was no significant difference in the remaining cytokines.

Irrespective of the group, overall production of some cytokines was significantly different from one time point to another. IL-2 was increased from T1 to T2 (p<0.001) and from T1 to T3 (p<0.001) (Figure 4A). IL-17A was increased from T1 to T2 (p=0.009) but declined fron T2 to T3 (p=0.022) (Figure 4D). Secretion of IL-6 was decreased fromt T1 to T2 (p=0.001) and T1 to T3 (p=0.001) (Figure 19B). IL-10 proressively declined from T1 to T3 (p<0.001) and T2 to T3 (p <0.001) (Figure 19C).
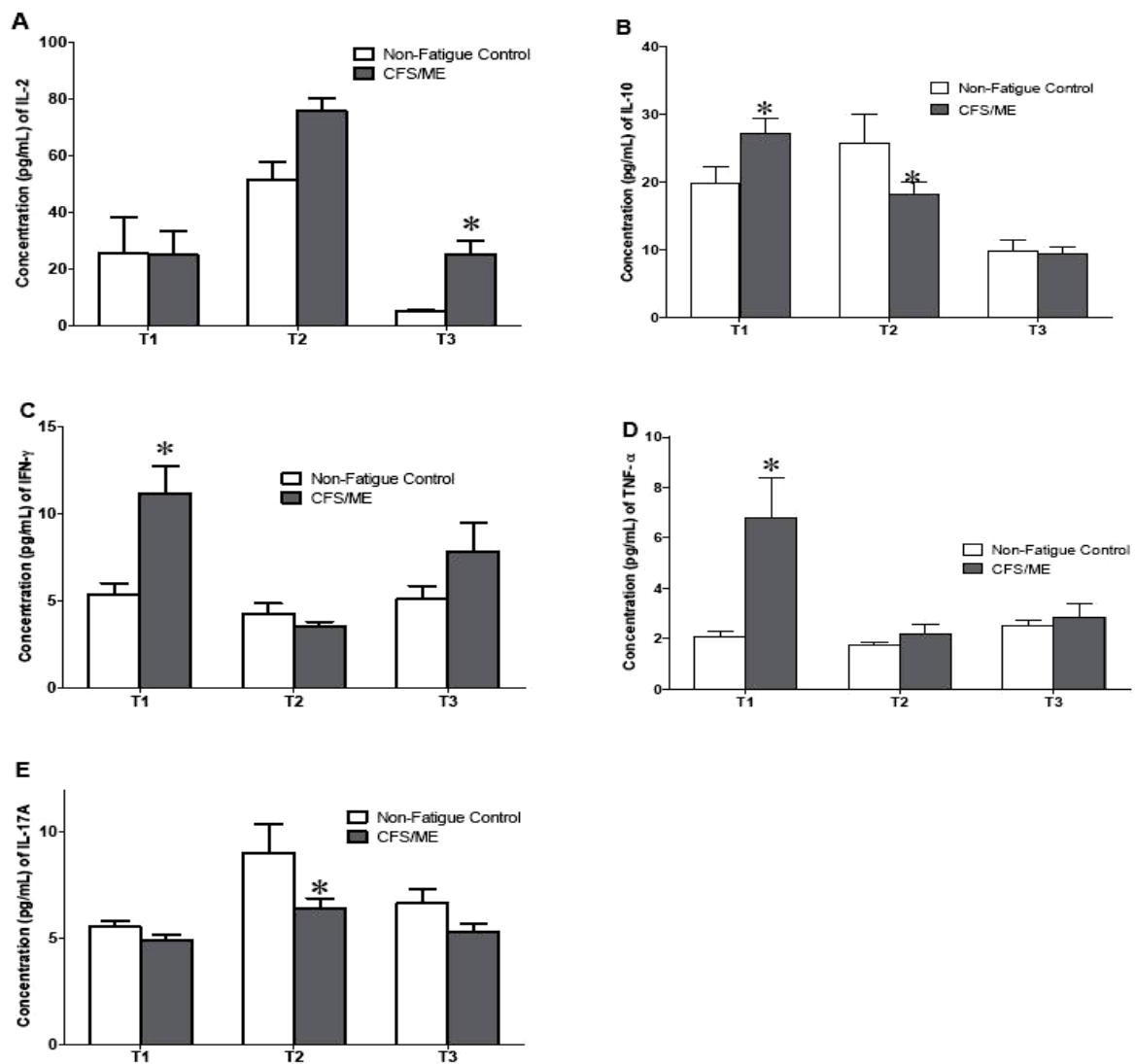
Figure 18: Between group differences in cytokine production overtime. The secretion of IL-2 (A) was significantly increased in the CFS/ME patients at the T3, (B) IL-10 increased at the T1 and dropped significantly at the T2 in the CFS/ME group. (C) IL-17A was reduced at the T1 while (D) IFN-γ and (E) TNF-α were increased significantly only at the T1. The CFS/ME data are signified by the black bars and non-fatigued controls the white bars. *Symbolizes statistically significant results were considered where $p \leq 0.05$. The results are expressed as the mean concentration at each time point ± SEM.
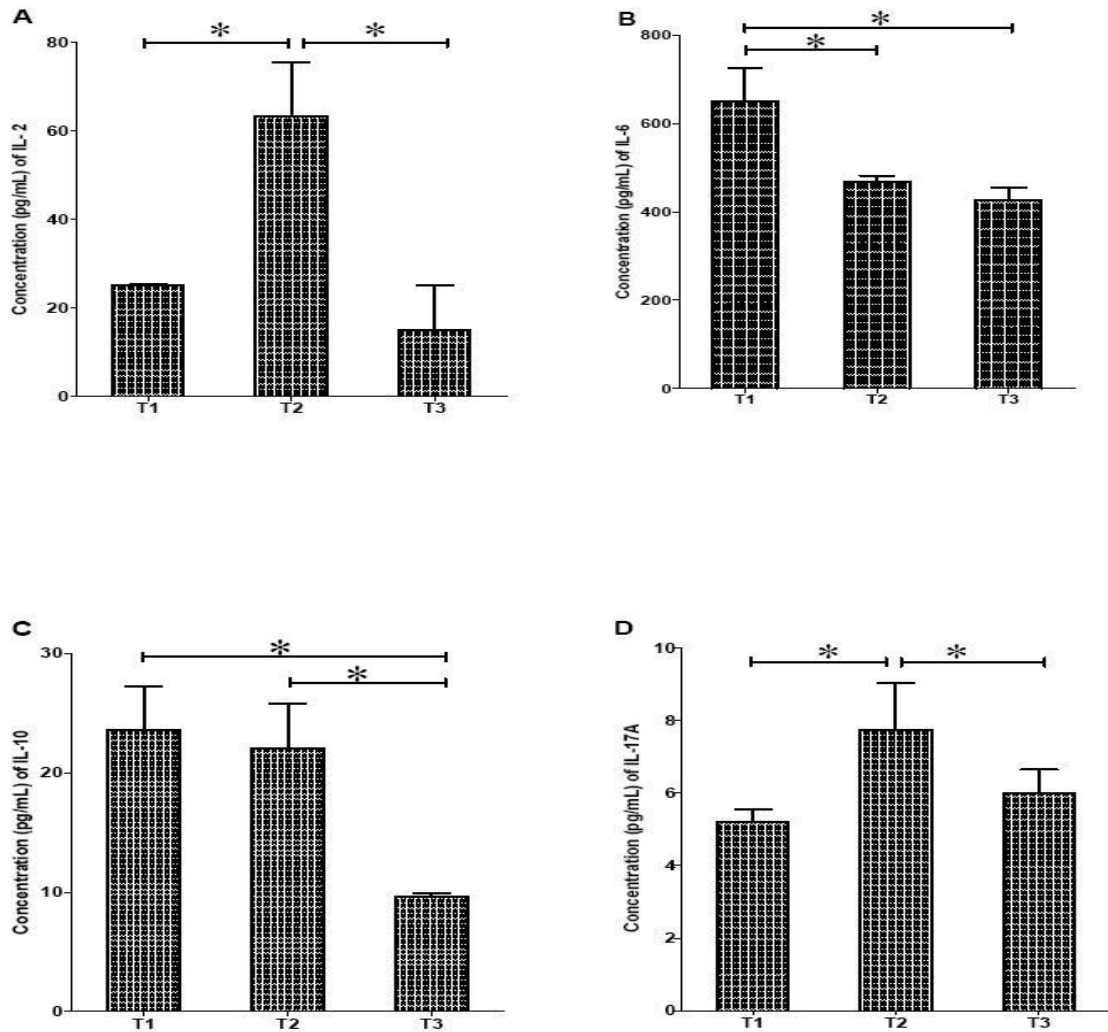
Figure 19: Overall Cytokine Secretion with Respect to Time. (A) Cytokine production within group was significantly different in IL-2 with high levels noticed at T1 compared to the T2 and T3. (B) A similar pattern was noticed in IL-2. (C) IL-10 was however significantly decreased at T3 compared to the T1 or at T2. (D) IL-17A tended to be higher at T2 within group. Data are presented in the form of log of the total events collected via flow cytometery ± SEM. *Designates statistical significant results relative to controls.

4.4.5. Parameter stability

In this study the stability of the immune parameters over time was assessed using Pearson and Spearman's correlation analysis, where significance was set at P less than or equal to 0.05. The results were highly significant for NK activity data, a positive correlation was perceived at all time points for NK activity (T1-T2; $p=0.003$, T1-T3; $p=0.032$). Although, CD56^{bright}CD16$^-$NK cells were not significantly decreased at T3 the correlation between time points was significant (T1-T2; $p<0.0001$, T1-T3; $p<0.0001$, T2-T3; $p<0.0001$). Using eta squared, significant main effects ($\eta^2=0.44$) were noticed for NK activity. Of note, cytokines were not correlated at any time point. However decreases in IFN-γ, TNF-α and IL-10 were correlated at T1.

4.5. Discussion

Our investigation is the first study to demonstrate that NK cytotoxic activity remains consistently decreased in CFS/ME patients during the course of the disease. However, other immune parameters, especially cytokine secretions fluctuate at different time points and therefore demonstrate inconsistencies in their distribution pattern during the course of the disease. The purpose of this investigation was to identify immune markers that can be possibly used as biomarkers for CFS/ME in a longitudinal manner.

Primarily, this longitudinal study has illustrated that NK cytotoxic activity is potentially useful as biomarkers for CFS/ME, since it was the most stable in the CFS/ME patients over the 12 months period of the study. Decreases in cytotoxic activity occur in CFS/ME, in some cases this is associated with differential expression in the levels of cytotoxic molecules (Klimas et al., 1990, Klimas and Koneru, 2007, Brenu et al., 2010, Robertson et al., 2005). These cytotoxic proteins and their genes including perforin (*PRF1*), granzyme A (*GZMA*), granzyme B (*GZMB*) and granzyme K (*GZMK*) have been shown to be either increased or decreased in CFS/ME (Brenu et al., 2011, Maher et al., 2005, Saiki et al., 2008). For example, the perforin gene (*PRF1*) may increase in some cases of CFS/ME while intracellular perforin protein may be decreased in other CFS/ME patients (Brenu et al., 2011). Such profound differences in protein and mRNA can reduce the effectiveness of NK cells to induce lysis/cytotoxic activity of viral infected cells. Hence, defective cytotoxic activity in CF/ME patients may be due to perturbations in the expression of cytotoxic factors resulting in reductions in cytotoxic proteins required for effective lysis of viral and microbial particles. Importantly, NK

cells have both activating and inhibitory receptors, successful killing of target cells occurs through integrated signalling by activating and inhibitory receptors, and co-receptors. Inhibitory receptors are activated through the recognition of MHC class I proteins and this inhibits NK cytoxic activity (Biassoni, 2009, Biassoni et al., 2001). Activating receptors are important for eliminating tumours, and other microbes through intracellular signal transduction mechanisms that connect them to immunodominant tyrosine based activation motif (ITAMS) adaptor proteins (Moretta et al., 2001, Biassoni, 2009, Biassoni et al., 2001). Certain viruses can affect NK receptor signalling thus reducing cytoxic activity. For example, the cytomegalovirus viral genes can regulate NK inhibitory receptor expression preventing the induction of activating receptors (Reyburn et al., 1997). An increase in viral load occurs during the course of CFS/ME, may trigger defective cytotoxic receptor activations hence resulting in malfunctioning of NK cytotoxic activity. The overall decrease in cytotoxic activity at T3 is not explicitly known.

The exact consequence of alterations in CD5^{6bright}CD16$^-$ NK cells in CFS/ME is not fully known, however, patterns of CD56^{bright}CD16$^-$ NK cells were affected by seasonal changes which may affect NK cytokine production. Incidentally gene expression of *IFNG* which is an important NK cytokine was significantly decreased in the NK cells of CFS/ME patients (Brenu et al., 2011), which may be related to the decrease in CD56^{bright}CD16$^-$ NK cells. Decreases in CD56^{bright}CD16$^-$ NK cells have been observed in coronary heart disease, allergic rhinitis and juvenile rheumatoid arthritis while in

diseases such as Chronic Obstructive Pulmonary Disease (COPD) CD56^{bright}CD16$^-$ NK cells have been reported to be increased (Scordamaglia et al., 2008, Hak et al., 2007).

Interleukin 2, a pro-inflammatory cytokine produced by Th1 cells (Zhu and Paul, 2008) is required for naïve CD4$^+$T cell differentiation into Th2 and regulatory T cells (Tregs) in the presence of IL-4 and transforming growth factor beta (TGF-β) respectively (Zhu and Paul, 2008). Binding of IL-2 to its high affinity receptor IL-2R induces the proliferation of T cells and memory CD4$^+$ and CD8$^+$T cells (Thornton et al., 2004, Williams et al., 2006, Yamane et al., 2005). It is also has important roles in generating effector functions for B cells, CD56^{bright}NK and CD8$^+$T cells (Fehniger et al., 2003). IL-2 regulates Treg cells and interestingly, CD4$^+$CD25$^+$Foxp3$^+$Treg cells, have been reported to be significantly increased in the CFS/ME patients in comparison to non-fatigued controls (Brenu et al., 2011). An increase in IL-2 may suggest a shift towards Th1/ pro-inflammatory immune response in CFS/ME patients.

Anti-inflammatory IL-10 exerts inhibitory effects on cytokine secretion and acts as an inhibitor to pro-inflammatory cytokine secretion by multiple cells including Th1 cells (IFN-γ), macrophages/monocytes (IL-1, IL-T2, IL-8, IL-12 and TNF-) and NK cells (IFN-γ and TNF-α) (Commins et al., 2010). A decrease in IL-10 favours an increase in pro-inflammatory responses and this may increase the prevalence of Th1 like cytokines. IL-17A is expressed by Th17 cells, it recruits and activates neutrophils, stimulates the generation of pro-inflammatory cytokines, chemokines and increases antimicrobial gene expression (Weaver et al., 2007, Fossiez et al., 1996, Liang et al., 2006, Ouyang et al.,

2008). IL-17A is an important immunoregulator during microbial infections as it activates immune cells to secrete pro-inflammatory factors. A decrease in IL-17A may contribute to the prevalence of infections. A possible explanation for the observed changes in the secretion of this cytokine may be related to TGF-β which at optimal levels directly promotes IL-17A generation while reducing IL-2 (Cejas et al., 2010). Thus, in the CFS/ME patients, TGF-β may be decreased causing an increase in IL-2. Therefore, cytokine release in CFS/ME patients undergoes shifts during the course of the disease where patients may present with either an amplified or depressed anti-inflammatory or pro-inflammatory cytokine profile. These alterations in cytokine secretion may occur during the course of the disease and at different times causing either a shift towards a predominant Th1 or Th2 immune response in CFS/ME (Brenu et al., 2011, Broderick et al., 2010, Fletcher et al., 2009, Skowera et al., 2004, Swanink et al., 1996). This makes it difficult to establish a unique CFS/ME-like inflammatory cytokine profile. The observed pattern of cytokine distribution among our CFS/ME patients is consistent with equivocal findings in the literature (Patarca et al., 1994, Linde et al., 1992, Lloyd et al., 1992, Peakman et al., 1997, Gold et al., 1990). In adolescents with CFS/ME cytokine secretions have been observed to be correlated with seasonal variations (ter Wolbeek et al., 2007). Therefore, CFS/ME may be associated with oscillations in pro and anti-inflammatory cytokines, supporting the heterogeneity and multifactorial nature of the disease and the diversity in symptom presentations.

4.6. Conclusion

In conclusion, altered regulation of immunological function, in particular reduced cytotoxic activity, is a key component of CFS/ME. This longitudinal study has identified NK cytotoxic activity and possibly $CD56^{bright}CD16^{-}$ NK cells as potential biomarkers for diagnosing CFS/ME. The observation of immune dysregulation made in this study in relation to CFS/ME patients have been observed in various immunological diseases. This suggests the need for further investigations into the underlining disrupted mechanism of decreases in cytotoxic activity. It is important to note that these cytokine profiles were measured following mitogenic stimulation of PBMCs, serum measurements of cytokines may display different results. Further studies are therefore required to investigate whether changes in cytokine secretions from activated PBMCs and/ or serum levels are associated with severity and progression of the complex clinical presentations in CFS/ME pathology.

5. Project Three: Cytotoxic Lymphocyte MicroRNAs as Potential Biomarkers for Chronic Fatigue Syndrome /Myalgic Encephalomyelitis Patients

5.1. Abstract

Background: Immune dysfunction associated with disease often has a molecular basis. A novel group of molecules known as microRNAs (miRNAs) have been associated with suppression of translational processes involved in cellular development and proliferation, protein secretion, apoptosis, immune function and inflammatory processes. MicroRNAs may be implicated in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME), where immune function is impaired. The objective of this study was to determine the association between miRNAs in cytotoxic cells and CFS/ME.

Methods: Natural Killer (NK) and CD8+T cells were preferentially isolated from peripheral blood mononuclear cells from all participants (CFS/ME, n=28; mean age=41.8±9.6 years and controls, n=28; mean age=45.3±11.7 years), via negative cell enrichment. Following total RNA extraction and subsequent synthesis of cDNA, reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) was used to determine the expression levels of nineteen miRNAs.

Results: There was a significant reduction in the expression levels of *miR-21*, in both the NK and CD8+T cells in the CFS/ME sufferers. Additionally, the expression of *miR-17-5p, miR-10a, miR-103, miR-152, miR-146a, miR-106, miR-223* and *miR-191* was significantly decreased in NK cells of CFS/ME patients in comparison to the non-fatigued controls.

Limitations: The results from these investigations are not yet transferable into the clinical setting, further validatory studies are now required.

Conclusions: Collectively these miRNAs have been associated with apoptosis, cell cycle, development and immune function. Changes in miRNAs in cytotoxic cells may reduce the functional capacity of these cells and disrupt effective cytotoxic activity along with other immune function in CFS/ME patients.

5.2. Introduction

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a multi-symptom, multi-factorial and heterogeneous disorder. CFS/ME affects about 1-4% of individuals worldwide and is characterised by deficits in short term memory and concentration, tender lymph nodes, muscle pain, severe headaches, sleep disturbances, profound fatigue and postexertional malaise (Fukuda et al., 1994). Immune markers have been identified including decreased cytotoxic activity (Klimas et al., 1990, Maher et al., 2005, Brenu et al., 2010). Differential expressions of genes involved in immunological, neurological and metabolic processes have also been implicated in CFS/ME (Kerr, 2008, Lin and Hsu, 2009, Saiki et al., 2008, Kaushik et al., 2005, Kerr et al., 2008a, Kerr et al., 2008b). A number of these genes encode transcription factors known to regulate immune function such as cytotoxicity, cytokine secretion and apoptosis and have been shown to be decreased in CFS/ME patients (Brenu et al., 2012, Faria and Weiner, 2006, Schaefer et al., 2007, Matsuoka and Jeang, 2005).

Other regulatory molecules have been identified that may have characteristics similar to transcription factors, these molecules are known as microRNAs (miRNAs). It is predicted that the human genome may encode over 1,000 miRNAs (Griffiths-Jones, 2010). MicroRNAs are highly conserved non-coding RNA molecules 18-24 nucleotides in length that preferentially target 3' untranslated regions of their target mRNAs (Sun et al., 2010). MicroRNAs are endogenously expressed and transcribed into a primary miRNA from introns of protein-/non-coding sequences of exclusive miRNA genes or host genes (Sun et al., 2010). The pri-miRNA formed is processed into a pre-miRNA

and integrated into an RNA-Induced Silencing Complex (RISC) (Hammond et al., 2000). The miRNA-RISC either cleavages complementary mRNA molecules or inhibits protein translation resulting in the decrease of *de novo* synthesis of the corresponding protein (Behm-Ansmant et al., 2006). The interactions between miRNAs and mRNAs are important in maintaining coherent physiological processes such as immune function.

MicroRNAs are required during development, maturation, proliferation, antigen recognition, apoptosis induction and cytokine secretion of immune cells (Tili et al., 2007, O'Connell et al., 2007, Taganov et al., 2006, Liston et al., 2010a). Dysregulation in the expression of miRNAs may adversely affect immune homeostasis. For example, deficiencies in *miR-155* encourage a shift towards T helper 2 (Th2) anti-inflammatory immune responses (Rodriguez et al., 2007, Thai et al., 2007), while in the absence of *miR-101* autoreactive T cell mediated autoimmunity occurs (Yu et al., 2007). MicroRNAs are also essential for modulating immune responses to bacterial and viral infection. Alterations in these miRNAs may significantly affect immune reactions such as cytotoxic activity which are known to be compromised in CFS/ME.

The purpose of this study was to assess the possible role of miRNAs in cytotoxic cells of the innate (NK cells) and adaptive (CD8^{+}T cells) immune system in CFS/ME patients. We hypothesised that as miRNAs can either increase or decrease the expression of various genes, they may also be involved in the regulation of cytotoxic cells in CFS/ME patients. To the best of our knowledge this is the first study to explore the role of miRNAs in cytotoxic cells of CFS/ME patients.

5.3. Method

5.3.1. Subject Recruitment

This study was approved by the Bond University Human Research Ethics Committee (R0852A). Participants (n=56) for the study were recruited from a database of patients from the South East Queensland region of Australia. The inclusion criteria for CFS/ME (n=28; age=42.0±9.4 years) was based on the Centers for Disease Control and Prevention (CDC) 1994 case definition while the non-fatigued healthy control (n=28; age=45.0±14.0 years) were participants with no medical history or symptoms of prolonged fatigue or illness of any kind (Fukuda *et al.*, 1994).

5.3.2. Sample Collection and Cell Isolation

Venous blood samples (40mL) from all participants were collected into EDTA tubes and analysed within three hours of collection. Peripheral blood mononuclear cells were isolated from 20mL of whole blood for each cell type using Ficoll-hypaque (GE Healthcare, Uppsala, Sweden). Enrichment of NK or CD8^{+}T cells was performed using NK and CD8^{+}T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched NK or CD8^{+}T cell purity was examined on the FACSCalibur flow cytometer (BD Bioscience, San Diego, CA) after staining with CD16/CD56 or CD8/CD3 monoclonal antibodies (BD Bioscience, San Diego, CA). Flow cytometry and haemocytometer assessment were used to determine the purity of the cells isolated for miRNA expression analysis. The recovery of isolated cells was calculated based on the observation that NK and CD8^{+}T cells represent 2% and 5% of peripheral blood lymphocytes respectively (Dorfman and Raulet, 1998,

Banerjee et al., 2005) hence; there are more CD8^{+}T cells in circulation compared to NK cells. Thus recovery was defined as the ratio of percentage of the total number of cells (i.e. NK or CD8^{+}T cells) isolated to the percentage of cells (ie. NK or CD8^{+}T cells) present in the volume of blood collected. Enriched cells were snap frozen in liquid nitrogen and stored at -80°C until further assessment.

5.3.3. RNA Extraction and cDNA Synthesis of NK and CD8^{+}T Cells

Total RNA (containing miRNA) was extracted from isolated NK and CD8^{+}T cells using the miRNeasy isolation kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Concentration and purity of RNA was determined using the NanoDrop 3300 (Thermo Scientific, Waltham, MA). Synthesis of cDNA from 250ng of miRNA was performed using the NCode™ miRNA First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Synthesized cDNA were diluted 1:20 and stored at -20°C prior to RT-qPCR.

5.3.4. RT-qPCR

A panel of 19 miRNAs expressed in NK and CD8^{+}T cells, was selected based on their involvement in immune cell function (Table 7). Six small non-coding RNA genes (*SNORD25, SCARNA17, SNORA73A, RNU5A, RNU1A* and *RNU6B*) (Qiagen, Hilden, Germany) were assessed using GeNorm to determine their usability as reference genes (Etschmann et al., 2006). *RNU1A* was found to be the most stable and therefore served as the endogenous reference control for all miRNAs assessed in this study. RT-qPCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

The final reaction volume (10μL) included 1× iQ SYBR-Green Supermix (Bio-Rad, Hercules, CA), 200nM of each primer and 4μL of diluted cDNA. Primer pairs consisted of a miRNA-specific forward primer (Zhang et al., 2009) and the universal RT primer (Invitrogen, Carlsbad, CA). The RT-qPCR conditions were 95°C for 3 minutes, followed by 45 cycles of 95°C for 10s, 57°C for 30s and generation of melt curves at 65°C to 95°C for the detection of secondary amplicons (Mestdagh et al., 2009). All reactions were performed in triplicate with each reaction plate containing an equal number of CFS/ME and non-fatigued controls, a calibrator control derived from a pool of all cDNA samples and a no template control (NTC). PCR amplification efficiencies (85-100%) for each primer pair was calculated using a 4-log serial dilution of the calibrator sample and efficiency correction was applied to the data during analysis.

Table 7: Summary of miRNA investigated, including their targeted messenger RNAs and function.

Gene	mRNA Targets	Function	
miR-10a	*BCL2L2, BCL6, E2F3*	Induction of cell survival; inhibition of transcription necessary for germinal centre formation; cell cycle regulation	**Apoptosis**
miR-16	*BCL2, CDK6*	Anti-apoptotic; regulation of cell cycle	
miR-15b	*CDK6, BCL2, ARL2*	Anti-apoptotic; cell cycle progression; regulation of STAT3	
miR-107	*PTEN*	Regulates AKT-mTOR signalling; tumor suppression	
miR-128b	*APAF1, BAG2*	Anti-apoptotic	
miR-146a	*NFKB1*	Inflammation; cell differentiation; apoptosis	
miR-191	*IL2RA, BCL214*	Regulation of apoptosis	
miR-21	*TLR4, PITX2, EGR2*	Activation of NFκB; regulation of gene expression; regulation of embryo development	**Cell proliferation**
miR-223	*E2F1,NFIA, MEF2C*	Regulation of cell proliferation; regulation of cell cycle genes; activation of transcription and replication	
miR-17-5p	*MAPK9, RBL2, TNFRSF21, TGFBR2*	Phosphorylation of transcription factors; chromatin Maintenance; activation of immune factors	
miR-150	*C-MYB, NOTCH3, EIF4B*	Activation of transcription factor; regulation of receptor function	
miR-103	*ARL2, DICER, TGIF2*	Formation of RISC complex; repression of transcription	**Immune functions**
miR-106b	*STAT3*	Transcription activation	
miR-126	*ITGA6*	Cell adhesion	
miR-142-3p	*IL6ST*	Signal transduction	
miR-146-5p	*EGFR*	Phosphorylation of proteins	
miR-152	*HLA-G, DNMT1, EIF4G3*	Antigen presentation; methylation	
miR-181	*EGR1, HOXA11, TCL1, BCL215*	Regulation of transcription; phosphorylation	
Let 7a	*E2F2,*	Activation of transcription	

5.3.5. Data and Statistical Analysis

The PCR data were analysed using the CFX Manager v1.6 (Bio-Rad, Hercules, CA). Baseline subtractions and threshold settings above background were applied to all data. The calibrator sample was used to normalize inter-assay variations, with the threshold coefficient of variance (CV) for intra-assay and inter-assay replicates <1% and <5% respectively. Following efficiency correction normalized expression ($\Delta\Delta$Cq) was calculated with miRNA expression normalized to *RNU1A* levels and the calibrator control. Expression values were $\log_2$ transformed and the median expression value for the non-fatigued group was set to zero. Data was analysed using the software packages SPSS 18 for Windows (SPSS Inc., Chicago, IL) and GraphPad Prism 5 (GraphPad Software, San Diego, CA). For miRNA analysis unpaired groups of values were compared according to the non-parametric Mann-Whitney test. Statistical significance was set at $P \leq 0.05$. ANOVA was used in analysing the results in Table 1 where results with $P \leq 0.05$ were classified as significant.

5.4. Results

5.4.1. Attributes of Participants

Participants from this study were randomly selected from a cohort of participants with known decreases in NK and CD8^{+}T cell cytotoxic activity (Brenu *et al*., 2011). Full blood counts were performed prior to cell isolation and gene expression. There were no significant changes in white blood cell counts between CFS/ME patients and controls, however, mean platelet volume was significantly different between the two groups (Table 8).

Table 8: Characteristics of CFS/ME and Non-fatigued Control Participants

Parameters	Control (n=30)	CFS/ME (n=30)	*p*-values
Sex: Female	22	23	
Male	8	7	
Mean Age	45.3±11.7	41.8±9.6	0.11
Height (cm)	167.47±13.2	167.9±8.9	0.16
Weight (lbs)	169.8±140.7	159.8±46.5	0.11
White Blood Cells	6.16 ± 0.25	5.17 ± 0.19	0.17
Lymphocyte (%)	35.02 ± 1.37	38.18 ± 0.10	0.10
Monocytes (%)	5.72 ± 0.30	6.00 ± 0.33	0.56
Granulocyte (%)	59.28 ± 1.48	54.99 ± 1.52	0.07
Lymphocyte ($x10^3/\mu L$)	2.07 ± 0.11	2.12 ± 0.08	0.75
Monocytes ($x10^3/\mu L$)	0.32 ± 0.02	0.33 ± 0.02	0.67
Granulocyte ($x10^3/\mu L$)	3.71 ± 0.22	3.21 ± 0.17	0.09
Red Blood Cells ($x10^6/\mu L$)	4.56 ± 0.04	4.36 ± 0.05	**0.01***
Haemoglobin (g/L)	136.30 ± 1.41	131.70 ± 1.52	**0.05***
Haematocrit (%)	41.57 ± 0.59	38.77 ± 0.50	**0.00***
MCV	84.78 ± 1.61	88.44 ± 0.73	**0.05***
MCH	33.74 ± 1.91	30.36 ± 0.23	0.09
MCHC	321.65 ± 10.30	341.29 ± 1.35	0.07
RDW	13.94 ± 10.91	12.58 ± 0.14	0.06
PLT	238.30 ± 10.40	237.93 ± 8.19	0.98
MPV	7.02 ± 0.08	7.24 ± 0.12	0.18

*significance set at $p \leq 0.05$

5.4.2. Cell Purity and Recovery

High levels of purity (>93%) were observed following isolation and enrichment. The rate of recovery was measured as a percentage of the cells isolated following enrichment to the expected number of NK or CD8$^+$T cells present in the volume of venous blood collected (Figure 20). The rate of recovery was higher for the NK (>90%) cells in comparison to the CD8$^+$T cells (>83%) (Figure 20).
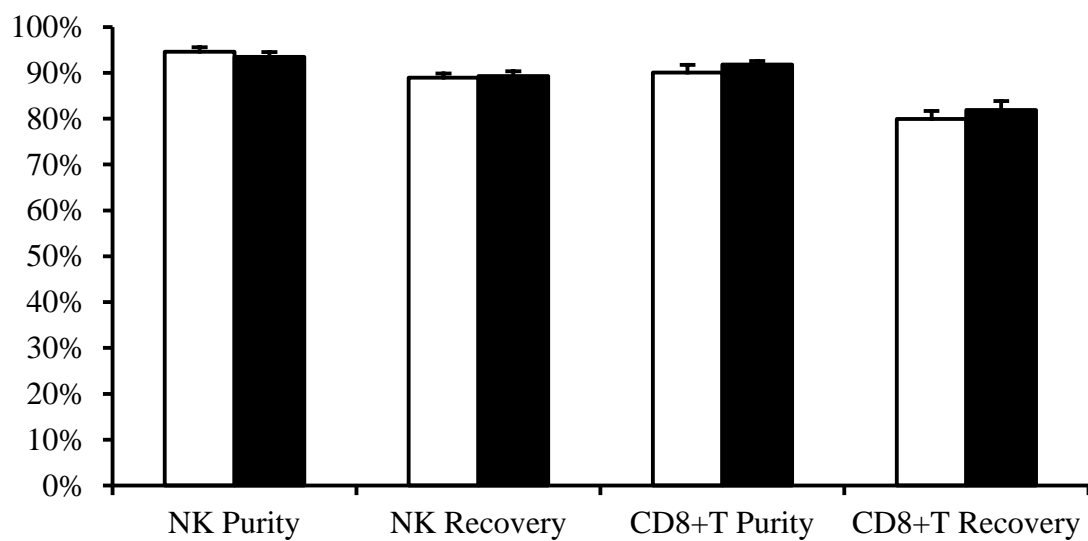
Figure 20: The purity of NK and CD8$^+$T cells. Minimal contamination from other cells types was observed as represented by the graphs. The white bars denote the non-fatigue control group (n=30) while the black bars depict the CFS/ME group (n=30). Results are presented here as means ± SEM.

5.4.3. RT-qPCR Results

The choice of a stable reference gene is critical for accurate gene experiment analysis, therefore a number of the reference genes were examined to determine the best possible gene to use. Expression of non-coding RNAs varied in both the NK and the CD8$^+$T cells (Figure 21). In both the NK and CD8$^+$T cells despite the slight discrepancies in expression, there were no significant differences between the two groups on measures of expression of the reference genes. *RNU1A* was determined to be the most stable reference gene for both cell types; in addition its relative abundance was most similar to the average abundance of the miRNAs investigated. Stability of reference genes were determined using the M values. M values refer to the average pair wise deviations of one reference gene to other reference genes. This deviation relates to the differences in the log2-transformed expression standard deviation of one gene to another. Therefore the stability of reference gene decreases at high M values compared to low M values. The M values for four snRNAs were much lower in both the NK and CD8$^+$T cells.
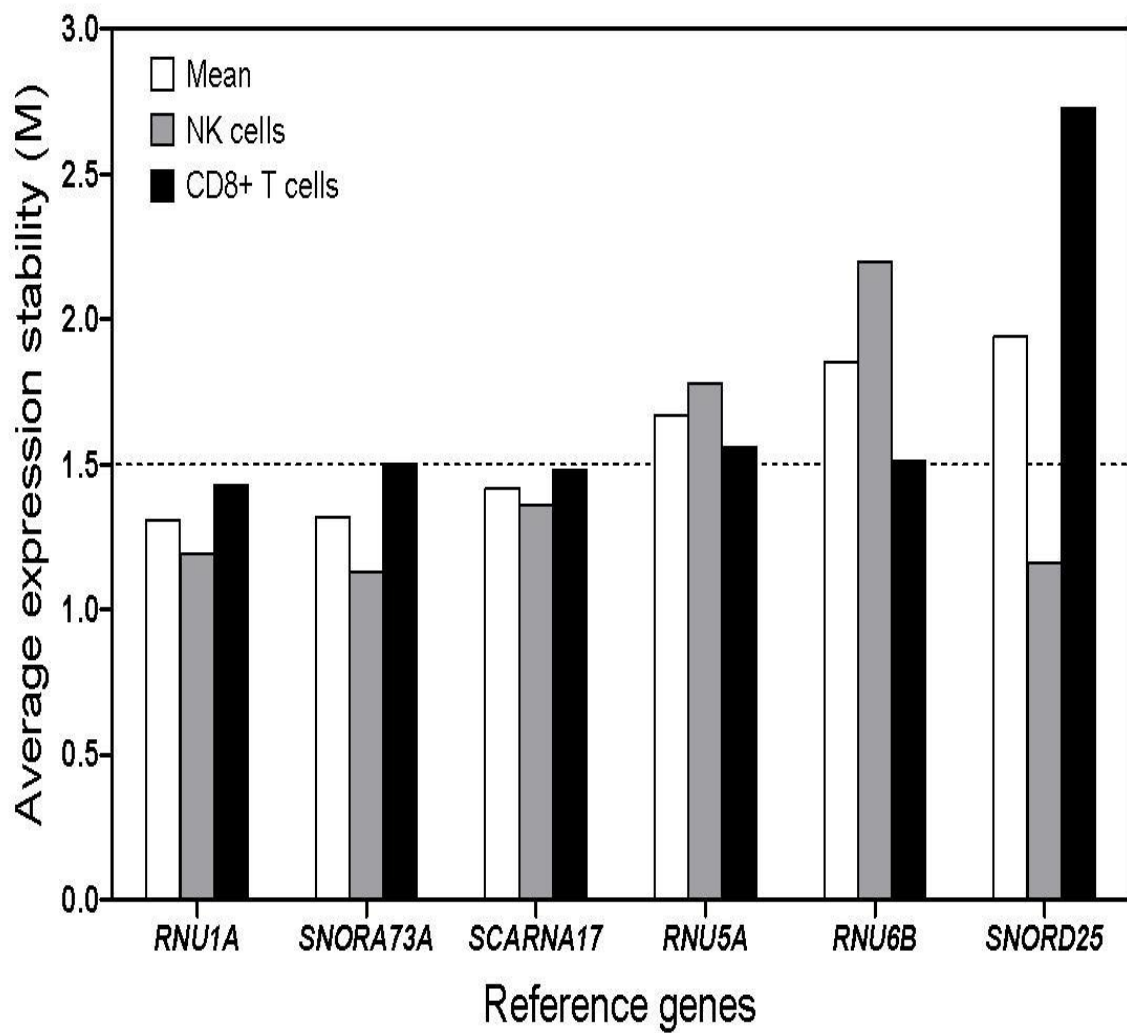
Figure 21: Expression profile of non-coding snRNAs in NK cells and CD8^{+}T cells. The figure represents all the snRNAs tested for use as a stable reference gene. GeNorm threshold value (1.5) is indicated with a dashed line.

The expression levels of 19 miRNAs were compared between CFS/ME and non-fatigued controls. Overall, NK cells had the most downregulated miRNAs in the CFS/ME population compared to the non-fatigued controls. Of the miRNAs selected, eight have regulatory roles involving apoptosis, and of these, four (*miR-10a, miR-146a, miR-191 and miR-223*) were found to be significantly decreased (Figure 22A) in the NK cells while none were significantly decreased in the CD8$^+$T cells (Figure 22B). Similarly of the six miRNAs involved in cell proliferation that were investigated, four (*miR-17-5p, miR-21, miR-106 and miR-152*) were significantly reduced in the NK cells (Figure 23A). A two-fold decrease in *miR-21* was also observed in the CD8$^+$T cells of in the CFS/ME group (Figure 23B). Of the remaining five miRNAs with broad function in immunity, only miR-103 was significantly decreased in NK cells (Figure 24A and 24B).
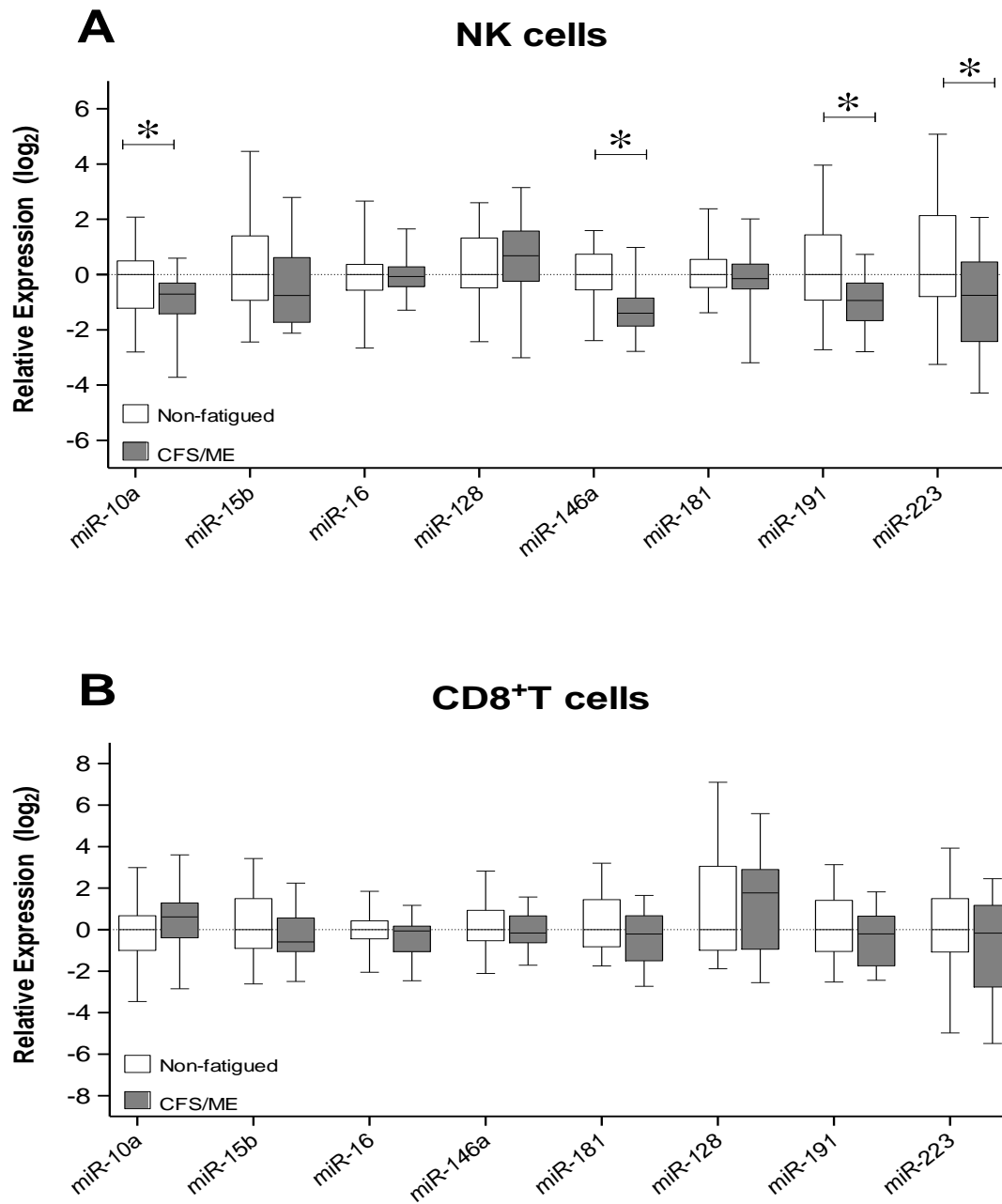
Figure 22: Expression profile of miRNAs involved in apoptosis. Relative expression data presented as boxplots for miRNAs involved in cell proliferation in A) NK cells and B) CD8+T cells. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values.*P < 0.05 vs. non-fatigued control.
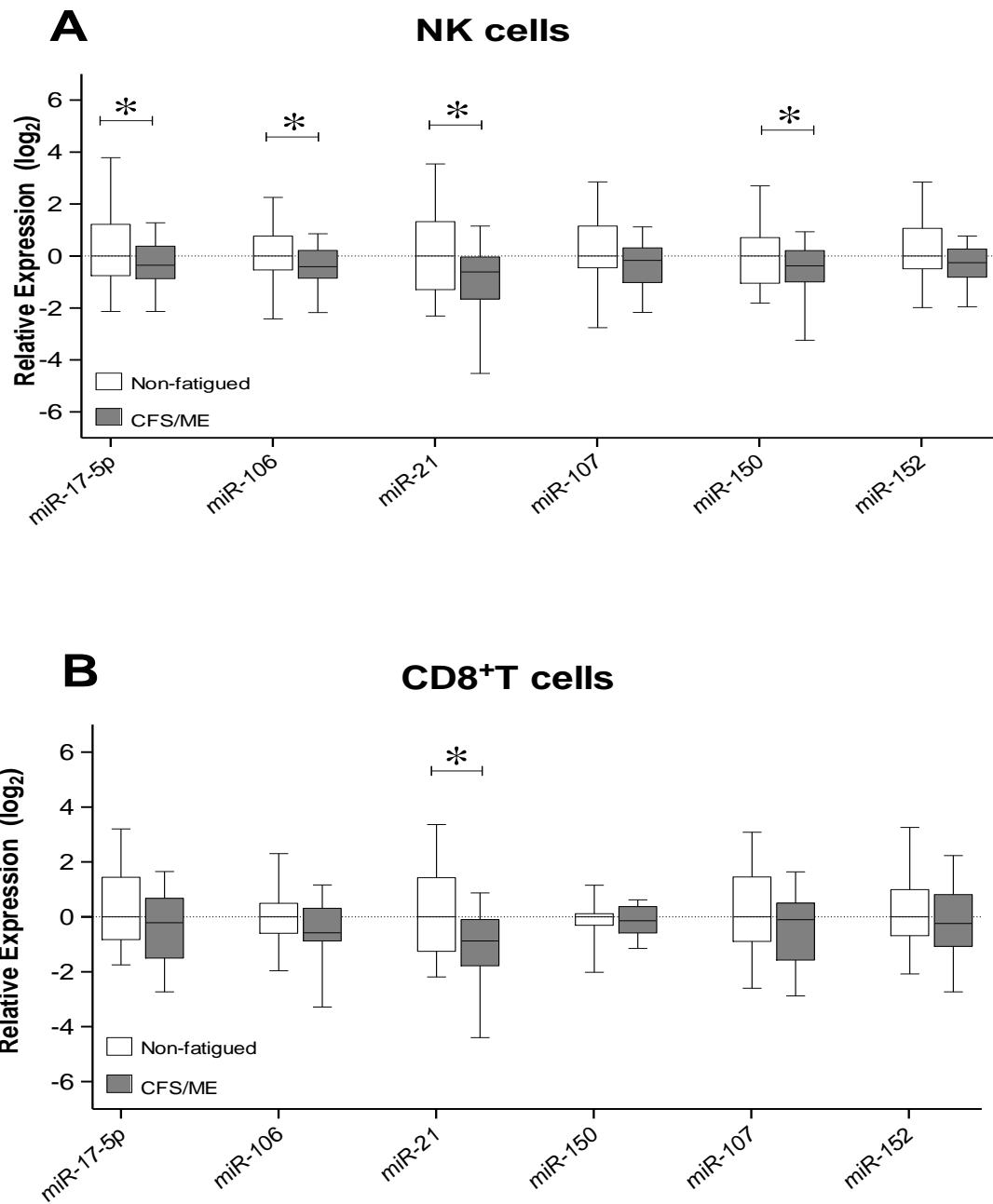
Figure 23: Relative expression data presented as boxplots for miRNAs involved in cell proliferation in A) NK cells and B) CD8+T cells. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values.*$P < 0.05$ vs. non-fatigued control.
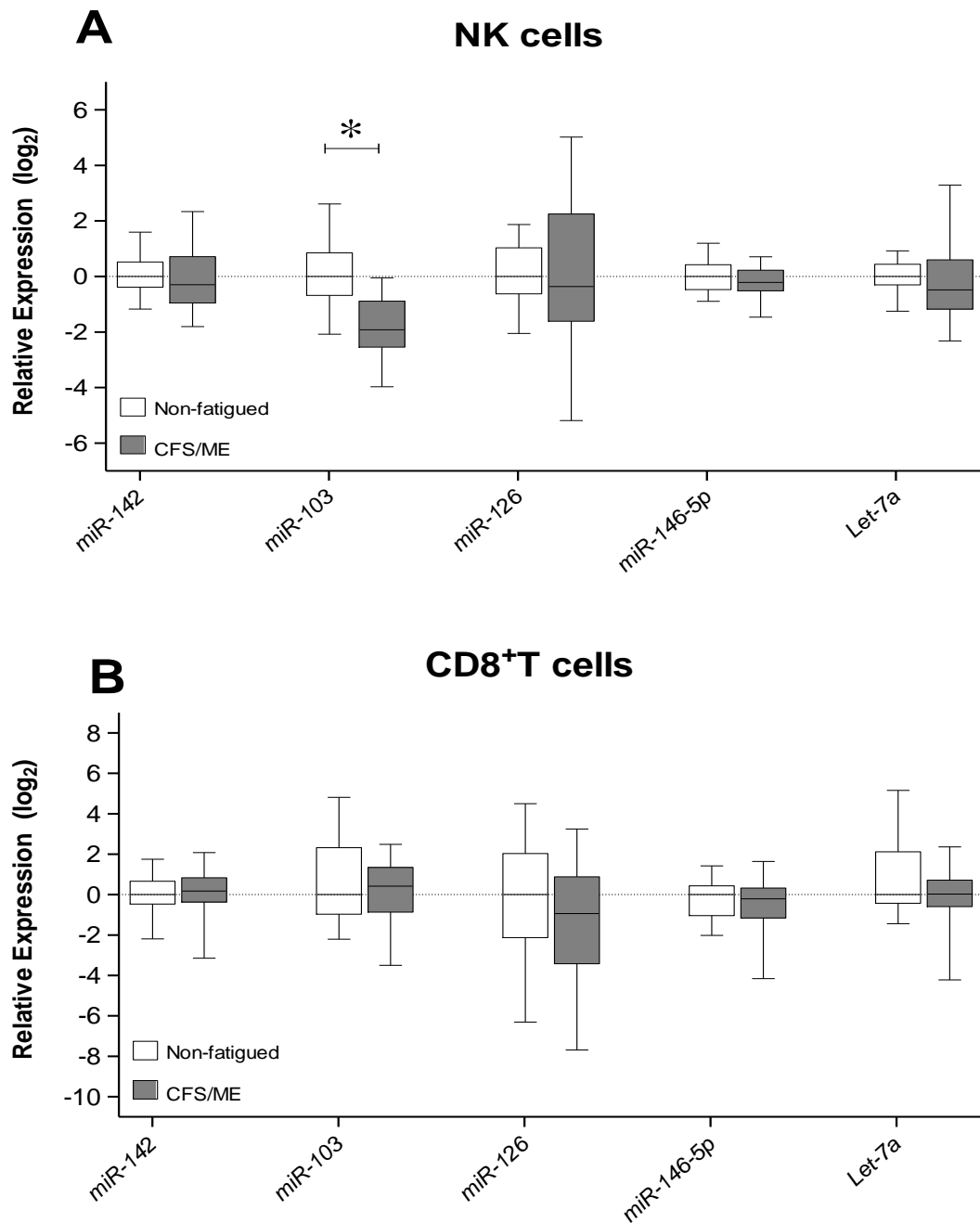
Figure 24: Relative expression data presented as boxplots for miRNAs involved in immune function in A) NK cells and B) CD8^{+}T cells. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values.*$P < 0.05$ vs. non-fatigued control.

5.5. Discussion

We examined the expression of miRNAs and contribution of miRNAs to the immune profile of cytotoxic cells, that is NK and CD8^{+}T cells, in CFS/ME patients. Non-coding RNA molecules affect physiological processes and certain pathological conditions. Our patient groups had similar cell numbers signifying that CFS/ME is not associated with changes in cell numbers. Similarly, there were no significant differences in the number of cells isolated from each group. However, mean platelet volume was reduced in the non-fatigued control in comparison to the CFS/ME patients; this may be due to individual characteristics and the heterogeneous nature of CFS/ME. Mean platelet volume has been reported elsewhere to be decreased in CFS/ME patients and this was not associated with heightened platelet activation or a hypercoagulable state (Kennedy et al., 2006). This study has shown significant down-regulation in the expression of miRNAs in CFS/ME population in comparison to a non-fatigued control group. These findings implicate miRNAs in the pathogenesis of CFS/ME, possibly relating to the cytotoxic function of NK and CD8^{+}T cells which has consistently been shown to be decreased in patients with CFS/ME (Brenu et al., 2010, Klimas et al., 1990, Maher et al., 2005, Fletcher et al., 2010). If cytotoxic activity of NK cells is deemed a possible biomarker for CFS/ME (Fletcher et al., 2010), then it is imperative to determine all components of this process that may be compromised.

Natural Killer and CD8^{+}T cells are important apoptosis inducers, therefore, a down regulation in *miR-10a, miR-146a, miR-223* and *miR-191* may affect the efficiency of these cells to induce apoptosis of target cells (Subramanian and Steer, 2010). These

miRNAs promote apoptosis by targeting the 3'UTR region of *BCL2*, *CDK6* and *BAX,* thus inhibiting their expression at the post-transcriptional level and potentially leading to increased programmed cell death (Nishi et al., 2010). Although the action of these miRNAs in cytotoxic cells is not explicitly known, the genes targeted by these miRNAs (Table 2) may suggest an involvement in the cytotoxic activity of NK cells (Xia et al., 2009, Satzger et al., 2010). Hence a decrease in their expression may reduce the induction of cell death and potentially affect the commitment of these cells to trigger apoptosis of viral infected cells in CFS/ME. Interestingly, *miR-146a* is modulated by NFκB and suppresses the expression of Toll-like receptor signalling related proteins TRAF6 and IRAK1 in immune cells (Taganov et al., 2006, Kawai and Akira, 2007, Shen et al., 2008, Hurst et al., 2009). This results in a decrease in TNF-α and helps to maintain inflammatory tolerance promoting cell survival and preventing the occurrence of hyperactive immune system (Nahid et al., 2009). In our CFS/ME patients TNF-α secretion by PBMCs after mitogenic stimulation was significantly increased at the time these measurements were performed (Brenu et al., 2011). NFκB also regulates the expression of pro-inflammatory cytokines TNF-α, IFN-γ, IL-1β, IL-2 and IL-12 (Nakasa et al., 2008), hence a decrease in *miR-146a* may be associated with decreases in NF-κB thus influencing cytokine expression. Incidentally, significant decreases in *IFN-G* were observed in the NK cells from our CFS/ME cohort compared to the non-fatigued controls (Brenu et al., 2011). This suggests a possible decrease in *IFN-G* induced cytotoxic activity in CFS/ME patients.

Additionally, *miR-191* facilitates p53 function by targeting inhibitors of p53 such as Mdm4 (Wynendaele et al., 2010), hence low levels of *miR-191* may affect cell cycle events related to p53 regulation in CFS/ME patients. Using the microrna.org (http://www.microrna.org) database we also found potential target sites for *miR-191*, these include *BCL214* and *IL2RA*. A direct association of miRNAs and cytotoxic activity may be associated with *miR-152* which targets inhibitory human leukocyte antigen–G (Zhu et al., 2010). CFS/ME patients have reduced cytotoxic cell function and *miR-152* may be implicated in this pathway. Importantly, *miR-223* has been shown to target the 3'UTR region of the *GZMB* mRNA in mice (Fehniger et al., 2011). Although it is not known what other miRNAs target granzymes, in our previous study a reduction in *GZMA* and *GZMK* was observed in the CFS/ME group in comparison to the non-fatigued controls (Brenu et al., 2011). Granzyme function and regulation are perhaps associated with the presence of certain miRNAs at the 3'UTR regions of these granzyme genes.

The miRNAs, *miR-21, miR-106, miR-152* and *miR-17-5p* promote cell proliferation (Chung et al., 2010, Pan et al., 2011, Subramanian and Steer, 2010). They inhibit apoptosis by negatively regulating apoptotic tumour suppressor related genes *PDCD4*, *PTEN*, *TMPI* and *RECK* (Li et al., 2009, Asangani et al., 2008). The observation of decreases in these miRNAs is counter-intuitive as similar findings were observed in the apoptotic promoters. This presupposes that these miRNAs may be involved in other aspects of immune function in CFS/ME. Similarly, these miRNAs also regulate certain cytokines produced by NK cells. For example *miR-21* down regulates IL-12 which

inhibits cytotoxic activity (Mehrotra et al., 1993), thus a down regulation in *miR-21* results in an over-expression of IL-12 which may severely dampen cytotoxic activity in NK cells. *miR-21* is important during the development and differentiation of effector CD8^{+}T cells (Salaun et al., 2011a), although the purpose of this increase is unclear. During pathogen invasion or immune insults CD8^{+}T cells and NK cells may be primed by *miR-21* and this may facilitate the cytotoxic activity of these cells as they both showed a similar decrease in *miR-21*. *miR-103* was significantly decreased in the NK cells of CFS/ME patients. It may have a role in the creation of the RISC (Tan et al., 2011).

Parallels can be drawn between the findings in this study and our previous work. In particular, *miR-146a* and *miR-21* regulate FOXP3 and Treg function (Rouas et al., 2009), which was observed to be compromised in our previous study (Brenu et al., 2011). Similarly, decreases in the expression of *miR-152* and *miR-21* may be responsible for the reduced cytotoxicity of NK and CD8^{+}T cells respectively in CFS/ME patients (Brenu et al. 2011). Heightened levels of *miR-152* may promote effective NK cytolytic activity while *miR-21* is vital for the delineation of CD8^{+}T cells in to effector cells (Salaun et al., 2011; Zhu et al., 2010).

The above mentioned miRNAs may serve as potential biomarkers for diagnosing and identifying CFS/ME patients. The methodology described in this paper may be tedious in clinical settings, however, identification of miRNA biomarkers using plasma or serum may be more useful. Analysis of circulating miRNAs in plasma and serum can

serve as biomarkers for cancer and other autoimmune diseases (Chen et al., 2008). Identification of miRNAs as biomarkers for CFS/ME may increase our understanding of the disease presentation and establish better diagnostic strategies for these patients.

5.6. Conclusion

In summary, miRNAs may play a role in immunological changes associated with CFS/ME. This study has identified miRNAs that may be used as potential biomarkers for CFS/ME, specifically, *miR-146a*, *miR-223* and *miR-21*. Although, the current literature on the influences of these miRNAs on NK and CD8$^+$T cell related lytic proteins, receptors and cytotoxic activity is limited, the presence of similar expression levels of miRNAs in both cells suggests a common pathway of the miRNAs in these cells. Cells that are regulated by these miRNAs could be important in understanding the mechanism of CFS/ME. Further studies are required to determine the exact genes regulated by these miRNAs in cytotoxic cells and their longitudinal expression patterns.

6. Final Discussion and Conclusion

This project has identified NK cytotoxic activity and NK phenotypes as potential biomarkers for CFS/ME. Additionally, this project has also for the first time identified novel immune parameters involved in the dysregulated immunological pathway in CFS/ME. These parameters include FOXP3, VPACR2 and miRNA and the relevance of these findings to CFS/ME is discussed below.

The establishment of decisive biomarkers for CFS/ME facilitates proper diagnosis and alleviates patient distress and financial burdens. Generally, biomarkers are key cellular or molecular events that are used as indicators of a biological state such as a specific disease. They can be measured in human body fluids as an indicator of normal biological functions, pathogenic processes and pharmacological responses to therapeutic intervention using available technologies (Naylor, 2003). For a biomarker to be useful they have to display certain important characteristics which include determination of the duration of a disease, recognition of possible pathways related to the disease, detect variability and modifications, decrease incidences of misclassification, are highly reliable, valid and repeatable (Mayeux, 2004). This study has identified a number of parameters with properties that make them potential biomarkers for CFS/ME diagnosis.

The results from this project illustrate significant decreases in cytotoxic activity, NK phenotypes, *GZMA, GZMK, IFNG* mRNA and miRNA genes with significant increases in *PRF1* mRNA, FOXP3 and VPAC2R proteins in CFS/ME patients compared to non-fatigued controls. Similarly in CFS/ME patients, compared to the non-fatigued controls, cytokine levels were inconsistent with no exact pattern yet discernible. Most gene

studies on CFS/ME have focused substantially on mRNA expression with little focus on small non-coding miRNAs. Despite the limited knowledge on miRNAs, a number of molecules have been implicated to be important immune and physiological functions; therefore they may have a role in CFS/ME.

Overall, the results emanating from this study strongly support an inept immune system in CFS/ME exemplified by decreases in cytotoxic activity, granzyme gene expressions (*GZMA* and *GZMK*), NK phenotypes and miRNAs with increases in perforin gene expression (*PRF1*), FOXP3, VPAC2R and differential expression of cytokines. The immune markers investigated were selected based on their salient role in innate and adaptive immune function. The heterogeneity and persistence of CFS/ME over many years or even decades with differing levels of severity, necessitates an inquiry into the condition of immune compromises during the course of the disease. Moreover, as this project explores biomarkers for CFS/ME it is obligatory to establish the pattern and stability of these markers over time before we can classify them as clinically useful markers for CFS/ME. Therefore a number of these immune parameters, specifically, cytotoxic activity, NK phenotypes and cytokines, were assessed over a period of 12 months.

6.1.Impaired Cytotoxic Activity in CFS/ME

The observation of a decrease in cytotoxic activity has been previously reported (Klimas *et al*. 1990; Fletcher *et al*. 2010; Maher *et al*. 2005). Recently, in cross-sectional studies cytotoxicity has been identified as an important marker in CFS/ME (Fletcher *et al*., 2010). The significance of the present findings is that these cytotoxic changes were confirmed at different time points in the course of the disease, which has not been previously shown. Thus, in the present study, NK lysis activity remained consistently decreased throughout the duration of the study, i.e. at baseline, 6 and 12 months. This not only confirms an involvement of cytotoxic activity in CFS/ME but also demonstrates that this persists overtime. Although, the causal factor eliciting these changes is not fully known, a few propositions can be put forward. These include decreases in cytolytic molecules mRNA and protein levels (Maher *et al*. 2005, Saiki *et al*., 2008). At baseline cytotoxic activity was measured in conjunction with granzyme and perforin. The mRNA expression levels of granzyme A (*GZMA*) and granzyme K (*GZMK*) were significantly decreased while perforin (*PRF1*) was increased in the CFS/ME patients compared to non-fatigued controls (Brenu *et al*. 2011). Perforin and granzymes are important lytic proteins required for the granule dependant cytotoxic activity pathway (Dustin and Long, 2010). Perforin protein has been suggested to act as a medium to facilitate the entry of granzymes into the target cells where granzymes induce apoptosis by inducing the release of anti-apoptotic BCL2, caspase activation or double and single stranded DNA nicks (Cullen *et al*., 2010).

This is the first study to observe a decrease in *GZMK* gene expression in CFS/ME patients. At the protein level, both GZMA and GZMK act independently of caspases and induce the accumulation of reactive oxygen species (ROS) in the mitochondria (Beresford *et al*., 1999). *GZMA* shares homology with *GZMK*, they are tryptases and are both found co-located at chromosome 5q11 (Bovenschen *et al*., 2009, Hua *et al*., 2009, Jenkins *et al*., 2008). GZMA acts by activating the caspase-independent programmed cell death pathway, using the substrate enzyme pro-interleukin 1β which activates pro-inflammatory IL-1 (Irmler *et al*., 1995). The exact role of *GZMK* is not fully known, nonetheless it is thought that in the absence of *GZMA*, *GZMK* is able to salvage the caspase-independent programmed cell death pathway and where they are both decreased, it may suggest failure to effectively remove pathogens (Pardo *et al*., 2009). Additionally, *GZMA* and *PRF1* are polymorphic, thus, phenotypic differences within the gene may affect their effectiveness to induce effectual pathogen lysis (Girnita *et al*., 2009, Padovan *et al*., 2011). Cytokines such as IL-2 regulate the expression of *GZMA* and *PRF1*. Differential expression in these cytokines may affect the expression of these genes and consequently cytolysis in all NK phenotypes (Janas *et al*., 2005). Transcriptional and post-transcriptional processes contribute to differential expression in *GZMA*, *GZMK* and *PRF1* for example activation of T cell receptor and co-stimulation from cytokines are fundamental mechanisms for inducing the expression of these molecules (Ruike *et al*., 2007). Similarly, signal transduction pathways involving the interaction between cytolytic genes and transcription factors initiate the expression of these molecules and their transcripts (Chowdhury and Lieberman, 2008). This suggests that the aberrant expression of these molecules in CFS/ME patients entails

other molecular networks that are probably dysregulated in CFS/ME. As mentioned previously mRNA expression is not necessarily representative of protein expression. However, in the occurrence of differential expression of *GZMA*, *GZMK* or *PRF1* cytotoxic activity can be severely compromised.

The decrease in *GZMA* and *GZMK* gene expression was not correlated with a decrease in *PRF1* and this is consistent with the observation that perforin and granzymes genes may work through different pathways (de Saint Basile *et al*., 2010). These results may translate into decreases in granzyme proteins however, as mRNA expressions are not wholly indicative of protein levels this may not apply in the case of *PRF1*. Perforin proteins have been shown to be significantly decreased in some cases of CFS/ME (Maher *et al*. 2005).

A decrease in cytotoxic activity corroborated with an increase in *PRF1*, which may reflect inadequacies in other regulatory mechanisms such as transcription factors that control the expression of these lytic genes. These transcription factors may be disproportionately expressed, thus translating into differential expression of these genes. In certain melanomas, elevated levels of *PRF1* have been reported and these were associated with deficits in cytotoxic activity (Guillot *et al*., 2005). CD2 has recently been shown to play a pivotal role in the reduced NK cytotoxic activity noticed in NK cells of CFS/ME patients. Importantly, CD2 acts as a co-stimulatory molecule and stimulates NK cells. This activates the src-family kinases leading to the production of inositol triphosphates and efficient target killing (Cheent and Khakoo, 2009). Other

possible contributors to the reduced cytotoxic activity may be related to cytotoxic receptors such as the killer cell immunoglobulin-like receptors (KIRs). In some CFS/ME patients, there is evidence indicating an increase in KIR3DS1 with indications for a loss in HLA-Bw4Ile80 binding motif for the KIR3DL1 and KIR3DS1 receptors (Pasi *et al*., 2011). A high incidence of inhibitory KIR receptors in combination with a deficiency in binding motifs may oppose efficient cytolysis and increase disease prevalence (Blackwell *et al*., 2009). Generally, HLA-B and its clusters tend to have protective effects that suppress infections. Similarly, a decrease in cytokines such as IL-18 can affect perforin and granzyme production and effective lysis (Hyodo *et al*., 1999).

Natural Killer cytotoxic activity was not affected by seasonal variations in CFS/ME patients as it remained consistent over time confirming that NK cytotoxic activity is a hallmark of CFS/ME patients. However, there may be a strong association between cytotoxic activity and miRNA expression pattern as these molecules were shown to be decreased in the cytotoxic cells (as detailed in Chapter 5). MicroRNAs regulate the transcription of genes and it is highly plausible, that most of the genes they modulate are responsible for NK and CD8^{+}T cell lysis of pathogens. A down regulation makes these cells redundant in the overall immune function of CFS/ME individuals.

The observation that the reduced cytotoxic activity remains stable over time makes it a useful biomarker for CFS/ME and thus advocates its use for diagnosing CFS/ME. The use of cytotoxic activity may encourage early detection of CFS/ME. Incidentally cytotoxic activity may be the hallmark of CFS/ME and its inefficiency ultimately

generates other immune function deficits as noted in this study. Additionally, using cytotoxic function as a primer for diagnosing CFS/ME may assist in differentiating subtypes of CFS/ME patients. CFS/ME patients have been previously subgrouped based on their symptoms and severity of illness. Subtyping of patients under these conditions when applied to genomic studies has successfully identified both universal and distinctive physiological pathways that are associated with these subtypes of CFS/ME patients (Kerr *et al*., 2008). Subtypes of CFS/ME patients may exist in the studied population and this may be related to cytotoxic activity. Additionally, in-depth clustering analysis of miRNAs may be associated with disease severity and cytotoxic activity.

6.2.NK Phenotypes

NK phenotypic studies demonstrated significantly lower levels of CD56^{bright}CD16^{negative}NK cells in CFS/ME patients compared to the non-fatigue controls. CD56^{dim}CD16^{positive}NK cells remained unchanged in both CFS/ME and non-fatigued controls. The CD56^{bright}CD16^{negative}NK cells are highly proliferative, less cytotoxic and produce significant amounts of cytokines, IL-10, IL-13, IFN-γ, TNF-α and GM-CSF compared to the CD56^{dim}CD16^{positive}NK cells (Cooper *et al*., 2001). The CD56^{dim}CD16^{positive}NK cells are more cytotoxic and secrete less cytokines (Cooper *et al*., 2001). It would be expected that a decrease in cytotoxic activity may transpire from reductions in the main cytotoxic NK cells, that is, CD56^{dim}CD16^{positive}NK cells. However, this was not the case in this cohort of CFS/ME patients. Considering the function of CD56^{dim}CD16^{positive} and The CD56^{bright}CD16^{negative}NK cells, reduced levels of CD56^{bright}CD16^{negative}NK cells are detrimental to efficient innate immune function. CD56^{bright}CD16^{negative}NK cells activate dendritic cells and monocytes to respond to pathogen invasion (Vitale *et al*., 2004, Dalbeth *et al*., 2004). Additionally, the adhesion molecule CD2 is strongly expressed on CD56^{bright}CD16^{negative} NK cells and facilitates the migration of these NK cells into the secondary lymphoid tissues (Cooper *et al*., 2001, Poli *et al*., 2009). Perhaps in CFS/ME CD2 expression is decreased and this may be related to a limited number of CD56^{bright}CD16^{negative} NK cells in these sites.

Another possible explanation for the decrease in CD56^{bright}CD16^{negative} NK cells may be related to the notion of an increase in lymphocyte apoptosis among some CFS/ME patients (Kennedy *et al*., 2004). This is uncertain as CD56^{bright}CD16^{negative} NK cells are

more resistant to apoptosis owing to their ability to resist reactive oxygen species (Harlin *et al*., 2007). Reductions in CD56^{bright}CD16^{negative} NK cells may reflect the low expression of *IFN-γ* in the NK cells of the CFS/ME patients. IFN-γ produced by NK cells plays a pivotal role in T cell activation by promoting Th1-like immune responses, generation of cytotoxic and memory T cells, while suppressing inflammation and infection by suppressing Th17 cytokines (Agaugue *et al*., 2008, Mailliard *et al*., 2003, Martin-Fontecha *et al*., 2004, Robbins *et al*., 2007, Lo *et al*., 2008).

Immune cell phenotyping to date in general has not been the most reliable marker for differentiating patients with CFS/ME from patients without the condition. This may have occurred due to the lack of exclusivity within the lymphocyte phenotypes. Most of the markers used in delineating lymphocyte phenotypes are partially expressed on most of these cells. Selection techniques that preferentially distinguish lymphocyte populations as used in this study may be the most optimal method of determining the exact phenotype within the major lymphocyte subsets that show inequality in their levels. Currently, this implies a potentially costly biomarker, therefore, administering it in the clinical setting may be difficult. These findings also open avenues for targeting new treatment options where the aim might be to develop pharmaceutical targets that are cell specific.

6.3. Impaired Distribution of immune regulators

The FOXP3 and VPACR2 protein expressions were increased in the CFS/ME patients in comparison to the non-fatigued controls. Profound increases in FOXP3 are suggestive of amplifications in the suppressive activities of regulatory T cells. This implicates possible increases in both anti- and pro-inflammatory immune reactions in CFS/ME patients. Levels of FOXP3 are correlated with the suppressive function of Tregs. Hence, it is possible that FOXP3 increased in an attempt to decrease potential incidences of over expressed pro-inflammatory reactions in the immune system of CFS/ME patients. At baseline an increase in IL-10 occurred in conjunction with an increase in FOXP3. This to some extent is indicative of heightened immune activation in CFS/ME. Heightened levels of FOXP3 have been used as markers for certain diseases with compromised immune function such as HIV where FOXP3 levels were increased. This was correlated with disease severity (Suchard *et al*., 2010). Additionally, these increases in FOXP3 may be related to compromises to receptors that reduce the suppression. Presently, it is not known which types of Tregs were increased in the CFS/ME patients. It is possible that these were adaptive Tregs, differentiated from conventional Tregs due to the presence of high levels of antigens in the circulation (Sakaguchi *et al*., 2008). Deficiencies in FOXP3 typically suggest an autoimmune disease while heightened levels of FOXP3 have been observed in a number of human carcinomas (Wang *et al*., 2009). An increase in the levels of FOXP3 may be detrimental as this can severely decrease viral elimination by overly suppressing important anti-inflammatory immune responses required for pathogen clearance (Belkaid, 2008).

Heightened levels of FOXP3 reduces IL-17 as the available number of cells, i.e. the Th17 cells, are reduced in the presence of high levels of FOXP3 (Ziegler and Buckner, 2009). This may explain the observed reduced levels of IL-17 cytokines at 12 months. High levels of FOXP3 can also inhibit the activation of $CD4^+T$ and $CD8^+T$ cells, which consequently decreases $CD8^+T$ cell cytotoxicity (Xu *et al*., 2009, Guo *et al*., 2010). FOXP3 over-expression also reduces the following cytokines IL-2, IL-4, IL-17 and IFN-γ (Zhu and Paul 2008). Elevations in FOXP3 Tregs confers a hypo-active immune state (Yun *et al*., 2010) and in our CFS/ME patients this possibly affects all other known immune functions modalities including NK cell and $CD8^+T$ cell cytotoxic activity (Hinz *et al*., 2007). Whilst TGF-β was not measured in this study, elevations in FOXP3 may suggest an increase in TGF-β which subsequently inhibits the activity of NK cells by repressing the action of IFN-γ, TNF-α and GMCSF, and also reducing the action of cytotoxic receptors NKp30 and NKG2D (Marie *et al*., 2006, Bellone *et al*., 1995, Castriconi *et al*., 2003). These may therefore have additional effects that contribute to the decrease in cytotoxic activity.

In Treg cells, the RNase Dicer is an important enzyme that determines the type of phenotype that is produced, and deletion of Dicer results in a more aggressive phenotype (Liston *et al*., 2008). This suggests an important role of miRNAs in regulating Treg development and function. Regulation of *FOXP3* expression may be associated with *miR-21* and *miR-31* which confer agonistic and antagonistic function on *FOXP3* respectively (Redouane *et al*., 2009). Importantly, *miR-146a* is most abundant in Tregs and regulates the functional capacity of Tregs required to control Th1 related

responses including production of IFN-γ by dampening STAT1. Hence *miR-146a* is an important molecule ensuring effective Treg suppressor function (Lu *et al*., 2010). In this study, a down-regulation of *miR-146a* was observed in NK cells and this may likely affect the function of these cells in CFS/ME. The exacerbated expression of FOXP3 expression in CFS/ME may not necessarily correlate with excessive suppression.

Additionally, a compromise in VN function may be a hallmark of CFS/ME. VNs during aberrant immune regulation are able to decrease TNF-α, IL-6 and increase Il-10. In the current project, the increase in IL-10 and VPAC2R may be due to the action of VNs to reducing the pro-inflammatory state occurring as a result of an increase in TNF-α and IFN-γ. However since a decrease in TNF-α and IFN-γ did not occur it is likely that this mechanism is compromised in CFS/ME, that is the anti-inflammatory reaction from IL-10 and receptor activations are not enough to inhibit over-reactive pro-inflammatory reactions (Abad *et al*., 2001). VPAC receptor activation stimulates downstream second messenger systems including adenosine and the PKA pathways, the result of which is the production of cAMP. Activation of a high number of VPAC receptors may increase adenosine and consequently intracellular cAMP which weakens cytotoxic activity (Hoskin *et al*., 2008). VPACR regulation is related to the normal circadian function and changes in their expression may perturb the circadian rhythm (Pretzmann *et al*., 2008), possibly explaining the sleep disturbances among CFS/ME patients. However elevation in *VPAC2R* may not necessarily mean an increase in VIP as in some cardiomyopathies increases in *VPAC2R* mRNA was not associated with an increase in VIP (Dvorakova *et al*., 2006). The exact role of high VPAC2R is currently not known and most measures

of VPAC2R were performed on brain samples hence it is very difficult to understand how these may have contributed to the immune profile in CFS/ME patients. Overexpression of VPAC2R may decrease cytotoxic activity in particular FasL directed cytotoxicity by inhibiting the expression of FasL on CD8$^+$T cells (Delgado and Ganea, 2000a). Similarly, VIP inhibits IL-12 and this acts to decrease cytotoxicity of NK and T cells (Delgado *et al*., 1999c).

Heightened immune activation is present in CFS/ME and in an attempt to modulate these abnormal reactions, the immune system inadvertently increases known mechanisms of suppression. In CFS/ME patients these suppressive reactions persist for a longer duration as the immune system is not able to effectively clear these heightened immune reactions. However, the converse may be true where discrepancies in the pro-inflammatory mechanisms alter the immune system. Moreover, high antigen load due to pathogen prevalence may account for these observations. Persistent viral or microbial antigen load initially activates the pro-inflammatory reactions. These reactions are dampened by the anti-inflammatory reactions and at the same time anti-inflammatory reactions are activated to restore immune balance. However this overstrains other immune activities thus allowing pathogens to persist and avoid elimination. Therefore, further studies are required to elucidate and validate the exact role of VPAC2R in CFS/ME patients.

Longitudinal assessment of cytokine secretion has previously been measured in a cohort of adolescents with severe cases of fatigue. Similar to the results presented here, the

cytokine distribution was not consistent over time and also varied at the three different time points at which they were measured (ter Wolbeek *et al*., 2007). The pattern of cytokine distribution followed an initial increase in the production of cytokines at the initial time point, in the fatigued adolescent this was followed by a drop in cytokine levels at 6 months and an increase at 12 months (ter Wolbeek *et al*., 2007). Similar to our findings, anti-inflammatory cytokine IL-10 was significantly increased in the fatigued patients compared to the controls at baseline (Brenu *et al*., 2011, ter Wolbeek *et al*., 2007). Additionally, a cohort of CFS/ME patients within the ter Wolbeek *et al*. (2007) study were shown to have an anti-inflammatory bias in cytokine secretion where CFS/ME patients had significantly lower levels of TNF-α and IFN-γ in comparison to the fatigued and the non-fatigued controls (ter Wolbeek *et al*., 2007). This is in contrast to the findings from this project where IFN-γ and TNF-α were increased in the CFS/ME group relative to the controls (Brenu *et al*. 2011). The pattern of cytokine secretions was attributed to seasonal variations (ter Wolbeek *et al*., 2007). In the present project variations in cytokine distribution were noticed between the two subject populations for the duration of the study. The differences in findings between the ter Wolbeek *et al*. (2007) study and this project can be explained by the stimulant used. In our study the cells were stimulated with PHA which induces mitogenesis, while in the ter Wolbeek (2007) study cells were stimulated with LPS to produce inflammation. Additionally, the cytokines at baseline may have increased because of other cell types such as the Treg cells. Although cytokine secretions from the Treg cells were not measured, the elevated levels of anti-inflammatory IL-10 can be explained by a substantial increase in the levels of FOXP3.

The fluctuations in cytokine levels suggest that they may not be the best biomarkers for CFS/ME. Nonetheless, this may be an indicator for other aspects of the disease such as disease progression and disease severity. Per chance cytokine fluctuations correspond to disease severity as the patient gets better or worse the cytokine distribution changes in that order (Bozza *et al*., 2005, Cheung *et al*., 2002, Gardlund *et al*., 1995, Jacob *et al*., 2003, van Deuren *et al*., 1995). This further suggests that cytokine skewness either towards a predominant pro- or anti-inflammatory secretion does not necessarily exist in CFS/ME; however, this may potentially reflect the severity of the disease. In our present study disease severity was not investigated and this may be a topic for further studies.

Th1/Th2/Th17 cytokine studies were not confined preferentially to CD4^{+}T cells but to PBMCs, it is possible that production of cytokines by other cells may have contributed to the cytokine milieu in the CFS/ME patients this study. NK cells in particular secrete high levels of IFN-γ which can increase Th1 immune response and thus cause them to secrete both IFN-γ and TNF-α (Martin-Fontecha *et al*., 2004). They can also regulate Th1 immune response by producing IL-10 to decrease Th1 immune reactions (Deniz *et al*., 2008), while causing an increase in Tregs through the production of TGF-β (Horwitz *et al*., 1999, Horwitz *et al*., 1997). Over-expression of FOXP3 can lower cytotoxic activity (Trzonkowski *et al*., 2006) while an increase in Th17 cells may increase lysis through the secretion of IL-21 (Liu *et al*., 2009).

Natural Killer cell interaction with other immune cells (in particular T cells) is dependent on a number of co-stimulatory molecules. Alterations in the expression of

these molecules are known to affect T cell function (Sharpe, 2009). For example, binding of CD80, CD86, CD70, Ox40 ligand and 2B4 on the NK cells to CD28, CD27, Ox40 and CD48 on the T cells translates into effective cytotoxic and memory T cells, expansion and proliferation of T cells (Hanna and Mandelboim, 2007). Hence, further studies are now required to determine the association of cytokines and disease severity in CFS/ME patients.

Moreover, these attributes may relate to the advancement in the disorder. Fluctuations in CFS/ME over time presuppose that a unique cytokine profile exists at the initial stages of the disease and with the advancement or progression in symptoms these become obscured. Hence, cytokines may be useful during early detection of CFS/ME and in this way they may contribute to the biomarker panel for CFS/ME. However, FOXP3 and VPAC2R are likely candidates for generating CFS/ME biomarkers. These parameters in general provide information on the pro- and anti-inflammatory reactions in the immune system and are therefore important in regulating cytokine distribution patterns. The lack of alignment between FOXP3, VPACR and cytokine pattern in CFS/ME presupposes distinct pathways in maintaining and activating these proteins. Further validity test are required to confirm these parameters.

6.4. The Profile of miRNAs in CFS/ME

MicroRNAs have only recently been discovered and therefore their exact role in all cell types has not yet been characterised (Sun *et al*., 2010). In this project miRNA gene expression was investigated in NK and CD8^{+}T cells. Although, the data presented here suggests a role for these molecules in immune function especially in cytotoxic cells, the exact genes targeted by these miRNAs are yet to be fully determined. Little is known currently about these miRNAs in relation to the cells that were measured, but they have been examined in cancer and cardiovascular diseases and therefore we have suggested similar roles in NK and CD8^{+}T cells (Lu *et al*., 2008). The results from this study suggest that miRNAs could be used as biomarkers for diagnosing CFS/ME. As these molecules are more stable than mRNA, their role in CFS/ME may be important for diagnosis. MicroRNAs could be responsible for the decreases in cytotoxic activity noticed in cytotoxic cells.

The observation of a decrease in miRNA levels on CFS/ME patients compared to controls may alter their effects on particular genes and possibly promote either a hyper or hypo state in immune function by targeting particular genes (Pauley and Chan, 2008). The importance of these miRNAs relates to their functional connotations where most of them regulated cellular apoptosis, cell proliferation and varying immune related pathways. Therefore, an inadvertent decrease in their optimal levels affects their function, alters gene expression and possibly affects the regulatory effects of the protein they target.

Importantly, the observed roles of some of these miRNAs in the regulation of apoptosis suggest an essential role of miRNAs in the activation and control of cytotoxic activity. For example, *miR-223* which showed marked reductions in CFS/ME patients compared to non-fatigued controls, targets and regulates the expression of *GZMB* which is a necessary factor in the granule induced exocytosis pathway (Fehniger *et al*., 2011). In the present study *GZMB* was not measured, however, decreases in the expression of *GZMA* and *GZMK* were observed (Brenu *et al*., 2011). It is plausible to infer that *miR-223* possibly also regulated these granzyme genes and this may account for their diminished expression. Per chance, a down regulation in *miR-223* negatively affects the expression pattern of *GZMA* and *GZMK* and this may also manifest in the form of diminished cytotoxic activity. Notably these patterns of expression in *miR-223* and *GZMA* and *GZMK* were similar in both the NK and CD8^{+}T cells indicating that cytotoxic activity within these cells entails analogous pathways that are regulated by miRNAs. *MiR-152* may be important for NK cell cytolysis as increased levels of *miR-152* is beneficial to NK cytolytic activity (Zhu *et al*., 2010). Nonetheless, phenotypic differences within the cells may also account for the lack of efficient cytotoxicity in the CFS/ME cohort.

Lymphocyte differentiation, proliferation and expansion are also regulated by miRNAs (Belver *et al*., 2011). For example, *miR-21* modulates the differentiation of CD8^{+}T cells in to the various subsets, thus inefficient cytolysis might ensue from the type of CD8^{+}T cells present in the circulation (Salaun *et al*., 2011b). Low expression of *miR-21* possibly contributes to the generation of CD8^{+}T cells with less cytotoxic properties or

poor memory CD8$^+$T cells. CD56^{bright}CD16^{negative}NK cell decreases in CFS/ME may to some extent ensue from the diminished expression in miRNAs that regulate the differentiation of NK cells and possibly transcription factors responsible for NK delineation.

Activation of the T cell receptor in CD8$^+$T cells is an important component of the cytotoxic mechanism, however, this also stimulates *miR-21* which regulates TCR signalling by targeting the *RASGRP1* (Sonkoly and Pivarcsi, 2009, Wu *et al*., 2007b). *MiR-21* is also induced in other inflammatory diseases (Takagi *et al*., 2010). If *miR-21* is essential for regulating TCR activities their down-regulation may affect many T cell activities such as pathogen recognition, cytokine secretion and cytolysis which are dysregulated in CFS/ME. Importantly, *miR-21* regulates cytokine production such as IL-12 which is responsible for Th1 immune responses (Lu *et al*., 2009b). In addition, *miR-21* is activated by NFκB, however it can act to decrease or dampen the NFκB by targeting *PDCD4*, which dampens the activity of NFκB and the induction of pro-inflammatory responses (Shin *et al*., 2011, Sheedy *et al*., 2010). Other miRNAs, including *miR21, miR-15b, miR-146a* and *miR-223* have an involvement in the NFκB signalling pathway (Ma *et al*., 2011). Although, it is not known whether NFκB has an effect in CFS/ME, their role in pathogen recognition is imperative for immune function. They regulate many immune related molecules including chemokines and cytokines (Ma *et al*., 2011). Activation of the NFκB signalling pathway is dependent on the type of stimulus present (Miyamoto, 2011). The observation that *miR-15b, miR-21 and miR-223* are down-regulated could suggest an impaired NFκB signalling in these cells.

Cytokine distribution in the present study was differentially expressed with no singular pattern over time that may be specific for CFS/ME. Despite these shortcomings, atypical NFκB signalling either directly or downstream from this pathway as a consequence of low miRNA expression may produce perturbations in the inflammatory pathways. Anti and pro-inflammatory signalling can be mediated by miRNAs given that these molecules regulate a vast majority of genes and their function may be different in each cell type. For example, the action of miR-21 on certain genes such as *PDCD4* and *IL-12p35*, *STAT3* and *TLR4,* regulates both anti and pro-inflammatory pathways necessary to maintain homeostasis (Kumarswamy *et al*., 2011). Although the present findings are limited to NK and CD8$^+$T cells, given that miRNAs regulate a plethora of immune genes they may be differentially expressed in other immune cells. Suggestively, miRNAs may be implicated in the immune compromises in the CFS/ME patients. Correspondingly, *miR-21* directly regulates FOXP3 expression in regulatory T cells (Rouas *et al*., 2009). These extensive properties of *miR-21* in immune function strongly suggest a role of this miRNA in most of the altered immune activities in CFS/ME. Decreases in *miR-21* expression may affect the differential expression of cytokines, cytotoxic activity and possibly facilitate the over–expression of FOXP3 via other pathways. Thus, *miR-21* could be a candidate for a biomarker for CFS/ME.

Additionally, differential expression of miRNAs may exist in other cell types in CFS/ME patients and this may prospectively explain the differential cytokine distribution and heightened FOXP3 and VPAC2R expression noticed at baseline. Importantly, *miR-525-5p* has been noted to target and down-regulate *VPAC1* gene in

monocytes (Cocco *et al*., 2011). As expressions of most miRNAs were decreased in our CFS/ME patients, it can be postulated that a similar trend may occur in other immune cells and this may prompt an increase in VPACR1 in CFS/ME. Given that miRNAs are stable molecules they may also remain stable over time. Here the present study provides only baseline data on the miRNAs, however, stability of miRNAs over time can be inferred from the maintenance of reduced cytotoxic activity.

Although, only recently discovered, miRNAs are emerging as putative biomarkers in a number of neuroimmune disorders and cancers (Duroux-Richard *et al*., 2011, Yu *et al*., 2011). These non-protein coding molecules can be identified both in the intracellular and extracellular environment and in both instances they have shown to be reliable biomarkers. Importantly, in lung cancer *miR-1254* and *miR-574-5p* have been identified as indicators for early onset of non-small cell lung cancer (Foss *et al*., 2011). Similarly, detection of differential expression of miRNAs at different stages of prostate cancer has been useful in determining disease progression (Long *et al*., 2011). In breast cancer over expression of *miR-10b* and *miR-34a* in association with a reduced expression of *miR-195* and *let 7a* has helped to distinguish between cancer patients and non-cancerous tumours (Andorfer *et al*., 2011, Weigel and Dowsett, 2011). Identifiers for detecting hepatocellular carcinoma include *miR-21, miR-221, miR-222* and *miR-155* (Yoon *et al*., 2011). Hence, miRNAs are useful markers for the detection of various cancers. In other diseases such as autoimmune Sjogren's syndrome, *miR-768-39* and *miR-574* are candidates for diagnosing this disease (Alevizos *et al*., 2011). Equally, miRNAs are relevant in neurological disorders such as Alzheimer's disease where at least eight of

these miRNAs (*miR-9, miR-132, miR-146b, miR-145, miR-29a, miR-129b, miR-423 and miR-98*) have been identified as candidates for differentiating patients with or without Alzheimer's disease (Cogswell *et al*., 2008). Not only have they been identified in neurological disease but a vast majority of research has also focused on the role of miRNAs in cardiovascular disorders. Studies have identified biomarkers that may be associated with myocardial infarction and heart failure (Meder *et al*., 2010).

MicroRNAs may be recommended as biomarkers for CFS/ME given the present data. This is mainly related to the stability of these molecules. MicroRNAs are found in the blood plasma and serum samples and unlike the mRNA these miRNAs are stable and highly resistant to RNases (Mitchell *et al*., 2008, Chen *et al*., 2008). Uncovering of these molecules in the extracellular environment may be a faster and more effective way of determining their targets. Moreover, the correlation between miRNAs and different stages of cancer or onset of a particular disease may potentially be useful in determining and defining subclasses of CFS/ME. In principle, a general trend of reduced miRNA expression permits the establishment of miRNA finger printing which may be useful in generating a complex of biomarkers with regulatory effect that are specific in CFS/ME. Furthermore, significant reductions in similar miRNAs in both cell populations, i.e. NK and CD8^{+}T cells, signify the existence of analogous pathways that are targets of these miRNAs. MicroRNAs may be a more robust approach to biomarker development for CFS/ME in view of the fact that CFS/ME is a disorder with a heterogeneous presentation. Additionally, miRNA expression patterns may be related to the time

course of the disease, perhaps miRNA progressively decreases in expression significantly as the disease often persists over decades.

Identification of the exact genes that these miRNAs regulate in NK and CD8^{+}T cells may help explain the reduced cytotoxic activity in CFS/ME patients.

6.5. Limitations

The study design of the present project has a number of shortcomings. The patient group comprised CFS/ME patients who had CFS/ME for many years. Conceivably, a subject group that was recently diagnosed with CFS/ME may have been more ideal in determining the time course and mechanism of CFS/ME. Although, cytokine measurements have demonstrated abnormal levels of Th cytokines in CFS/ME patients these were performed on PBMCs, hence they are still not specific and do not preferentially provide information on $CD4^+T$ cell cytokines. Thus, the cytokine secretions are representative of the total cytokines secreted by not only T cells but also by other PBMCs. Further studies are required to investigate whether the changes and elevated levels of FOXP3 noticed in this study are related to receptors such as inhibitory CTLA-4. The cytokines examined in the CFS/ME project were based on activated cells and this is not representative of the cytokines present in the circulation on the day of sample collection. Analysis of cytokines in unstimulated samples may have provided a more clear indication of the inflammatory immune profile in CFS/ME. The project did not provide information on the cell counts of the different phenotypes of lymphocytes investigated. In CFS/ME, the levels of lymphocyte subsets are inconsistent and are therefore poor markers for CFS/ME. Additionally, although the miRNA studies suggest a role of miRNAs in CFS/ME, it is still not known which genes are targeted specifically by these miRNAs. Knowledge of the immune related genes modulated by these miRNAs may be important for diagnostic purposes. All the CFS/ME participants in this study were taking different medications at the time of the project and medications were not part of the exclusionary criteria for this project. Hence, it is likely that some of the

immune changes noted in this project may have being influenced by medications or supplements taken by the patients.

6.6. Further Research

Following these important findings, further research is now required to examine the immunological parameters investigated in this study, in other chronic disorders that present with persistent fatigue (such as rheumatoid arthritis or multiple sclerosis) to determine if the markers are specific for CFS/ME. Similarly, gene expression of miRNAs necessitates longitudinal assessment to determine whether these are stable markers for CFS/ME and also the role of these markers in other immune cell types. This study focused on only VIP related receptors, however, PACAP receptor PAC1 has been associated with many diseases and it may be important to ascertain whether or not CFS/ME is related to compromise in all VN receptors. Severity and type of onset (gradual or sudden) were not investigated in this research, these may be important components of the disease that determine immune presentation, especially cytokine distribution as previously highlighted. Lastly, CFS/ME patients are known to take an array of medications and supplements in the quest to attain full health. Most of these medications have side effects that may affect immune related activities, thus a study examining CFS/ME on or off medications may also be necessary. CFS/ME patients present with a wide spectrum of symptoms that are identified in many diseases. Although, an increase in FOXP3 was noted it is still not known whether this translates into increase in suppressive actions by Tregs, thus a further in depth examination may disclose the type of phenotype of FOXP3 that is highly expressed in these patients. The results have provided salient information on the status of CFS/ME patients that can assist in the development of important biomarkers. However, transference of these results in to useable biomarkers may be difficult as the techniques and reagents used are

costly and time consuming. For example, most of the gene studies were performed on isolated cells, and the techniques involved in these isolations are time consuming and expensive. Therefore miRNA biomarkers for CFS/ME can only be useful where whole blood samples, plasma or serum are used without further isolation of cells. This necessitates further investigations into the role of circulating miRNAs in CFS/ME. Similarly much work is now required to specifically focus on the status of CD4$^+$T cells in CFS/ME. The present investigations related to CD4$^+$T cells cytokine profiles were performed PBMCs, it will be important for future studies to exclusively focus on isolated CD4$^+$T cells to determine their role in regulating the Th immune complex.

6.7.Conclusion

Diagnosis of CFS/ME based on well established laboratory procedures may not be out of reach as our data have highlighted a number of specific immunological markers that could be used as biomarkers for CFS/ME. Previous research suggests that CFS/ME is characterised by decreases in cytotoxic activity, granzyme A, B and perforin with differential expression of genes involved in immune, cellular, metabolic and neurological function. However, inconsistencies have been shown across research on measures of cytokines and lymphocyte subsets.

The present study adds to the available scientific knowledge on CFS/ME. The results accumulated from this research ultimately indicate the importance of longitudinal assessment of immune cell markers in CFS/ME. This study has shown for the first time consistent decreases in cytotoxic activity and NK phenotypes over a period of 12 months. This is also the foremost study to report a decrease in granzyme K and an increase in perforin in cytotoxic cells of patients with CFS/ME. Similarly, this study has illustrated compromises to the VN mechanism characterised by significant increases in VPAC2R and FOXP3 expression over time in CFS/ME. Further, the observations of inconsistencies in cytokine distribution in CFS/ME patients, observed in other studies were confirmed in this study, even though the previous studies measured only at one point. This is also the first study to advocate for a role of miRNAs in the reduced activity of cytotoxic cells and consequently immune function owing to the decreases in miRNA expression in cytotoxic cells, CD8$^+$T and NK, of CFS/ME patients. The above

data demonstrates that immunological impairments are an important component of the mechanism of CFS/ME.

Importantly, this study has further identified novel immunological pathways that may be important in understanding the dysfunctional immune presentations in CFS/ME. These pathways are in relation to FOXP3 and VPACR2, important suppressors of pro-inflammatory episodes. Similarly, the observation of reduced miRNA expression in CFS/ME suggests a role of these molecules in dysregulated expression of genes related to various aspects of immune function. Lastly, the consistency in certain immunological parameters over time has not yet been reported in CFS/ME and this further verifies the notion of immune compromise. However, further validatory studies are now required to establish a suite of biomarkers that can be tested in the clinical setting.

7. References

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8. Appendix

A. Chronic Fatigue Syndrome Questionnaire adapted from the Centre for Disease

    Prevention and Control. (Fukkudah *et al*. 1994)

Participant Name: _____

Contact Details

    Phone Number: _____

    Email Address: _____

1. Age _____ years

2. Weight_____ kg/lb

3. Height _____ cm

4. Sex _____

Please circle the appropriate answer.

5. Have you felt generally "unwell" for more than 6 months?

    a. Yes

    b. No

6. Do you have severe fatigue of that has persisted for 6 months or longer?

    a. Yes

    b. No.

7. Can you still do most or all of the physical and mental activities that you did before you began feeling unwell?

    a. Yes

    b. No

8. Has your illness had a major impact on work, social, and/or educational activities, to the extent that you have had to make adjustments in your lifestyle in an effort to avoid relapsing or becoming more ill?

    a. Yes

    b. No

9. Have you had at least four (4) of the following eight (8) symptoms for 6 months or longer? Circle which 4.

- Weakness and exhaustion, lasting more than 24 hours, following mental or physical activity

- Unrefreshing sleep

- Substantial impairment of short-term memory or concentration

- Muscle pain

- Pain in the joints, without swelling or redness

- Headaches of a new type, pattern or severity

- Tender armpit and/or neck lymph nodes

- Sore throat

10. Did your feelings of unwellness begin suddenly, within a period of hours or a couple of days?

    a. Yes

    b. No

11. Have you ever been diagnosed with one or more of the following conditions? Please circle which ones.

- Fibromyalgia

- Multiple chemical sensitivity or environmental illness

- Irritable bowel syndrome

- Orthostatic intolerance in any form, including fainting, vasovagal syncope, neurally mediated hypotension or postural orthostatic tachycardia syndrome

- Chiari 1 malformation

- Depression

- Anxiety disorder

- Autoimmune disorder

- Anaemia

12. In the next section please rate your symptoms on a 100 point scale, with 0= no pain or problem and 100= severe pain. Please rate these symptoms during your periods before the onset of illness, 6 month period of illness, your worst period during those six months or more and also rate the symptoms for how you are experiencing them today.

	Symptoms	Pre-date illness	6 or more months	Rating during the worst period	Rating today
a.	Fatigue				
b.	Post-exertional malaise lasting more than 24 hours				
c.	Sore throat				
d.	Tender neck or axillary lymph nodes				
e.	Muscle pain				
f.	Multiple joint pain without swelling or redness				
g.	Headaches of a new type, pattern or severity				
h.	Unrefreshing sleep				
i.	Impairments in short term memory or concentration				

13. Circle which of the following type(s) of cognitive difficulties you experience

   a. Slowness of thought

   b. Absent-mindedness

   c. Confusion/disorientation

   d. Difficulty reasoning things out

   e. Forgetting what you are trying to say

f. Difficulty finding the right word

g. Difficulty following things

h. Difficulty understanding

i. Slow to react

j. Poor hand to eye coordination

k. New trouble with math

l. Concern with driving

m. Other:_____

14. Circle which one(s) of the following mood difficulties you experience

a. Anxiety/tension

b. Easily irritated

c. Depression

d. Mood swings

e. Other: _____

15. Do you smoke or have you smoked in the past two years?

a. Yes

b. No

16. Please   specify   the   medications   you   are   currently   taking.

_____

_____

_____

_____

_____

_____