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**The role of immune responses, focussing on
herpes virus specificity and interferon-
gamma, in Myocardial Infarction with
reperfusion and in Chronic Fatigue
Syndrome**

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This thesis is submitted for the degree of

Doctor of Philosophy



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Abstract

Background

Immune responses targeting microbes can contribute to chronic inflammation, particularly when the microbes are persistent such as herpes-family Cytomegalovirus (CMV) and Epstein-Barr virus (EBV). Such persistence of antigens can cause T cell exhaustion characterized by loss of appropriate effector functions, expression of inhibitory receptors, as well as failure to return to homeostatic pre-inflammatory conditions. This results in immune senescence and dysregulation which may cause disrupted cell populations, homing and cytokine productions that mediate immunopathology and compromise anti-microbial defences.

Aims

The aim of the study was to determine whether microbe-specific particularly, interferon-gamma immune responses measured in 2 disease states, where a herpes viral inflammatory aetiology and immune dysregulation are suggested, acute Myocardial Infarction (MI) with reperfusion and Chronic Fatigue Syndrome (CFS), are indicative of disease presence and severity.

Patients

MI occurs due to blockage of the coronary artery, and treatment involves stent insertion, allowing reperfusion (R), which has associated immunopathology due to T cell homing. A total of 52 MI patients were studied. Blood samples were taken before and after reperfusion to investigate the dynamics of specific T cell homing to the heart, that may contribute to disease severity (reperfusion damage). For 50 CFS patients with measured disease levels, blood samples were taken to examine immune responses including those against microbes implicated in disease (CMV & EBV) compared to healthy controls.

Methods

T cell immunity was measured by ELISpot and flow cytometry, and cytokine levels in cell culture supernatants were measured using multiplex technology. Statistical analyses were carried out for normality in data sets. The Mann-Whitney test or unpaired t test was used to determine the difference between two unrelated groups. Differences between repeat or paired measurements were determined by Wilcoxon signed rank tests or paired t tests. Associations between measurements were investigated using Spearman correlation.

Results and Discussion

In MI patients, compared to before reperfusion, levels of the following cell populations fell significantly after reperfusion: terminally-differentiated CD8⁺ PD-1⁺effector memory cells (p=0.016) and CMV-specific IFN γ -secreting CD4⁺ T cells (p=0.002) the latter also being associated with injury. This suggests specific pathogenic T cell homing to the heart during reperfusion.

In CFS patients, increased disease severity was associated with increased non-specific IFN γ production (p=0.008), and reduced percentage of NK cells (p=0.0047). NK cell deficiency may reduce antiviral defences and allow detrimental viral reactivation.

Conclusion

T cell responses against CMV appeared to have greater involvement in MI + R than CFS. Immune responses involving IFN- γ are demonstrated in both conditions as being associated with disease, and so this cytokine may be considered a disease biomarker and a therapeutic target for both.

DECLARATION

This thesis is submitted for the degree of Doctor of Philosophy at Northumbria University, Newcastle Upon Tyne, UK. Research was conducted at Northumbria University and at the Institute of Genetic Medicine, Newcastle University under the supervision of Professor Stephen Todryk, Chair in Immunology at Northumbria University between August 2013 and April 2018. All work described in the thesis is my own, unless otherwise stated. The research was funded by both Northumbria University and Newcastle University. Research was performed under the ethical guidelines set by the NHS.

I declare all research submitted is my own and has not been submitted by me previously at this university or any other university.

Santosh Murali

August 2019

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Publications

Publications as a co-author

Hoffmann, J., Shmeleva, E.V., Boag, S.E., Fiser, K., Bagnall, A., Murali, S., Dimmick, I., Pircher, H., Martin-Ruiz, C., Egred, M. and Keavney, B., 2015. Myocardial ischemia and reperfusion leads to transient CD8 immune deficiency and accelerated immunosenescence in CMV-seropositive patients. *Circulation Research*, 116(1), pp.87-98.

Shmeleva, E.V., Boag, S.E., Murali, S., Bennaceur, K., Das, R., Egred, M., Purcell, I., Edwards, R., Todryk, S. and Spyridopoulos, I., 2015. Differences in immune responses between CMV-seronegative and-seropositive patients with myocardial ischemia and reperfusion. *Immunity, Inflammation and Disease*, 3(2), pp.56-70.

Jaat, F.G., Hasan, S.F., Perry, A., Cookson, S., Murali, S., Perry, J.D., Lanyon, C.V., De Soyza, A. and Todryk, S.M., 2018. Anti-bacterial antibody and T cell responses in bronchiectasis are differentially associated with lung colonization and disease. *Respiratory Research*, 19(106), pp.1-12.

Note: I am a co-author on the above publications.

Manuscript preparation for two journal articles (as a first author) focusing on lymphocyte migration to the myocardium and immune responses to chronic fatigue syndrome is currently ongoing.

Poster Presentations

Presented a poster entitled “T cells and CMV in acute Myocardial Infarction” at the Immunology North East Annual Symposium in Durham, UK in June 2015.

Presented a poster entitled “T cells and CMV in acute Myocardial Infarction” at the European Congress of Immunology (ECI), Vienna, Austria in September 2015.

Presented a poster entitled “Immune responses in Chronic Fatigue Syndrome” at the UK CFS/ME Research Collaborative, Newcastle upon Tyne, UK in October 2015.

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List of Abbreviations

ADCC- Antibody-dependant cell cytotoxicity

MI- myocardial infarction

AMI- Acute myocardial infarction

APC- Allophycocyanin

APC- Antigen presenting cells

APC-Cy7- Allophycocyanin conjugated with cyanine dye7

BCR- B cell receptor

Bregs- Regulatory B cells

BV421- Brilliant Violet 421

BV510- Brilliant Violet 510

CDI- *Clostridium difficile*

CFS- Chronic Fatigue syndrome

CHD- Coronary Heart Disease

CMV- Cytomegalovirus

CTLA-4- Cytotoxic T cell associated antigen

DAMP- Damage-associated molecular patterns

DMSO- Dimethyl sulfoxide

EBV- Epstein-Barr Virus

ELISA- Enzyme-linked immunosorbent assay

ELIspot- Enzyme-linked immunospot assay

EU/ml- Endotoxin units/millilitre

FCS- Fetal calf serum

FIS- Fatigue Impact Scale

FITC- Fluorescein Isothiocyanate

HLA- Human leukocyte antigen

Ig-Immunoglobulin

IL-17- Interleukin 17

IL-2- Interleukin 2

IL-5- Interleukin 5

ITIM- Immunoreceptor tyrosine inhibition motif

KIR- Killer Immunoglobulin receptor

LDL- Low Density Lipoprotein

LPS- Lipopolysaccharide

MHC- Major Histocompatibility complex

MPO- Myeloperoxidase

MSD- Meso Scale discovery

MVO- Microvascular obstruction

NK cell - Natural Killer cell

NKT cell - Natural Killer T cell

NO- Nitric oxide

PAMP- Pathogen-associated Molecular patterns

PBMC- Peripheral blood mononuclear cell

PBS- Phosphate buffered saline

PCR-Polymerase chain reaction

PD-L- Programmed cell death ligand

PE- Phycoerythrin

PE-Cy7- Phycoerythrin conjugated with cyanine dye7

PerCP-Cy5.5- Peridinin-chlorophyll protein conjugated to cyanine dye5.5

PHA- Phytohemagglutinin

PMAi- phorbol 12-myristate 13-acetate with ionomycin

PPCI- Primary percutaneous coronary intervention

PRR- pattern recognition receptor

PWM- Pokeweed mitogen

ROS- Reactive oxygen species

SDS-PAGE- sodium dodecyl sulphate polyacrylamide gel electrophoresis

SHM- Somatic Hypermutation

STEMI- ST Segment elevated myocardial infarction

TCR- T cell receptor

T_{EMRA}- Terminally differentiated effector memory cells (CD45RA⁺)

Th1- T helper 1 cell

Th17- T helper 17 cell

Th2- T helper 2 cell

IT- Ischemic time

TL- Telomere length

TLR- Toll like Receptor

TNF- Tumour necrosis factor

VCAM-1- Vacular cell adhesion molecule-1

VEGF- Vascular endothelial growth factor

VLDL- Very low-density lipoprotein

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CHAPTER ONE

Introduction and Literature review

1.1 Scope of thesis-setting the scene

Uncontrolled or excessive inflammation is central to many of today's major diseases, including heart disease, autoimmune and degenerative diseases, and fatigue-inducing conditions; which may have direct or indirect microbial involvement. The understanding of such diseases involves the study of diseased patients themselves as well as animal models of disease, and *in vitro* analysis of key mechanisms involved or implicated. The combination of such research avenues contributes to improved disease diagnosis, monitoring and treatments. This thesis examines the role of immune responses, mainly T and B cell responses, focussing especially on specificity for Cytomegalovirus and Epstein-Barr virus, in two disease states: acute myocardial infarction with reperfusion (MI+R); and Chronic Fatigue Syndrome (CFS). Such anti-microbial immunity has been implicated in both these diseases. It is important to set the scene for these disease states and to introduce the immune processes investigated in this thesis.

In acute MI, commonly known as a "heart attack", the coronary artery becomes blocked usually due to the rupture of an atherosclerotic plaque (see page 29). The muscle of the heart, the myocardium, becomes starved of oxygen (ischemia) and local cells die causing inflammation and damage the muscle at the endothelium. Blood clotting will also be initiated. Cardiac surgeons will insert a stent (an expandable metal cage) which opens the artery and re-establishes blood flow. The damaged myocardium is, however, prone to reperfusion injury, believed to be in part caused by the homing of inflammatory T cells to the region. CMV and EBV reactive T cells are the most abundant T cells in the body (of sero-positive individuals) and are thought to be major contributors to the immunopathology, and hence the subject of the first study in this thesis.

CFS is a condition in which sufferers endure debilitating fatigue accompanied by pain in muscles, and hence its other name myalgic encephalomyelitis (ME). Whilst fatigue can accompany a range of well-known diseases (such as infections and autoimmunity), CFS is defined as having an unknown cause (idiopathic). There are likely to be many mechanistic pathways that converge to give rise to inflammatory cytokines in the central nervous system and “sickness behaviour”. CFS has often been observed following an infection and clinical researchers have proposed that sufferers have dysregulated immune responses against microbes that cause inflammation, reduced protection from infection, and potentially a vicious cycle of infection, inflammation and dysregulation. A greater understanding of immunity in CFS, particularly against persistent infections such as CMV and EBV, may reveal mechanistic pathways and suggest ways to improve disease management. This is the subject of the second study of this thesis.

The common theme of the thesis is the characterization of immune responses, particularly interferon-gamma, against persistent microbes such as CMV and EBV, and their possible contribution to diseases presence or severity.

1.2 Introduction to the immune system

The environment is abundant with pathogenic microbes, commensal microbes, toxic substances and allergic proteins. The immune system thus uses a wide variety of mechanisms to defend from pathogens and tolerate harmless molecules to maintain homeostatic balance (Chaplin, 2010). Anatomical and physiological barriers, innate immunity and adaptive immunity constitute the three arms of host defence. Intact skin, mucosal-ciliary clearance, low pH of the stomach and lysozyme in secretions are the hallmarks of the anatomical and physiological barriers (Turvey & Broide, 2010). The protection conferred by the anatomical and physiologic barriers are augmented by the immune responses of the innate immune system.

The innate immune system broadly recognizes molecular patterns on pathogens and toxic substances that are not expressed by the human body (Turvey & Broide, 2010). Some of the microbial patterns recognized by the innate immune system are complex polysaccharides, glycolipids, lipoproteins, nucleic acids and nucleotides (Iwasaki & Medzhitov, 2015). The innate immune system swiftly encounters invading pathogens and usually eliminates them. The adaptive immune system comprises adaptive immune cells (T and B cells) that somatically rearrange gene segments to produce antigen receptors that bind to antigenic molecules with high specificity. Cells of the adaptive immune system are long lived and mount a stronger, faster effective immune when re-encountered with the same pathogen, this being known as immune memory (Chaplin, 2010).

1.3 Innate Immune System

The innate system is the first line of defence against invading pathogens after physical barriers are breached. Cellular internalization mechanisms and inflammation and protein cascades (including complement) constitute the main arms of the innate immune system. The innate immune system broadly identifies pathogen-associated molecular patterns (PAMPs) using germline encoded pattern-recognition receptors (PRRs) expressed by immune cells. The innate immune system is thought to not undergo lasting changes following exposure to the pathogen i.e. there is no difference in the quality of immune response during the first and second exposure to the same pathogen (Sompayrac, 2015). However, recent research points to a memory component of the innate immune system. The memory component of innate immunity is acquired due to transcriptional changes and epigenetic reprogramming whereas immunologic memory in the adaptive immune system is acquired due to somatic rearrangement of genes and clonal expansion (Netea *et al.*, 2016).

1.3.2 Inflammation

The presence of pathogens or dead cells within host tissues causes a 'state of inflammation' which is characterized by the presence of innate and adaptive immune cells and their secretory products. If infections cannot be contained by the innate system, the adaptive arm of the immune system is recruited to combat the infections. The adaptive arm of the immune system takes more time to develop and is more potent than the innate immune system. The PRRs of tissue resident immune cells such as macrophages and mast cells, recognize PAMPs or damage-associated molecular patterns (DAMPs) on dead or damaged cells. Once activated, the PRRs initiate a signalling cascade reaction which results in the secretion of cytokines. These cytokines play a major role in the recruitment of immune cells into the area of infection or damage (Newton & Dixit, 2012). Induction of inflammation by pathogen recognition initiates

the production and release of inflammatory mediators which cause changes in the blood vessels and the influx of immune cells. Vasoactive amines such as histamine and serotonin, which are released due to mast cell and basophil degranulation, have both potent vasodilating and vasoconstricting effects. Other molecules such as kinins, bradykinin and fibrin also cause vasodilation and allow increased vascular permeability which allow the entry of leukocytes into the inflamed area (Medzhitov, 2008).

Neutrophils are polymorphonuclear leukocytes that are the first immune cells to respond to acute inflammation. Neutrophils are produced in the bone marrow from myeloid progenitor cells (Kolaczkowska & Kubes, 2013). Neutrophils possess granules which contain a host of pro-inflammatory proteins such as myeloperoxidase, lactoferrin and matrix metalloproteinase 9 (MMP9). Neutrophils can kill phagocytosed pathogens by lowering pH, secreting reactive oxygen species (ROS), degranulation (can be intra or extracellular) and by neutrophil extracellular traps (NET). The NET consists of a DNA scaffold on which histones and granular products are attached and in which pathogens are trapped and killed (Kolaczkowska & Kubes, 2013).

Natural killer cells (NK) are considered group 1 innate lymphoid cells. Group 1 innate lymphoid cells are those that secrete IFN- γ but are unable to secrete T_H2 and T_H17 associated cytokines (Spits *et al.*, 2013). Main functions of NK cells involve antibody-dependent cell cytotoxicity (ADCC), the recognition of lost MHC expression on tumour cells or virally-infected cells, secretion of cytokines and chemokines to influence other immune cells and to offer costimulatory support for the activation of T cell and B cells (Toledo *et al.*, 2010).

During their development NK cells acquire CD122, which binds to its ligand IL-15 allowing differentiation into immature NK cells. The acquisition of CD94, NKG2A, NKp46 marks the transformation to CD56^{bright} NK cells. CD56^{bright} NK cells express CD122, produce IFN- γ as

well as expressing T-bet transcription factor. It is suggested that CD56^{dim} NK cells arise owing to CD56^{dim} having shorter telomeres than CD56^{bright} cells (Yu *et al.*, 2013). Min-Oo *et al.*, 2013 suggested three possible ways NK memory cells could develop, i) cytokine stimulation, ii) exposure to CMV and iii) hapten-specific for liver localized NK cells. Romee *et al.*, in 2012 showed that NK cells pre-activated with IL-12 and IL-18 showed increased production of IFN- γ after 21 days of *in vitro* resting followed by subsequent reactivation with IL-12+IL-15 and IL-12+IL-18. They also found that CD107a expression in pre-activated NK cells and control NK cells were similar. Thus, increased production of IFN- γ and similar levels of CD107a expression suggest that NK cells exhibit memory like characteristics. In addition, both CD56^{bright} and CD56^{dim} NK cells were also found to be capable of memory. O' Sullivan *et al.*, in 2015 showed that during viral infection, autophagy and mitochondrial changes are induced in NK cells. The authors transferred purified NK cells expressing Ly49H receptor that recognizes m157 glycoprotein of mouse cytomegalovirus (MCMV) into mice deficient in Ly49H receptor. The transferred NK cells underwent rapid proliferation which was accompanied by mitochondrial depolarization and increase in mitochondrial reactive oxygen species. However, from week 2 to week 4 after the adoptive transfer of NK cells, there was a pronounced drop in the mitochondrial dysfunction, which the authors attributed to NK cells entering the memory phase. In addition, Cyto-ID (autophagy detection reagent) staining and transmission electron microscopy showed the presence of autophagosomes in NK cells during active proliferation, which sequentially decreased during the transition from effector phase to the memory phase. Moreover, selective ablation of Atg3 gene in mice by the introduction of tamoxifen led to the significant reduction of Ly49H⁺ NK cells in tamoxifen-induced mice after the start of the contraction phase. Thus, the authors showed that autophagy was required for the formation of memory NK cells.

NK cells can be broadly divided into two main subsets, CD56^{bright} and CD56^{dim} NK cells. The CD56^{bright} cells are only present in small numbers in peripheral blood but are more numerous in secondary lymphoid tissues. The CD56^{bright} cells provide a persistent supply of cytokines to support immune responses against pathogens. However, unlike CD56^{dim} NK cells, they possess limited cytolytic capacity. On the other hand, CD56^{dim} cells have lower proliferative capacity and lower telomere length than CD56^{bright} cells but secrete substantial amounts of IFN- γ and carry out potent cytolytic functions upon activation (Bellora *et al.*, 2014). NK cell functioning is carried out by the presence of activating and inhibitory receptors on the cell surface. The activating receptors of NK cells are called natural cytotoxicity receptors (NCRs) which include molecules such as NKp30, NKp44, NKp46, DNAM-1, NK2G2D and CD16. All these cell surface receptors share an immunoreceptor tyrosine activating motif (ITAM) and adaptor proteins such as CD3 ζ , FcR γ and DAP12. NK2GD associates itself with DAP10 adaptor protein. The binding of these receptors to their specific ligand causes the phosphorylation of tyrosine residues within ITAM, which in turn causes a signalling cascade resulting in downstream effector functions (Cheent & Khakhoo, 2009). A prime example of this mechanism is the antibody dependent cell cytotoxicity (ADCC). The Fc region of an IgG-coated cell, marked for destruction, binds to the Fc γ RIIIA receptor (CD16) on NK cells and induces the phosphorylation of tyrosine residues in ITAMs by kinases of the Src family. This activates tyrosine kinases, SYK and ZAP70, causing a signalling cascade that result in the release of cytolytic granules (Perforin and Granzyme B) and cytokines (IFN- γ). However, it is important to note that the action of CD244 and DNAM-1, in conjunction with CD16, is vital for efficient ADCC to take place (Bryceson *et al.*, 2011). Inhibitory receptors such as killer immunoglobulin receptors (KIRs) and CD94/NKG2A contain immunoreceptor tyrosine inhibitory motifs (ITIMs). The binding of a ligand to its specific inhibitory receptor causes the activation of the Src kinases, which in turn causes the phosphorylation of tyrosine residues on

ITIMs. This causes the recruitment of tyrosine phosphatases, which dephosphorylate the protein substrates of tyrosine kinases involved in the activation pathway and dampens the functioning of activating receptors. Recognition of target cells with no or deficient MHC class I molecules on the cell surface is one of the most important functions of NK cells. The inhibitory receptors bind to MHC class I molecules on normal cells and dampen the activating signals. However, on tumour cells, or virally infected cells, which have suppressed MHC class I expression, the inhibitory receptors cannot associate with the MHC molecules and this leads to a full-fledged engagement of the activation receptors.

1.3 Antigen Presenting Cells (APCs) of the immune system

It is widely accepted that extracellular pathogens and antigens are processed by APCs and presented on MHC class II molecules for CD4⁺ T cells of the adaptive immune system to recognize (Kambayashi & Laufer, 2014). On the other hand, intracellular antigens, particularly viral, are processed and expressed on MHC class I molecules for the CD8⁺ cytotoxic T lymphocytes (CTLs) to recognise (Leone *et al.*, 2013). Professional APCs prime naïve CD8⁺ T cells through the process of cross presentation. Thus, dendritic cells (DCs) express high levels of MHC class II molecules but are also capable of cross presentation on MHC class I molecules (Joffre *et al.*, 2012). DCs can be divided into two major classes, 1) conventional or classical DCs and 2) Plasmacytoid DCs (pDCs). The cDCs can be further classified into two main subsets, i) CD1c⁺ cDCs and ii) CD141⁺cDCs. CD1c⁺ DCs are specialized in presenting antigen to CD4⁺ T cells and support Th2 and Th17 mediated immune responses against extracellular pathogens, whereas CD141⁺ DCs are specialized in mediating Th1 immune responses and in the cross presentation and in the priming of CD8⁺ T cells (Breton *et al.*, 2016). Plasmacytoid DCs are a distinct lineage of cells that arise from the bone marrow derived cells. pDCs, during steady state conditions are present in the blood and lymphoid organs. However,

during infections, these cells are also found at the site of infection. The characteristic feature of pDCs is the ability to rapidly secrete high amounts of IFN- α . Apart from the primary function of secreting IFN- α , pDCs are involved in a myriad of functioning including natural killer activation, immunocyte recruitment, promoting CD8⁺ T and CD4⁺ T cell responses and antigen presentation (Swiecki & Colonna, 2015).

Macrophages, like DCs, are a myeloid leukocyte population originating in the bone marrow from progenitor cells. Monocytes, precursors of macrophages, are released into circulation and differentiate into macrophages upon migration to tissues. Macrophages possess a wide variety of cell surface receptors including toll-like receptors and scavenger receptors to regulate immune response and phagocytose pathogens (Murray & Wynn, 2011). Macrophages have a plethora of functions. They are involved in pro- and anti-inflammatory activities, help in tissue organization, contribute to the differentiation of T cells into specific subsets, have anti-tumour activity, anti-microbial activity and anti-parasitic activity. Two main subsets of macrophages exist, the M1 macrophage and the M2 macrophage. The M1 macrophage is pro-inflammatory whereas M2 macrophages are anti-inflammatory (Mills, 2015). Macrophages that metabolize arginine to nitric oxide and citrulline, catalysed by nitric oxide synthase (NOS), become the pro-inflammatory macrophages (M1) and the macrophages that that convert arginine into ornithine and urea via the enzyme arginase become anti-inflammatory macrophages (M2) (Rath *et al.*, 2014).

1.5 MHC & Antigen processing pathways

Major histocompatibility complex (MHC), also called human leukocyte antigen (HLA), contains more than 100 genes and is present on chromosome 6 in humans. The genes in the MHC locus can be divided into MHC class I, class II and class III. The class I MHC molecules comprise three joined α chains, α_1 , α_2 and α_3 , and assemble with β_2 microglobulin (Wood, 2006). The α_1 , α_2 chains make up the N terminal region of the molecule and form an anti-parallel β -pleated base on which two parallel α chains can rest and form a narrow peptide binding pit which allows only peptides 8 to 11 amino acids long to bind. While the α_1 and α_2 chains are polymorphic, allowing a wide range of foreign peptides to bind, the α_3 domain is conserved and has a binding site for CD8 receptor and is non-covalently linked to the β_2 microglobulin (β_2m). All the chains fold to form an Ig domain-like structure stabilized by disulphide bonds (S-S). The MHC class I molecule also has a hydrophobic section that passes through the plasma membrane, and a cytoplasmic tail.

The main classes of MHC class II genes are HLA-DP, DQ and DR. MHC class II molecules have two α chains and two β chains which are non-covalently bound to each other. The N terminal regions of the α_1 and β_1 chains interact to form a large peptide binding pit which can accommodate peptides 12 to 20 amino acids long. The β_2 chain contains a binding site for CD4 receptor. The base of the peptide-binding pit is formed from 4 β pleated strands and 1 α -helix strand of the α_1 chain, and 4 β pleated strands and 1 α -helix strand from the β_1 chain. The α_1 and β_1 chains exhibit polymorphism while the α_2 and β_2 chain are non-polymorphic and adopt an Ig domain like structure supported by a disulphide bond. The MHC class II molecule has two hydrophobic residues (one each for α chain & β chain) spanning the plasma membrane with two cytoplasmic tails (Abbas, Litchman & Pillai, 2012). The MHC polymorphism is vital to defend the body against a wide range of pathogens. Each MHC molecule can present an extremely large numbers of peptide sequences. The presence of three to six MHC class I

molecules and three to twelve MHC class II molecules indicate that in theory, MHC class I could present up to $6 \times 20^{6-7}$ and MHC class II molecules could present up to 12×20^{10} peptides. However, the combination of the presence of only 200,000 MHC class I molecules and 20,000 MHC class II molecules in T and B cells and the increased presentation of certain peptides indicate that the actual number of peptides presented by a single cell is likely to be around 10,000 (Rock *et al.*, 2016).

As mentioned earlier, antigen processing pathways can be of two types depending upon the location of foreign pathogens. Intracellular pathogens are mainly processed via the endogenous antigen processing pathway involving MHC class I molecules. In contrast, the pathogens that originate from outside the cells are taken up by cellular internalization mechanisms such as phagocytosis, macropinocytosis and receptor mediated endocytosis, and are processed via the exogenous antigen processing pathways; and peptide peptides are presented by MHC class II molecules. MHC class I molecules are present on all nucleated cells, which makes evolutionary sense as all nucleated cells are prone to infection, and present peptide to $CD8^+$ T cells through the peptide-MHC class I complex leading to cytotoxic mechanisms and death of the infected cell. MHC class II molecules are only expressed by specialised cells (professional antigen presenting cells) which are capable of engulfing pathogenic material and presenting peptides through the peptide-MHC class II complex to $CD4^+$ T cells, which on activation release cytokines and initiate an immune response which engages several arms of the immune system. It makes evolutionary sense to limit the expression of MHC class II molecules to a few select cells to limit the inflammatory response which otherwise would be extremely powerful for the immune system to control (Neefjes *et al.*, 2011).

1.6 Adaptive Immune system

The adaptive immune system came into existence approximately 500 million years ago through the evolution of somatic recombination. It provides a more focussed, potent response to pathogenic challenge compared to the innate immune system, as well as generating larger responses to counter pathogens. The adaptive immune system is mainly comprised of T cells and B cells, although natural killer (NK) cells can act as adaptive immune cells in the absence of T cell and B cells (discussed later). The recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) of the innate immune system kick-starts an inflammatory reaction that contributes to activate the adaptive arm of the immune system (Flajnik & Kasahara, 2010).

1.6.1 B cells

B cells are derived from the stepwise differentiation of the hematopoietic stem cell (HSC) in the bone marrow, apart from B1 cells, which develop in the fetal liver. The expression of paired box protein 5 (Pax5) on common lymphoid progenitor (CLPs) cells imprints B cell lineage. The bone marrow stromal cells provide an important cocktail of chemokines, cytokines and growth factors for the maturing B lymphocyte. B cell development progresses through VDJ recombination of immunoglobulin gene segments from pro-B cell to pre-B cell to immature B cells. The immature B cells, after the central tolerance mechanism, transport to the spleen where they differentiate into transitional (T1 and T2) B cells. After undergoing peripheral tolerance mechanism and under the influence of B cell activating factor (BAFF), alternative NF- κ B signalling, NOTCH2 transcription factor and Bruton's tyrosine kinase (BTK), the T2 B cells differentiate into mature marginal zone B cells (MZ) and mature follicular B cells (FO). The marginal zone B cells are retained in the spleen whereas the follicular B cells seed the secondary lymphoid organs. (Hoffman *et al.*, 2016). The immature B1 cells travel to the spleen

where they undergo transformation into transitional B1 cells, like the one mentioned above. The transformation from transitional B1 cell to mature B1 cell is dependent on classical NF- κ B signalling effectors such as BTK and NF- κ B1. It is important to mention that B1 cells and MZ B cells are innate-like, whereas FO B cells constitute the adaptive arm of the B lymphocytes (Montecino-Rodriguez and Dorshkind, 2011). These naïve B cells that encounter foreign antigens become mature B cells. The mature B cells then leave circulation and enter the germinal centre (GC) in the spleen and become long lived, class switch-capable memory B cells and short-lived effector plasma cells.

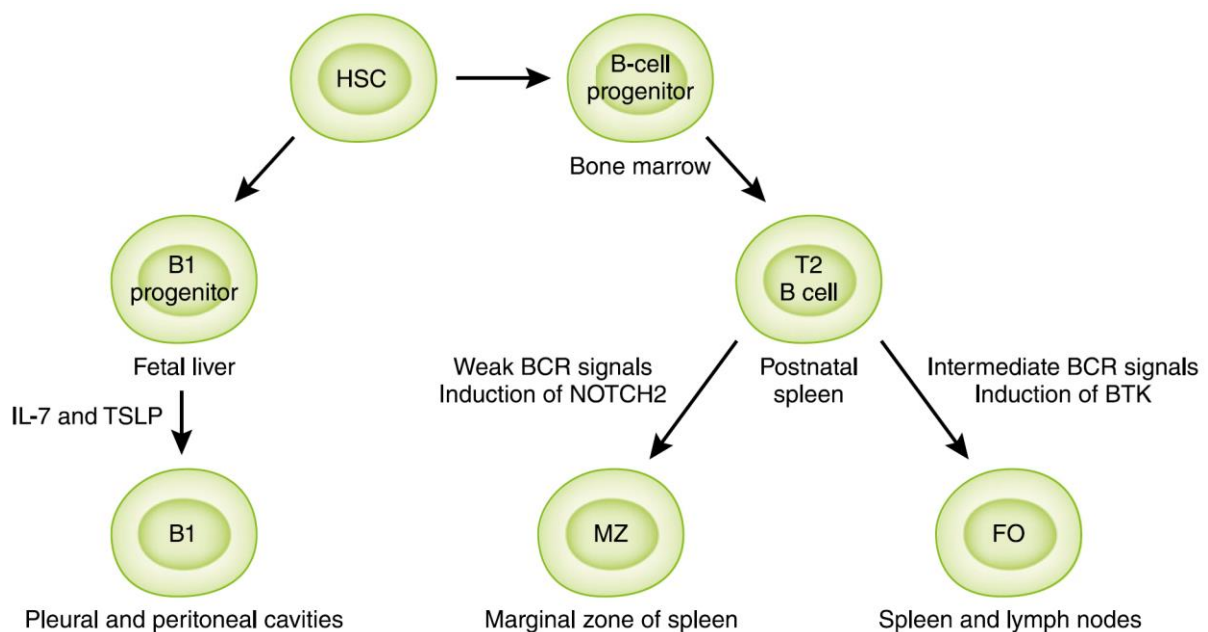


Figure 1.1 B cell generation from HSC and diversification. B cell progenitors develop from HSC and seed the Pleural and peritoneal cavities, the marginal zone of spleen and Lymph nodes where the different subsets of B cell develop (Taken from Hoffman *et al.*, 2016)

B1 cells have the following cellular phenotype: $CD27^+CD43^+CD20^+CD70^-$. B1 cells also secrete IgM and IgA without the requirement of T cells for activation (Martin *et al.*, 2001). So, the cellular phenotype of B cells is $CD27^+CD43^+CD20^+CD70^-IgM^+IgA^+$. The Marginal Zone B cells have immunophenotype: $IgM^+IgD^{low}CD21^{high}CD23^-CD1d^{high}$ and follicular B cells have the immunophenotype: $IgM^{low}IgD^{high}CD21^{medium}CD23^+CD1d^{low}$ (Cerutti *et al.*, 2013).

Regulatory B cells (Bregs) represent the immunosuppressive counterpart in B cells of regulatory T cells. The signature molecules secreted by Bregs are IL-10, TGF- β and IL-35. In an inflammatory setting, Bregs function by dampening T cell mediated inflammatory exacerbation and skewing T cell differentiation into a regulatory type (Rosser & Mauri, 2015). Bregs also play an important role in the regulation of the regulatory T cell population. Mammary adenocarcinoma-induced B cell deficient mice were associated with marked reduced tumour growth and were also associated with increased expression of Th1 cytokines and cytotoxic T lymphocyte response. In addition, the percentage of regulatory T cells were also reduced in B cell deficient mice compared to immunocompetent mice. These reduced regulatory T cells showed decreased expansion as well as proliferation compared to wild type mice. These results thus indicate that regulatory B cells play a vital role in contributing to the control of regulatory T cells (Tadmor *et al.*, 2011). Bosma *et al.*, in 2012 identified an important role for regulatory B cells in the maintenance of invariant natural killer T cell homeostasis. Depletion of B cells from PBMC led to the defective expansion of iNKT cells. This reduced expansion was associated with decreased expression of CD25 and Ki67. Also, the blockage of the CD1d-iTCR interaction led to the marked inhibition of proliferation suggesting that CD1d⁺ B cells play an active role in the expansion, proliferation as well as the maintenance of invariant natural killer T cell population.

Table 1.1 phenotypes of B cells during different stages of development and differentiation

B cell type	Cellular phenotype	Reference
Pro-B cell	CD34 ⁺ CD19 ⁺ CD10 ⁺ CD79a ⁺	Blom & Spits, (2006)
Pre-B cell	CD34 ⁻ CD19 ⁺ CD10 ⁺ CD79a ⁺	
Immature B cell	IgM ⁺ IgD ^{-/lo} BAFF-R ^{lo} CD10 ⁺ CD19 ⁺ CD79a ⁺	Blom & Spits, (2006) and Pieper <i>et al.</i> , (2013)
Transitional B cells	CD45 ⁺ CD19 ⁺ CD10 ⁺ IgM ⁺ IgD ^{+/lo}	Agrawal <i>et al.</i> , (2013)
Marginal zone cells	IgM ⁺ IgD ^{low} CD21 ^{high} CD1d ^{high}	Cerutti <i>et al.</i> , (2013) and McCulloch <i>et al.</i> , (2017)
Follicular cells	IgM ^{low} IgD ^{high} CD21 ⁺ CD23 ⁺ CD21 ^{med} CD1d ^{low}	Cerutti <i>et al.</i> , (2013)
Short lived plasma cells	IgM ^{lo} CD38 ⁺⁺ CD138 ⁺ CD27 ⁺ BCMA ⁺	Pieper <i>et al.</i> , (2013)
Long lived plasma cells	IgA ^{lo} IgG ^{lo} CD38 ⁺⁺ CD138 ⁺ CD27 ⁺ BCMA ⁺	
Memory B cells	IgG ⁺ BAFF-R ⁺ CD21 ⁺ CD23 ⁺ CD27 ⁺ TACI ⁺	
Regulatory B cells (Bregs)	CD5 ⁺ CD1d ^{high}	Tedder <i>et al.</i> , (2017)

1.6.2 T cells

Early T cell progenitors (ETPs) derive from HSCs in the bone marrow or fetal liver and seed the cortex-medulla junction (CMJ) of the thymus through the expression of P-selectin (Ladi *et al.*, 2006). The migration of double negative (DN, for CD4 and CD8) thymocytes to the subcapsular region of the cortex from CMJ occurs by chemotaxis with the help of CCL25 (ligand for CCR9, expressed on DN thymocytes), CXCL12 (ligand for CXCR4, expressed on DN thymocytes), VCAM-1 and CCRL1 (Nitta & Suzuki, 2016) expressed on thymic epithelial cells. ETPs in the thymus initially have the phenotype, CD4⁻CD8⁻CD25⁻CD44⁺. VDJ recombination involving the formation of β , γ , δ chains of TCR occurs when the DN thymocytes have the phenotype, CD4⁻CD8⁻CD25⁺CD44⁻. The final stage of differentiation of DN thymocytes is characterized by the phenotype, CD4⁻CD8⁻CD25⁻CD44⁻. The final stage of DN thymocytes differentiation occurs at the subscapular region of the cortex. The generation of the β chain allows DN thymocytes to proliferate into double positive (CD4⁺CD8⁺) thymocytes and imprints ETPs for $\alpha\beta$ T cell lineage. VDJ association of α chain with β chain generates $\alpha\beta$ TCR, which is expressed on double positive thymocytes (Nitta & Suzuki, 2016). Double positive thymocytes are then exposed to self-peptide-MHC complexes (spMHC) on thymic epithelial cells (TECs). Reactivity of thymocytes to spMHC complexes determines the fate of these cells. High level of reaction to spMHC complexes leads to negative selection and the eventual apoptosis of thymocytes. Only double positive thymocytes that show low levels of reactivity to spMHC complexes survive and differentiate into single positive CD4⁺ and CD8⁺ thymocytes (Klein *et al.*, 2014). The single positive thymocytes (SPT) downregulate CXCR4 and express CCR7. Ligands of CCR7, CCL19 and CCL21, bind to CCR7 and help in the migration of SPT into the medullary section of the thymus (Halkias *et al.*, 2014). Autoimmune regulator- (AIRE) dependent selection of single positive thymocytes takes place in the medulla. AIRE ensures the presentation of tissue specific antigens to single positive

thymocytes by medulla-specific thymic epithelial cells. Double positive thymocytes that have escaped the tolerance mechanism of cortex region and single positive thymocytes in medulla that have high reactivity to tissue specific self-antigens undergo apoptosis. Single positive thymocytes that have low reactivity to tissue specific self-antigens complete the maturation process and become single positive CD62L^{hi}CD69^{low} naïve T cells (Takahama, 2006)

Table 1.2 Important cell surface receptors present on CD4+ T cell subsets

Cell type	Cell surface molecule	Signature cytokine	Function	References
Th1	CXCR3, CCR5	IFN- γ , TNF- α	Pro-inflammatory reaction and macrophage mediated phagocytosis.	Zhu & Paul, (2008)
Th2	CCR3, CCR4, CCR8	IL-4, IL-5, IL-13	Allergy specific immune responses and against helminths	
Th17	CCR4, CCR6	IL-17A, IL-17F, IL-22	Clearance of extracellular pathogens, but implicated in the exacerbation of autoimmune diseases	Lee <i>et al.</i> , (2012)
T _{FH}	CXCR5, CD84, OX40, ICOS	IL-21, IL-4	Maturation of B cell in the germinal centre.	Ma <i>et al.</i> , (2012)
Cytolytic	CD11a, CD11b, CD57	Perforin, granzymeB and FasL.	Killing of infected cells	Soghoian & Streeck, (2010)
Tregs	CD25, CTLA4, GITR, CCR9	IL-10	Dampens inflammation	Sakaguchi <i>et al.</i> , (2010)

Molecules prefixed CXCR or CCR are chemokine receptors controlling T cell homing.

Table 1.3 Important cell surface receptors present on CD8+ T cell subsets

Cell type	Cell surface molecule	Signature cytokine	Function	References
Tc1	CD44, CD127, CD27, KLRGI	IFN- γ , TNF- α	Clearance of intracellular pathogens	Kaech & Cui 2012); Mittrucker <i>et al.</i> , (2014)
Tc2	CD3, CD8, IL-4	IL-4, IL-5, IL-13	Development and progression of rheumatoid arthritis	
Tc17	CCR6, CD161, CCR5, IL-23R	IL-17, IL-21	Ambivalent functionality in tumour micro environment Adopt a pro-inflammatory profile during transplantation and in autoimmune disorders.	Mittrucker <i>et al.</i> , (2014) and Srenathan <i>et al.</i> , (2016)
Tc9	CCR6, PD-1	IL-9, IL-10	Dampen CD4 ⁺ T cell activation through IL-10 production	Chang <i>et al.</i> , (2013)
TcRegs	Foxp3, CCR6, CCR7	IL-10, TGF- β , IFN- γ	Supresses the activation of T cells as well as promotes the differentiation of Th1 by secreting IFN- γ .	Li <i>et al.</i> , (2011)
T _{FC}	CD8, CXCR5, CCR7 ^{lo}	MIP-1 β , IFN- γ , TNF- α	Killing of infected B cells and follicular helper T cells as well as the killing of HIV infected T cells	Leong <i>et al.</i> , (2016); Petrovas <i>et al.</i> , (2017)

The low expression of CD69 on mature naïve T cells is accompanied by high expression of sphingosine-1 phosphate receptor (S1PR1), which binds to sphingosine phosphate produced by perivascular cells of the cortex-medulla junction and circulating blood, and in a chemotactic manner allow exit from the thymus. Among the naïve T cells produced are also the regulatory T cells (Tregs). Epithelial cells of the Hassal's corpuscles in the thymus produce thymic stromal lymphopietin (TSLP), which activate medullary dendritic cells (mDC). Activated DCs express high levels of CD80 and CD86 and promote MHC dependent transformation of CD4⁺CD25⁻ single positive thymocytes into Foxp3⁺CD4⁺CD25⁺ naïve Tregs cells. The presence of forkhead transcription factor and CD25 cell surface marker is an indication of regulatory T cell imprint (Ladi *et al.*, 2006).

Naive T cells, apart from circulating in the blood stream, are confined to the secondary lymphoid organs such as periarteriolar lymphoid sheath (PALS) in the spleen, Peyer's patches and paracortex of the lymph nodes, due to the presence of tissue specific receptors (CCR7) and selectins (CD62L). Irrespective of place of pathogen entry, antigen presenting cells such as DCs present processed pathogen 'antigens' to naïve CD4 and CD8 T cells in the associated lymph nodes.

The VDJ recombination, in T cells, takes places in a similar fashion to the process that occurs in B cells. The D and J segments are rearranged to form DJ complex. The DJ complex then associates with the variable region to complete the VDJ recombination process. The T cell receptor (TCR) β chain is assembled first and it is interesting to note that progenitor T cells that do not make a productive rearrangement modify their second allele to undergo productive rearrangements. The TCR β chain then associates itself with a surrogate TCR α chain (Pre-T α). This pre-receptor complex is formed in double negative thymocytes. The transformation of double negative thymocytes into double positive thymocytes is accompanied by the association of TCR α chain to the pre-existing TCR β chain. The formation of TCR $\alpha\beta$ on double positive

thymocytes signals the differentiation of these cells into single positive immature T cells (Bassing *et al.*, 2002).

Whilst macrophages and B cell play a 'secondary' role, dendritic cells are the primary antigen presenters to naive T cells (Jenkins *et al.*, 2001). Mempel and colleagues in 2004, using two-photon microscopy, showed that activation of naïve CD4⁺ and CD8⁺ T cells by dendritic cells take place in three distinct phases. The first phase involved short contacts between T cells and LPS-activated DCs in the lymph node, 8 hours after homing. T cells also showed an upregulation of CD44 and CD69, while the expression of CD25 was low. There was also no expression of IL-2 and IFN- γ . In phase two of naïve T cell priming, stronger and more prolonged interactions between DCs and T cells were observed from 12 to 20 hours. The levels of IL-2 and IFN- γ were highest after 20 hours and CD69 and CD44 were also highly activated. Phase 3 of T cell priming commenced 24 hours after homing to the lymph node. T cells migrated from the lymph node, proliferated, whilst maintaining a high expression of CD44⁺ and lower expression of CD25 and CD69. Once activated, CD4⁺ and CD8⁺ T cells transform into effector cells, which provide an immediate immune response against infections, and cause inflammation, by the production of a wide variety of cytokines, and once infection is resolved, become long lived memory cells. Two subsets of memory T cells are the effector memory T cells and central memory T cells.

Effector memory T cells possess a surface receptor profile that allows their preferred homing to peripheral tissues. CD62L⁻ CCR7⁻ but CD45RO⁺. On the other hand, central memory T cells possess receptors that promote their homing to the lymphoid tissues: CD62L⁺ CCR7⁺ and CD45RO⁺. Naïve T cells possess markers that also allow homing to lymphoid tissues- CD62L⁺ CCR7⁺ but CD45RO⁻ and CD45RA⁺. A population of terminally differentiated T cells which are CD62L⁻ CCR7⁻ but CD45RA⁺ are referred to as the T_{EMRA}.

1.6.2.1 T cell polarization

1.6.2.1.1 CD4⁺ T cells

The polarization of activated naïve CD4⁺ T cells into different subsets primarily depends upon cytokines acting on CD4⁺ T cells during activation. The action of cytokines activates specific transcription factors, which leads to production of a particular subset of CD4⁺ T cell. Apart from the traditional Th1, Th2 and Th17 subsets, there are induced T regulatory cells (iTreg), follicular T helper cells (T_{FH}) and cytolytic CD4⁺ T cells. There is also considerable plasticity within CD4⁺ T cell subsets depending upon the infectious state of the host (Swain *et al.*, 2012) (Figure 1.2).

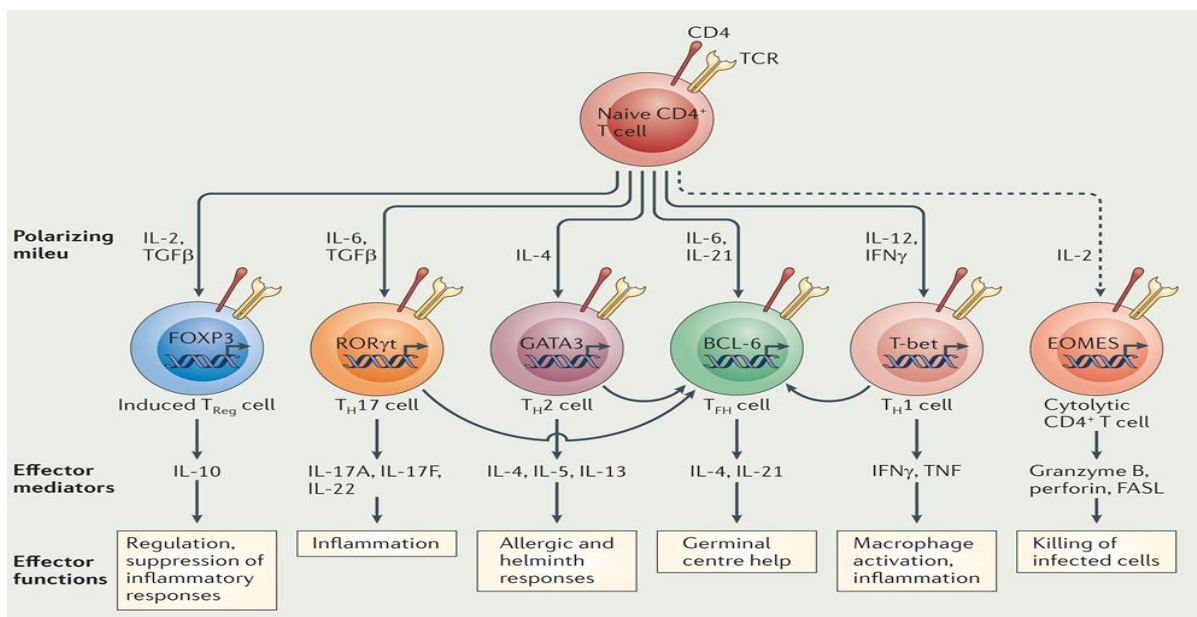


Figure 1.2. Polarization and Subset polarization of activated CD4⁺ T cells. Different cytokines activate different transcription factors which cause the generation of various subsets of CD4⁺ T cells and each of these subsets have specific functions. FOXP3 forkhead box P3, RORγT Retinoic acid related orphan receptor-γT, GATA-3 GATA binding protein 3, BCL-6 B cell lymphoma 6, T-bet T box transcription factor, EOMES eomesdermin (Taken from Swain *et al.*, 2012).

Th1 cells, characterized by the presence of transcription factor T-bet, are vital for mounting pro-inflammatory immune responses against intracellular pathogens via the secretion of IFN- γ , the signature cytokine in Th1 cells. In the absence of T-bet, eomesdermin assumes responsibility for the induction of IFN- γ . As well as these transcription factors, signal transducer STAT-4 and transcriptional repressor Runx3, also amplify and induce the release of IFN- γ from Th1 cells. IL-12 and IL-18 act in a synergistic manner to induce the production of IFN- γ in activated Th1 cells. Other major cytokines produced by Th1 cells are IL-2, TNF- α and lymphotoxin. (Zhu & Paul, 2008). IFN- γ produced by Th1 cells activate macrophages to release matrix metalloproteinase (MMPs), nitric oxide and pro-inflammatory cytokines to kill pathogens that have been ingested. Th1 cells, through the release of pro-inflammatory cytokines, also recruit CD8⁺ T cells and NK cells to the sites of pathogenic infection (Annunziato *et al.*, 2015), Th1 cells are also implicated in the exacerbation of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disorders (IBD) and collagen induced arthritis (Cosmi *et al.*, 2014) and chronic inflammatory diseases such as atherosclerosis (Hoffmann *et al.*, 2015). Th2 cell differentiation from naïve T cells require the activation of the GATA-3 transcription factor. GATA-3 is expressed only by Th2 cells and continued expression of GATA-3 is required for the maintenance of Th2 phenotype (Zheng & Flavell, 1997). The expression of GATA-3 is controlled by IL-4 and the signal transducers STAT5 and STAT6 (Paul & Zhu, 2010). The main function of Th2 cells is to confer protection of the host from extracellular parasites such as helminths, but they also mediate allergic reactions. The presence of allergens and parasites induce the expression of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) from epithelial cells which activate type 2 innate lymphoid cells (ILC2). ILC2 induce dendritic cells to activate Th2 immune responses. Th2 cells, apart from releasing their signature cytokines IL-4, IL-5 and IL-13, also release IL-9 and IL-10. IL-4 induces immunoglobulin class switching to IgE in B cells. The IgE immune

complexes then bind to FcεRI receptor on basophils and mast cells. This results in the release of various products such as histamine, proteases, serotonin and heparin. The release of these products leads to smooth muscle constriction, vascular permeability and migration of leukocytes to the sites to infection and leading to allergic responses. Release of IL-5 leads to the recruitment of eosinophils in asthma. IL-4 and IL-13 released by Th2 cells also promote the development of M2 macrophages that are anti-inflammatory, promote tissue repair but also help in the removal of parasites (Licona-Limon *et al.*, 2013).

Activated naïve CD4⁺ T cells, responding to IL-6, activates the bcl6 transcription factor which directs the development of Follicular T helper cell (T_{FH}) cells. Inducible costimulator (ICOS) directs follicular helper T cells to the B cell follicle. These cells express the chemokine receptor, CXCR5, and lack CCR7, and play a major role in T cell dependent B cells responses to infection. Presentation of antigens by B cells in the light zone of the germinal centre (GC) activates T_{FH} cells. Activated T_{FH} cells help in the survival and proliferation of B cells. B cells then travel to the dark zone where they undergo CSR and SHM. B cell then return to the light zone for further multiple rounds of signalling with T_{FH} cells. Thus, these T cells help in the generation of high quality antigen specific B cells, which produce immunoglobulins to neutralize pathogens (Crotty, 2014).

Harrington *et al.*, in 2005 showed that Th17 cell are a distinct T helper cell subset that develop distinctly from Th1 and Th2 cells. IL-23 stimulation had little effect on Th1 and Th2 cells but led to the development of Th17 cells from naïve CD4⁺ T cells. The Th17 cells had minimal expression of IFN-γ and expressed high levels of IL-17F. Furthermore, it was also shown that development of Th17 cells did not require the transcription factor T-bet. Ivanov *et al.*, in 2006 showed that the transcription factor, orphan receptor RORγt, is indispensable for the development of Th17 cells. IL-6 also upregulates the expression of RORγt and IL-23 receptor, thus promoting the generation of Th17 cells. Cytokines released by Th17 cells drive

contribution to autoimmune disorders such as multiple sclerosis, psoriasis, inflammatory bowel disease and rheumatoid arthritis but also confer protective cover against pathogens such as *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, *Candida albicans* and *Bordetella pertussis* (Korn *et al.*, 2009). Only T cells in the thymus expressing Foxp3, which bind to self-peptide MHC complex with a higher affinity than other T cells, develop into natural T regulatory cells (Tregs). Foxp3⁺ interacts with nuclear factor of activated T cells (NFAT) to repress the formation of IL-2 and upregulate CD25, cytotoxic T cell associated antigen (CTLA-4) and glucocorticoid induced TNF-receptor (GITR). IL-2, apart from activating Th1 and Th2 cells and playing a vital role in the functioning of CD8⁺ T cells and natural killer cells, also induces the production of Tregs through CD25(IL-2 α chain). Thus IL-2 maintains a negative feedback control mechanism whereby pro-inflammatory T cells are controlled by immunosuppressive regulatory T cells.

Regulatory T cells express a range of chemokine receptors and adhesion molecules for homing into lymphoid tissue and sites of infection. Although some of the cell surface receptors expressed by Tregs are CCR7, CCR4, CCR5, CCR6, CD103, CXCR4 and CXCR5, the main markers are CD25 and FoxP3. Functions of regulatory T cells include suppressing immune responses towards self-antigens, tumour cells and non-self-antigens, establishing immune tolerance towards organ grafts and preventing host vs graft disease. Naïve T cells in the periphery can also be induced to differentiate into induced regulatory T cells (iTregs) by the action of IL-2 and transforming growth factor (TGF- β) (Sakaguchi *et al.*, 2008).

Cytolytic CD4⁺ T cells make up the cytotoxic arm of T helper cells. Cytolytic CD4⁺ T cells, unlike Th1 cells, lack CD27, CD28, express CD11a, CD11b, CD57⁺ are CCR7^{lo} and produce perforin and granzymeB. Thus, it has been suggested that the phenotype of cytolytic CD4⁺ T cells resemble that of a terminally differentiated effector cell. Cytolytic CD4⁺ T cells perform their cytotoxic functions in several ways. The Fas ligand (FasL) on CD4⁺ cytolytic T cells

binding to its receptor (Fas) on target cells causes the apoptosis of the infected target cells by caspase activation. These cells also kill target cells by releasing perforin and granzymeB cytotoxic granules. Furthermore, it is also suggested that cytolytic CD4⁺ T cells also cause apoptosis of target cells through Tumour necrosis factor apoptosis inducing ligand (TRAIL) (Soghoian & Streeck, 2010). In addition to all T helper cell subsets mentioned above, Zhang *et al.*, in 2013, discovered a novel T helper subset, ThGM, which express high levels of CD45, CD95 and CD69, produce copious amount of GM-CSF and induce other T helper subsets to produce their signature cytokines and thus, might play an important role in the regulation of the immune response.

1.6.2.1.2 CD8⁺ T cells

Naïve CD8 T cells are activated by dendritic cells in a TCR dependant manner which is accompanied by changes to their migration patterns, gene expression and functionality. Naïve CD8 T cells proliferate and transform into cytotoxic T lymphocytes. TCR affinity to its specific antigen is a critical factor in determining the time spent by CD8 T cells in the lymph nodes (Halle *et al.*, 2017). Cells with low affinity acquire S1PR1 and leave the lymph nodes faster (Breart & Bouso, 2016) whereas the cells with a much stronger affinity for its cognate antigen migrate to the interfollicular regions of the lymph nodes via CXCR3 for further TCR stimulation (Ozga *et al.*, 2016). After sufficient priming CD8 T cells leave the lymph nodes and enter the lymph as well as the blood stream to enter peripheral tissues to combat pathogens. Once CD8 T cells enter the tissue, they acquire tissue resident memory phenotype (T_{RM}) typified by the expression of CD69, CD103 and increased expression of granzyme B. These tissue resident cells are however unable to re-enter circulation and so provide immediate immune protection to the tissues (Bergsbaken & Bevan, 2015). The survival of tissue resident memory T cells is dependent upon the uptake of exogenous lipid. Tissue resident memory CD8 T cells in the skin of experimental mice expressed high levels of Fatty acid binding proteins 4

and 5. Furthermore, the targeted deletion of FABP4 and FABP5 led to low survival rate of these cells *in vivo*.

Joshi *et al.*, in 2007 suggested that CD8⁺ T cells could be divided into short-lived effector cells (SLEC) and memory precursor effector cells (MPEC). Naïve CD8⁺ T cells primed by antigenic stimulation differentiate into short-lived effector and long-lived memory cells. These effector cytotoxic T cells (CTLs) kill infected cells and pathogens by releasing proteins such as perforin and granzyme B. They also release IFN- γ and Tumour necrosis factor (TNF) which mediate pathogen removal. After the elimination of pathogen, effector CD8⁺ T cells are rapidly removed from circulation by apoptosis and a small percentage of effector cells transform into memory CD8⁺ T cells (Sarkar *et al.*, 2008). Effector cytotoxic CD8⁺ T cells, with the phenotype CD44^{hi}CD62L^{low}CD27^{low}KLRG1^{hi}IL7R α ^{lo}, Memory precursor CD8⁺ T cells have the phenotype: CD44^{hi} CD27^{hi} KLRG1^{low} IL7R α ^{hi} and lack CD62L. Apart from Type 1 Cytotoxic CD8⁺ T cells (Tc1 cells), other subtypes of CD8⁺ T cells are Tc2 (CD8⁺ T cells secreting IL-4, IL-5), Tc17 (IL-17 secreting CD8⁺ T cells), Tc9 (IL-9 secreting CD8⁺ T cells) and CD8⁺ regulatory T cells. Tc2 cells are similar to Th2 cells, but aggravate allergy and autoimmune disorders (Mittrucker *et al.*, 2014). Hamada *et al.*, in 2009 identified a unique Tc17 subset of CD8⁺ T cells similar to Th17 cells belonging to the subset of CD4⁺ T cells in the lungs of experimental mice. Cell suspensions of spleen, lung, bronchoalveolar lavage (BAL) and lymph nodes indicated the expression of IL-17 by CD8 T cells. The authors also used anti-IL-17 antibody to deplete IL-17. This resulted in the reduction of immunity to influenza in mice. Tc17 cells promote autoimmunity, ambivalent response in tumour microenvironment and elicit a strong pro-inflammatory response against influenza infection (Mittrucker *et al.*, 2014).

Visekruna *et al.*, in 2013 identified a novel subpopulation of CD8⁺ T cells, Tc9 cells. The author showed that in the absence of 'skewing cytokines' CD8⁺ T cells were able to develop

an effector phenotype characterized by the production of granzyme B and IFN- γ . However, the addition of TGF- β and IL-4 suppressed the production of granzyme B and induced the release of IL-9, while the production of IFN- γ did not differ. The authors also performed quantitative RT-PCR to understand the transcriptional process in Tc9 cells. It was found that Tc9 cells produce low levels of IL-5, IL-13 and IFN- γ and displayed lower levels of cytotoxicity compared to cytotoxic T lymphocytes or Tc2 cells. As for the role Tc9 play in immunity, the authors showed that they play a role in the exacerbation of allergic airway disease along with Tc2 cells. Similar to the work by Visekruna group, Lu *et al.*, (2014) also showed diminished expression of IFN- γ , granzyme B and perforin as well as transcriptional controllers such as *Eomes* and *Tbx21*, while showing increased expression of *IRF-4* and *Pu.1* These Tc9 cells also showed enhanced expression of IL-2 and IL-9. Apart from these cytokines, these cells also secrete IL-4, IL-10 and IL-17.

1.6.2.1.3 Plasticity of T cells

Differentiated CD4⁺ T cells, in the absence of external influence by cytokines, retain their phenotypic stability aided by epigenetic modifications and microRNA expression. However, there exists considerable plasticity among the T helper populations. Th2 cells, in the presence of IL-12, can be stimulated to produce IFN- γ , the signature Th1 cytokine. Th2 cells can also be transformed into Th9 cells in the presence of TGF- β . Furthermore, Th17 cells induce insulin dependent diabetes mellitus (IDDM) only after acquiring Th1 phenotype. In addition, IDDM in NOD/SCID mice was abrogated when injected with anti-IFN- γ antibody but not with anti-IL17A antibody (Cosmi *et al.*, 2014). CD8⁺ T cells, like CD4⁺ T cells, show considerable plasticity. Tc2 cells, counterpart of Th2 cells, can produce IL-4, IL-13 and IFN- γ . Similarly, Tc17 cells produce both IL-17 and IFN- γ in experimental autoimmune encephalomyelitis. Tc1

cells produce IL-10 to dampen inflammatory response and CD8⁺ regulatory T cells produce IL-17 and IFN- γ (Mittrucker *et al.*, 2014).

1.7 Cardiovascular Disease

Cardiovascular disease accounts for 30% of all mortalities across the globe and costs the world economy \$862 billion; and the cost for treatment is projected to increase further in the next two decades with an ageing population. Atherosclerosis is the major pathological process that leads to cardiovascular diseases, particularly myocardial infarction (Swirski & Nahrendorf, 2013). It is therefore imperative to increase the understanding the mechanisms of disease and translate this into improved diagnosis, treatments or cures.

Atherosclerosis is a chronic inflammatory disease caused by the retention of low density lipoproteins (LDL) in the intima of the arteries. Cardiovascular risk factors such as smoking, diabetes, hypertension, autoimmune disorders, dyslipidaemia and other chronic inflammatory conditions cause endothelial dysfunction and lead to the increased retention of lipids in the intima (Patel & Blazing, 2013). Endothelial injury causes changes in the structural integrity of the endothelium leading to the disappearance of the elastin layer and the subsequent exposure of the proteoglycans. It is the binding of Apo-lipoprotein B100 (ApoB100) present in the LDL particles, to the proteoglycans that causes the retention of the lipids. The inflammatory responses of the immune cells toward the retained lipids lead to the accumulation of apoptotic cells, cell debris and cholesterol crystals that contribute to the formation of the atherosclerotic plaque. A fibrous layer comprised of collagen and vascular smooth muscle cells usually covers the plaque. The lesions, due to sustained inflammatory response, grow and lead to the narrowing of the blood vessels. Thrombosis occurs following plaque rupture (Weber & Noels, 2011) which blocks the vessel leading to myocardial ischemia, starving of oxygen supply to the heart muscle. If the vessel blocked is the coronary artery, then a heart attack will usually occur (Refer to **Figure.1.3**).

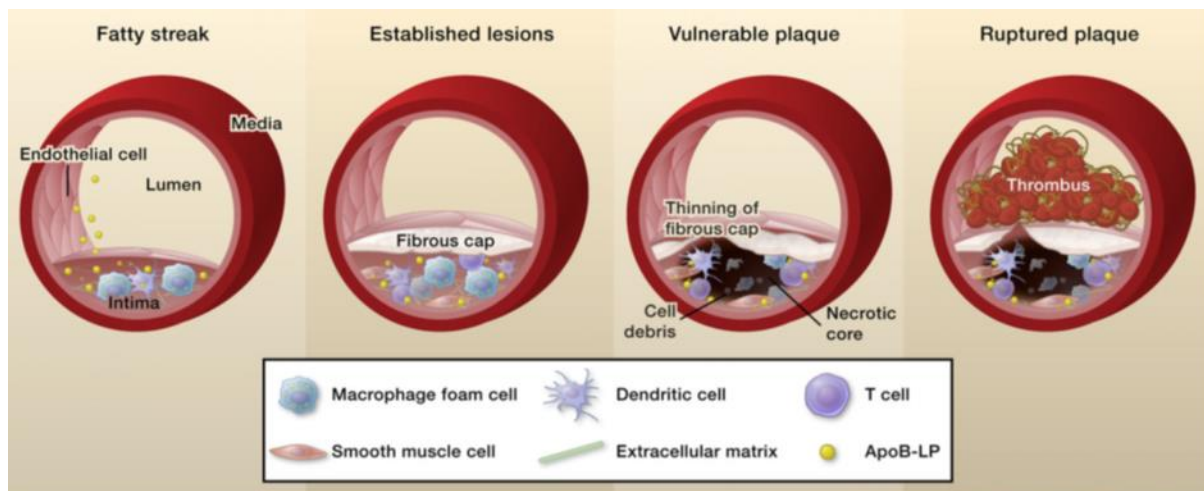


Figure 1.3 Stages of atherosclerotic lesions in an impaired endothelium. Early fatty streak lesions characterized by retention of Apo-lipoproteinB-100 in the endothelial wall provokes the migration of inflammatory immune cells which further increases and establishes the lesion size leading to the eventual rupture and blocking of arterial walls (Taken from Moore & Tabas, 2011).

Cardiovascular disease risk factors like smoking, diabetes, hyperlipidaemia and hypertension cause ‘oxidative stress’ which causes an increase in the concentration of reactive oxygen species (ROS) in the arterial intima. This leads to a marked reduction in the bio-availability of nitric oxide (NO), a potent vasodilator. The decrease in concentration of nitric oxide gives rise to endothelial dysfunction, which is a major factor in the advent of atherosclerosis. Reduced NO leads to pro-inflammatory, vasoconstrictive and defective anti-coagulatory properties of the endothelium (Bonetti *et al.*, 2003). This functional impairment of the endothelium leads to the retention of lipids in the intima. A study by Morel *et al.*, (1984) showed that human endothelial cells and smooth muscle cells, *in vitro*, in the presence of butylated hydroxytoluene (BHT, an antioxidant) did not modify LDL particles but modified them in the absence of BHT. This may suggest dysfunctional endothelial cells *in vivo* might be involved in the minimal oxidation of LDL in the intima of the arteries. Minimally modified LDL particles, due to dysfunctional endothelial cells, induce the endothelial cells to express adhesion molecules and release chemokines that causes the recruitment of immune cells such as monocytes and T cells.

This influx is responsible for the development of early lesions called the ‘fatty streak’. The monocytes present in the intima differentiate into macrophages under the influence of macrophage colony stimulating factor (M-CSF) released by the activated endothelial cells. The macrophages then take up the oxidized-LDL (ox-LDL) through their scavenger receptors and transform into lipid-filled ‘foam cells’ which, after apoptosis, contribute to the build-up of the atherosclerotic plaque (Hansson & Hermansson, 2011).

Studying mouse models of atherosclerosis is extremely important to gather insights into atherosclerosis and myocardial infarction mechanisms in humans. Researchers have consistently used two major models: *ApoE*^{-/-} and *Ldlr*^{-/-} mice to study atherosclerosis. The apo-lipoprotein E model involves the targeted deletion of the lipid transporter gene ‘ApoE’. The ApoE is involved in the metabolism and transport of very low-density lipoproteins (VLDL) from the myocardium. The deletion of the gene coupled with the mice being fed a high fat diet, leads to the retention of lipids (hyperlipidaemia) at the endothelial layer of the myocardium and contributes to the increase in the size of atherosclerotic lesions (Pendse *et al.*, 2009). The *Ldlr*^{-/-} model involves the targeted disruption of the low-density lipoprotein receptor (Ldlr). Ishibashi *et al.*, in 1994 used this model to show the formation of atherosclerotic lesions and xanthomatosis in mice. The deletion of LDL receptor causes the retention of VLDL, LDL and intermediate density lipoproteins (IDL) and exacerbates lesion formation which coincided with marked increase in plasma cholesterol from 246 to >1500 mg/dl (dl-decilitre). The plasma cholesterol levels of wild type control mice remained <160mg/dl. Both wild type control mice and *Ldlr*^{-/-} mice received a 1.25 % cholesterol diet (Normal Chow diet infused with cholesterol, cocoa butter, casein and sodium cholate).

1.7.1 Innate immune responses to Atherosclerosis

Van Leeuwen *et al.*, (2008), investigated the role of neutrophils in the development of atherosclerosis. *Ldlr*^{-/-} mice were fed a high fat diet and the atherosclerotic lesions were

investigated for neutrophils by staining with monoclonal antibodies for Ly-6G, a marker for murine neutrophils, and myeloperoxidase (MPO) specific monoclonal antibody to test the expression of MPO in the tissues. Neutrophils were present abundantly in the intermediate and advanced atherosclerotic lesions but were not present in early lesions. There was also a marked increase in the expression and plasma levels of MPO, which directly correlated with the increase in atherosclerosis development. Double staining for Ly-6G and MPO markers revealed that neutrophils were the predominant producers of MPO, whereas the monocytes weakly expressed MPO. Ionita *et al.*, in 2010 stained human carotid atherosclerotic plaques with mouse anti-human CD66b, anti-MPO and anti-naphthol AS-D chloroacetate esterase to identify neutrophils. It was found that mainly neutrophils and a subset of plaque macrophages present in the plaque were responsible for the production of MPO. Other studies have also shown neutrophils are the predominant producers of MPO. Naruko *et al.*, (2002) analysed coronary artery segments from AMI patients as well as atherectomy sections of patients with stable and unstable angina pectoris. In AMI patients with plaque rupture and Plaque erosion, double immunostaining with monoclonal antibodies for CD66b, elastase, MPO-7 and CD68 (cell surface molecule on macrophages), CD11b and MPO showed that majority of MPO producing cells were neutrophils.

Neutrophils which extravasate into the tissues in response to inflammation release preformed granule proteins including LL-37 and azurocidin, which activate and recruit monocytes into the lesions. Neutrophils release MPO, which reduces Nitric oxide content thereby causing 'oxidative stress' which then leads to dysfunction of the endothelium. Neutrophils also release lipid mediators such as leukotriene B₄ that increases the expression of adhesion molecules on the endothelial cells and helps maintain steady neutrophil inflammatory functions in the atherosclerotic tissues.

Neutrophils, apart from being potent pro-inflammatory cells, also quench inflammation by releasing molecules such as protectins (neuroprotectins) which reduce the expression of VCAM-1 on endothelial cells, which then subsequently leads to reduced leukocyte recruitment, and smaller plaques. Another potent molecule released is the lipoxinA₄ that reduces the generation of reactive oxygen species and decreases the expression of P-selectins on endothelial cells (Baetta & Corsini, 2010).

The striking feature of macrophages in the atherosclerotic lesions is their multifunctional capability. The macrophages release inflammatory mediators, which attract other immunocytes, further impair the endothelial cells, and release proteinases that cause the dysfunction of the extra-cellular matrix (Swirski & Nahrendorf, 2013). Nahrendorf *et al.*, in 2007 found that monocytes and macrophages also resolve inflammation. Ly-6C^{hi} monocyte subset dominates the initial stages of myocardial injury in atherosclerotic C57BL/6 mice by displaying inflammatory functions whereas the Ly-6C^{lo} monocyte subset resolves inflammation by promoting angiogenesis and granular tissue formation through extra cellular matrix deposition during the later stages of myocardial injury (4 to 7 days after myocardial infarction)

Erbel *et al.*, (2007) showed that dendritic cells are present in an activated state in the atherosclerotic plaque. A comparison between symptomatic and asymptomatic patients undergoing endarterectomy (a procedure for the removal of lesions in the artery) for ischemic conditions was done using carotid artery tissues. The target cells in the tissues of symptomatic patients showed a marked increase in the expression of not only CD83, a marker for mature dendritic cells but also CD86, a co-stimulatory molecule, which is involved in T cell activation. The activated dendritic cells also release chemokines like CCL19 and CCL21 that recruit the CCR7-expressing naïve as well as central memory T cells into the atherosclerotic plaque. The quantitative PCR reaction performed also showed a very high number of IFN- γ and TNF- α

transcripts that directly correlated with the T cell presence in the plaque tissue. Thus, the dendritic cells present in the plaque tissues play a vital role in the inflammatory processes and lead to the advancement of the atherosclerotic lesions.

1.7.2 Adaptive immunity in Atherosclerosis

Chronic inflammation, caused by the adaptive immune system (T and B lymphocytes) in the heart tissue is the driving force behind the occurrence of atherosclerosis. The local cells of the innate immune system release cytokines and chemokines that attract T and B lymphocytes into the lesions and exacerbate inflammation. Different subsets of T and B cells have contrasting inflammatory actions on atherosclerotic lesions, which further complicate the understanding of coronary heart disease (CHD). T_H1 cells are pro-inflammatory, the roles of T_H2 and T_H17 cells are indefinite and Regulatory T cells (Tregs) confer suppression of inflammation (Oufella *et al.*, 2014). IL-13, a T_H2 cytokine, when administered into *Ldlr*^{-/-} mice was shown to decrease the expression levels of VCAM-1, thereby, reducing monocyte influx into atherosclerotic lesions. In addition, IL-13 also induces the activation of anti-inflammatory M2 macrophages over the pro-inflammatory M1 macrophages. Binder *et al.*, in 2004 reported that IL-5 deficiency led to increased atherosclerotic development. Also, IL-5 induced the production of IgM from B 1 cells, which are atheroprotective. In contrast, transfer of bone marrow stem cells from *IL-4*^{-/-} mice into *Ldlr*^{-/-} led to the decrease in the atherosclerotic development. This suggests that the deficiency of IL-4 led to the decrease in atherosclerosis development. Similar to T_H2 cells, the roles of T_H17 cells are also unclear. Breeding of *IL-17A*^{-/-}*ApoE*^{-/-} and *IL-17RA*^{-/-}*ApoE*^{-/-} mice led to the reduction in atherosclerotic lesion size in the aorta. Van Es *et al.*, in 2010 used dendritic cells transfected with mRNA coding for Foxp3 to understand the role regulatory T cells essay in atherosclerosis. *Ldlr*^{-/-} mice were vaccinated with transfected DCs to induce a cytotoxic immune response against Foxp3. It was found that the percentage of Foxp3 Tregs in *Ldlr*^{-/-} transfected with Foxp3 was 27% lower than *Ldlr*^{-/-} mice transfected with

GFP. This reduction also led to the increase in atherosclerotic lesion formation in *Ldlr*^{-/-} mice. George *et al.*, in 2012 showed that patients who suffered from two myocardial infarctions have significantly lower levels of CD4⁺CD25⁺ regulatory T cells and IL-10 than controls who underwent elective coronary angiography. Furthermore, Klingenberg *et al.*, (2013) used *Ldlr*^{-/-} mice that received bone marrow from depletion of regulatory T cells (DEREG) mice and were injected with diphtheria toxin (DT) to deplete Foxp3⁺ T regulatory cells. This depletion of regulatory T cells leads to the significant increase in atherosclerosis as well as increase in very low-density lipoproteins (VLDL).

With respect to B cells, Kyaw *et al.*, in 2010 showed that CD20 antibody mediated depletion of B cells from *ApoE*^{-/-} mice ameliorated atherosclerosis development. In addition, the transfer of conventional B2 B cells into mice led to the increase in lesion size, whereas the adoptive transfer of B1 cells did not support the formation of lesions. Thus, the authors concluded that the B2 subset was pro-atherogenic whereas B1 subset was anti-atherogenic. Moreover, a report by Oufella *et al.*, in 2010 showed that CD20 antibody mediated depletion of B cells in *ApoE*^{-/-} led to the reduction in the development of atherosclerosis. Another report by the Kyaw research group in 2012 showed that BAFF receptor deficient *ApoE*^{-/-} mice showed a reduction in B2 cells but not B1 cells in blood, spleen, peripheral lymph nodes and peritoneal cavity. This reduction in B2 cells also led to the decrease in atherosclerotic lesion size as well as IgG1 and IgG2a levels. Thus, it is important to research the functions of different subsets of T and B lymphocytes at the atherosclerotic lesions to understand the role of lymphocytes and improve disease state by devising treatments.

1.7.2.1 T cells in Atherosclerosis

T cells in the atherosclerotic lesion regions are recruited in parallel with the monocytes by chemokines and via the adhesion molecules on the impaired endothelium. Dendritic cells also

play a major role in presenting oxidised LDL (antigens) within lymph nodes where the T cells reside, and program them to 'home' to the plaque regions. The activated T cells in the plaque regions release pro-inflammatory mediators, which promote the growth of lesions and exacerbate atherosclerosis. T helper1 (T_h1) cells are the major promoters of atherosclerosis due to their release of IFN- γ which causes increased expression of inflammatory mediators and enhanced expression of adhesion molecules on dysfunctional endothelial cells (Hansson & Hermansson, 2011). The pro-atherogenic nature of IFN- γ is evident in the reduction in lesion size when the expression of IFN- γ is reduced. Research by several groups prove the major role for IFN- γ . Gupta *et al.*, in 1997 showed that in *ApoE*^{-/-} mice, there was substantial influx of CD4⁺ and CD8⁺ T cells in the intima of the arterial walls, whereas in *ApoE*^{-/-}*IFN- γ R*^{-/-} double knockout mice showed a 59% reduction in lipid content in the lesions compared to the *ApoE*^{-/-} mice. Buono *et al.*, in 2003 showed that in *IFN- γ* ^{-/-}*Ldlr*^{-/-} mice, the atherosclerotic lesions size reduced by 43% in the aortal arch and 65% in the descending aorta after 20 weeks of high cholesterol diet than in control mice. Another important observation is that the deletion of the *Tbx21* gene which codes for the transcription factor T-bet also led to the marked reduction of lesion size in *Ldlr*^{-/-} mice. It is widely known that activation of T-bet leads to the differentiation of the naïve T cells into T_h1 cells (Hansson & Hermansson, 2011).

The role of T_h2 cells is debatable as the presence of IL-33 (a potent activator of T_h2 cells) leads to reduced atherosclerosis (Hansson & Hermansson, 2011). Contradictory to the theory that T_h2 cells reduces atherosclerosis, a study by Davenport & Tipping, (2003) showed using a double knockout *ApoE*^{-/-}*IL-4*^{-/-} mouse model that the deficiency of IL-4 leads to reduced plaque formation in the aortic arch at 30 weeks but there was no significant difference in the lesion size with *ApoE*^{-/-} only mice at 45 weeks. IL-4 increases the expression of monocyte chemoattractant protein 1 (MCP-1) which leads to the recruitment of monocytes. Increase in

levels of IL-4 also leads to the upregulation of adhesion molecule VCAM-1 and promotes the formation of oxidized lipoproteins (Davenport & Tipping, 2003).

The role of T_h17 cells in CHD, like T_h2 cells, is also debatable. A study by Smith and Colleagues in 2010 implicates IL-17A in an atherogenic capacity. The mRNA level of IL-17A was significantly higher in *ApoE*⁻ mice than in C57BL/6 control mice and directly correlated with increased lesion size. The IL-17A level was also strikingly higher than other cytokines in the IL-17 family. It was further shown that blocking IL-17A using a fusion protein reduced atherosclerotic plaque development. Thus, while the role of T_h1 cells is clearly atherogenic, the roles of T_h17 and T_h2 cells remain unclear.

Kyaw *et al.*, (2013) showed that depletion of CD8⁺ T cells reduces atherosclerosis. The depletion of CD8 α ⁺ T cells and CD8 β ⁺ T cells in *ApoE*⁻ mice showed a profound decrease in lipid accumulation, atherosclerotic lesion size and macrophage accumulation. The results of the TUNEL assay and hematoxylin and eosin staining performed on plaque sections showed that CD8⁺ T cells contribute greatly to the increase in apoptotic cells and cell necrosis by releasing perforin and granzyme B, which demonstrates their role in atherogenic effects of CD8⁺ T cells.

The molecules programmed death ligand 1(PD-1) and PD-2 on T cells inhibit the activation (both CD4⁺ & CD8⁺) through binding of ligands on tissue cells. The mouse model PD-L1/2^(-/-) *Ldlr*^(-/-) mice had a marked increase in the number of T cells compared to control mice (*Ldlr*^(-/-)) and increased atherosclerotic lesions. Thus, PDL1 and PDL2 play a vital role in regulating T cell population in the plaque regions (Gotsman *et al.*, 2007). Regulatory T (Tregs) cells have an atheroprotective role in atherosclerosis. These cells are the major producers of TGF- β and IL-10, which suppress inflammation and confer protection against inflammation-induced injuries (Hansson & Hermansson, 2011). Oufella *et al.*, (2006) showed that the number

of Tregs cells was reduced in CD80⁻CD86 and CD28 deficient *Ldlr*⁻ mice that points to the possible role of regulatory T cells having an atheroprotective role in atherosclerosis.

T cell homing in atherosclerotic lesions involves a wide variety of chemokines. The role of CCR7 on T cells, and its ligands CCL19 and CCL21, in homing and migration of T cells into the heart is contradictory. Lesion size increased in *CCR7*⁻*ApoE*⁻ mice whereas lesions size decreased in *CCR7*⁻*Ldlr*⁻ mice suggesting conflicting results when two different mouse models were used. Li & Ley in 2015 suggested the use of T cell specific CCR7 knockout mice to study atherosclerosis. The use of NB-74330 as an antagonist for CXCR3 in *Ldlr*⁻ mice reduced lesion size by 53% compared to control mice, which indicates that the presence of CXCR3 is vital for the migration of pro-inflammatory cells into the lesion area. The increased number of regulatory T cells in the lesion region also suggested a shift from a pro-inflammatory to anti-inflammatory environment (Van Wanrooij *et al.*, 2008). Thus, while the roles of Th1 cells are clearly atherogenic, the role of Th2 and Th17 cells in atherosclerosis is less clear.

1.7.2.2 Role of T cells in Myocardial Infarction and Reperfusion (MI+R) injury

Thygesen *et al.*, in 2012, defined myocardial infarction as death of myocardial cells due to ischemia (lack of oxygen supply to the heart). Stent or balloon inflation by primary percutaneous coronary intervention (PPCI) causes transient ischemia irrespective of chest or ST elevated changes. It is important to note that myocardial injury with necrosis may occur as the result of pre-PPCI procedure or due to microvascular plugging and atherosclerotic plaques. This procedure results in inflammation of the myocardium, thus the myocardial infarction-related reperfusion injury is usually associated with elevated troponin levels. Studying the role of T cells in the myocardium might provide important insights into the immunological aspects of myocardial infarction, potentially indicating diagnostic and therapeutic targets.

Lluberas *et al.*, in 2015 showed that, in ST segment elevated myocardial infarction (STEMI) patients, CD4⁺CD28^{null} T cells preferentially accumulated in the intracoronary regions at the plaque rupture site compared to peripheral blood. In addition, they also compared T cells levels with evolution of STEMI ~6 hours (from pain to balloon time). It was found that T cell numbers were higher near the plaque regions than in peripheral blood. Moreover, CD4⁺CD28^{null} T cells were significantly higher in patients with higher creatine kinase levels, thus suggesting that myocardial injury is increased with the increase in T cells in the plaque regions. The levels of IFN- γ and IL-10 were also increased. This shows how the immune system maintains the balance between potent pro-inflammatory reaction and potent anti-inflammatory reaction. Mathes *et al.*, (2016) showed that MHC class II KO mice, which are incapable of developing MHC class II restricted CD4⁺ T cells, had significantly lower infarct size and improved microvascular reperfusion. Also, the ejection fraction as determined by *in-vivo* magnetic resonance tomography was better preserved in MHC class II KO mice. The research group also used CD4 KO mice and reconstituted them with Foxp3⁺ and Foxp3⁻ CD4 T cells. It was found that CD4 KO mice with CD4⁺ CD25⁺ T cells had a significant increase in infarct size similar to MHC class II KO mice. Interestingly, using immunofluorescence to carry out histological examination, the authors found out that CD4⁺ T cells remain in the microvascular bed rather than infiltrate or enter the myocardium, 24 hours after reperfusion.

The myocardium, under ischemic pressure, undergoes ventricular remodelling to maintain blood flow to the heart. However, persistent inflammation in the heart tissue leads to impaired ventricular remodelling, damage to extracellular matrix and apoptosis of immune cells. Persistent inflammation is caused by pro-inflammatory cytokines and infiltration of pro-inflammatory immune cells. Regulatory T cells induce the differentiation of anti-inflammatory M2 macrophages, secrete TGF- β , and dampen the inflammation by inhibiting TNF- α , MMPs. The dampening of inflammation helps in repairing the injured myocardium (Fang *et al.*, 2015).

Hoffmann *et al* in 2012 showed, 24 hours after PPCI, the central memory compartment of CD4⁺ T cells was highly elevated whereas the levels of naïve and T_{EMRA} cells dropped. The elevation and drop in cell numbers was attributed to the both the blockage in the arteries as well as reperfusion injury caused by PPCI. The research group also developed a hierarchical clustering analysis, which identified a memory CD4⁺ T cell expressing CD57⁺ which was negative for KLRG1. Further, the mean fluorescent intensity of CD57⁺ increased whereas MFI for KLRG1 and PD-1 remained unchanged. Further, the MFI for naïve CD4⁺ and CD4⁺ central memory cells did not differ from each other whereas the MFI for effector memory cells and TEMRAs were significantly higher than MFI of naïve CD4⁺ T cells which indicates that the expression of CD57 occurred after cell divisions. Among the CD4⁺ sub population, the MFI of effector memory and T_{EMRA} were higher than in naïve T cells. The mean telomere lengths of CD4⁺ T cells was shorter in STEMI patients than in healthy controls indicating cellular ageing. Interestingly, there was also a selective depletion of CD4⁺CCR7⁺ T cells during the first 30 minutes after reperfusion. However, this population started to increase between the 30 minutes to the 2-hour time point. Thus, the expression of CD57⁺ and depletion of CCR7⁺ cells represent changes in the CD4⁺ T cell compartment due to myocardial infarction and reperfusion injury. Damas *et al.*, in 2007 also showed that in patients with stable and unstable angina pectoris, there was a significant drop in CD4⁺CCR7⁺ T cells compared to healthy controls. This drop in CD4⁺ also coincided with the increase in serum levels of CCL19 and CCL21, both of which are ligands for CCR7.

ApoE^{-/-} mice with advanced atherosclerotic lesions showed increased levels of CCL19, CCL21 and CCR7 expression within the atherosclerotic plaques. In addition, patients undergoing carotid endarterectomy also showed increased levels of CCL19, CCL21 and increased expression of CCR7 in the atherosclerotic lesions, which strongly indicate the homing of CCR7⁺ T cells in the plaque regions. Carotid endarterectomy is a surgical procedure designed

to remove fatty acid deposits from the carotid arteries that supply blood to the head and neck regions in patients suffering from carotid artery stenosis, one of the main reasons for the occurrence of ischemic stroke (National health service, 2018). The strong role CCR7 plays in the exacerbation of atherosclerosis was studied by Luchtefeld *et al.*, in 2010 in great detail. A cross of mice lacking the low-density lipoprotein receptor (*Ldlr*^{-/-}) and CCR7 was fed with a high cholesterol diet. It was found that *Ldlr*^{-/-} *CCR7*^{-/-} mice showed greater than 50 percent decrease in atherosclerotic lesion size compared to the lesion size in *Ldlr*^{-/-} mice. The drop in lesion size was also accompanied by a significant decrease in the release of IFN- γ in *Ldlr*^{-/-} *CCR7*^{-/-} mice compared to *Ldlr*^{-/-} mice. Thus, these results suggest that CCR7 mediated migration of T cells into the myocardium or atherosclerotic lesions leads to the exacerbation of heart disease.

Low circulating lymphocyte counts in STEMI patients after PPCI was associated with mortality. 90 minutes after PPCI-mediated reperfusion, effector memory and terminally differentiated effector memory T cells (both CD4⁺ and CD8⁺) showed a larger drop in percentages than naïve and central memory T cells. The drop in effector T cell and effector memory (T_{EMRA}) correlated with level of microvascular obstruction (MVO). Furthermore, the level of Fractalkine receptor CX3CR1 correlated with the drop in T cell counts. Serum fractalkine levels were highest at 90-minute time point which coincided with the loss of T cells from the myocardium. Thus, it is also suggested that fractalkine levels might play a vital role in the level of microvascular obstruction (MVO) (Boag *et al.*, 2015).

1.8 Ageing and Immune senescence is rapid in CMV seropositive patients

The telomeres are DNA-protein complexes present in the ends of chromosomes that prevent the ends of chromosomes from recombination and degradation. Telomeres are tandem repeats of TTAGGG sequences. Telomere length shortens with each cell division owing to the inability of the DNA polymerase to replicate the 5' end of the strand. Thus, the telomeres are considered

molecular clocks in ageing (Blasco, 2005). Immune senescence is the age-related dysfunction of the immune system that leads to the decrease in the naïve T cell population and a decrease in the range and functions of the T cell subsets thereby leading to impaired ability of the immune system in the elderly to mount effective defenses against pathogens (Pawelec *et al.*, 2009).

1.8.1 A primer on Cytomegalovirus

Cytomegalovirus (CMV) is a ubiquitous β -type 5 human herpes virus. CMV is the largest member of the human herpes virus family. The double stranded DNA of CMV is enclosed by protein-rich tegument matrix, which is itself enveloped by a lipid bilayer. The infection causing genes of CMV are located in the tegument matrix (Crough & Khanna, 2009). The seroprevalence of CMV in Asia and Africa is considered 100 %, whereas in Europe, the seroprevalence is approximately 80% (Rosa & Diamond, 2012). The main modes of CMV transmission through individuals are sexual contact, placental transfer, solid organ transplantation and stem cell transplant. After CMV enters the host, it establishes lifelong latency in an immunocompetent individual. CMV establishes latency through latency genes before proceeding to activate genes that cause infection. The conversion from latency mode to infection mode occurs due to inflammation, stress, and other infections (Crough & Khanna, 2009). CMV establishes latency due to the suppression of lytic genes such as the immediate early (IE) genes. This results in the lack of production of infectious viral particles and viral gene expression is primarily focussed on the expression of latency genes (Poole *et al.*, 2014). It is widely known that active CMV infection results in the modulation of numerous cell activities such as transcription, translation and immune surveillance, however, it is interesting to note that CMV latency also elicits a host of beneficial cell activities such as metabolism, cell signalling, transcription and translation (Poole & Sinclair, 2015). Mason *et al.*, in 2012 screened cell culture of supernatants of CD34⁺ progenitor cells infected with Merlin, a clinical

CMV strain, as well as a UV-inactivated version of Merlin to account for changes resulting from viral binding. The authors identified a significant increase in both CCL8 and CCL2 during the latent infection of CD34⁺ cells compared to UV-inactivated infection of CD34⁺ cells. The increased expression of CCL8 induced the migration and recruitment of CD4⁺ T cells via CCR5 and CCR3. There was also no observable migration of NK cells, CD8⁺ T cells and B cells. Although, the latent infection caused the migration of CD4⁺ T cells, latently infected CD34⁺ progenitor cells also secreted cellular IL-10 and TGF- β which causes the dampening of the immune activity of CD4⁺ T cells. These results indicate how the CMV maintains latency despite being under immune surveillance.

Transmission of CMV from mother to child occurs via three routes, 1. intrauterine transmission, 2. intrapartum transmission and 3. post-natal transmission. Intrauterine transmission of CMV comprises either the direct entry of CMV into the placenta or amniotic fluid. It could also occur due to the leucocyte transmigration into the placenta. Intrapartum transmission is caused by viral shedding by CMV seropositive mothers during delivery. Post-natal transmission of virus is the entry of CMV into neonates through breast milk feeding (Joseph *et al.*, 2018). Most neonates (85-90 percent) have asymptomatic infection whereas the remaining 10-15 percent of neonates show clinical manifestations. Some of the clinical manifestations are jaundice, petechial rash, hepatosplenomegaly, microcephaly, atopic atrophy and lethargy (Boppana *et al.*, 2013). Congenital CMV infection poses a significant global burden where children are born with, or develop over a period of time, permanent disabilities such as hearing loss, cerebral palsy and loss of eyesight. The prevalence of congenital CMV is much higher in developing countries than it is in developed countries (Fowler & Boppana, 2018). Detection of CMV in neonates through rapid culture or PCR is thus imperative to prevent long term disability (James & Kimberlin, 2016). The prevention of CMV infection using CMV hyperimmune serum has been suggested. Pregnant women were given

hyperimmune globulin every 4 weeks till 36 weeks or until the detection of CMV in the amniotic fluid. A randomized double-blind study involving placebo controls showed no statistically significant difference between the placebo group and the group comprising of pregnant women (Revello *et al.*, 2014). The current accepted treatment for congenital CMV infection is the administration of valganciclovir. Kimberlin *et al.*, in 2015 compared the efficacy in the use of valganciclovir for 6 weeks and for 6 months. Although both groups did not differ in the hearing levels in neonates at the 2-year period, the neonates belonging in the 6-month groups showed better neurological development as evidenced by better scores on the Bayley scales of toddler and infant development.

CMV also causes significant mortality and morbidity in solid organ transplantation (SOT) in immunocompromised recipients. Lytic CMV replication occurs either due to latent CMV infection or due to *de novo* primary CMV infection (Han, 2017). The CMV seronegative patients who receive organs from CMV seropositive donors face the highest risk for CMV disease. Other major risk factors involved in solid organ transplantation are history of graft treatment as well as type of immunomodulatory drugs administered (Beam & Razonable, 2012). The Burden of CMV infection in D⁺R⁻ SOT patients could be reduced by anti-viral prophylaxis (Han 2017). In the absence of adequate anti-CMV immunity in D⁺R⁻ SOT patients, the risk of late onset CMV disease is highly increased. Limaye *et al.*, in 2016 tested the efficacy of plasma IL-10 levels in determining the propensity of SOT patients to develop late onset CMV disease. CMV specific T cell responses were detectable during VALGAN prophylaxis when the IL-10 levels were undetectable. Thus, the extension of VALGAN administration beyond the initial treatment period could delay or reduce the incidence of the late onset CMV disease in SOT patients. Another important factor that could influence plasma IL-10 could be the presence of anti-graft rejection immune suppressing drugs. So, SOT patients who are forced to consume immune suppressing drugs may be more prone to late onset CMV disease.

Apart from all the negative aspects of CMV infection in SOT, it is also important to note there might be beneficial effects of CMV infections. Litjens *et al.*, (2018) recently published an excellent review on the potential benefits of CMV infection due to transplantation. In a cohort of allogeneic hematopoietic stem cell transplantation (HCT) patients with acute myeloid leukaemia (AML), it was found that pre-emptive anti-CMV therapy showed a significantly reduced risk for the AML to relapse within 10 years after transplantation. However, this protection correlated only for AML and not for acute lymphoblastic leukaemia and chronic myeloid leukaemia.

1.8.2 Role of CMV in immunosenescence

Immunosenescence or ageing of the immune system is characterized by loss of proper functioning of immune system, chronic inflammation and autoimmunity, all of which are associated with comorbidities and mortality (Müller *et al.*, 2017). It is suggested that altered transcription and altered epigenetics are major influences in immunosenescence. CD28⁻ memory T cells had elevated levels of T-bet and EOMES than CD28⁺ memory T cells. In addition, epigenetic mechanisms such as DNA methylation, modifications to histone proteins, and structural changes in chromatin and microRNA regulation are thought to play a vital role in the alterations to T cell repertoire. Furthermore, nutrition, environmental factors and lifestyle too play a role in accelerating or decelerating immunosenescence (Tu & Rao, 2016). However, in this thesis, the focus will be on the role of CMV in immunosenescence. Koch research group in 2008 used multicolour flow cytometric analysis to identify changes in T cell homeostasis in younger and elderly cohort. For the CD4⁺ T cell compartment, the loss of naïve CD4⁺ T cells in elderly populations did not show a significant change compared to the younger cohort, furthermore, there was an increase in proportion of CD4⁺ central memory T cells in the elderly population. Whereas for CD8⁺ T cell compartment, there was a significant drop in naïve cell count but a significant gain in terminally differentiated effector memory count (T_{EMRA}) in the

elderly population. This suggested that the drop in naïve CD8⁺ count was accompanied by the increase in terminally differentiated effector memory CD8⁺ T cell count. This research group, however, did not consider the CMV serostatus of these individuals and did not include the impact of active CMV on the changes to the CD8⁺ T cell compartment. The drop in naïve CD8⁺ T cell count and increase in CD8⁺ T_{EMRA} T cell count is a strong indicator of immunosenescence. Weinberger *et al.*, in 2007 showed that although percentages of naïve and memory CD8⁺ T cells dropped and percentages of CD28⁻CD8⁺ effector T cells in elderly CMV seropositive individuals were higher than in elderly CMV seronegative individuals, CD28⁻CD8⁺ effector T cells negatively correlate with CD28⁺CD45RA⁺ naïve and CD28⁺CD45RA⁻ memory cells irrespective of CMV serostatus. This indicated ageing was the primary cause of the changes in the T cell pool. With respect to CMV seropositive CD4⁺ T cells, the effector cells negatively correlated with memory cells but not naïve cells. Also, in the elderly cohort with latent CMV presence, the CD8⁺ effector cells positively and negatively correlated with CD4⁺ effector and memory cells, respectively. Thus, they suggested that the presence of CMV induces changes in both CD4⁺ and CD8⁺ T cell compartment (Weinberger *et al.*, 2007). Consistent with Weinberger and Koch research group, Wertheimer *et al.*, in 2014 showed that, in CMV seronegative healthy individuals, naïve CD8⁺ T cell drop was accompanied by the increase in the effector memory and central memory CD8⁺ T cells with age and central memory CD4⁺ T cells also showed a slight significance increase in cell numbers with age. However, apart from percentages of T cells, absolute numbers of T cells were also considered unlike in the Koch and Weinberger research group. Absolute numbers of naïve, central memory and effector memory CD4⁺ T cells in CMV seronegative individuals did not change with age. Furthermore, absolute numbers of naïve CD8⁺ T cells showed a steep drop whereas numbers of effector memory (EM) and central memory (CM) CD8⁺ T cells showed no change or decreased minimally. In CMV seropositive healthy individuals, the drop in absolute numbers

of naïve CD8⁺ T cells was steeper but not significantly than in CMV seronegative healthy individuals. Absolute counts of CD4⁺ EM and CD8⁺ EM cells was significantly higher in CMV seropositive individuals than in CMV seronegative individuals. Thus, this research group attributed the loss of naïve CD8⁺ T cells to ageing and the increase in EM CD8⁺ and CD4⁺ T cells to CMV seropositivity (possibly due to persistent activation of CMV). Thus, this study concluded that ageing and CMV seropositivity jointly influence the changes in the T cell compartment. Turner *et al.*, (2014) showed that CMV seropositive young healthy individuals (Mean age: 21; BMI:22.7) show initial signs of Immunosenescence. CMV seropositive healthy individuals had significantly higher lymphocyte count and lower CD4/CD8 ratio than CMV seronegative healthy individuals. Among lymphocytes, effector memory and late differentiated in both CD4⁺ and CD8⁺ T cells were higher in CMV seropositive individuals. In addition to differences in lymphocyte counts, plasma levels of IL-6 were also higher in CMV seropositive individuals. However, Turner and Wertheimer groups did not study the effects of CMV on telomere lengths of T cells in their study cohort. Van de Berg research group in 2010 studied the changes in telomere lengths of T cell populations in CMV seropositive renal transplant patients. The drop in naïve CD27⁺CD8⁺ T cell count was accompanied by the increase in CD8⁺CD27⁻ differentiated T cells in CMV seronegative transplant patients receiving renal transplants from CMV seropositive donors (Primary CMV infection). The lymphocyte pool did not differ in CMV seronegative patients receiving renal transplants from CMV seronegative donors. In CMV seropositive renal transplant patients, the mean telomere lengths of lymphocytes significantly dropped after primary CMV infections whereas the drop in telomere length of lymphocytes in CMV seronegative renal transplant patients did not achieve significance. Telomere length of naïve CD27⁺CD8⁺ T cells was longer than the telomere length of CD8⁺CD27⁻ differentiated T cells. Even among the healthy cohort, telomere length of CMV seropositive individuals were shorter than CMV seronegative individuals and the drop in

telomere length in CMV seropositive individuals positively correlated with differentiated CD4⁺ and CD8⁺ T cells. Thus, these studies clearly indicate that in ageing, the presence of CMV cause the decrease in telomere length and changes in the circulating T cell pool.

The role of CMV-induced immunosenescence in CHD is profound. A study found that telomere lengths of monocytes, granulocytes, CD4⁺ T cells and CD19⁺ B cells were approximately 500 base pairs shorter in CHD patients than in age-matched healthy controls (Spyridopoulos *et al.*, 2009). However, the telomere length of CD8⁺ T cells, more specifically, CD28⁻CD8⁺ T cells in CHD patients was 1000bp shorter than in age-matched controls; also, telomere lengths of CD8⁺CD27⁻CD28⁻CD45RA⁺ T cells correlated with left ventricular function (ejection fraction) in CHD patients, suggesting a link between CD8⁺ T cells and myocardial dysfunction in CHD patients or high-risk individuals. Moreover, only telomere lengths of CD8⁺CD28⁻ T cells in CMV seropositive healthy controls was higher than in CMV seropositive CHD patients. Thus, continued CMV-specific immune response in CHD patients causes profound changes to the telomere lengths of lymphoid cell population, especially, the CD8⁺ T cells (Spyridopoulos *et al.*, 2009). Analysis of 749 peripheral blood samples by Spyridopoulos research group in 2016, of octogenarians revealed interesting results. CMV seropositive participants were more prone to ischemic heart disease. There were also profound changes to the T cell pool: CD4/CD8 ratio was lower with the increased presence of CD4 and CD8 T_{EMRA} cells. There was a significant association between CMV seropositivity and cardiovascular mortality in participant cohort. In addition, CD4⁺CD45RO⁺CD28⁻CD27⁻ cells (senescent-like) and CD8⁺CD27⁺ (non-senescent) cells were also associated with cardiovascular mortality. Consistent with Spyridopoulos *et al.*, 2016; in elderly participants (age above 65), median life expectancy in CMV seropositive population was also lower than in CMV seronegative population CMV seropositivity was associated with 40% mortality. In addition, cardiovascular death was the most common cause of mortality among CMV

seropositive participants and mortality from other causes did not correlate with CMV seropositivity. Thus, CMV seropositivity in individuals above the age of 65 is strongly associated with cardiovascular mortality (Savva *et al.*, 2013).

In conclusion, studies have shown that the homing of immune cells, particularly EM T cells, to the damaged myocardium, contribute to injury during, and following, myocardial infarction and reperfusion. CMV positivity enhances the numbers of EM T cells and induces their chronic stimulation and proliferation leading to premature aging of the cells (immune senescence) together with immune dysregulation in the form of chronic inflammation through cytokine secretion.

1.9 Chronic Fatigue Syndrome (CFS)

Early research into CFS was typified by the presence of numerous definitions for CFS used by research groups, the use of incorrect medical tests to diagnose CFS patients, and the lack of subgrouping for cohort populations, making it difficult for comparative studies to be conducted. Thus, Fukuda *et al.*, in 1994 designed a conceptual framework that eliminated all the above-mentioned negatives. According to the framework, only persistent fatigue lasting more than 6 months would be classified as ‘chronic fatigue’ and diagnosis of CFS would only be made after a comprehensive mental, physical and laboratory examination. Patients with depression-related conditions such as bi-polar disorder, schizophrenia, alcohol and or drug abuse, and medical conditions that have chronic fatigue as a major symptom, such as untreated hyperthyroidism, can be classified as having chronic fatigue. However, patients with conditions such as fibromyalgia and anxiety disorders that cannot be confirmed by laboratory tests, or any condition for which there is definitive treatment options, require further diagnosis to be classified as having chronic fatigue. Severe fatigue is a symptom that is associated with a wide variety of disorders such as sleep disorders, infections, immunopathology, autoimmunity, cancer, and endocrine and metabolic disorders. CFS may be associated with pathogenic

signalling pathways that converge at the neurological axis (Jones *et al.*, 2009) and is most often associated with chronic inflammation. In most cases fatigue is idiopathic i.e. the cause of fatigue in patients with CFS/ME is unknown and cannot be identified by conventional lab tests (Holgate *et al.*, 2011). Extensive research points to CFS as a condition that occurs after an infection by a pathogen, thus testing immune responses of CFS patients against a wide range of implicated pathogens such as Influenza, Cytomegalovirus, Epstein-Barr virus, Human herpes virus 6, 7, and mycoplasma is warranted (Cameron *et al.*, 2010). Chapenko *et al.*, in 2012 used the 1994 CDC criteria to diagnose CFS patients and used the fatigue severity scale (FSS) to measure fatigue in patients. They measured the frequency of HHV-6, HHV-7 and parvovirus B19 viral transcripts in CFS patients. CFS patients and healthy controls had a high seroprevalence as well as comparable levels of HHV-6 and B19 IgG and IgM antibodies. However, there was a significant increase in active HH6, HHV-7 and B19 viral infection in CFS patients compared to healthy controls. In disagreement with Chapenko group, Oakes *et al.*, (2013) found using qPCR that viral copy numbers of HHV-6 and HHV-7 did not differ between healthy controls and chronic fatigue syndrome patients. Furthermore, they also showed that the viral copy numbers of human endogenous retroviruses (HERV) did not differ between the two groups. Thus, analysis of viral influence in CFS patients revealed contrasting results. Treatment of patients with valacyclovir and valganciclovir, in response to elevated serum levels of EBV, HHV 6, 7 and CMV, led to improvements related to immunologic and neurological functioning. The use of Isoprinosine, an immunostimulatory drug, increased NK cell activity and increased levels of CD4⁺ T cells. In addition, the clinical trial involving the use of Rintatolimod led to the production of type 1 interferon and led to the reduction of fatigue symptoms in patients. Thus, the successful action of these drugs indicates a role of microbial pathogens in contributing chronic fatigue in patients. However, the action of acyclovir had no effect on CFS patients. Moreover, a research report by the Reeves research group in 2000 and

Burbelo *et al.*, in 2012 identified no associations between viral infections and CFS. Therefore, it is possible that the presence and active replication of EBV, HHV 6, 7 and CMV will be due to underlying conditions rather than active involvement with CFS. The use of different disease classifications and use of different analytical methods might also play a vital role in the contrasting results. There is also emerging evidence to suggest CFS might have an autoimmune component. Rituximab is usually used to treat rheumatoid arthritis, but it was used by Fluge group to show disturbances in the immune system of CFS patients. Navaneetharaja, *et al.*, 2016 suggested that intestinal microbiota might be a trigger for CFS and identification of biomarkers would add to the understanding of CFS as well as help in generating therapeutics to treat patients through alteration of this microbiota. An intestinal dysfunction is supported by the research by Fremont *et al.*, (2013) who used high throughput 16S rRNA sequencing to test for changes in intestinal microbiota in CFS patients from Belgium and Norway. It was found that the levels of genera *Lactonifactor* and *Asaccharobacter* were significantly higher and lower respectively in Belgian CFS patients. Genera *Roseburia* and *Holdemania* showed a significant increase in Norwegian CFS patients whereas genera *Bacteroides*, *Alistipes*, *Barnesiella*, *Parabacteroides* and *Prevotella* were significantly reduced when compared to levels in Norwegian controls.

The innate arm of the immune system is non-specific and elicits the same magnitude of response when encountered with a second bout of infection as the first, whereas the adaptive arm of the immune system is highly specific and elicits a stronger immune response when the same pathogen is encountered again. Innate immune cells such as macrophages express TLRs which recognize common molecules on invading pathogens and release an assortment of cytokines which attracts other immune cells to the site of infection. In contrast, immune cells of adaptive immune system such as T cells have cell surface T cell receptors that are highly specific for a complementary molecule of a pathogen. The T cells of the Th1 phenotype release

IFN- γ to combat intracellular pathogens, Th2 cells produce cytokines like IL-4, IL-5 to combat worms, and Th17 cells produce IL-17 to fight extracellular pathogens. Once the pathogen is neutralized, the inflammation is resolved by regulatory T cells (T_{regs}) which produce IL-10 and TGF- β to dampen the immune response. In a normally functional immune system, a homeostatic balance is maintained between inflammation-causing and inflammation-resolving immune cells. Chronic inflammation is caused by an over-reactive immune system due to the presence of persistent pathogens like CMV, which is also associated with immune senescence, accelerated immune ageing and increased mortality among the elderly population (Macaulay *et al.*, 2013).

Limited studies have attempted to define the immunology behind CFS that could throw a light on the mechanism of disease and act as biomarkers and therapeutic targets (Lorusso *et al.*, 2009; Klimas *et al.*, (2012). From a wide variety of published results, consistent markers observed have been increased inflammatory cytokines such as IFN- γ , TNF- α , IL-1, IL-12 in blood (Kerr *et al.*, 2008) and serum, and upregulation of related receptors and signalling pathway molecules (NF- κ B, JAK-STAT) which strongly suggest increased inflammation in individuals with CFS/ME. Persistent viruses such as EBV and CMV are also thought to play an important role in the exacerbation of fatigue in patients. Studies have also suggested increased Th2 responses in CFS patients (Skowera *et al.*, 2004). CFS patients who are seropositive for Epstein-Barr virus had lower levels of NKH1 T3⁻ cells compared to healthy controls. Consequently, NK cells of CFS patients also showed reduced cytotoxic activity. Infusion of recombinant IL-2 increased the cytotoxic activity of NK cells against K562 cells; however, despite the infusion of IL-2, NK cells of patients could not lyse EBV infected B cells (Caligiuri *et al.*, 1987). There is also a suggestion that CFS may be an autoimmune disease (Elfaitouri *et al.*, 2013), where the immune system attacks healthy tissue. Some studies have researched targeted therapies for CFS including the use of antivirals (Lerner *et al.*, 2007) and

B cell-depleting antibodies [rituximab] (Fluge *et al.*, 2011). Thus, a more comprehensive investigation into the immunology behind CFS will expand our knowledge and help us understand the pathology of chronic fatigue.

Nakatomi *et al* in 2014 showed through positron emission tomography (PET) scanning that 11C-(R) -(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxamide (11C-(R)-PK11195) bound to translocator protein (TSPO) 45% to 199% higher in CFS than in healthy controls. 11C-(R)-PK11195 is a ligand for TSPO. The presence of higher percentage of TSPO suggests activated astrocytes, microglia and glial cells indicating neuro-inflammation. The non-displaceable binding potential values (BP_{ND}) (affinity of a ligand to its receptor) values of this motif positively correlated with pain score, cognitive impairment score and depression score, thus strongly supporting a neuronal link to CFS. However, one drawback of this study was the small sample size, only 9 CFS patients and 10 healthy controls were used. As for treatment options for CFS, cognitive behaviour therapy (CBT) and graded exercise therapy (GET) are more likely to lead to recovery from CFS than specialist medical care alone (SMC) and adaptive pacing therapy (APT) (White *et al.*, 2013). Thus, a more comprehensive research approach encompassing both the immune system and nervous system must be taken to understand CFS.

1.9.1 Characterization of CFS: Fatigue Impact Scale (FIS)

The fatigue impact scale (FIS) was created to measure a patient's perception of functional limitations that they attribute to the symptoms of fatigue. The foundation of the FIS is laid on two pillars, questionnaires and interviews. The FIS is determined on the cognitive, physical and psychosocial functioning of patients. Along with fatigue impact scale, the sickness impact profile (SIP) was also generated to decipher the link between chronic fatigue and general health. One of the most important findings of the study by Fisk *et al.*, 1994 was that the FIS

was able to clearly distinguish between CFS patients and multiple sclerosis (MS) patients although fatigue is a common factor in both ailments.

Flachenecker *et al.*, (2002) concluded that fatigue was influenced by numerous factors and felt the necessity for developing a clear definition for CFS as well as develop more reliable tools to conclusively measure fatigue in patients. Fatigue severity scale (FSS) and modified fatigue impact scale (MFIS) were better tools to measure fatigue than the visual analog scale (VAS) or multiple sclerosis specific-FSS. FSS and MFIS, nevertheless had wide variations amongst themselves thus necessitating the requirement for a better understanding of fatigue in patients with MS so that wide variations could be reduced to a minimum. This process could also help measure accurate fatigue levels in patients with chronic fatigue. Tellez *et al.*, (2005) showed that cognitive and psychosocial aspects of fatigue were better measured by MFIS than FSS. The physical subscale of MFIS correlated strongly with FSS and the linear regression analysis of the physical subscale of MFIS was the only independent predictor of the FSS. Thus, the authors suggested that while the physical subscale of MFIS correlated with FSS, the cognitive and psychosocial aspects of fatigue are better analysed by MFIS.

Despite the establishment of numerous fatigue scales, the FIS still remains invaluable, robust and widely used among researchers and doctors (Newton & Frith, 2010).

1.9.2 Innate & Adaptive Immune Responses in CFS

1.9.2.1 Innate Immune cells in CFS

Hardcastle *et al.*, (2015) conducted experiments that measured the activation markers CD80 and CD86 on DCs but found no significant differences between healthy controls and patient cohorts. There were also no significant differences in the phagocytic potential determined by calculating the ‘respiratory burst’ of neutrophils and monocytes which did not differ between the healthy controls, moderately affected patient and severely affected patients. The expression

of lytic proteins such as perforin and granzyme B also showed no significant correlations between the participating groups. However, in a contrasting report, Brenu *et al.*, (2010) reported reduced neutrophil function in CFS patients. The 'respiratory burst' of neutrophils was decreased in patients when compared to healthy controls. It was suggested that decrease in neutrophil functions was due to the increase in levels of apoptotic neutrophils which impair immune monitoring activities of neutrophils. It is, however, important to mention only 10 patients and 10 healthy controls were used for this study. CD177, a cell surface molecule of neutrophils, used for migration into inflamed tissues, was reduced in patient samples compared to healthy controls which may indicate impaired migration of neutrophils to sites of infection or reduced CD177 expressing neutrophils in the periphery (Brenu *et al.*, 2013). Thus, sub-par respiratory burst of neutrophils along with reduced CD177 expression seem to have a negative effect on CFS patients and so more research is required to understand the role of neutrophils in CFS patients.

NK cells showed reduced cytotoxic capabilities in the CFS patient group compared to healthy controls (Brenu *et al.*, 2013). Type I IFN is also known to activate NK cells (Siegal *et al.*, 1999). Reduced NK cytotoxicity in CFS patients occurs despite the possible increase in serum type I IFN- α level, indicating that other factors might contribute to the reduced NK cell cytotoxicity. T_{regs} are known to have immunomodulatory and immunosuppressive effects during acute or chronic infections and the increased cell percentages of T_{regs} in patients may explain the reduced NK cell cytotoxicity. It is interesting to note that while NK cell cytotoxicity was reduced, the NK degranulation levels remained high suggesting NK cells, despite being in an activated state, are not functionally able to combat pathogens, which might be due to NK cell anergy. Brenu *et al.*, in 2010 once again demonstrated the changes in NK cell phenotype. CD56^{bright}CD16⁻ NK cells were lower in the patient cohort, and NK cells as a whole showed reduced cytotoxic potential in CFS patients. However, in a contrasting report, the Curriu

research group in 2013 reported that although NK cell percentages and cytotoxicity did not statistically differ between patients and healthy controls, the expression levels of activation markers CD69 and NKp46 in NK cells were higher and CD25 levels were lower for CFS patients when compared to healthy controls.

Hardcastle *et al.*, in 2015 compared the moderately and severely affected CFS patients for irregularities in immune function. NK cells showed increased expression levels of CD150 (SLAM receptors) and NKp46 in moderately affected patients compared to severely affected patients. NKp46 is a member of the cytotoxicity activating receptor family of NK cells (Pessino *et al.*, 1998). The low levels of expression of NKp46 in severely affected patients may be one of the factors that leads to low NK cytotoxicity levels. The paucity of SLAM expression in severely affected patients may indicate a lack of immune regulation, thus leading to immune dysregulation.

1.9.2.2 Adaptive Immune system in CFS

Few comprehensive investigations into the role played by the innate and adaptive immune cells have been conducted in CFS. Plasmacytoid DC (pDCs) and immature B cells showed a decrease in cell numbers, and NK cells showed reduced cytotoxic capabilities in the CFS patient group compared to the healthy controls. T_{regs} and CD4⁺CD73⁺CD39⁻ T cells were elevated in the CFS patient group (Brenu *et al.*, 2013). The higher expression of CD73 leads to the increase in adenosine levels which further increases the immune suppressing capabilities of Tregs, thus dampening the pro-inflammatory immune responses (Kobie *et al.*, 2006). Disturbances in T cell homeostasis were prevalent in CFS patients. The cell percentages of T_{regs} were elevated in CFS patients, while the proliferative potential of CD4⁺ T cells was lower in patients evidenced by the low expression of Ki67 marker. Also, the CD38 marker was reduced in the CD45RO⁺ memory T cell compartment. CD5 marker was highly expressed on patient

CD8⁺ T cells (Curriu *et al.*, (2013). CD5 expression is associated with self-antigen tolerance of T cells whereby they are unable to mount an immune response to antigen *in-vivo* (Hawiger *et al.*, 2004) thereby indicating CD8⁺ T cell anergy.

The decrease in naïve or immature B cell numbers and increase in memory B cells in CFS patients suggests a lack of homeostatic balance in CFS patients that is similarly observed in patients with rheumatoid arthritis (Fekete *et al.*, 2007) and patients with multiple sclerosis (Hartung & Keiseier, 2010). Thus, CFS may have an autoimmune component to pathogenesis. As previously mentioned, the research by Fluge *et al.*, (2011) showed that depleting B cells using the anti-CD20 antibody rituximab improved clinical response of CFS patients. However, contrastingly, studies on B cell phenotypes and cell numbers by Curriu *et al.*, (2013) showed that there were no significant changes in absolute B cell numbers within Transitional, Marginal zone and Plasma/ Plasmablast populations, as did Hardcastle *et al.*, (2015) who reported no significant differences in B cell receptor levels and regulatory B cells levels (B_{regs}) between moderately and severely affected patients

1.9.3 Cytokine Signatures in CFS

Landi *et al.*, in 2016, using patient serum samples approved from the “Solve ME/CFS biobank”, found significant differences in levels of cytokines and chemokines between the patients and healthy controls. Levels of IL-6, IL-7, VEGF-A, CX3CL1 and CXCL9 all showed reductions in patient samples, whereas only levels of eotaxin-2 (CCL24) increased. Maes *et al.*, (2012) used the fibromyalgia and chronic fatigue syndrome (FF) scale to measure fatigue in patients. The patient groups were ME/CFS and CF (patients with fatigue for more than 6 months but not satisfying all diagnostic criteria as entailed by Fukuda *et al.*, in 1994). Serum levels of IL-1, TNF- α , neopterin, lysozyme and PMN-elastase in ME/CFS were significantly higher than in CF patients and healthy controls. Hardcastle *et al.*, (2015) found increased levels of IL-1 β and RANTES in moderately affected patients while severely affected patients had

higher levels of IL-7, IL-8 and IFN- γ . No associations were found for VEGF, TNF- α , IL-2, IL-4, IL-5, IL-10, IP-10, GM-CSF, IL-13, IL-17, IL-1ra, IL-6, IL-9, IL-12p70, FGF, eotaxin, G-CSF and PDGF-BB. So, increase and decrease in serum cytokine and chemokine levels are due to the disturbances in the homeostasis of the immune system. Brenu *et al.*, in 2012 conducted a longitudinal analysis of cytokine levels of 65 CFS patients at T0 (baseline), T6 (after 6months) and T12 (after 12 months). The blood samples of test subjects were collected at the time of CFS diagnosis (T0) and at 6 months and 12 months after diagnosis. It was interesting to note that cytokine levels were different at different time points. IL-10, IFN- γ and TNF- α were significantly higher in CFS patients at T0, but at T6, there was a significant drop in IL-10 and IL-17A levels in CFS patients. At T12 only IL-2 showed a significant increase in CFS patients. Thus, the increase and decrease in cytokine levels at different time points follow a pro-inflammatory trend. The drop in IL-10 levels from T0 to T6 showed a drop in anti-inflammatory functioning whereas the increase in IL-2 at T12 pointed to a pro-inflammatory trend. Thus, to summarize, CFS patients show a significant increase in pro-inflammatory cytokines which suggests that CFS is characterized by chronic inflammation.

Different research groups, using different fatigue scales to measure fatigue in patients, have found contrasting results when the immune measurements and the fatigue scale scores were checked for correlations. This was underlined in the research by Brenu *et al.*, in 2013, who reported different levels of expression of NK killer immunoglobulin receptors and NKG2D in patients diagnosed by 1994 CDC (Centre for Disease Control) and ICC (International Consensus Criteria) CFS criteria. The ICC patients had higher KIR2DL1, KIR3DL1, NK2G2D and KIR2DL2. This complicates arriving at a definite immunological explanation to chronic fatigue. A consensus must be achieved to have a comprehensive yet universal fatigue scale scoring system to better define immunology of CFS patients. This might lead to therapeutic treatments at a much faster rate than the present scenario.

Overall conclusion to literature review

Immune dysregulation, due in part to unresolved immune responses to persistent microbes such as CMV and EBV, are believed to be central to the immunopathology at the centre of 1. myocardial damage associated with reperfusion treatment for MI; and 2. cytokine-induced sickness behaviour which is a major part of CFS. Hence this thesis examines both diseases for discernible differences in immune responses from healthy individuals, and how the magnitude of such responses associate with disease parameters.

Hypothesis of this work

The overall hypothesis of this work is that, in two disease states (MI+R & CFS), immune responses can be measured and characterised that are associated with the disease process with clinical parameters, or that can discern diseases for non-diseased state.

Aims

To identify immune characteristics that are indicative of disease and could enlighten disease aetiology and serve as disease biomarkers.

Objectives

- 1). Measure features of T cells, particularly reactivity against herpes viruses and other microbes, in patients undergoing MI+R or in patients with CFS.
- 2). Look for relationships of immune measurements with disease parameters or compare responses between diseased and non-diseased groups.

2.0 CHAPTER TWO

Materials & Methods

2.1 Patient Cohort data in this thesis

2.1.1 Myocardial Infarction patient group

Professor Ioakim Spyridopoulos (MD) at the Institute of Genetic Medicine, Newcastle University and Dr. Stephen Boag (Newcastle University) recruited 59 patients with AMI for the study. Only patients with chest pain within 6 hours of ST-elevation myocardial infarction (STEMI) were included. Patients with chronic infection, autoimmune disorders, active infection and previous MI were excluded. Local NHS Health Research Authority Ethical committee (HRA) (REC 12/NE/0322) (Appendix) and the ethical committee of Northumbria University (RE-HLS-13-140219-53048a) approved this study. The attending physicians obtained written informed consent from the patients taking part in the study. Whole heparinized blood from the peripheral veins of patients was collected from the clinicians at the Freeman Hospital at 0 minutes (before reperfusion), 90 minutes after PPCI, 24 hours after PPCI, and at 3 months after PPCI.

2.1.2 Chronic Fatigue Syndrome patient group

For the CFS project, Prof. Wan Fai Ng and Prof. Julia Newton of the Institute of Cellular Medicine, Newcastle University, and Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK provided access to their patients. Dr. Gavin Spickett, consultant Immunologist and Ms Victoria Hindmarsh, Phlebotomist at the Royal Victoria Infirmary, Newcastle Upon Tyne, UK recruited a total of 50 CFS patients for this study. The local NHS Research Ethics Committee (12/NE/0225) and ethical committee of Northumbria University (RE-HLS-13-140219-53048a) approved the study. The Fukuda Criteria was used to diagnose all patients giving each patient a Fatigue Impact Score (FIS) value. Whole heparinized blood samples of

patients were obtained from the clinicians at the Clinics for Research and Service in Themed Assessments (CRESTA) clinic at the General Hospital, Newcastle upon Tyne and the Royal Victoria Infirmary (RVI). The non-fatigued healthy controls were recruited at the clinical site. Furthermore, it is important to mention that the number of healthy controls did not match with the CFS patient number, and healthy controls were not age and sex matched with CFS patients.

2.2 Isolation of PBMC from Whole Blood & Cell counting

All handling of blood and cells was carried out under sterile conditions within a laminar flow hood (Telstar Bio-II-A).

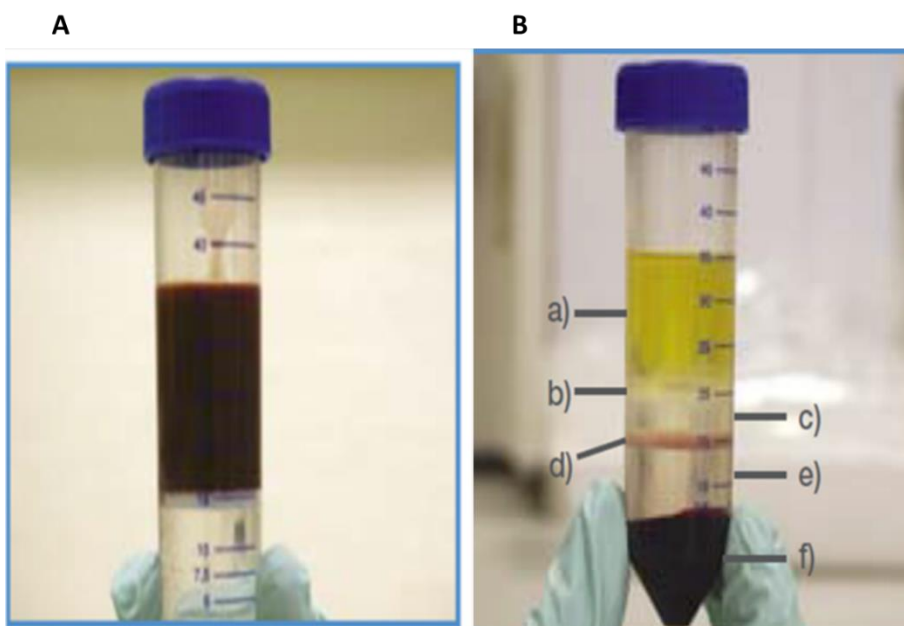


Figure 2.1 PBMC isolation from whole blood. A. Heparinized blood is carefully layered onto Lymphoprep in the Leucosep tubes containing the filter (left), B. A typical image after separation of whole blood (right). a). plasma layer, b). Interface between the plasma layer and PBMC layer, c). PBMC layer, d). High grade polyethylene barrier, e). Lymphoprep, f). Granulocytes and Erythrocytes.

Whole, heparinised blood was layered on to Lymphoprep (Axis-Shield, Norway), a density gradient medium, in Leucosep tubes (Greiner Bio-one, Stonehouse, UK) which contains a high-grade polyethylene filter, which makes sure the blood and lymphoprep do not mix. The tubes

with the blood were spun in a Megafuge16 (Thermo scientific, Loughborough, UK) at 2100 rpm for 12 minutes with brake at zero. It is important to put the brake at zero to make sure the Plasma and PBMCs layers do not mix after separation. The plasma obtained was stored at -20°C for future use. The PBMCs were washed with RPMI-1640 medium (R0) (Sigma, Dorset, UK) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2mM) (Invitrogen, Paisley, UK) and centrifuged (Megafuge 16) at 1700 rpm for 10 minutes with the brake on. The PBMC pellet obtained was resuspended in freezing medium (90%FCS, 10% DMSO (Sigma Aldrich, Poole, UK) and stored at -80°C in Mr Frosty to ensure slow freezing and then further stored at -150°C (Revco freezer, Thermo). For immediate use, cells were resuspended in R0 medium supplemented with 10% v/v heat inactivated Fetal Calf serum (BioSera, Sussex, UK) (i.e. R10 medium).

2.2.1 Cell counting using CASY cell counter

The PBMCs obtained from different study participants were counted using the CASY cell counter (Roche diagnostics, GmbH, Mannheim, Germany). 10 μl of cells was added to 10 ml of CASYTON solution in a CASY counting tube and placed in the machine containing the probe. After the appropriate command ('START') was initiated, the cell count was displayed on the cell counter display screen. All cell counts were adjusted to 8×10^6 cells/ml to obtain optimal immune responses and maintain uniformity among samples (as previously established by Prof. Stephen Todryk). The CASY clean solution was used to clean the cell counter system before and after each patient sample run. The CASY counter was set up to measure cell size ranges for lymphocytes of 5.4 μm -9.2 μm and monocytes 9.3 μm -20 μm . The CASY count settings were validated by flow cytometric separation of lymphocytes and monocytes.

2.3 Ex-vivo and Cultured ELISpot

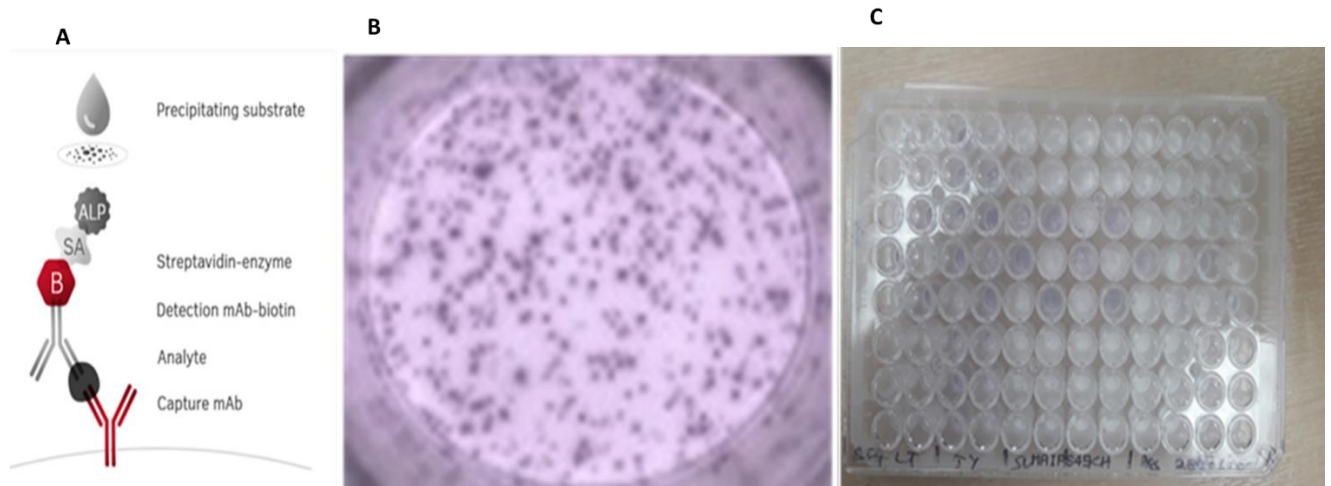


Figure 2.2 The ELISpot assay principle. A. Cytokine produced by PBMCs is bound to capture antibody and then to detector antibody. The addition of alkaline phosphatase to the streptavidin-biotin-labelled detector antibody follows. Addition of substrate reveals 'spots' B. Typical formation of spots depicting production of cytokines by T cells, each spot represents a T cell that has produced a cytokine. The size of the spot indicates the amount of cytokine produced by the cells. C. ELISpot plate with varied spot profile when PBMCs get stimulated with a wide variety of selected peptides and non-specific stimulants.

Enzyme-linked Immunospot assay (ELISpot) was performed to measure T cell responses in study participants. Capture antibody (CAP-Ab) for the desired cytokine was diluted in bicarbonate buffer. 50µl of capture-antibody (CAP-Ab)-Bicarbonate buffer mixture was added to each well on the ELISpot plate (Millipore, Watford, UK), a day before the assay and incubated at 4⁰C overnight. The ELISpot plates were then washed three times with sterile phosphate buffer saline (PBS) and blocked with R10 for 1-4 hours. Cryopreserved PBMC were quickly thawed by gentle shaking of the cell vials in a 37⁰C water bath, washed with R0 solution and resuspended in R10 medium. Fresh or cryopreserved PBMC were set up for ex-vivo ELISpot at 8x10⁶cells/ml. After the blocking solution was flicked off, 50µl of antigenic stimulus and 50µl of cells were added to the plates and incubated in the 5% CO₂ incubator (Thermo Scientific). The length of incubation ranges from 18-20 hours for IFN-γ and 36-48

hours for other cytokines being tested (See table). ELISpot kits were purchased from Mabtech (Nacka, Sweden). After incubation, the contents of the plate were flicked off and the plate was washed 6 times with PBS. 50µl detector antibody-Biotin complex was added to each well and incubated for 1 hour at room temperature. The plates were then washed 6 times with PBS and 50µl of streptavidin-alkaline phosphatase (SA-ALP) (Mabtech) (5µl in 5ml of PBS) was added and the plate, incubated for 1 hour at room temperature. The strong bond formation between biotin and streptavidin is crucial for successful plate development. The contents of the plates were once again flicked off and 50µl of the substrate solution (see appendix) was added to each well and incubated at room temperature for 5-15 minutes following which the plates were washed in tap water and allowed to dry. The ELISpot plates were then read using ELISpot plate reader.

For cultured ELISpot, on day 0, PBMCs were placed in a 24 well corning plate. 500µl each of patient PBMC (1M/ml) along with 500µl of CD4⁺ and CD8⁺ specific CMV peptide pool and EBV CD4⁺ and CD8⁺ specific peptide pool in separate wells (1000µl in total for each sample). On day 3, 500µl of cell culture supernatant was carefully removed and 500µl of 100U/ml IL-2 solution (previously optimized by Todryk *et al.*, 2009) was added. The same process was then repeated on day 7. On day 9, the cells were gently scraped from the wells, washed 3 times with 2ml of R0, resuspended in 1 ml of R10 in 5ml round bottomed polystyrene tubes. The tubes with cells were left overnight in a CO₂ incubator at 37⁰C. The lids on the tubes were not tightly closed to maintain neutral pH and proper air circulation. ELISpot plates were also coated and kept at 4⁰C. On day 10 (next day), cells were counted using the CASY counter. The cells were washed with 2ml of R10, resuspended in 1ml of R10 and the ‘protocol for the ex-vivo ELISpot’ was followed, i.e. the CMV and EBV antigens were added to the ELISpot wells followed by the cells in the round bottomed polystyrene tubes. The ELISpot plates containing the mixture

of antigen and cells were incubated overnight in the CO₂ incubator. The plates were then developed the following day.

For the NK cell assay, PBMCs of healthy controls were stimulated with K562 cells (American type culture collection ATCC, Middlesex, UK) to measure baseline IFN- γ spot forming cells/M.

2.3.1 ELISpot coating and detection antibody concentrations and setting up ELISpot reader

Carbonate-bicarbonate buffer capsule was added to 100ml of sterile H₂O. The sterile water was then taken in a BD Plastipak 10ml syringe attached to a sterile filter and collected in a 50ml falcon tube. The process was repeated until all the coating buffer was collected. The coating antibodies were then mixed with bicarbonate coating buffer and added to the ELISpot wells. The coating concentration for coating antibodies for IFN- γ and IL-5 was 1 in 100. Coating concentration for IL-17 was 1 in 500. Coating concentration for IL-2 was 1 in 50. The detector antibody concentration for IFN- γ and IL-5 was 1 in 1000, 1 in 500 for IL-17 and 1 in 60 for IL-2. The detector antibody solution was prepared by mixing antibody with PBS. R10 medium only was used for the unstimulated background. The CMV and EBV viral antigenic stimuli used were pools of known CD8 epitope peptides (10 mg/ml) for CD8 responses and pools of known CD4 epitope peptides (10 mg/ml) plus viral lysates for CD4 responses. Phytohaemagglutinin (PHA, 5 μ g/ml) and α -CD3 (1 in 200) were used as positive controls in all assays. Measurement of polyclonal secretion of IL-5, IL-17 and IL-2 was carried out using PHA as the stimulus, and ELISPOT reagents from Mabtech (IL-5), eBioscience (IL-17), or R&D Systems (IL-2). Data analysis involved the use of spot forming cells/10⁶ PBMC (net antigen-stimulated spots minus medium). The Spots were read using ELISpot plate reader (Autoimmun Diagnostika, GmbH, Strassberg, Germany). The thresholds for spot counting were as follows:

	Intensity	Size	Gradient
MIN	4	3	1
MAX	255	500	90

Emphasis: Tiny

Speed of reading = maximum

The protocol underwent minor changes for the CFS project. All experiments were performed with fresh PBMCs. Peptides and lysates belonging to a wide variety of pathogens were tested at optimal concentrations. Apart from PHA, other positive controls used were pokeweed mitogen (PWM), Phorbol 12-myristate 13-acetate with ionomycin (PMAi) and α -CD3. Measurement of polyclonal secretion of IL-5 and IL-17, but not IL-2 was carried out with α -CD3 as the positive control. K562 cell line was cultured and used as a “stimulus” to test IFN- γ production by NK cells.

2.3.2 Calculation of ELISpot result

The ELISpot plate was read and printout containing the ‘spot count’ was obtained. The spot counts for negative control (medium only) were subtracted from spots counts obtained from antigenic stimulus and positive controls (PWM, PMAi, PHA and α -CD3). The response to positive controls were used to determine initial experimental success. All cell counts were adjusted for 50 μ l of cells from the starting concentration of 8M/ml. To obtain spot forming cells/10⁶, we divided 10⁶ by the cell count for 50 μ l of cells added (4 Million). Thus, we obtained a multiplication factor of 2.5 for all stimulants except the positive controls. The multiplication factor for PWM, PHA and α -CD3 was 12.5 since only 10 μ l of cell were added

(for 50µl of cells, the multiplication factor is 2.5 and so for 10µl of cells, the multiplication factor would become 12.5(2.5 times 5)). The multiplication factor for PMAi was 62.5 since only 2µl of cell were added. The volume of cells added for positive controls were lower than other stimulants to obtain countable results and avoid obtaining a 'blackout' due to high levels of cytokine production by the cells. The number of spots obtained for each stimulant was multiplied with the respective multiplication factor to obtain spot forming cells /Million.

2.4 Enzyme linked immunosorbent assay (ELISA)

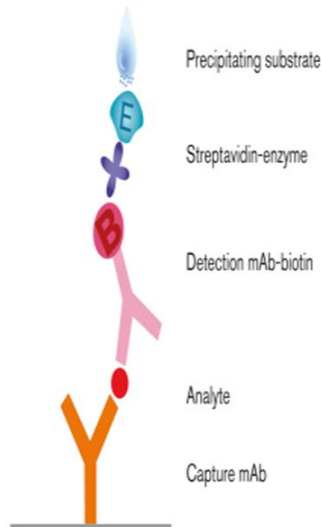


Figure 2.3 ELISA principle. Addition of Plasma or Serum to the ELISA plate coated with antigen allows the plasma to be tested for the presence of antibodies. The biotin-labelled detection antibody binds to immunoglobulin produced. Streptavidin-horse radish peroxidase (HRP) binds to biotin forming a strong bond. The addition of substrate leads to colour change, which is measured by ELISA plate reader at 490nm.

2.4.1 EuroImmun ELISA

Euroimmun ELISA kits were used to quantify the CMV (IgG, IgM) and EBV (IgG, IgA) specific antibody responses. The presence of IgG in patient sera indicated established immunity to CMV and EBV whereas IgM antibody titres indicate recent infection to CMV. The IgA antibody titre was used as an indicator of primary EBV infection. The CMV antigen used in

ELISA was obtained from the inactivated cell lysates of MRC-5 cells infected with AD169 strain of cytomegalovirus while the EBV antigen used was obtained from inactivated cell lysates of human B cells infected with P3HR1 strain of EBV. The results obtained from using these ELISA kits were used to determine the viral serology of the CFS patients. The Euroimmun plates are pre-coated with the antigen of interest. 100µl of calibrators along with patient samples and positive and negative controls were added to the well and incubated for 30 minutes at room temperature. The plates were washed 3 times with the kit specified wash buffer (dilution to 1X from 10X is necessary). 100µL of the secondary antibody-enzyme conjugate was added and the plates incubated for 30 minutes at room temperature. The plates were then washed again. 100µl of substrate solution was added and incubated for 15 minutes at RT following which 100µL of stop solution was added to stop the reaction. The plates were finally read at 450nm to measure the absorbance (EL808, Bio-Tek Vermont, USA).

2.4.2 Meso Scale Discovery (MSD)

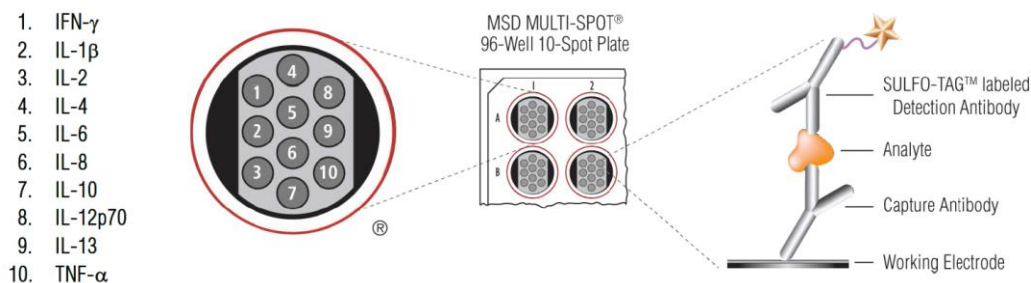


Figure 2.4 Principle of MSD with a typical profile of MULTI-SPOT MSD plate. Each well in the MSD plate consist of 10 spots with each spot coding for a specific cytokine. The analyte produced from serum samples of study participants is added to detector antibody labelled with electro-chemiluminescent SULPHO-TAG. Upon the application of electricity at the carbon electrodes, the tags emit light. The intensity of light is directly proportional to the amount of analyte produced.

Quantification of cytokine levels in patient sera or cell culture supernatants was determined by a highly sensitive multiplex cytokine assay. MSD uses an electro-chemiluminescent principle,

which leads to the production of light when samples containing the analyte are stimulated with electricity. In MSD, carbon electrodes that have 10 times higher binding capacity to reagents than polystyrene are used. The detection antibody is conjugated with SULPHO tags, which are electro-chemiluminescent and produce light once electricity is applied at the carbon electrodes. The intensity of the light generated is directly proportional to the quantity of analyte in the cell culture supernatant. Unlike ELISA, MSD can quantify multiple analytes in a single well with low background. The labels used are non-radioactive, stable and bind to biological molecules with ease. Multiple excitation levels of labels allow increased sensitivity of the assay. Labelling reporters with ruthenium complexes (tris (bipyridine) ruthenium (II); $[\text{Ru}(\text{bipy})_3]^{2+}$), which are stimulated to produce photons when a small electrical charge is applied. The electrically excited complex emits light at 620 nm as it falls back to its resting state. Output is regenerated in the presence of TPA (Tripropylamine) which allows repeated triggering of the output signal, improving sensitivity. This stimulation and regeneration occurs at the solid-liquid interface at the binding surface of the MSD plate.

We used three multispot 96 well plates and quantified the cytokines and chemokines of interest (see Appendix 7). A proinflammatory panel, a cytokine panel as well as a chemokine panel were tested. These three 10-plex panels were a part of the “V-PLEX human cytokine 30-Plex kit”. 1000 μl of assay diluent (diluent 2) was added into the calibrator bottle. 100 μl of the mixture was added to seven Eppendorf tubes each containing 300 μl of assay diluent by doubling dilution. The proinflammatory panel and the cytokine panel had different calibrators and thus two standard curves were generated. The detector antibody solution was prepared using the diluent 3 solution in the MSD kit. 60 μl of the desired detector antibody was added to 2400 μl of diluent solution to generate a 1X working solution from a 50X detector antibody solution. The wash buffer was prepared by adding 15ml of wash buffer in 285ml of distilled

water. 10ml of read buffer (4X) was added to 10ml of distilled water to generate a working solution of the read buffer (2X).

Both plates were washed 3 times with 150µl of wash buffer and 50µl of doubly diluted calibrators and patient samples were added to the appropriate wells and sealed with the adhesive tape. The plates were then incubated with shaking for 2 hours at room temperature. The plates were washed again with 150µl of wash buffer and 25µl of detector antibody solution was added to each well. The plates were covered with an adhesive tape and incubated at room temperature for 2 hours with shaking. The plates were washed with 150µl of wash buffer and 150µl of read buffer was added to the well and read on the MSD plate reader at 620nm (Meso Scale Discovery, Rockville, MD, USA). The concentration (ng/ml) of cytokines in the patient samples was calculated using the standard curve.

2.5 Flow Cytometry

All FACS analysis was performed on an 8 colour FACS Canto II flow cytometer with FACS Diva software. A minimum of 10,000 events for a population of interest were recorded for each sample. The 8 fluorochromes used for the experiments are as follows: FITC, PE, PerCP-Cy5.5, PE-Cy-7, APC, APC-Cy7, BV421 and BV510. Further details on the fluorochromes are furnished in **Table 2.5**.

Cell surface markers on PBMCs that are indicative of infection, antigen-specific responses, immune senescence and regulation were determined by flow cytometry. A panel containing antibodies against a wide range of cell surface markers specific for T cells, NK cells, Treg and B cells bound to fluorochromes was used to stain the PBMCs, which were then analysed using the flow cytometer.

Cryopreserved patient PBMCs were thawed and 300µl of 1×10^6 cells were washed with PBS and the supernatant poured off. The master mix antibody for FACS was made in an Eppendorf

tube (2µl Ab/sample +50µl brilliant stain buffer/sample). 66µl of the master mix was added to designate FACS tubes, 50 µl of cells was also added, and tubes kept at 4°C for 30 minutes in the dark. 2ml of PBS was then added to each FACS tube and centrifuged at 1500 revolutions per minute (RPM) for 5 minutes and cells resuspended in 300 µl of PBS and acquired by the Flow cytometer and the results were analysed. The panel of markers tested for the MI and CFS project are described in table 2.2 and table 2.3. Measurement of CMV specific CD8⁺ T cells in eight patient PBMCs were done by tetramer analysis. HLA typing database (performed by our research collaborators) of patient cohort were used to identify the specific HLA typing for each patient sample. HLA type A*0101, A*0201 and A*0301 were used.

2.5.1 Setting up the Flow Cytometer

FACS tubes were labelled for each fluorochrome to be tested and 2µl of each antibody was added to the appropriate FACS tube. Only negative compensation beads were added to the tube marked 'negative control'. To optimize compensation, one drop of negative compensation beads and one drop of anti-mouse Ig, κ positive compensation beads (BDTM CompBeads, BD Biosciences, Oxford, UK) was added to each tube. This was done to help distinguish positive and negative background for analysis of data. The BDTM CompBeads anti-mouse Ig κ particles bind to κ light chain Ig whilst negative compensation beads do not bind to antibodies. Tubes were vortexed and incubated for 15 minutes in the dark at RT or incubated for 30 minutes at 4°C. 2ml of PBS was then added to each tube and the tubes were centrifuged at 1500rpm for 5 minutes. The supernatants from all tubes were discarded and the beads were re-suspended in 300µl of PBS. The beads were then acquired on BD FACScantoTM II Flow cytometer (BD Biosciences, Oxford, UK), using automated compensation program, which is a part of the BD FACSDivaTM software to measure the spectral overlap. The gating strategy used for the CFS project is shown in detail in section 4.7 of chapter 4).

2.5.2 Tetramer analysis of patient PBMCs

HLA typing of patients were carried out by NHS blood and transplant, Newcastle upon Tyne and the HLA typing results were obtained from our research collaborators at Newcastle University. CMV-specific T cells were identified in PBMCs samples using a CMV tetramer kit (Immudex, Denmark). The kit consisted of 8 CMV-specific tetramers that were conjugated to PE. The alleles used for main sub-group-B were A*0101, A*0201, A*0301, A*2402, B*0702, B*0801 and B*3501. Before staining process with tetramers, PBMCs were thawed quickly in a water bath at 37⁰C for 1-2 minutes. PBMC samples were then washed twice in a PBS buffer containing 5% fetal calf serum (PBS-5 % FCS). The cells were then resuspended in PBS-5 % FCS. The viability of Vi-CELL Cell Viability Analyser (Beckman Coulter). 1x10⁶ cells were resuspended in a total volume of 50µl PBS/5%FBS and incubated with 10µl appropriate MHC dextramer in the dark at room temperature. After a 10-minute incubation, appropriate amounts of CD3-FITC, CD27-APC, CD8-APC-H7, CD45RA-PacificBlue monoclonal antibodies were added to the sample and incubated for at least 20 minutes at room temperature. Finally, cells were washed twice with PBS/5%FBS and assessed by multiparametric FCS (BD FACS Canto II). The same procedure was carried out for cohort C. the alleles used for cohort C were A*0101, A*0201 and A*0301. The monoclonal antibodies used for testing cohort C were CX3CR1-PE-Cy7, S1PR1-APC, C-met-FITC, CXCR3-PerCP-Cy5.5, CCR5-APC-Cy7 and CCR4-BV421. The experiment was performed to test three measurements. 1). CD8⁺ Tetramer cells, 2). CD8⁺ non-tetramer cells and 3). CD8⁺ non-tetramer T cells within the CX3CR1 population.

2.6 Production of Bacterial lysates for Antigens

We grew *Pseudomonas aeruginosa* in 10ml of broth and placed in an orbital shaker at 200 rpm and incubated overnight at 37⁰C. The following day, 1 ml of culture was transferred into a 200ml flask containing nutrient broth. The flask was placed on an orbital shaker at 200rpm

at 37°C for 2 hours. The flasks were kept in incubation until an optical density of 0.8 (mid-log of the growth phase) was reached. Bacterial culture was transferred into 50ml centrifuge tubes and centrifuged at 4000 RPM for 5 minutes at 4°C, and the supernatant discarded. The bacterial cell pellets were resuspended and washed with PBS 3 times and finally resuspended in 5ml of PBS. Bacterial cells in PBS were sonicated (Soniprep 150, Henderson Biomedical, UK) at high intensity for 10 seconds with 10 second intervals. This process was carried out three times. The sonicated bacterial cells were transferred into Eppendorf tubes and centrifuged at 13000 RPM for 20 minutes at 4°C to remove unwanted cell debris. Desired number of aliquots were made by carefully removing the supernatant from the Eppendorf tubes and stored at -20°C for future use. The resulting pellet was discarded.

2.7 Determination of Protein Concentration by Bradford Assay

100µl of the original bacterial lysate was diluted in PBS in 10-fold steps and incubated for 30 minutes at room temperature. 5 µl of each concentration were added to labelled 96 well ELISA plate in duplicate. To produce the standard curve, 10 dilutions of BSA (Bovine serum albumin) in 500 µl of deionised water were made ranging from 0.1–1.4 mg/ml. 5 µl of these samples were added to the plate. Subsequently, 250 µl of Bradford Dye Reagents was added to each well. The plate was incubated for 30-45 minutes at room temperature and read on ELISA Plate Reader (EL808, Bio-Tek Vermont, USA) at 595 nm. The concentration of unknowns was calculated from the standard curve.

2.8 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

Two glass plates were placed on top of each other and clamped at the edges. The two glass plates were then placed on a casting tray. A 12% separating gel was prepared and mixed well albeit gently (refer appendix for contents). The separating gel was carefully layered into the space between the clamped glass plates and care was taken to make sure the top layer of the stacking gel was horizontal and not slanted (a layer of distilled water was added to the top layer

of stacking gel to ensure horizontal layer was achieved). Furthermore, addition of the water layer also aids in proper polymerization of the gel protecting it from oxidation. The casting tray with separating gel was kept aside for polymerization. After 45 minutes, the water at top was removed by gently inserting a filter paper without affecting the separating gel. The stacking gel was then gently layered onto the space between the glass plates until overflow. A comb was inserted gently into the glass plates avoiding air bubbles. The stacking gel with the comb was allowed to polymerize for another 45 minutes. The Comb was gently taken out to reveal the wells. The glass plates were removed from the casting tray and placed in an electrophoresis buffer dam. The electrophoresis buffer was poured into the dam until an overflow (buffer level higher near the glass plates than on the outside). 20 μ l of samples was mixed with 5 μ l of loading buffer and placed in boiling water for 5 minutes. The samples were pipetted into the wells carefully and care was taken to avoid overflow of samples. The first well was reserved for the molecular weight maker. Power supply and voltage were set at 120 mA and 150V respectively. The apparatus was run for 45 minutes. After sufficient protein migration, the power supply was switched off. The gel was carefully removed from the glass plates and placed inside a container with coomassie brilliant blue stain for 10 minutes (with gently shaking). The gel was then, placed in a different container with destaining solution overnight (with gentle shaking). The gels were photographed using Bio-Rad Gel Doc2000 gel documentation system using Quantity One software. Hard copies of the gel photographs were printed using a Mitsubishi video copy processor attached to the gel documentation apparatus (see chapter 4, section 4.2.4).

2.9 Lipopolysaccharide (LPS) assay

The presence of endotoxins in antigenic stimulus was tested using the Pierce LAL chromogenic endotoxin quantitation kit (Thermo Scientific, Rockford, USA). The presence of LPS in antigenic peptides catalyses the production of proenzymes of Limulus Amebocyte Lysate (LAL). The catalysed proenzyme then proceeds to catalyse the cleavage of p-nitroaniline

(pNA) of the substrate. Higher the presence of endotoxin in the sample, the higher is the colour intensity of the reaction. The standard curve was plotted by preparing standard stock solution of LPS (Refer Chapter 3). 50µl of samples to be tested was added to the microplate along with 50µl of LAL reconstituted in endotoxin free water. Endotoxin free water alone was used as the blank. The microplate was then incubated at 37°C for 10 minutes following which 100µl of substrate was added to the plate. The plate was then gently rocked and incubated for 6 minutes. 50µl of 25% acetic acid was added to all the wells to stop the reaction. The microplate was read at 410 nm in plate reader (EL808, Bio-Tek Vermont, USA) to measure the absorbance. The blank absorbance values were subtracted from the absorbance values of the samples tested. The net absorbance values were inserted into the standard curve to obtain the concentration of endotoxin present in the test samples (EU/ml).

2.9.1 Preparation of Stock solution and generation of standard curve

The endotoxin present in the kit was added to 1ml of endotoxin free water to make 26EU/ml stock. 0.05ml of this stock was then added to 1.25 ml of endotoxin free water to produce 1EU/ml stock solution (Taken from instruction sheet obtained within the kit)

Table 2.1 Dilutions for preparing the endotoxin standard stock solutions

Vial number	Endotoxin free water added(ml)	Final stock concentration(EU/ml)
1	1.25	1
2	0.25	0.5
3	0.75	0.25
4	0.9	0.1

Standard concentration of 1EU/ml of was prepared by the addition of 0.05ml of the standard stock to 1.25ml of endotoxin free water. To prepare 0.5EU/ml stock solution, 0.25ml of endotoxin free water was added to 0.25 ml of 1EU/ml stock solution. 0.25EU/ml stock solution was prepared by the addition 0.25 ml of 1EU/ml stock to 0.75ml of endotoxin free water. 0.1EU/ml stock was prepared by the addition of 0.1ml of 1EU/ml of stock solution to 0.9ml of endotoxin free water. (**Table 2.1**). The standard curve generated for the assay was used to measure the endotoxin levels of test antigens (refer **Figure 4.2.1**).

The limulus Amebocyte lysate was reconstituted with 1.4ml of endotoxin free water. Furthermore, the chromogenic substrate in the kit was reconstituted with 6.5ml of endotoxin free water to make up a final concentration of 2mM. The complete list of antigens tested in the LPS assay and their endotoxin levels is listed in **Table 4.2**.

2.10 Statistical analysis

All statistical analysis was performed using IBM SPSS statistics version 24, Graphpad prism version 6 and Graphpad prism version 7. For the CFS project, normality tests such as the D'Agostino-Pearson omnibus K2 test was performed to assess whether the datasets were parametric or non-parametric. Thus, correlations were analysed by Spearman's correlation analysis as well as Pearson's correlation analysis. Non-parametric Mann-Whitney U test and parametric unpaired t test with welch correction were used to determine the relationship between two unpaired or independent groups and Wilcoxon signed rank test or paired t test was used to determine the relationship between two paired or dependant groups. Kruskal-Wallis test with Dunn's multiple comparison test or a one-way ANOVA with Tukey posthoc test was performed to test the relationship between three or more unrelated samples. Friedman test was performed to test the relationship between 3 or more matched groups. P values lower than 0.05 ($p < 0.05$) were considered significant. Data was presented as median with the inclusion of upper and lower quartiles unless otherwise indicated. The data on column graphs

were presented as mean+SEM. For the cultured ELIspot data analysis, the ‘zero’ values in the data sets were reassigned with a minimum value of ‘1’. This change did not affect the result of the statistical analysis and the logarithmic scale for the data sets were then obtained.

Table 2.2 Panel of Cell Surface markers used for MI project (Flow Cytometry)

CELL SURFACE MARKER	NAME	FUNCTION in MI	FLUROCHROME
CD11a	Integrin α L	Lymphocyte adhesion and migration	FITC
CCR5	C-C chemokine receptor 5	Regulates homing of CD4 ⁺ T cell into inflamed tissues	PE
CXCR3	C-X-C chemokine receptor 3	Recruitment of Th1 cells to site of inflammation binding to IP10	PerCP-Cy5.5
CX3CR1	CX3 C chemokine receptor 1	Fractalkine signalling plays an important role in increasing MVO	PE
CD107a	Lysosome associated membrane glycoprotein 1	Measurement of cytotoxic activity of CD8 ⁺ T cells	PE-Cy7
PD-1	Programmed cell death ligand-1	Apoptosis of activated T cells expressing high levels of PD-1	APC
c-MET	Hepatocyte growth factor	T cell recruitment to the myocardium	FITC
CD69	Early activation antigen 69	Marker of lymphocyte activation	BV421
CD8	T cell surface glycoprotein α	T cell killing of infected cells by binding to MHC class-I molecules on APCs.	BV510
CD4	T cell surface glycoprotein	Binds to MHC class-II molecules on APCs and causes immune activation.	APC-Cy7
CD45RA	Src kinase	Expressed on naïve as well as terminally differentiated T cells	PerCP-Cy5.5
CD27	T cell activation antigen	Identification of effector memory T cells.	PE-Cy7
CCR7	C-C chemokine receptor type7	Homing of T cell and DCs to lymph nodes	BV421
S1PR1	Sphingosine 1-phosphate receptor 1	Cell migration and trafficking from lymph nodes.	APC

Table 2.3 Panel of cell surface markers used for CFS project (Flow Cytometry)

CELL SURFACE MARKER	NAME	FUNCTION	FLUROCHROME
CD57	Human Natural Killer-1	CD57 ⁺ NK cells exhibit potent cytotoxic capabilities	FITC
CD161	Killer cell lectin-like receptor subfamily B member 1	Inhibits NK cell cytotoxicity	PE
TCR V α 24-J α 18	Invariant T cell receptor α chain	NKT cells use iTCR to bind to antigens presented by CD1d.	PE-Cy7
TCR V α 7.2	Semi-invariant T cell Receptor	Bind to antigens expressed on MHC molecule MR1.	APC
CD16	Fc γ IIIb Receptor.	Antibody dependant cell mediated cytotoxicity by NK cells	APC-Cy7
CD56	Neural cell adhesion molecule	Identification of Natural Killer cells	BV421
CD3	T cell surface glycoprotein	Activation of T cells and used to differentiate T cells from B cells.	FITC, BV510
CD95	Tumour necrosis factor receptor superfamily member 6	Apoptosis of Immune cells	PE-Cy7
TCR $\gamma\delta$	T cell receptor with $\gamma\delta$ chains	Bind to CD1d molecule containing α Galactosylceramide.	FITC
CD25	Interleukin 2 receptor subunit α	IL-2 binds to CD25 and causes a myriad of immune functions	PE
CD19	B cell antigen	Enhances B cell-antigen interactions.	PerCP-Cy5.5
CD127	IL-7 Receptor	IL-7 is important for B cell development	APC
CD38	ADP ribosyl cyclase	Marker of B cell activation	BV421

The following cell surface markers CD45RA, PD-1, CD4, CD8, CCR7, CD27, and CD69 are common for both studies.

Table 2.4 Chemokine receptors tested for the MI project by flow cytometry

<u>Cytokine</u>	<u>Name</u>	<u>Role in MI</u>
CCR4	Chemokine receptor 4	T cell trafficking of Th2 cells, and regulatory T cells (Nakagawa <i>et al.</i> , 2014)
CCR5	Chemokine receptor 5	Homing of CCR5 ⁺ T cells into atherosclerotic lesions by binding to CCL5 (Li & Ley, 2015).
C-met	Hepatocyte growth factor	T cell recruitment to the myocardium (Komarowska <i>et al.</i> , 2015)
CX3CR1	Fractalkine receptor	Involved the depletion of T cells from circulation and into the myocardium (Boag <i>et al.</i> , 2015)
CCR7	Chemokine receptor 7	Priming of naïve T cells and T cell migration.
CXCR3	CXC Chemokine receptor 3	T cells trafficking into sites of inflammation (Groom & Luster, 2011).

Table 2.5 Fluorochromes and their emission spectra used in flow cytometry experiments

Fluorochromes	Name	Excitation/Emission Spectra(nm)
FITC	Fluorescein Isothiocyanate	494/520
PE	Phycoerythrin	496-578
PerCp-Cy5.5	Peridinin Chlorophyll protein with cyanine5.5	482/695
APC	Allophycocyanin	650/660
APC-Cy7	Allophycocyanin with cyanine7	650/785
BV421	Brilliant violet 421	407/421
BV510	Brilliant violet 510	405/510

Data obtained from BD Biosciences Fluorochrome/Laser Reference Poster

CHAPTER THREE

The significance of anti-viral T cells and cytokines in patients with acute myocardial infarction (AMI) receiving reperfusion (R) treatment (AMI+R)

Abstract

Background

The sudden rupture of an atherosclerotic plaque leads to myocardial infarction (MI) and ischemia. Such coronary occlusion and reduced oxygen provision results in death of cardiomyocytes. Inflammatory responses follow, with the release of cytokines which play a crucial role in cardiac repair (Nah & Rhee, 2009). The reopening of blocked artery by PPCI is vital for survival. However, the restoration of blood flow causes reperfusion injury due to the influx of leukocytes, particularly T cells, into the cardiac tissue causing microvascular obstruction (MVO). MVO has been linked with improper left ventricular remodelling, increased infarct size and generation of oxygen free radicals (Hoffmann *et al.*, 2012). CMV seropositivity is shown to be associated with mortality due to myocardial ischemia (MI). Thus, the focus of this chapter is on the role of anti-viral T cells in myocardial ischemia and reperfusion injury.

Aims

The aim of this chapter was to investigate T cell immune responses, T cell populations, and serum cytokine concentrations, in cytomegalovirus (CMV) seropositive and seronegative, as well as Epstein-Barr virus (EBV) seropositive and seronegative, AMI patients during reperfusion (+R) treatment (AMI+R). The expression of chemokine receptors involved in the migration of T cells was also studied.

Methods

Techniques used included *ex-vivo* and cultured ELISpot to measure T cell responses in AMI patients before and after reperfusion treatment. Stimuli for T cells comprised CMV and EBV antigenic peptides and lysates, as well as positive controls. Multiplex assay was used to measure serum cytokine levels in patients. Flow cytometric analysis was carried out to identify the expression of chemokine receptors on patient T cells. Statistical tests involved testing data for normality followed by parametric or non-parametric analysis performed to test for correlations and significance. Comparison of two unrelated groups was performed using Mann-Whitney U test or the unpaired t test and paired t test or Wilcoxon signed rank test for related groups. Comparison of three or more groups were performed by one-way ANOVA followed by Tukey's post hoc test or the Friedman test. P values below 0.05 were considered significant.

Results

Polyclonal activation of T cells with PHA in ELISpot showed increased Th1 (IFN- γ) responses in CMV seropositive patients. The serum concentrations of IFN- γ , IL-10 and IP-10 in CMV seropositive patients was higher than in CMV seronegative patients after reperfusion. The ELISpot results did not correlate with total ischemic time. Studies involving flow cytometric analysis of programmed death ligand 1(PD-1) revealed the permanent depletion of terminally activated PD-1⁺ memory CD8⁺ T cells from circulation during MI+R. Furthermore, a significant drop in cell numbers at 90 minute time point in fractalkine receptor⁺ population of CD8⁺ T cells ($p=0.001$) was seen. No correlations were seen between MVO, a measure of reperfusion-induced disease, and CMV specific *ex-vivo* ELISpot immune responses. There were, however, correlations of MVO with CMV specific cultured ELISpot responses, along with EBV-specific *ex-vivo* and cultured ELISpot.

Conclusion

The results of this study suggest a chronic activation of the pro-inflammatory Th1 arm of the immune system together with the loss of CMV-specific terminally differentiated effector memory PD-1⁺ CD8⁺ T cells, likely via fractalkine-mediated homing to the myocardium. This might help explain the link between heart disease, CMV and immunosenescence.

Introduction

Myocardial infarction (MI) is one of the major causes of morbidity and mortality and across the world, although treatment and management of patients has improved considerably (Thygesen *et al.*, 2012). MI usually follows the rupture of an atherosclerotic plaque which blocks the coronary artery. Myocardial cell death or necrosis due to prolonged ischemia (lack of oxygen supply to the heart tissue) causes myocardial infarction MI, which can be identified by electrocardiograph (ECG), increase in levels of biomarkers associated with myocardial necrosis (e.g. Troponin), and by imaging (Thygesen *et al.*, 2012). Boag *et al.*, in 2015 used cardiac MRI to detect microvascular obstruction in MI patients. Some of the prominent cardiac biomarkers that are released from dead cells are cardiac troponin, myoglobin, creatine kinase and C reactive protein. Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) are considered reliable indicators of MI (Fathil *et al.*, 2015). Both subunits of troponin are released from 2-4 hours from the onset of MI and persist in the blood from 10-14 days after MI (Bahadir & Sezginurk, 2015). Hoffmann *et al.*, in 2015 also used initial and peak troponin levels in two patient cohorts for their study. Primary percutaneous coronary intervention (PPCI) using intra-arterial catheter/stent is considered the most effective procedure to remove blockages, widening the narrow arterial walls and restoring the blood supply to the heart (Reperfusion). Unfortunately, this results in reperfusion injury, characterized by the influx of leukocytes such as T cells and neutrophils which cause microvascular obstruction (MVO) and initiate inflammatory reaction in response to dead cells, damaged tissue and clot components

(Hoffmann *et al.*, 2012). Thus, elevated levels of troponin indicate the onset of MI and the extent of reperfusion injury after PPCI. In this research, the focus is mainly on the role of T cells in MI and reperfusion injury and the contribution of cytomegalovirus specificity of T cells. The main premise to the study is that T cells contribute significantly to heart disease generally, and to cardiac reperfusion injury, by homing to injured heart tissue during reperfusion. Furthermore, pro-inflammatory T cell subsets, particularly those specific for persistent viruses such as CMV, are abundant in the circulation of seropositive patients and home to the heart to contribute to immunopathology.

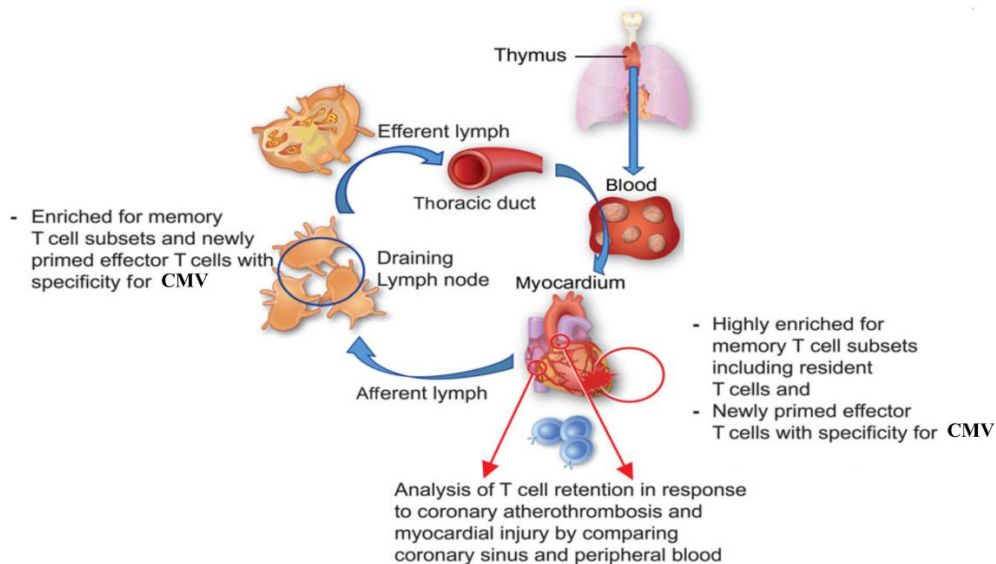


Figure 3.1 Migration of T cell through the myocardium. T cell migration and circulation from the blood stream into myocardium passing through the lymph node occurs periodically at steady state condition. During inflammatory conditions lymphocytes, mainly CMV-specific T cells are recruited into the myocardium and exacerbate disease conditions. (Image taken and modified from Hofmann & Frantz, 2015).

Naïve T cells and memory T cells circulate in the blood stream during steady state conditions. In the case of MI, the migration of T cells to the heart, and their retention, is controlled by chemokines and their receptors.

Several studies have shown the role of T cells in MI. RAG-1 KO mice which lack T cells were shown to have lower infarct size than the infarct size in control mice in an MI model (Yang *et al.*, 2006). It was also found that the depletion of CD4⁺ and not CD8⁺ led to the decrease in infarct size in mice. Further, the adoptive transfer of CD4⁺ T cells led to the loss in protection from the depletion of these cells. This strongly indicates an important role for CD4 T cells in the onset of MI. It was extremely interesting to note that the adoptive transfer of CD4⁺ T cells from IFN- γ KO mice did not abolish the protection which strongly suggest a critical role for IFN- γ produced by Th1 cells in the exacerbation of inflammatory damage. (Yang *et al.*, 2006). Hoffmann *et al.*, in 2012 showed a shift in CD4⁺ T cell population 24 hours after PPCI, where there was a drop in cell numbers of the terminally differentiated effector memory T cells expressing CD45RA (T_{EMRA}). The depleted cell populations were CCR7⁺, suggesting the role for CCR7 in the migration of T cells into the heart tissue. Ammirati *et al.*, in 2012 showed that effector memory T cells (T_{EM}) were significantly increased in patients with acute MI providing more cells with heart-homing potential. The central memory T cells (T_{CM}) express CCR7 but lack CD45RA, whereas effector memory T cells (T_{EM}) lack both CCR7 and CD45RA. Naïve T cells express both CCR7 and CD45RA (Ammirati *et al.*, (2012)). The T_{EMRA} express CD45RA but lack CCR7. Yu *et al.*, in 2015 performed a study to characterize the CD8⁺ CD57⁺ T cells subset in AMI patients and showed that these cells contributed to the pathology of AMI. It was found that the levels of CD57⁺ CD4⁺ and CD8⁺ T cells were significantly higher in the patient group that suffered cardiovascular mortality. When other factors such as left ventricular ejection fraction, age and gender were considered, cardiovascular mortality was associated with the CD8⁺ T cells compartment. In addition to the high expression of CD57, these cells also expressed high levels of PD-1, FasL, CCR5 and CX3CR1. Thus, these cells exhibited hallmarks of hyper-activation, senescence, and with capacity to home to the target tissue. The authors also studied the functionality of these cells. The frequency IFN- γ ⁺ TNF- α ⁺ cells were

significantly higher in the CD8⁺CD57⁺ T cell compartment, and this was accompanied by the high expression of cytotoxic granules such as perforin and granzyme B.

The role of other chemokines in MI have also been investigated. Komarowska *et al.*, in 2015 investigated the role of c-met, CCR4 and CXCR3 in the migration of T cells to the myocardium. It was found that the binding of hepatocyte growth factor receptor (HGFR=c-met) to myocardium-specific HGF during priming of naïve T cells in the lymph nodes imprints 'T cell cardiotropism'. This binding also results in the engagement of CCR5 with CCR5 ligand (released in an autocrine manner due to the binding of HGFR-HGF). Zamilpa *et al.*, (2011) showed that the deletion of CCR5 in macrophages results in impaired left-ventricular remodelling post-MI in a mouse model. In addition, only during inflammatory conditions and not during steady state conditions, are CCR4 and CXCR3 required for the recruitment of T cells to the myocardium (Komarowska *et al.*, 2015). Boag *et al.*, in 2015 showed that the drop in T cell counts correlated with the expression of fractalkine receptor (CX3CR1) on T cells. The amount of fractalkine in the serum of patients peaked at 90 minutes after reperfusion coinciding with the drop in T cell count at the same time period. Boag *et al.*, 2015 suggested fractalkine-mediated migration of lymphocytes to the myocardium could affect the extent of microvascular obstruction (MVO) in patients. So, studying the phenotype, migration and specificity of T cells will give better insights into the role of T cells in MI patients.

Thus, the aim of this study was to examine T cell phenotype and functionality, particularly in relation to CMV specificity, in MI patients before and after their reperfusion treatment. These findings may provide targets for treatment of T cell-mediated reperfusion injury as well as identifying patients who could benefit from T cell targeting therapies.

RESULTS

3.1 Patient demographics in acute myocardial infarction patients

The main cohort of patients was divided into three sub groups for investigation: initial sub group A (n=28), main sub group B (n=52) and tetramer sub group C (n=8) based on availability of patient samples, patient data and project chronology. The main subgroup B represents an addition of patient numbers to the initial sub group A. The sub group C was selected based on the presence of PBMCs from patients that were MHC-typed and matched the available tetramers. It was decided to test these remaining patient samples for cell surface receptors involved in the migration of T cells into the myocardium.

Baseline Characteristics of sub-group A

The sub group A patient cohort included 28 patients suffering from acute myocardial infarction and were divided according to CMV serostatus. 14 patients were CMV seropositive and 14 patients were CMV seronegative. All the patients were male and the mean age for CMV seronegative patients was 57.9 and for CMV seropositive patients, it was 57.2. There were no other significant differences between CMV seropositive and CMV seronegative patient population in other clinical parameters, including blood lipids, BMI, troponin levels and ischemic time (Onset to balloon). Total ischemic time is the total time the myocardium is deprived of oxygen supply. (**Table 3.1**).

Parameters	Cytomegalovirus seronegative patients Median (Q1; Q3)	Cytomegalovirus seropositive patients Median (Q1; Q3)	P value
Number of patients	14	14	
Age median range	57.2(52;61)	57.9(50;60)	0.87
Previous incidence of MI (%)	0	7	0.33
Number of Vessel disease, 1/2/3	11/2/1	7/5/2	0.29
Serum Creatinine (μmol/L)	94(88;100)	92(88;103)	0.96
HDL cholesterol (mmol/L)	1.1 (0.9;1.2)	1.1 (1.0;1.3)	0.44
LDL Cholesterol (mmol/L)	4.1 (3.5;4.8)	3.9 (3.5;4.3)	0.52
Triglycerides, (mmol/L)	1.3(0.9;2.0)	1.4(0.9;2.4)	0.98
BMI	28(26;31)	27(25;34)	0.92
Hypertension (%)	21	21	1
Active smokers (%)	50	50	1
Statin Treatment (%)	7	29	0.16
Initial troponin I (ng/L)	0.5(0.2;11.8)	1.0(0.1;1.8)	0.66
Peak Troponin I (ng/L)	50(28;50)	40(19;50)	0.18
Onset-to-balloon time (min)	153(96;240)	113(102;272)	0.68
Door to balloon time (min)	25(17;35)	19(16;29)	0.42
Sex (Male/Female)	14/0	14/0	1

Table 3.1. Baseline characteristics of sub group A. All p values were determined by non-parametric t test. AMI indicates acute myocardial infarction; HDL, high density lipoprotein; LDL, low density lipoprotein; BMI, Body mass index; Q1-first quartile, Q3- third quartile.

3.2 Higher IFN- γ response in CMV seropositive AMI patients

In order to address the aims of this study, the first objective was to find out if there were differences in the main Th subtype responses between CMV seropositive and CMV seronegative patients. Patient PBMCs were examined before reperfusion/PPCI by stimulation with PHA (a polyclonal activator of T cells) to look for global reactions. It was found that CMV seropositive patients had significantly higher Th1 (IFN- γ secreting cells) than CMV seronegative patients. Th2 (IL-5 secreting) cells were lower in CMV seropositive patients, though not significantly so, and no differences were found in Th17 (IL-17 secreting) responses (**Figure 3.2.1**). Although IL-4 was our preferred Th2 cytokine to be tested, the lack of optimized response in ELISpot experiments forced us to test IL-5, another Th2 cytokine.

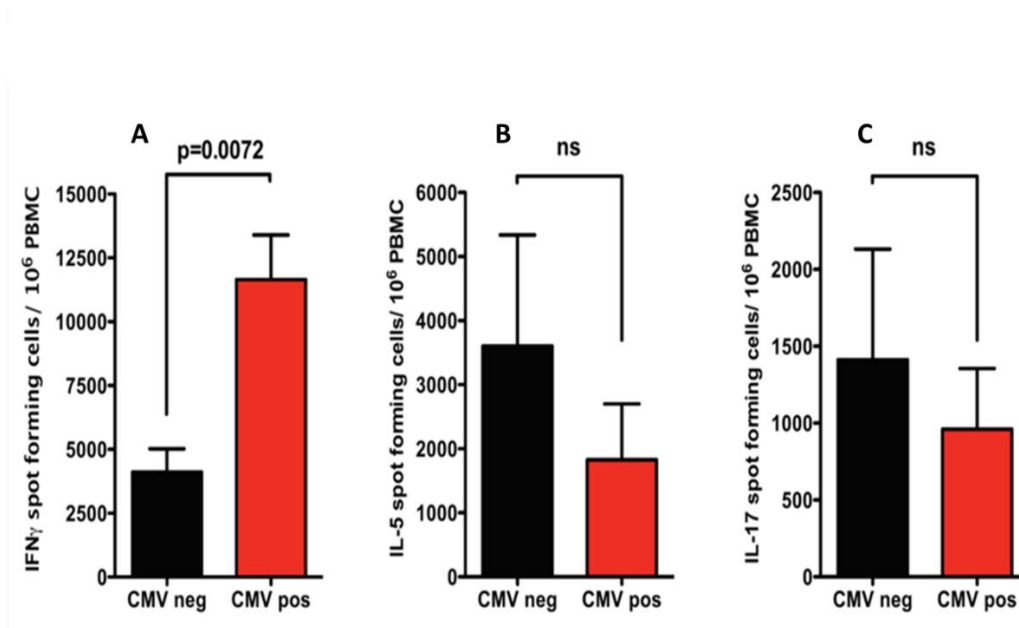


Figure 3.2.1 Polyclonal T cell activation in patients PBMC with PHA. A. There were more IFN- γ secreting Th1 cells in CMV seropositive patients than CMV seronegative patients ($p=0.0072$). B. There were no significant differences in T cells secreting IL-5 ($p=0.606$) and C. IL-17 ($p=0.670$), Th2 and Th17 cells, respectively, between CMV seropositive and CMV seronegative groups. Th1, T helper 1 cells; Th2, T helper 2 cells; Th17, T helper 17 cells. ns- Not significant. The bars indicate standard error of mean (+SEM), $n=28$. Mann-Whitney U test was carried out to test for significance between the two groups.

Next, we wanted to find out whether there was increased IFN- γ serum levels in a subset of patient samples shortly after the reperfusion process. Examination of patient serum samples by MSD, a multiplex cytokine assay, revealed CMV seropositive patients had higher levels of IFN- γ at 30 minutes post PPCI (**Figure 3.2.2**). There were no significant changes at the 15 minute and 90 minute time points. We found no other significant changes in the levels of other cytokines.

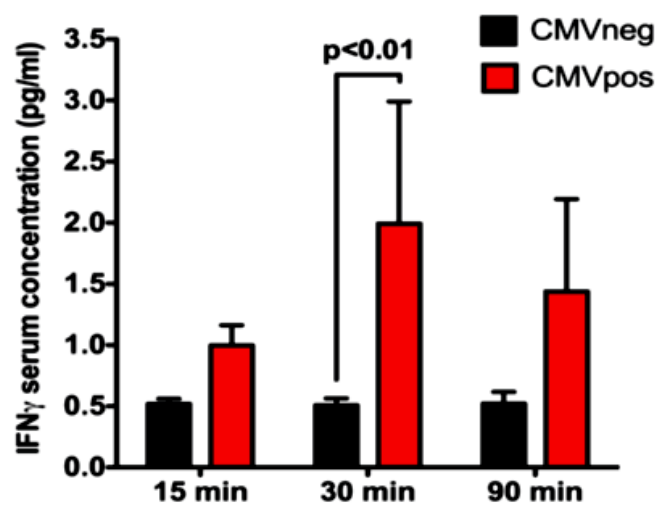


Figure 3.2.2 IFN- γ levels in patient serum samples. CMV seropositive patients had higher levels of IFN- γ at 30 minutes after reperfusion ($p < 0.01$). However, there were no changes between CMV seropositive and CMV seronegative groups at 15 and 90 minute time points. pg/ml, picograms/millilitre. The serum levels were measured by MSD. $n=12$ at 15 minutes, $n=14$ at 30 minutes and $n=14$ at 90 minutes. Unpaired t test was used to test for significance between the two groups.

3.3 Relationship between Total ischemic time and CMV serology in patients

The next step was to investigate the association between CMV seropositivity and the total ischemic time. The total ischemic times for the patients were obtained from our clinical collaborators and indicate the total time the heart is deprived of oxygen supply. In medical terminology, ‘door (onset of heart attack) to balloon (insertion of stent to remove blockage)’ is considered as total ischemic time. Total ischemic time may reflect the degree of atherosclerotic plaque blockage of the coronary artery, which may be dependent on a pre-existing inflammatory state, or may be simply the logistical time to deliver treatment. Just like we predicted, it was found that there was no significant difference in ischemic time between CMV⁺ and CMV⁻ serology. Thus, as expected, speed of treatment from the onset of heart attack determines the total ischemic time, and the presence or absence of cytomegalovirus does cause any changes in the total ischemic time.

Next, we decided to ascertain whether there were associations between total ischemic time and IFN- γ production by T lymphocytes in CMV seropositive and CMV seronegative patients. We decided to focus on IFN- γ as it is the main Th1 cytokine and high Th1 responses have been implicated in the progression of MI. It was found that the magnitude of Th1 response, evidenced by IFN- γ spot forming cells/10⁶ PBMC in response to PHA showed a positive correlation with total ischemic time ($p=0.0006$, $R= 0.84$) in CMV-seronegative patients. No correlations, however, were seen between magnitude of T cell response (IFN- γ spot forming cells/10⁶ PBMC in response to PHA) in CMV-seropositive patients ($p=0.47$, $R= 0.17$). Thus, it is entirely possible that T cells in CMV seropositive patients are highly activated and present in high numbers and has nothing to with total ischemic time.

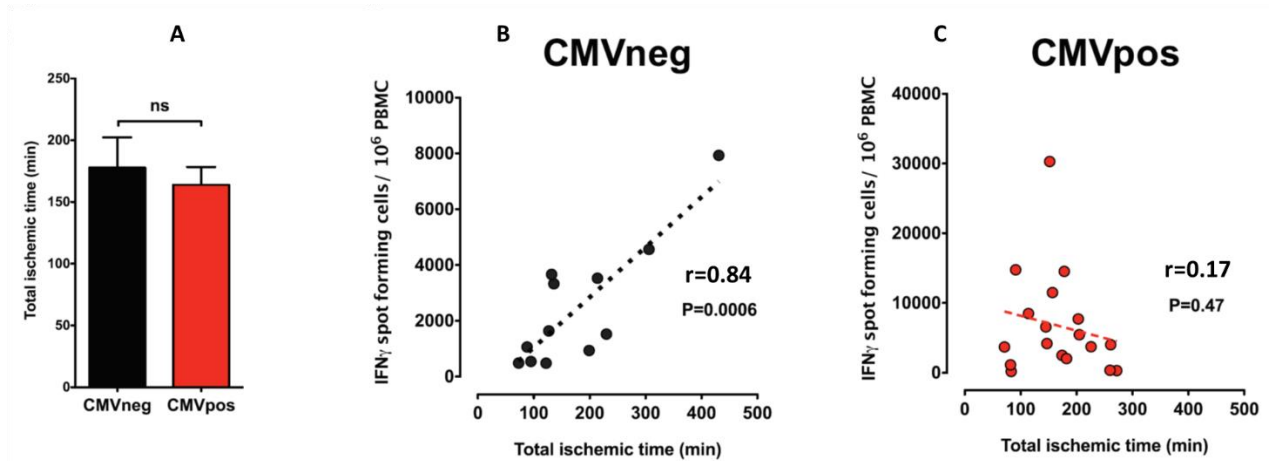


Figure 3.3.1 Relationship between CMV serology and IFN- γ levels with ischemic time. A. There was no association between CMV serology and total ischemic time. Total ischemic time indicates the total time the myocardium was deprived of oxygen supply. ns, not significant. n=28. B. The production of IFN- γ in CMV-seronegative patients was lower and correlated with ischemic time, n=12; C. The production of IFN- γ in CMV-seropositive patients was much higher and showed no correlation with total ischemic time, n=19. p-significance value; R, Spearman correlation coefficient. Patient PBMCs were stimulated with PHA.

Keeping in view of the above results, we hypothesize that the longer the myocardium remains deprived of oxygen supply, the more significant is the damage to the heart tissue. In CMV seronegative patients, we suggest the migration of T cells away from the heart tissues causes less damage. In CMV seropositive patients, the presence of high numbers of CMV-specific T cells indicate a lack of migration away from the heart tissues, consequently causing increased damage. This will be covered in more detail in the discussion section.

3.4 Tetramer analysis of CD8⁺ T cells before and after reperfusion

On examining absolute counts of leukocytes before reperfusion, we found that only CD8⁺ T cell compartment showed a significant increase in number in CMV seropositive patients. So, tetramer analysis of CD8⁺ T cells was one of the approaches carried out to examine the CD8⁺ T cell population in greater detail, since tetramers identify T cells with a CMV-specific TcR. In order to do this, we initially measured the percentage of CMV-specific CD8⁺ T cells present in patients PBMCs before PPCI. We found that there was considerable variability in the percentage of CMV-specific CD8 T cells (**Figure 3.4.1**). The percentages of tetramer⁺ T cells ranged from 0.2 to 18% for HLA specific tetramers.

Next, we focussed on the numbers of tetramer⁺ CD8⁺ T cells at 0 minutes, 90 minutes, 24 hours and 3 months after reperfusion. It was found that there was a significant decrease in CMV-specific T cells at the 90 minute time point compared to 0 minute time point ($p < 0.0001$) (**Figure 3.4.1**). Whilst the percentage of T cells was increased at 24 hours, they were still significantly decreased compared to T cell percentage at 0 minutes ($p < 0.05$). Whilst levels remained reduced at the 3 month time point, this did not reach significance.

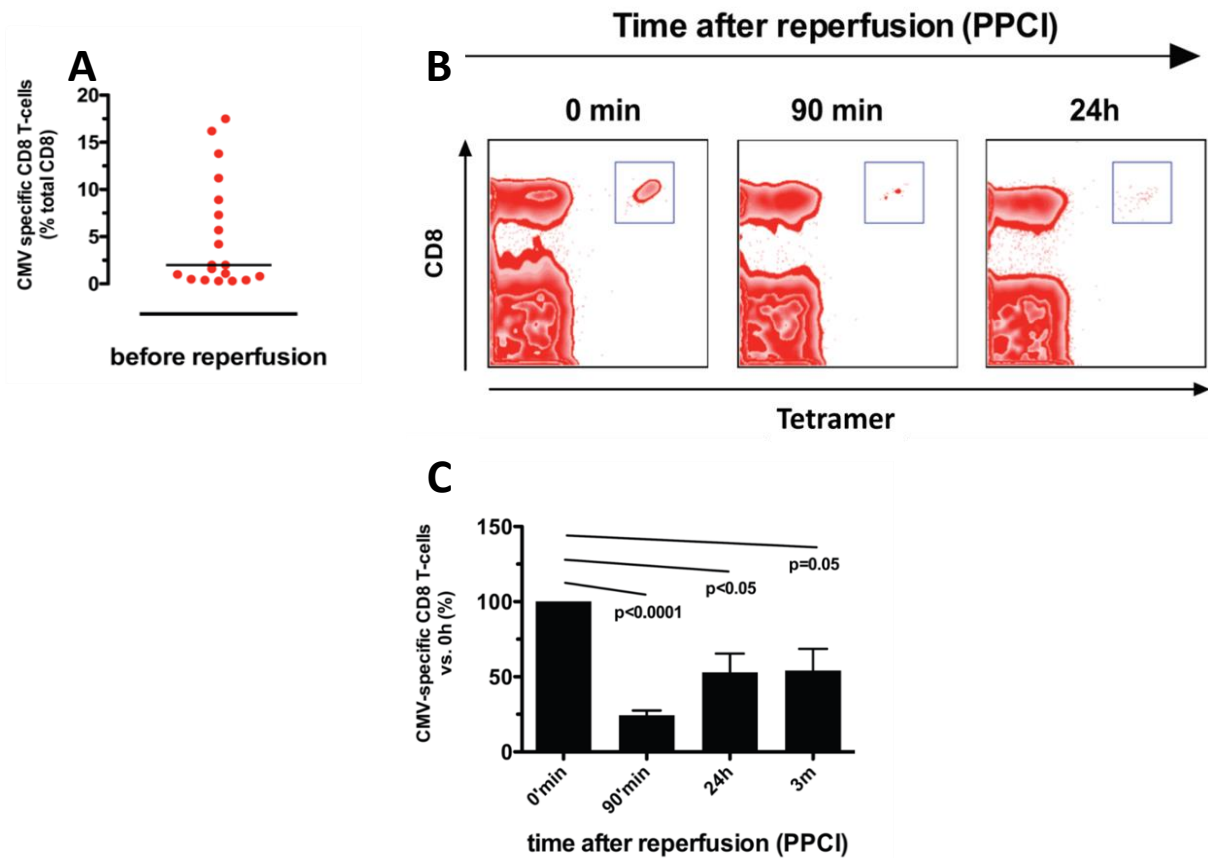


Figure 3.4.1 Percentage of CMV-specific T cells before and after reperfusion therapy. A. Tetramer analysis using HLA specific epitopes revealed considerable variability in CMV-specific CD8⁺ T cells in patients. The data for blood samples collected before PPCI was performed on the patient group. n=19. B. The highlighted sections on flow cytometric scatter plots show the CMV-specific tetramer⁺ CD8⁺ T cells at 0 minute, 90 minute and 24 hours post PPCI. There is clearly a decrease at the 90 minute time point. This is representative data for one patient. C. Compared to the relative proportion of T cells before reperfusion, there was significant decline to 24 % at 90 minutes after PPCI (P<0.0001), 53% at 24 hours (p<0.05) and 54% at 3 months (p=0.05). n=10. Friedman test was carried out to test for significant associations.

These results indicate the homing of T cells to the heart, possibly, via the sphingosine-1 phosphate receptor pathway into lymph nodes and to site of inflammation (myocardium). In addition, the loss of circulating CMV-specific T cells may lead to reactivation of latent CMV, thus causing long lasting low-grade inflammation.

It was then decided to investigate associations between tetramer-determined CMV-specific CD8⁺ T cells and functional CMV-specific CD8⁺ measured by ELIspot. Interestingly, there was a negative correlation between ELIspot CMV-specific CD8⁺ T cells and tetramer CMV-specific CD8⁺ T cells ($r=-0.79$, $p=0.006$) (**Figure 3.4.2**).

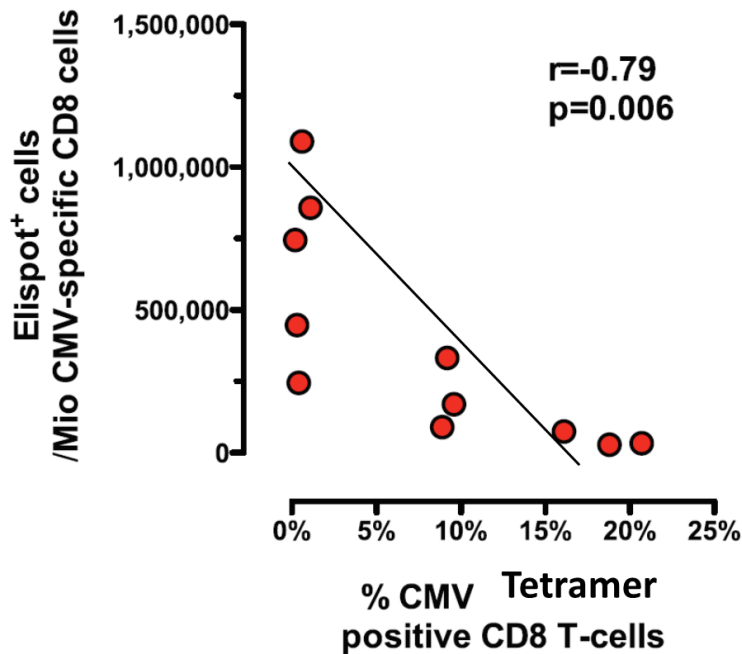


Figure 3.4.2 Relationship between ELIspot and tetramer+ CMV-specific CD8+ T cells. An inverse relationship was found between ELIspot and tetramer CMV specific CD8⁺ T cells. Higher the percentage of tetramer CMV specific T cells lower was the ELIspot CMV specific T cells. p value indicates value of significance; r-Spearman correlation coefficient.

The functionality of CMV specific tetramer CD8⁺ T cells before reperfusion is decreased as shown by the decrease in number of CMV specific CD8⁺ T cells measured by ELIspot. This suggests that the CMV-specific T cells are over-stimulated and have become anergic.

3.5 Depletion of PD-1⁺ terminally differentiated CD8⁺ effector memory T cells (T_{EMRA})

The next step was to identify the CD8⁺ T cell subset that accounted for the drop at the 90 minute time point and also to find out whether there was a permanent loss of CD8⁺ T cells since CD8⁺ T cells at the 24 hours and at the 3 month time point do not recover from the drop at 90 minutes to the levels immediately after reperfusion. It is also possible that the cells homed away from the myocardium and were anergic and not permanently lost.

We first identified the percentage of activated CD8⁺ T cell subsets before reperfusion. The expression of CD69 was used as an indicator of T cell activation. It was found that only the effector memory (T_{EM}) and terminally differentiated effector memory CD8⁺ T cells (T_{EMRA}) showed the highest activation levels of CD69 (**Figure 3.5.1**). In addition, there was a significant difference in the activation levels of naïve CD8⁺ T cells and T_{EMRA} population (p=0.016).

PD-1 is expressed on activated memory T cells to dampen T cell activity, thus it made sense to examine the loss of PD-1⁺ CD8⁺ T_{EMRA} cells after reperfusion. Interestingly it was found that at 90 minutes after reperfusion there was significant drop in PD-1⁺ CD8⁺ T_{EMRA} cells (**Figure 3.5.1**). The 15 minute time point was one of the time points included in this pilot experiment to determine the time point at which the most significant drop in T cell count occurred.

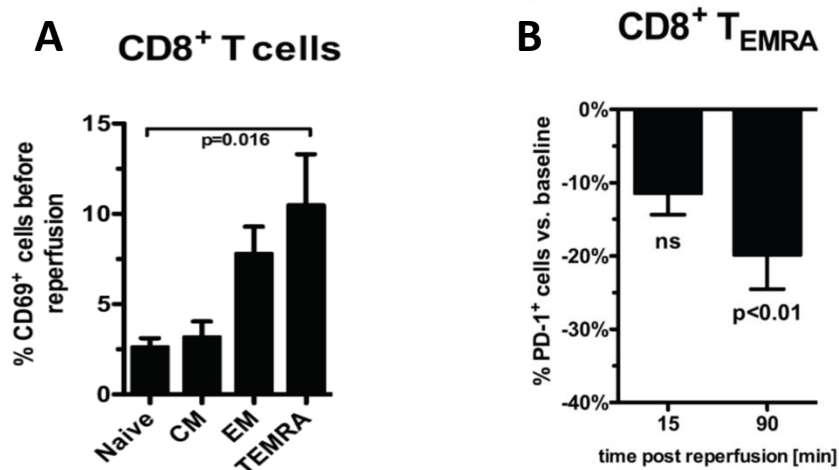


Figure 3.5.1 Percentage of activated CD8⁺ T cell subsets before reperfusion and Drop in PD-1⁺ CD8⁺ TEMRA cells. A. Staining of CD8⁺ T cells for memory (CD45RA/RO) and activation (CD69) markers revealed the activation levels of CD8⁺ T cell subsets before reperfusion. There was a significant difference between the activation levels of naïve CD8⁺ and T_{EMRA} CD8⁺ T cells ($p=0.016$). p value indicated significance. It must also be noted that the activated levels of effector memory cells (EM) and T_{EMRA} were higher than naïve and central memory cells (CM). There was no significant relationship between the other T cell subsets. $n=28$. One-way ANOVA followed by Tukey's post hoc test was carried out to test for significant associations. B. Compared to baseline CD8⁺ TEMRA, the PD-1 expressing CD8⁺ TEMRA showed a significant drop at 90 minutes after reperfusion ($p=0.016$). There was, however, no significance at the 15 minute time point. $n=28$. Wilcoxon signed rank test was carried out to test for significant associations.

The next aim was to measure PD-1⁺ CD8⁺ T_{EMRA} cell levels at 24 hours after reperfusion. The percentages of effector memory (T_{EM}) and T_{EMRA} cells were higher than naïve and central memory (T_{CM}) cells in both CMV seronegative and seropositive patients (**Figure 3.5.2**). In addition, in CMV seropositive patients, the percentages of PD-1⁺ CD8⁺ T_{EMRA} cells were significantly lower than PD-1⁺ CD8⁺ T_{EM} cells ($p=0.017$). The level of PD-1⁺ CD8⁺ T_{EMRA} in CMV-seropositive patients was significantly lower than the same population in CMV-seronegative patients ($p=0.04$).

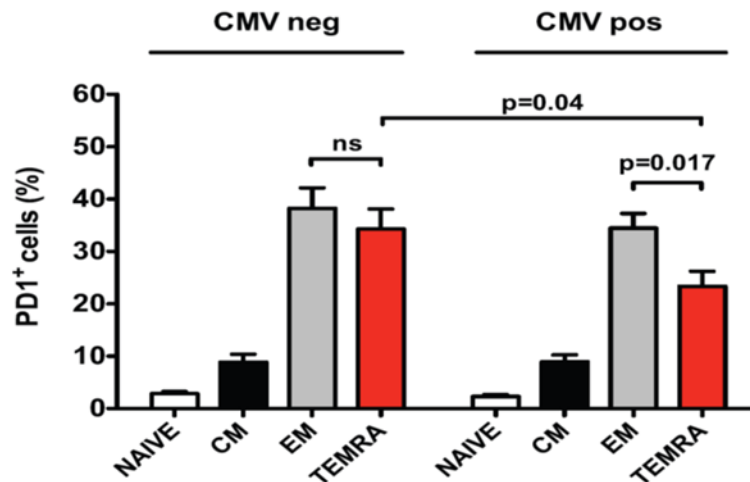


Figure 3.5.2 PD-1+ CD8+ T cell subsets in CMV seropositive and seronegative patients. In both CMV seropositive and CMV seronegative patient groups the effector memory cells and terminally differentiated effector memory cells were higher than naïve and central memory cells. The T_{EMRA} in CMV seropositive patients after 24 hours was significantly lower than T_{EMRA} in CMV seronegative patients (p=0.04). The T_{EMRA} population in CMV seropositive patients were also significantly lower than effector memory cells. CM, Central memory; EM, effector memory; T_{EMRA}, terminally differentiated effector memory cells; PD-1, programmed cell death ligand-1. p value indicates significance value. n=28. One-way ANOVA followed by Tukey's post hoc test was carried out to test for significant associations.

The presence of PD-1 on highly activated T cells prepares the cells for apoptosis, thus, it was decided to test the level of spontaneous and induced early; (Annexin V⁺) and late (7-AAD⁺ & Annexin V⁺) apoptosis on CD8⁺ T cells. For spontaneous apoptosis, uncultured patient PBMCs stained with Annexin V and 7-AAD were analysed by flow cytometry. The CD8⁺ T cells were split into PD-1 negative, PD-1 dim and PD-1 high cells. (**Figure 3.5.3A**). PD-1 negative cells showed only early apoptosis and no late apoptosis, whereas PD-1 dim and PD-1 high cells showed early and late spontaneous apoptosis (**Figure 3.5.3B**).

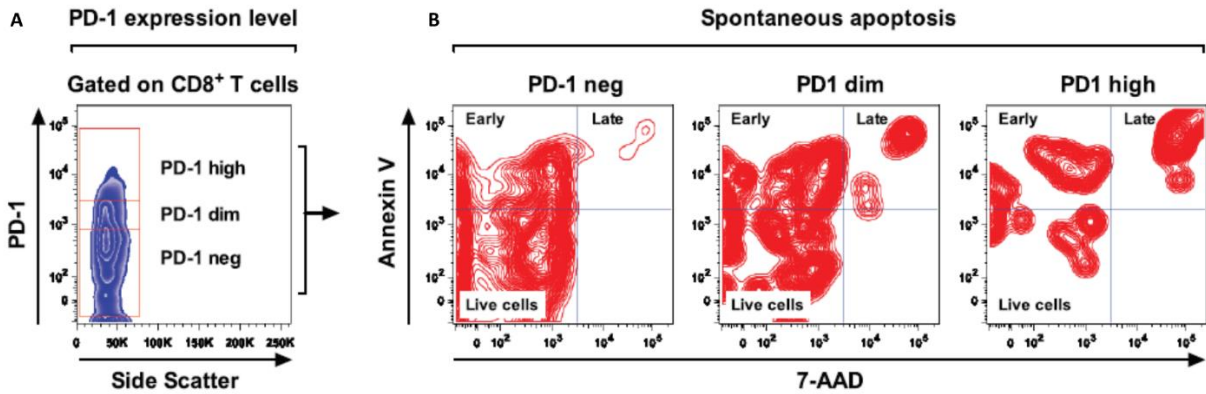


Figure 3.5.3 PD-1 expression levels and spontaneous apoptosis in activated CD8+ T cells. A. PD-1 high cells and PD-1 dim cells were more numerous than PD-1 negative cells. B. There was no late apoptosis in PD-1 negative cells indicated by the lack of presence of 7-AAD staining, whereas PD-1 high and PD-1 dim cells showed both early and late spontaneous apoptosis. A representative set of FACS data for one patient is shown. PD-1, Programmed cell death ligand-1; 7-AAD, 7-Aminoactinomycin D. n=28.

3.5.1 Gating strategy for different levels of PD-1 expression

The lymphocyte population was first gated by plotting forward scatter against side scatter; the gated lymphocyte population were further gated by plotting forward scatter (height) vs forward scatter (area) to exclude all doublet cells and include only singlet cells. Next, only the CD3 population were gated from singlet cells by creating FSC-A vs CD3 dotplot. The CD8⁺ T cell population was obtained from the CD3 population and the PD-1 expression was investigated in CD8⁺ T cells.

The high, dim and ‘negative’ expression levels of PD-1 were discriminated. We used a contour plot to identify PD-1 CD8⁺ T cells. We used a combination of statistics as well as fluorescent minus one (FMO) samples to make sure that the gating procedure is accurate and appropriate. The FMO sample was used to determine the negative control for the experiment. Next, we used statistics to split the positive population into high and dim populations. The PD-1⁺ cells above

the median were classified as 'high' whereas all the positive population below the median were classified as 'dim'.

We also measured the extent of induced early and late apoptosis in these cells. PD-1 negative cells and PD-1 dim cells showed higher percentage of early apoptosis compared to PD-1 high cells (**Figure 3.5.4**). The percentage of early apoptosis of PD-1 dim cells was significantly higher than PD-1 negative cells ($p < 0.05$) and PD-1 high cells ($p < 0.01$). There were changes during late apoptosis where the PD-1 high cells showed significantly higher apoptosis compared to negative and dim cells. Furthermore, the percentage of live cells expressing high levels of PD-1 was significantly lower than live PD-1 negative cells ($p < 0.0001$).

To confirm the role of PD-1 in the permanent loss of activated CD8⁺ T cells, we decided to test the level of apoptosis of T cells in the presence of PD-1-blocking monoclonal antibody. The loss of cell membrane integrity during cell death allows the intake of propidium iodide (PI) which can be detected by flow cytometric analysis. Interestingly, the percentage of late apoptosis in stimulated T cells incubated with the anti-PD-1-monoclonal antibody showed significantly lower apoptosis than cells with no blocking antibody (**Figure 3.5.4**).

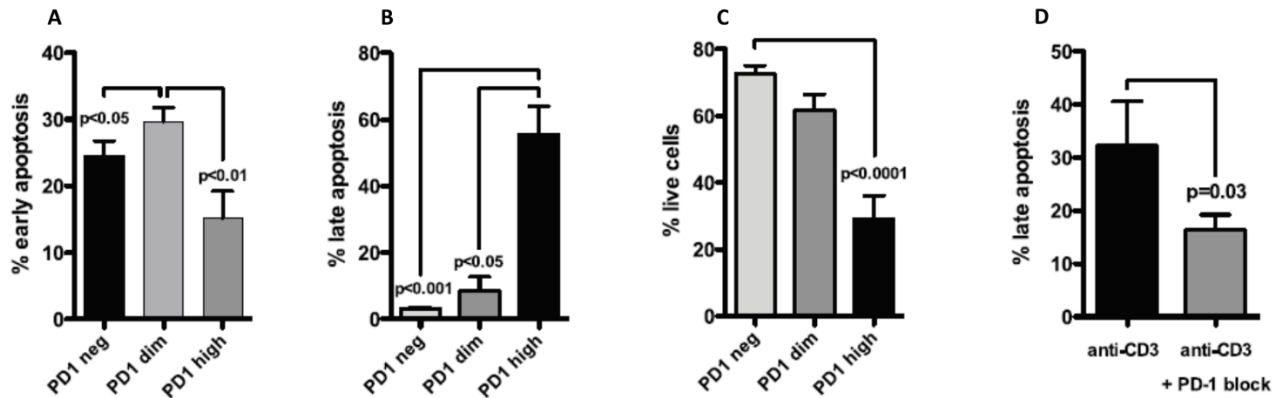


Figure 3.5.4 Induced early and late apoptosis in PD-1 negative, PD-1 dim and PD-1 high and

Apoptosis in anti-CD3 stimulated T cells.

A. The percentage of early apoptosis in PD-1 dim cells was significantly higher than PD-1 negative ($p < 0.05$) and PD-1 high cells ($p < 0.01$), B. The percentage of late apoptosis was significantly higher in PD-1 high cells than in PD-1 negative cells ($p < 0.001$) and PD-1 dim cells ($p < 0.05$). C. concurrently, the percentage of live cells expressing high levels of PD-1 is significantly lower than PD-1 dim and PD-1 negative cells. D. PD-1 high cells underwent a much higher percentage of apoptosis than dim ($p < 0.05$) and negative cells ($p < 0.001$). C). The percentage of live cells ($7AAD^-$) expressing high levels of PD-1 was lower than live cells with no PD-1 and less PD-1. Sample size, $n = 10$. B). Cells stimulated with anti-CD3 showed a significantly higher percentage of late apoptosis compared to cells incubated with anti-PD-1 monoclonal antibody. PI, Propidium Iodide. $p = 0.03$. $n = 6$. One-way ANOVA followed by Tukey's post hoc test was carried out to test for significant associations between the three groups (A, B, C) and the unpaired t test was carried out to compare the two groups (D).

Thus, it was shown that the expression of PD-1, predominantly in $CD8^+$ T_{EMRA} cells, led to increased late apoptosis and was responsible for the permanent loss of $CD8^+$ T_{EMRA} from circulation. The analysis of cells incubated and stained with anti-PD-1 monoclonal antibody, propidium iodine and 7-AAD confirmed this hypothesis.

3.6 Baseline characteristics of main sub group B

52 patients comprised the main sub-group B of the study. 29 patients were CMV seropositive and 23 patients were CMV seronegative. The CMV seropositive patients were significantly older than CMV seronegative patients (p=0.033). Moreover, there were more males than females (p=0.016). More CMV seropositive patients were EBV seropositive compared to CMV seronegative patients (p=0.04). The patient sub group did not vary in other parameters based on CMV-seropositivity.

Parameters	CMV-seropositive (n=29)	CMV-seronegative (n=23)	P value
Age	62 (54; 72)	56 (47; 63)	0.033
Sex: male/female	18 / 11	21 / 2	0.016
EBV-seropositive	28 (96.5%)	18 (78.3%)	0.040
White Blood Cells, (cells/ μ l)	11520 (9500; 14155)	12540 (10938; 14903)	0.392
Lymphocytes, (cells/ μ l)	2000 (1400; 3110)	1915 (1413; 2730)	0.669
Monocytes, (cells/ μ l)	800 (545; 910)	635 (463; 833)	0.278
Neutrophils, (cells/ μ l)	9220 (6800; 10615)	9240 (7775; 10865)	0.530
Eosinophils, (cells/ μ l)	100 (60; 170)	160 (65; 310)	0.397
Platelets, ($\times 10^3$ / μ l)	239 (204; 311)	240 (225; 326)	0.482
Serum Creatinine, (μ mol/L)	77 (65; 88)	81 (69; 95)	0.190
HDL cholesterol, (mmol/L)	1.2 (1.0; 1.4)	1.2 (1.0; 1.4)	0.795
Serum cholesterol, (mmol/L)	5.1 (4.5; 5.8)	5.0 (4.5; 6.1)	0.699
Glucose, (mmol/L)	7.9 (6.1; 9.2)	8.1 (7.3; 9.4)	0.161
Triglycerides (mmol/L)	1.3 (0.9; 2.4)	1.9 (1.1; 2.7)	0.483
Troponin, 12h sample (ng/L)	4800 (2274; 8461)	5098 (2102; 8238)	0.934

Table 3.2 Baseline characteristics of main sub group B. Continuous variables are represented as median (25% quartile; 75% quartile) and compared by Mann-Whitney test; categorical variables are compared by chi-square test. Listed parameters represent measurements before reperfusion, except for troponin levels (12h after reperfusion). HDL, high density lipoprotein; EBV, Epstein-Barr virus.

3.7 Cytokine production by CD8⁺ and CD4⁺ T cells in CMV and EBV seropositive AMI patients

One of the main objectives of this part of the study was to investigate antigen-specific cytokine production by patient PBMC using the *ex-vivo* ELISpot assay. We first analysed IFN- γ production by CD4⁺ and CD8⁺ T cells in CMV seropositive patients. There were no changes in the CD8⁺ T cell compartment across all time points whereas IFN- γ production in CD4⁺ T cells showed a significant reduction at the 90 minute time point after reperfusion ($p=0.002$) before recovering at 24 hours ($p=0.003$).

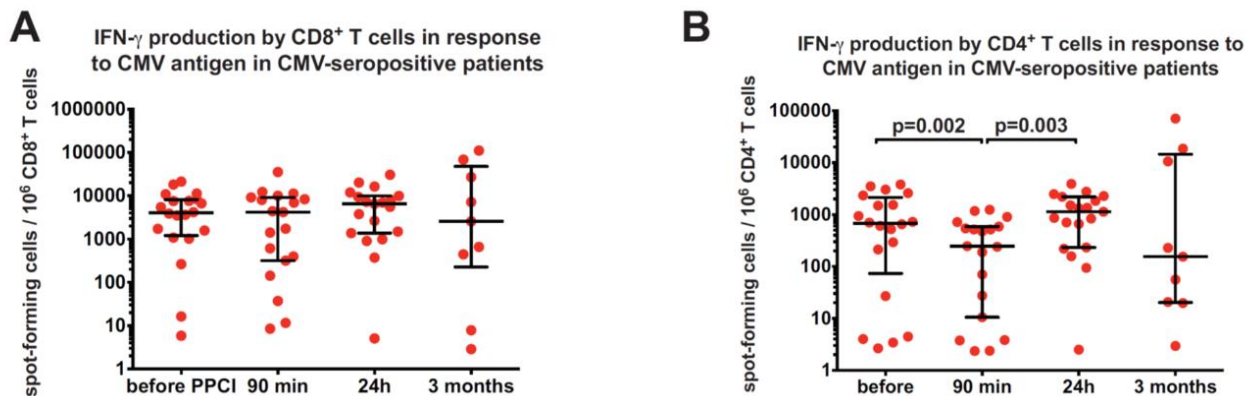


Figure 3.7.1 IFN- γ production by CMV-specific CD4⁺ and CD8⁺ T cells by *ex-vivo* ELISpot. A. Production of IFN- γ by CD8⁺ T cells across all time points after reperfusion did not show any significant changes. B. There was, however, a significant drop in the production of IFN- γ by CD4⁺ T cells at the 90 minute time point ($p=0.002$), but at 24 hours IFN- γ production had recovered ($p=0.003$). The y axis is shown as logarithmic scale (10). 90 min, 24h and 3 months indicate time points after reperfusion. The data sets include median with interquartile range. $n=20$ (before PPCI), $n=19$ (90 minutes), $n=19$ (24 hours) and $n=9$ (3 months) for CD8⁺ T cells. $n=20$ (before PPCI), $n=19$ (90 minutes), $n=19$ (24 hours) and $n=8$ (3 months) for CD4⁺ T cells. Wilcoxon signed rank test was performed to identify associations between each of the time points.

Performing cultured ELISpot is an indicator and measure of the memory T cell response. In contrast to *ex-vivo* ELISpot CD4⁺ T cell response, no changes were found across all time points in cultured CD4⁺ T cell response (**Figure 3.7.2**). The CD8⁺ T cell compartment showed some

changes by cultured ELISpot. IFN- γ production in CD8⁺ T cells showed no changes at 90 minute time point compared to baseline levels. However, IFN- γ levels at 24 hours was significantly higher compared to baseline levels (p=0.0074). (**Figure 3.7.2**)

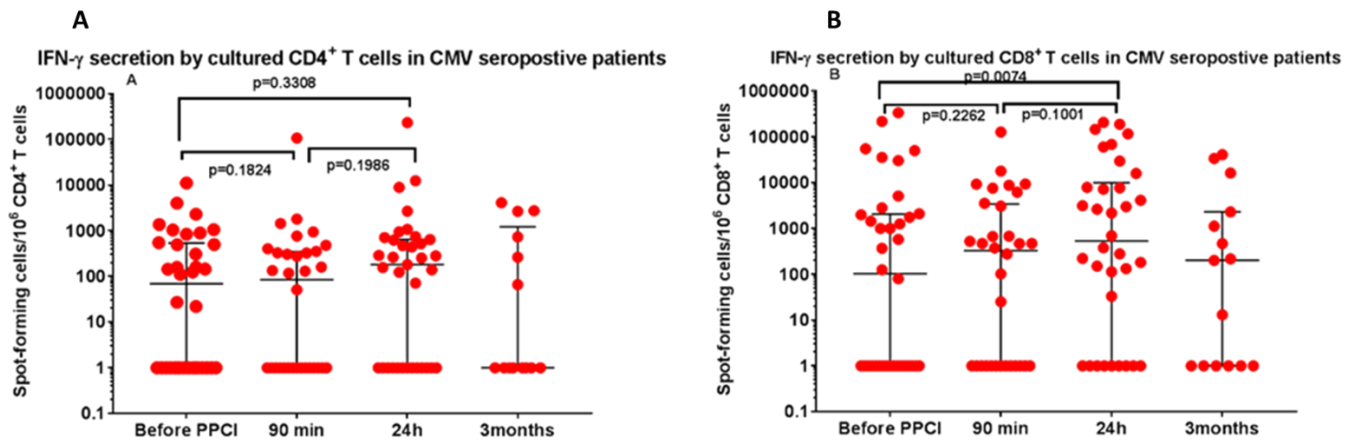


Figure 3.7.2 IFN- γ production by CMV specific CD4⁺ and CD8⁺ T cell by Cultured ELISpot. A. IFN- γ production in CD4⁺ T cells showed no changes at any time point. B. The cytokine production in CD8⁺ T cells was significantly higher 24 hours after PPCI compared to the secretion of IFN- γ before PPCI (p=0.0074). However, there were no further changes. The y axis is shown as a logarithmic scale. Data sets include median with interquartile range. n=35 (before PPCI), n=32 (90 minutes), n=34 (24 hours) and n=14 (3 months) for CD8⁺ T cells. n= 35 (before PPCI), n= 32 (90 minutes), n= 34 (24 hours) and n=15 (3 months) for CD4⁺ T cells. Wilcoxon signed rank test was performed to identify associations between each of the time points.

The focus then shifted to IFN- γ production by EBV specific CD4⁺ and CD8⁺ T cells (**Figure 3.7.3**). EBV has no known role in MI and thus testing EBV responses would give a control response against CMV response. Release of IFN- γ at 24 hours was significantly higher than the levels before PCCI (p=0.026) and at 90 minutes (p<0.001). The CD4⁺ T cell compartment showed a significant dip at 90 minutes (p=0.008) which recovered at 24 hours (p=0.02).

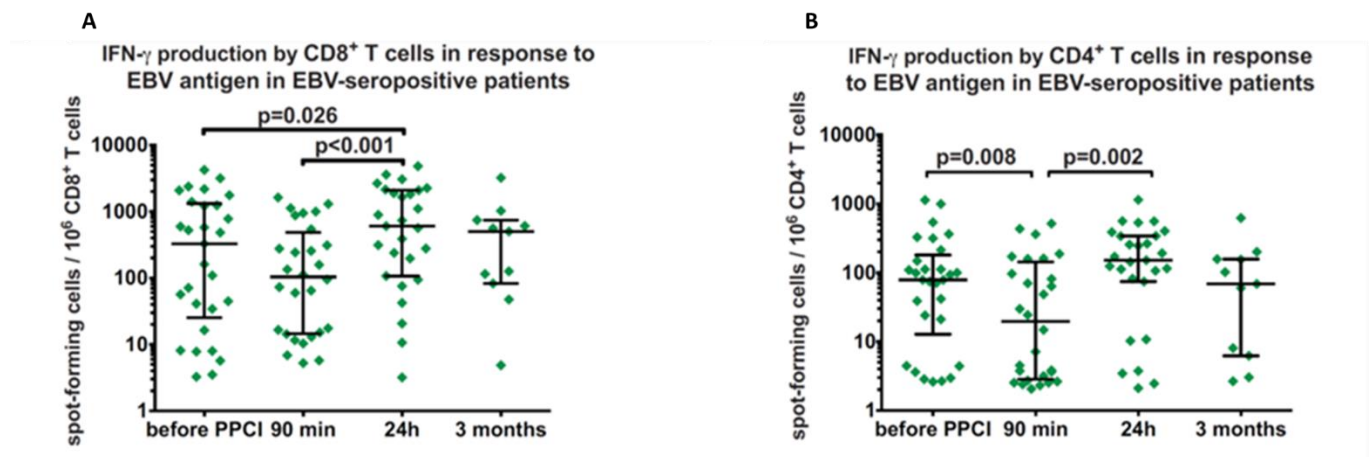


Figure 3.7.3 IFN- γ production by EBV-specific CD4⁺ and CD8⁺ T cells by *ex-vivo* ELISPOT. A.

There was a dip in CD8⁺ T cell responses at 90 minutes but was not significant. However, there was significant increase at 24 hours ($p < 0.01$). B. There was, however, a significant drop in the production of IFN- γ by CD4⁺ T cells at the 90 minute time point ($p = 0.008$), but at 24 hours IFN- γ production had recovered ($p = 0.002$). The y axis is shown as logarithmic scale (10). 90 min, 24h and 3 months indicate time point after reperfusion. The data sets include median with interquartile range. $n = 29$ (before PPCI), $n = 28$ (90 minutes), $n = 27$ (24 hours) and $n = 11$ (3 months) for CD4⁺ T cells. $n = 29$ (before PPCI), $n = 28$ (90 minutes), $n = 27$ (24 hours) and $n = 11$ (3 months) for CD8⁺ T cells. Wilcoxon signed rank test was performed to identify associations between each of the time points.

Next, the cultured ELISPOT responses for EBV were examined. IFN- γ production by EBV-specific CD4⁺ T cells at 24 hours was significantly higher than the production before PPCI ($p = 0.0027$) with no changes at 90 minutes. In the CD8⁺ T cell compartment, IFN- γ production at 24 hours was significantly higher than the production before PPCI ($p = 0.0010$) and the levels at 24 hours were also significantly higher than the levels at 90 minutes ($p = 0.0001$) (**Figure 3.7.4**).

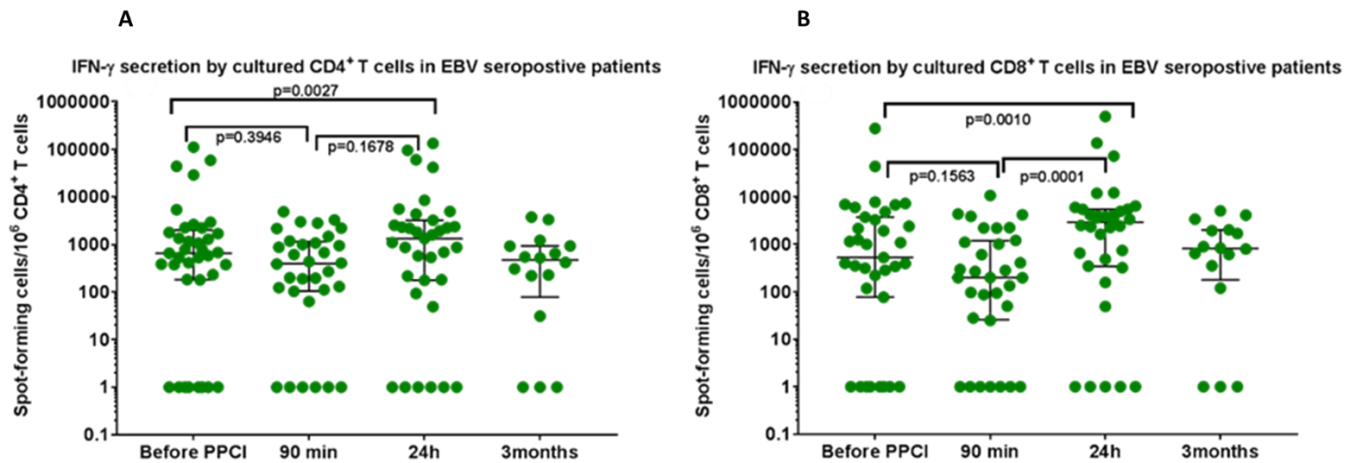


Figure 3.7.4 IFN- γ production by EBV specific CD4⁺ and CD8⁺ T cells by Cultured ELISpot. A. IFN- γ production in both CD4⁺ T cells showed an increase at 24 hours compared to baseline level, but no difference was seen at 90 minutes. B. IFN- γ production in both CD8⁺ T cells also showed an increase at 24 hours compared to baseline levels, but no difference was seen at 90 minutes. The y axis is shown as a logarithmic scale (log₁₀). Data sets include median with interquartile range. n=36 (before PPCI), n=32 (90 minutes), n=34 (24 hours) and n=16 (3 months) for CD4⁺ T cells. n= 34 (before PPCI), n= 32 (90 minutes), n= 33 (24 hours) and n=16 (3 months) for CD8⁺ T cells. Wilcoxon signed rank test was performed to identify associations between each of the time points.

It was then decided to measure non-specific T cell activation and other cytokine levels in patient PBMCs. Non-specific IFN- γ production in response to PHA stimulation was higher in CMV seropositive patients than in CMV seronegative patients (**Figure 3.7.5**). In addition, in CMV seropositive patients, there was a significant decrease in IFN γ production at 90 minutes (p=0.009) followed by an increase at 24 hours after PPCI (p=0.0329). A similar trend was seen for CMV seronegative patients. There was significant decrease at 90 minutes (p=0.048) followed by a significant increase at 24 hours (p=0.0067). There was a significant drop in IL-2 production in response to PHA stimulation at 90 minutes after PPCI (p=0.008). However, no changes were seen at 24 hours and 3 months after PPCI. IL-5 production in response to PHA stimulation at 24 hours after PPCI was significantly higher than at 90 minutes after PPCI. There were no changes at any other time points (Before PPCI and 3 months after PPCI). IL-17

production was significantly increased at 24 hours after PPCI compared to production before PPCI. There was no relationship at other time points (**Figure 3.7.5**).

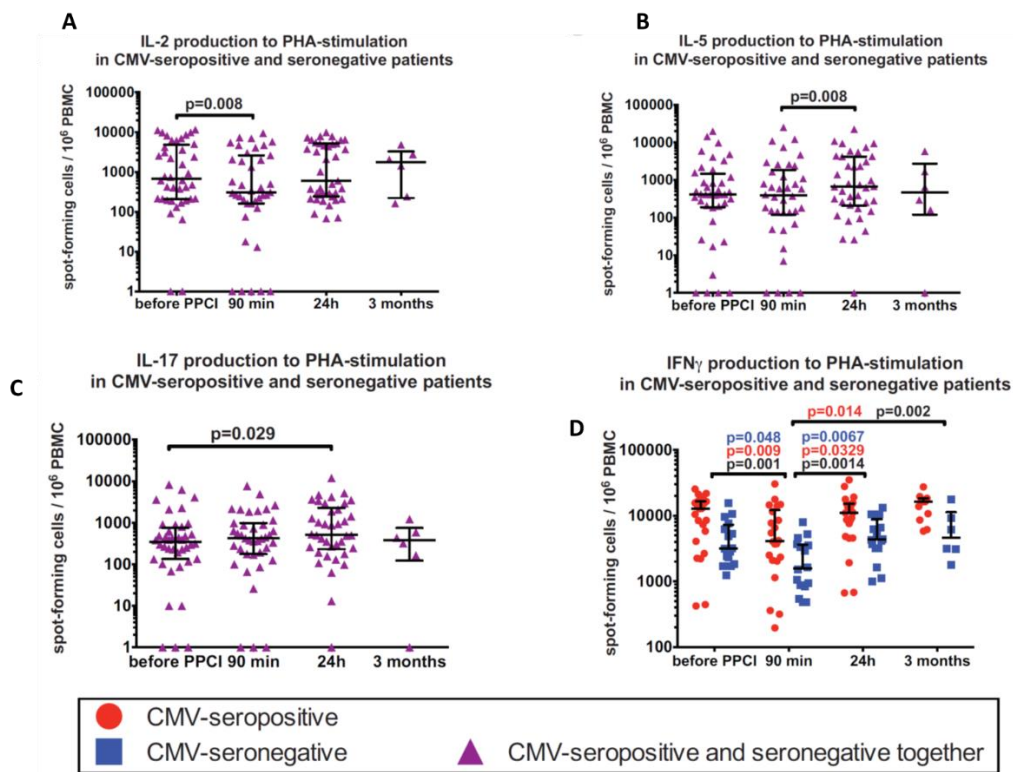


Figure 3.7.5 Measurement of cytokine levels and non-specific activation of PBMCs with PHA. A. Production of IL-2 (n=40 before PPCI, n= 39 at 90minutes, n=39 at 24 hours and n=6 at 3 months); B. Production of IL-5 (n=40 before PPCI, n= 38 at 90minutes, n=38 at 24 hours and n= 6 at 3 months); C. Production of IL-17 (n=40 before PPCI, n= 39 at 90minutes, n=38 at 24 hours and n=6 at 3 months); and D. Production of IFN- γ in responses to 24 h PHA-stimulation by *ex-vivo* ELISpot in CMV-seropositive and seronegative patients; D. For CMV seropositive patients, n =23 before PPCI, n=22 at 90 minutes, n=23 at 24 hours and n=11 at 3 months. For CMV seronegative patients, n=18 before PPCI, n=17 at 90 minutes, n=17 at 24 hours and n=6 at 3 months. P values are provided separately for CMV seropositive individuals (red), CMV-seronegative individuals (blue) otherwise, CMV-seropositive and seronegative individuals are given together (black). Data sets include median with interquartile range. IL-2, interleukin-2; IL-5, interleukin-5; IL-17, interleukin-17; PHA, phytohaemagglutinin. Y axis is shown as a logarithmic scale (log10). Due to the suboptimal performance in the use of IL-4 in our ELISpot experiment, we used IL-5, another Th2 cytokine. Wilcoxon signed rank test was used to identify associations between each of the time points.

The inflammatory process connected to myocardial infarction and reperfusion injury may be mediated through lymphocyte migration controlled by several cytokines that will manifest in the patient circulation/serum. So next, MSD was used to measure the levels of serum cytokines in patients. 11 different serum cytokines in CMV seropositive and CMV seronegative patients were tested using MSD. MSD was the preferred electrochemiluminescence multiplex platform owing to its minimum background levels, the use of carbon electrode plate that offers 10 times the binding capacity compared to normal polystyrene plates, the use of non-radioactive labels, signal amplification caused by multiples excitation levels allow for highly enhanced sensitivity. Among the 11, only IP-10 and IL-16 showed significant changes. The levels of IP-10, in CMV seropositive patients, at all times points after PPCI were higher than the level before PPCI (**Figure 3.7.6 A, B**). In contrast, IL-16 levels were lower than IL-16 levels for CMV seronegative patients. Also, the levels of IL-16 oscillated across all time points before and after PPCI. (n=25, 14-CMV seronegative, 11-CMV seropositive). Interestingly, IL-16 levels at 90 minutes had an inverse relationship with CD4⁺ T cells levels at 90 minute time point (**Figure 3.7.6 C**).

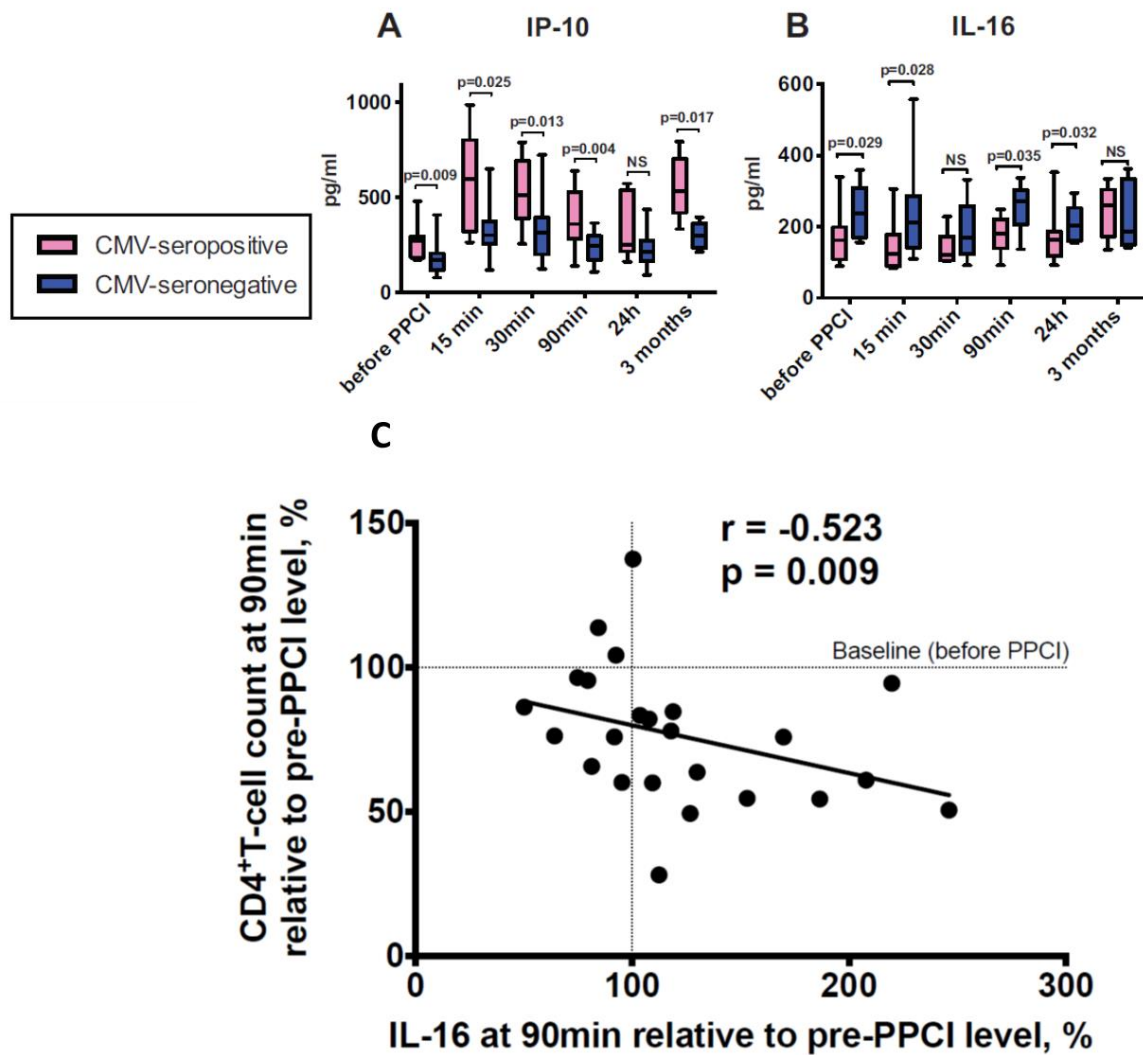


Figure 3.7.6 Serum cytokine levels in patient samples. A. Apart from the 24 hour time point after PPCI, the IP-10 levels in CMV seropositive patients were significantly higher than in CMV seronegative patients. n=25 at 0 minutes, n=22 at 15 minutes, n=24 at 30 minutes, n=24 at 90 minutes, n=23 at 24 hours and n=3 at 3 months. B. The IL-16 levels in CMV seropositive patients were significantly lower than the levels in CMV seronegative patients. n=25 at 0 minutes, n=22 at 15 minutes, n=24 at 30 minutes, n=24 at 90 minutes, n=23 at 24 hours and n=3 at 3 months. C. The percentage of CD4⁺ T cells at 90 minutes had an inverse association with IL-16 levels at the same time point. The drop in CD4⁺ T cell count was accompanied by the reduction in IL-16 levels, n=24. Boxplots represent median, upper and lower quartiles along with minimum and maximum values. P values were calculated using Wilcoxon signed rank test. For Figure C, Spearman analysis was used to test for correlation between the two variables.

3.8 Analysis of the relationship between Microvascular obstruction with *Ex-Vivo* and Cultured ELISpot

Microvascular obstruction (MVO), or no-reflow phenomenon, occurs in a proportion of patients after PPCI. MVO is characterised by structural and functional changes to the myocardium such as endothelial destruction, emboli of neutrophils and platelets, and release of free radicals, and is an indication of reperfusion-induced injury (Ito, 2009). So next, it was decided to test for association between MVO levels in patients and T cell responses in patients measured by ELISpot. The values of MVO were provided by our clinical collaborators at Freeman Hospital. MVO was quantified and detected on late gadolinium enhancement (LGE) cardiac MRI images as an area of hypoenhancement within the infarct core (Boag *et al.*, 2015). These values were then used to identify correlations between ELISpot and MVO levels in patients. Spot forming cells/ 10^6 PBMC obtained by *ex-vivo* ELISpot and cultured ELISpot for patient cohorts were compared to microvascular obstruction (MVO) and peak troponin levels by Spearman analysis to identify significant correlations. CMV-specific cultured ELISpot CD8⁺ T cell response at 24 hour point (p=0.044 for MVO volume, p=0.042 for MVO mass) and EBV-specific CD4⁺ T cells at 24 hour time point (p=0.026 for MVO volume, p=0.025 for MVO mass) showed a positive correlation with both MVO volume and MVO mass which was significant (**Figure 3.8.1**). No *ex-vivo* ELISpot responses correlated with either MVO volume or MVO mass. Furthermore, CMV-specific CD4⁺ T cell responses at 24 hours was just outside significance (p=0.071 for MVO volume, p=0.069 for MVO mass) and EBV specific CD4⁺ T cell response at 3 months after PPCI was just outside significance as well (p=0.05 for both MVO volume and MVO mass). For peak troponin levels, both CMV specific CD4⁺ T cell response at 0 minute time point (p=0.006) and CMV specific cultured CD8⁺ T cell response at 24 hours post PPCI showed a negative correlation (p=0.042).

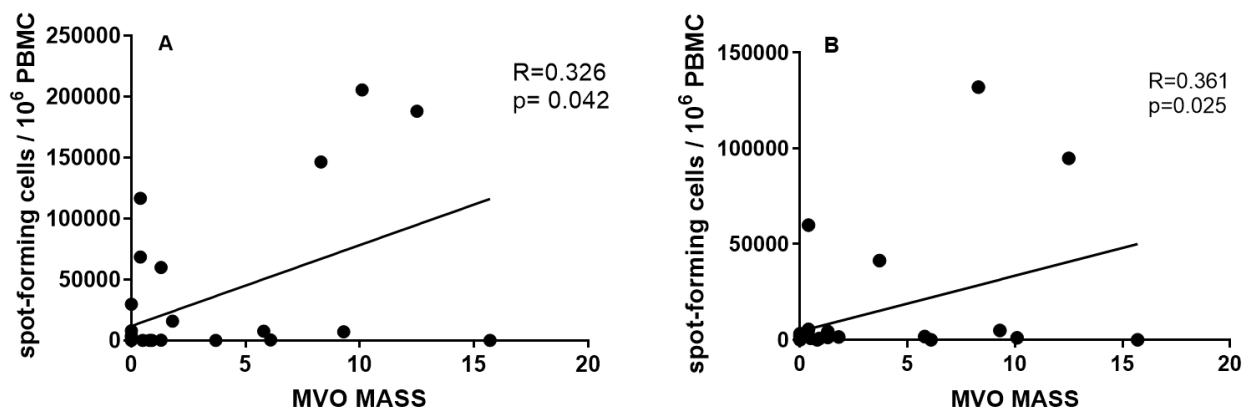


Figure 3.8.1 Associations between MVO mass and cultured ELISpot EBV and CMV-specific T cells 24 hours after PPCI. A. Cultured ELISpot CMV-specific CD8⁺ T cell response 24 hours after PPCI positively correlated with MVO mass ($r=0.326$, $p=0.042$); B. Cultured ELISpot EBV specific CD4⁺ T cell response 24 hours after PPCI correlated with MVO mass ($r=0.361$, $p=0.025$). PPCI-Primary percutaneous coronary intervention. Correlations were obtained using Spearman analysis. $n=33$ for A. and $n= 35$ for B.

In addition to the above analysis, the MVO mass in patients were split into ‘high’, ‘low’ and ‘none’ as in the research article by Boag *et al.*, 2015. These MVO levels were then analysed with ELISpot data at various time points. Patients were divided into the three groups depending upon the extent of MVO. Patients with MVO levels 3.5 and above were classified as high, patients with MVO levels higher than 0 but lower than 3.5 were classified as low and patients with MVO levels of 0 were classified as none. There were 9 patients classified as ‘high’, 7 patients classified as ‘low’ and 12 patients were classified as ‘none’. We first investigated the relationship between CMV-specific CD4⁺ and CD8⁺ T cells, at different time points with MVO. We found no associations between MVO and CMV-specific CD4⁺ and CD8⁺ T cell response.

We then performed the same analysis for EBV-specific T cells. Interestingly, the production of IFN- γ by EBV-specific CD4⁺ T cells at baseline levels (before PPCI) were significantly higher in the ‘none’ MVO patient group ($p=0.03978$). The trend continued at 90 minutes after

PPCI, the ‘none’ MVO patient group still had higher (but not significant) IFN- γ spot forming cells/M than patients with ‘high’ MVO ($p=0.05$), although this was lost at 24 hours after reperfusion (data not shown) (**Figure 3.8.2**) Moreover, for EBV specific CD8⁺ T cells, the IFN- γ production by ‘none’ MVO group significantly exceeded the high MVO group before PPCI ($p=0.0082$), however, this trend was lost at 24 hours ($p=0.092$). We also found that at 24 hours, the ‘none’ MVO group had significantly higher IFN- γ production compared to low MVO group.

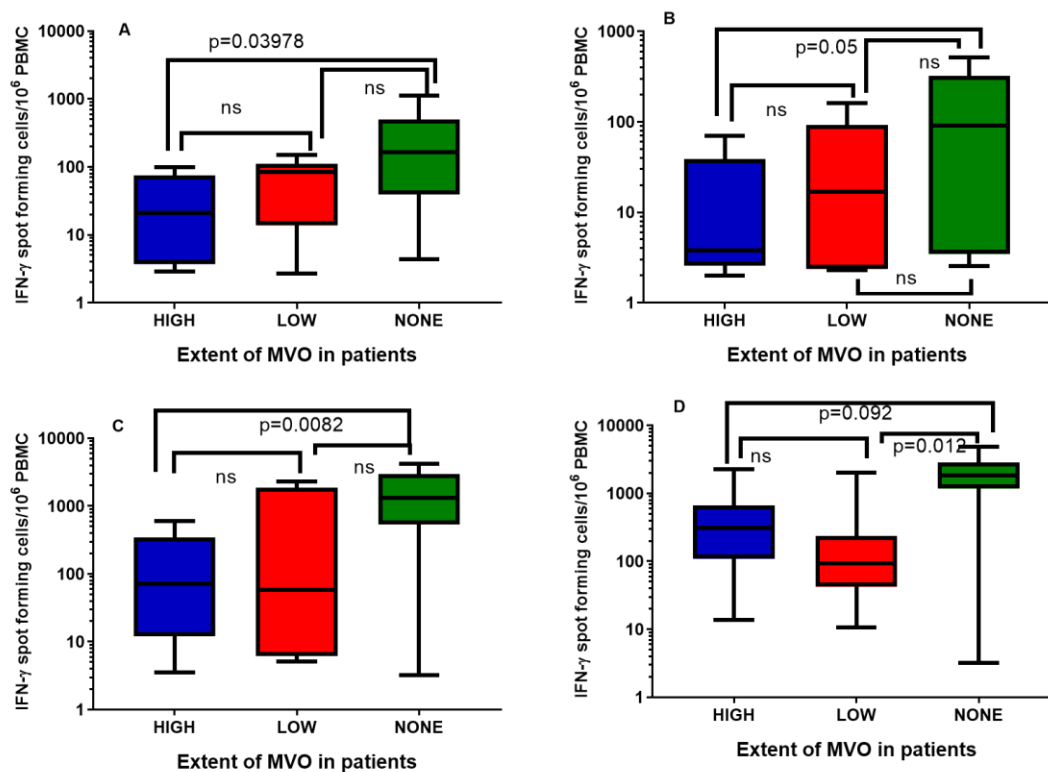


Figure 3.8.2 Relationship between MVO and EBV-specific T cells. A. Secretion of IFN- γ by EBV-specific CD4⁺ T cells before PPCI; B. Secretion of IFN- γ by EBV-specific CD4⁺ T cells at 90 minutes after PPCI; C. Secretion of IFN- γ by EBV-specific CD8⁺ T cells before PPCI; D. Secretion of IFN- γ by EBV-specific CD8⁺ T cells at 24 hours after PPCI. ns- Not significant. The y axis is shown as a logarithmic scale and the x axis indicates the extent of MVO (high, low or none) in the patients group. Blue-high MVO; red-low MVO; green-no MVO. n=9 for ‘high’, n=7 for ‘low’ and n= 12 for ‘high’. Kruskal-Wallis test with Dunn’s multiple comparisons test was used to compare the three groups.

Next, the cultured ELISpot responses for CMV and EBV were tested. We hoped this might offer insights into the relationship of MVO with *ex-vivo* as well as cultured ELISpot. For both CMV specific and EBV specific CD4⁺ T cells and CD8⁺ T cells, we found no associations between the extent of MVO in patients and level of IFN- γ produced by these cells.

After testing MVO levels against pathogen specific T cells, patient lymphocytes were also non-specifically activated by Phytohaemagglutinin (PHA) to test the production of IL-2, IL-5, IL-17 and IFN- γ . IL-5 and IL-17 are signature cytokine of Th2 and Th17 cells respectively, so it was interesting to test the relationship between different T cell subtypes with the extent of MVO in patients. We found IL-2, IL-5, IL-17 and IFN- γ production in response to PHA did not differ in all three MVO groups at all three time points.

There was no association between the different MVO patient groups and non-specific production of cytokines at all the three time points.

Apart from looking for correlations between MVO and ELISpot, associations between peak troponin levels and ELISpot responses (both *Ex-Vivo* & Cultured) were also studied. Spearman correlation analysis revealed both CMV-specific CD4⁺ cultured T cell response at 0 minute time point (p=0.003) and CMV-specific cultured CD8⁺ T cell response at 24 hours post PPCI showed a negative correlation (p=0.042) with peak troponin levels (**Figure 3.8.3**). Apart from these correlations, EBV-specific CD4⁺ and CD8⁺ T cell response at 0 minutes after PPCI was just outside significance (p=0.066 and p=0.064), respectively. In addition, cultured CD8⁺ T cell response at 0 minutes after PPCI was also just outside significance (p=0.056).

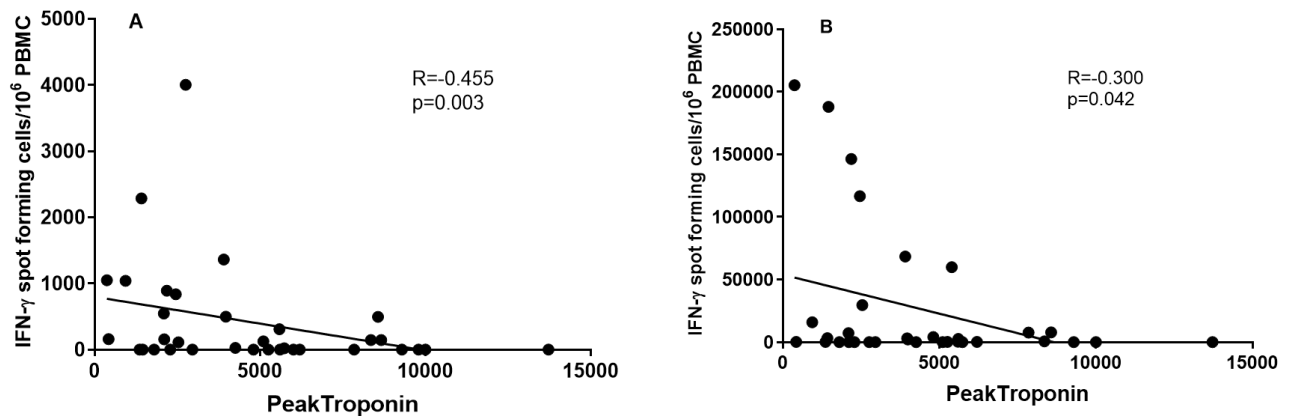


Figure 3.8.3 Relationship between Peak Troponin levels and ELISpot responses. A. Correlation between cultured CMV-specific CD4⁺ T cell response before PPCI and peak troponin (R=-0.455, p=0.003). B. Correlation between Peak Troponin and cultured CMV-specific CD8⁺ T cell response at 24 hours after PPCI (R=-0.300, p= 0.042); p value indicates value of significance. Correlations were obtained using Spearman analysis. n=35 for A. and n=34 for B.

Peak Troponin is considered a prognosticator of MI disease level before reperfusion, and Microvascular obstruction (MVO) is an indicator of reperfusion injury after PPCI. We clearly show that these two measurements are unrelated in our patient cohort. Therefore, identifying associations between MVO, Peak Troponin level and ELISpot responses gave an interesting insight into the influence of the immune response, especially by T cells.

3.9 Flow cytometric Analysis of Tetramer and Non-Tetramer CD8⁺ T cells

Boag *et al.*, in 2015 revealed that the increase in levels of fractalkine (CX3CR1) was strongly associated with the T cell drop in STEMI patients following PPCI/Reperfusion. Thus, it was decided to study the role of fractalkine in more detail. To this end, all remaining patient PBMC samples were surveyed to identify if PBMC samples were available at all time points. 8 patient PBMC samples (sub-group C) were selected and flow cytometric analysis of tetramer, non-tetramer and fractalkine positive CD8⁺ was performed on them. As well as fractalkine, it was

decided to test various other cell surface receptors implicated in myocardial injury such as CCR4, CCR5, CXCR3, CX3CR1, SIP1R and C-met.

3.9.1 Gating Strategy used for tetramer analysis of CD8⁺ T cells

A typical gating strategy to identify, measure and depict the levels of expression of cell surface receptors of tetramer CD8⁺ T cells is shown. Similar gating strategies were adopted to analyse non-tetramer CD8⁺ T cells and CX3CR1⁺ CD8⁺ T cells in patient samples. Each sample was analysed on a BD FACS Canto II cytometer with BD FACSDiva acquisition software. The samples were run until 10000 T cell events had been acquired, or until just before the sample ran dry in cases with low T cell numbers. Similar gating strategy was adopted for analysing levels of cell surface markers on non-tetramer CD8⁺ T cells as well as within CD8⁺ CX3CR1⁺ T cells.

- 1) Lymphocytes were gated according to their typical forward and side scatter characteristics (**Figure 3.9.1 A**).
- 2) Singlet cells were further analysed for CD3 expression focussing only on the T cell population. From the CD4⁺ and CD8⁺ T cell populations, the CD8⁺ T cell population was further analysed to measure tetramer PE recognition (**Figure 3.9.1 A**).
- 3) CD8⁺ T-BV510 cells were gated with tetramer-PE and only CD8⁺ T cell populations that recognized tetramer was taken for further analysis (**Figure 3.9.1 A**).
- 4) Cell surface markers such as CX3CR1, S1PR1, c-met, CCR5, CCR4 and CXCR3 were plotted against side scatter to ascertain the level of expression of these cell surface molecules in tetramer CD8⁺ T cells (**Figure 3.9.1 B**).

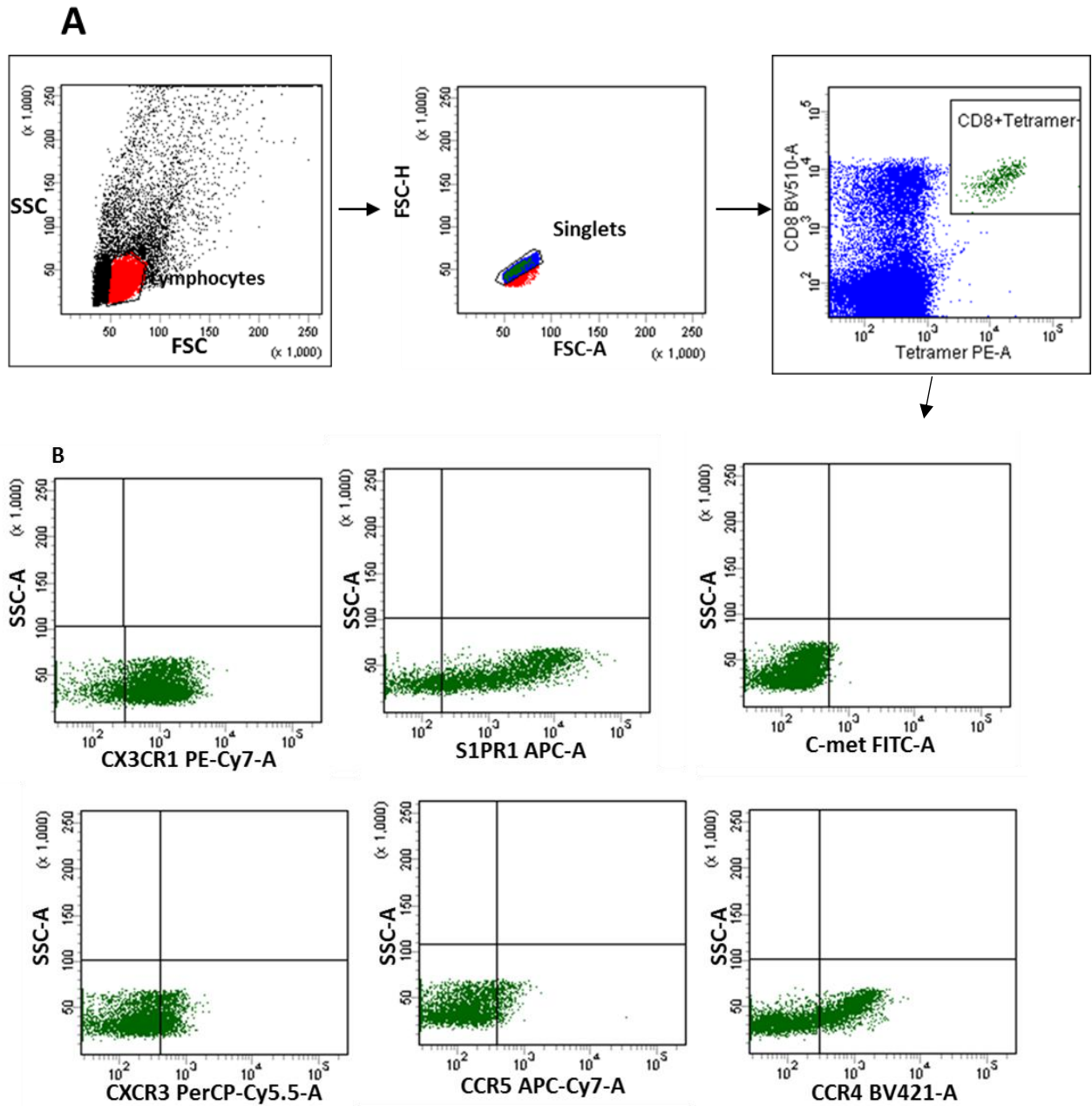


Figure 3.9.1 Gating strategy for tetramer CD8⁺ T cells. A. Lymphocytes were gated according to their forward and side scatter characteristics. Next, the lymphocytes were gated FSC-A against FSC-H to identify the singlets. The singlets were then gated on to identify CD3⁺ T lymphocytes. The CD4⁺ and CD8⁺ T cell populations were identified from the CD3⁺ population. The CD8⁺ T cell population was gated with tetramer-PE and double positive CD8⁺ T cell populations were considered for further analysis. B. All the cell surface markers shown were plotted against side scatter to measure the levels of these markers in CD8⁺ T cell tetramer population. This gating strategy is representative for a single patient sample and the similar process was performed all patient samples at all time points.

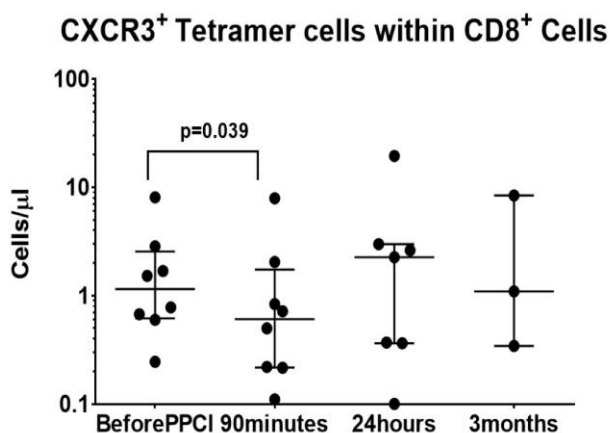


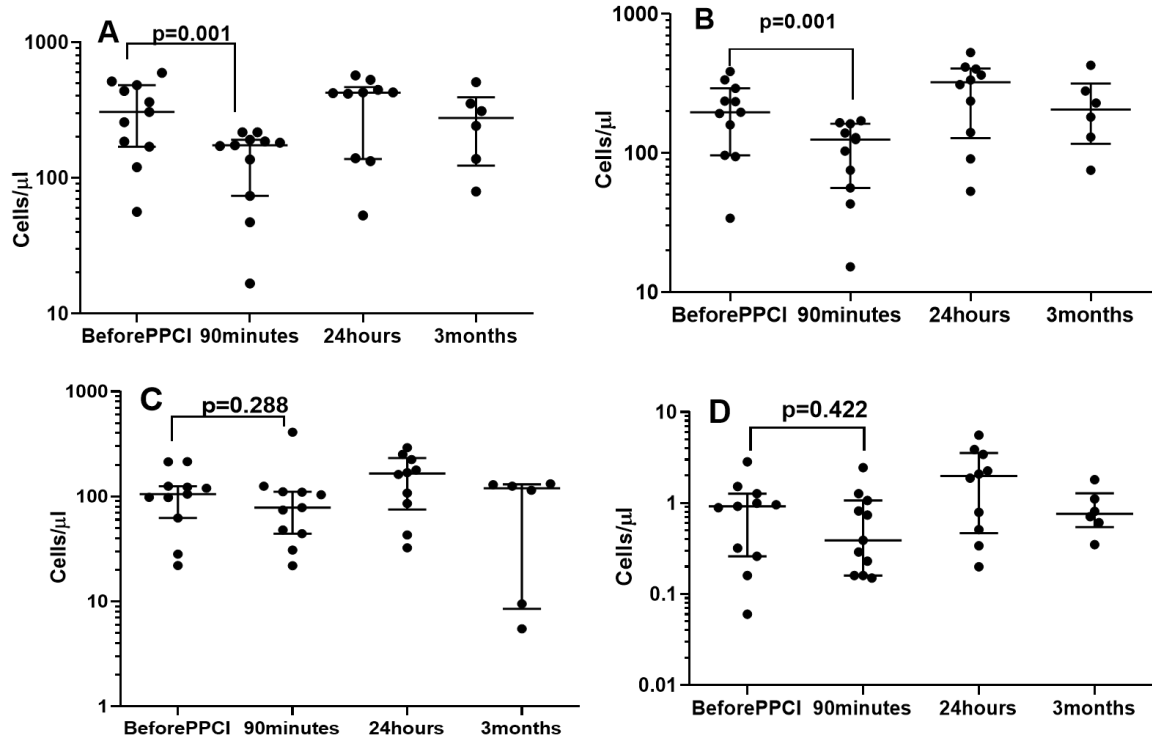
Figure 3.9.2 CD8⁺ Tetramer⁺ cells and various receptor positive cells within CD8⁺ Tetramer⁺ cells.

There were no significant changes in the CD8⁺ T cells before PPCI and 90 minutes, 24 hours and 3 months after PPCI except for CXCR3 tetramer⁺ T cells ($p=0.0391$). CX3CR1-fractakine receptor; S1PR1- Sphingosine -1 phosphate receptor; C-met- Hepatocyte growth factor receptor; CCR5- C-C chemokine receptor 5; CCR4- C-C chemokine receptor 4; CXCR3- G protein coupled receptor 9. p -value of significance. Y axis indicates cell/ μ l; PPCI- primary percutaneous coronary intervention. ($n=8$ for 0, 90, 24 hour time points) and $n=3$ for 3 months post PPCI). Wilcoxon signed rank test was used to test for significance between two time points.

The CD8⁺ tetramer⁺ T cells showed no correlation before PPCI/Reperfusion and 90 minutes after PPCI/Reperfusion ($p=0.1250$). Moreover, the levels of other chemokine receptors also did not show significant associations between ‘before PPCI’ and 90 minutes, 24 hours and 3 months after PPCI except for CXCR3⁺ tetramer⁺ cells which showed a significant drop at 90 minutes from pre-PPCI levels ($p=0.039$) (**Figure 3.9.2**).

In contrast to CD8⁺ Tetramer cells, the non-tetramer CD8⁺ T cells showed a significant drop in T cell count at 90 minutes after PPCI ($p=0.0010$) but by 24 hours and 3 months, had returned to pre-PPCI levels. CX3CR1⁺ cells within non-tetramer CD8⁺ cells also showed a significant drop at 90 minutes ($p=0.0010$). Furthermore, CXCR3⁺ ($p=0.012$), CCR4⁺ ($p=0.008$), CCR5⁺ ($p=0.033$) cells also showed a significant loss at 90 minutes before recovering at 24 hours. The

other cell surface receptors did not show any significant difference between the different time points.



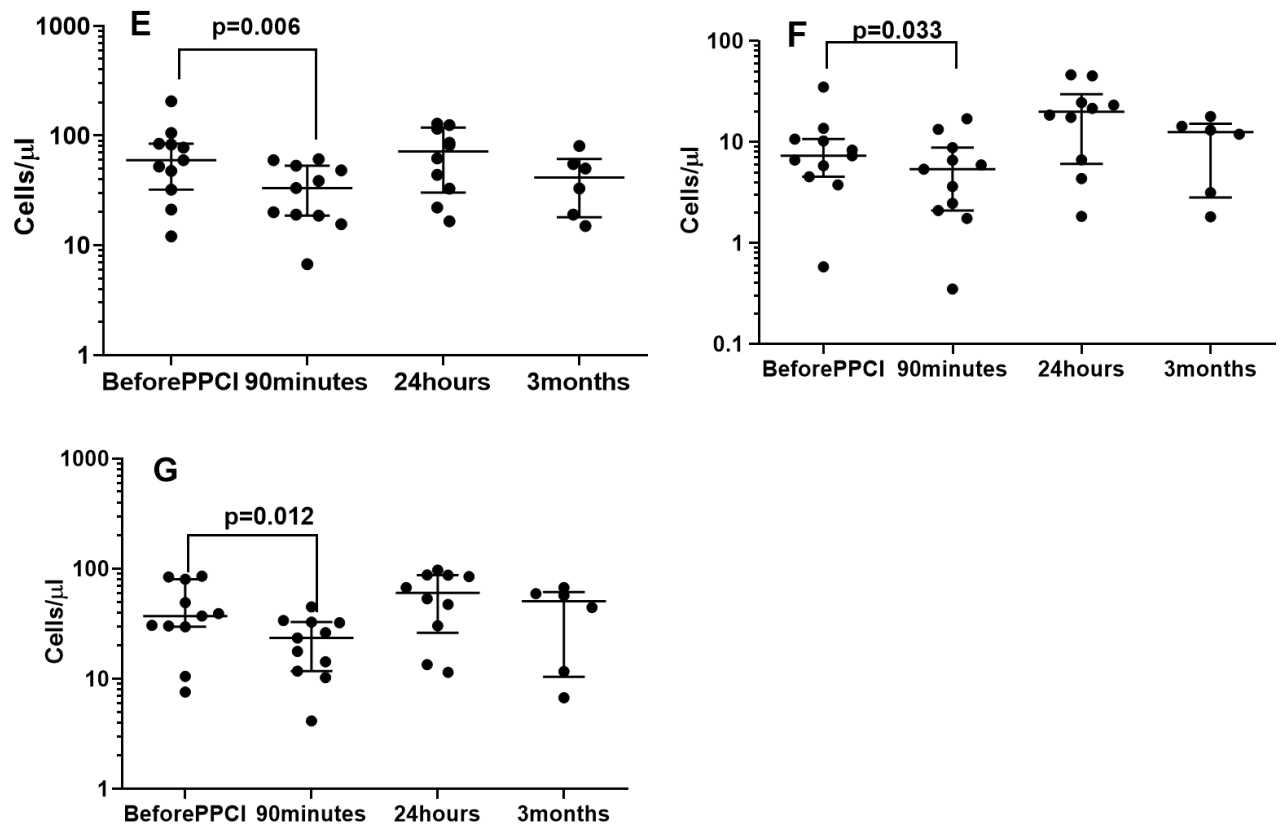


Figure 3.9.3 CD8⁺ Non-Tetramer cells and various receptor positive cells within CD8⁺ Non-Tetramer cells. A. CD8⁺ non-tetramer cells; B. CX3CR1⁺ non-tetramer cells; C. S1PR1⁺ non-tetramer cells; D. c-met⁺ non-tetramer cells; E. CCR4⁺ non-tetramer cells; F. CCR5⁺ non-tetramer cells; G. CXCR3⁺ non-tetramer cells. CX3CR1-fractalkine receptor; S1PR1-Sphingosine -1 phosphate receptor; C-met-Hepatocyte growth factor receptor; CCR5-C-C chemokine receptor 5; CCR4- C-C chemokine receptor 4; CXCR3-G protein coupled receptor 9. p- Value of significance. Y axis indicates cell/ μ l; PPCI- primary percutaneous coronary intervention. (n=8 for 0, 90, 24 hour time points) and n=6 for 3 months post PPCI). Wilcoxon signed rank test was used to test for significance between two time points.

Next, it was decided to test the presence of these cell surface receptors within the CX3CR1 positive cells since this population was found to be important previously (Boag *et al.*, 2015) and in agreement with Boag *et al.*, 2015, we found some interesting results (**Figure 3.9.4**).

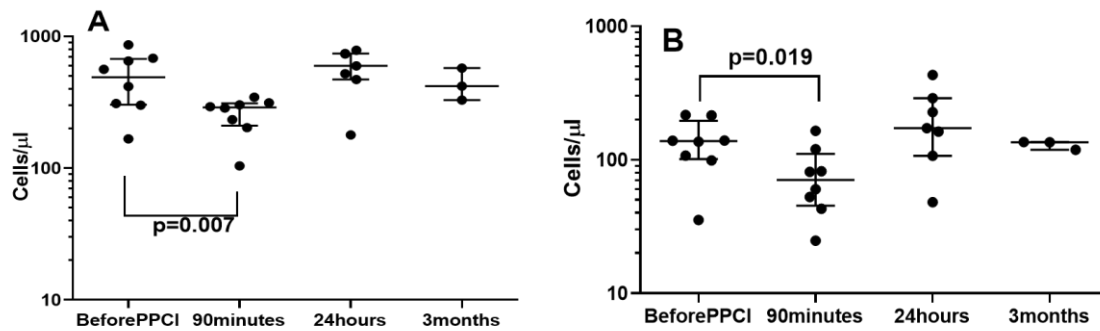


Figure 3.9.4 Presence of cell surface receptors within fractalkine-positive non-tetramer cells. A. CX3CR1⁺ cells; B. S1PR1⁺ cells. CX3CR1-fractalkine receptor; S1PR1-Sphingosine -1 phosphate receptor; p- Value of significance. Y axis indicates cell/μl; PPCI- primary percutaneous coronary intervention. (n=8 for 0, 90, 24 hour time points) and n=3 for 3 months post PPCI). Wilcoxon matched signed rank test was used to compare the two time points.

CD8⁺ T cells within the CX3CR1⁺ population also showed a drop in T cell numbers at the 90 minute (p=0.0078) time point before recovering at 24 hours. Naturally, CX3CR1⁺ cells also show a significant drop at 90 minutes before recovering at 24 hours. Amongst other cell surface receptors within CX3CR1 positive cells, only S1PR1 showed a significant drop in count at the 90 minute time point and recovered at 24 hours. Only S1PR1⁺ (p=0.0195) apart from CX3CR1⁺ (p=0.0078) showed a significant correlation between the 0 and 90 minutes. (n=8 for before PPCI, 90 minutes after PPCI and 24 hours after PPCI and n=3 for 3 months after PPCI).

DISCUSSION

Higher IFN- γ Responses in CMV-seropositive patient PBMCs

In response to PHA (non-specific polyclonal activator) stimulation, the CMV-seropositive STEMI patients' PBMCs produced higher levels of IFN- γ (Th1 cytokine) compared to CMV-seronegative STEMI patients immediately before reperfusion, whereas the levels of IL-5 (Th2 cytokine) and IL-17 (Th17 cytokine) (**Figure 3.2.1**) showed no differences between groups suggesting that Th1 cells are more dominant in CMV-seropositive patients during inflammatory conditions. Thus, there is greater potential in the events leading up to ischemia for Th1 cells to infiltrate the myocardium. Cheng *et al.*, in 2005 showed that acute MI patients had higher numbers of IFN- γ (Th1 cytokine) producing cells compared to patients with unstable and stable angina. The high production of IFN- γ was evident at even one month after ischemic attack. There was however, no differences in the production of IL-4 (a Th2 cytokine) among the three groups. This suggests a preference towards a Th1 phenotype rather than a Th2 phenotype during acute myocardial ischemia. The Cheng research group, however, did not investigate the role of Th17 cytokines. De Boer *et al.*, in 2010 showed the presence of IL-17A, IL-17F and neutrophils in thrombus specimens suggesting a role for IL-17 in the growth and stabilization of thrombus after myocardial ischemia. Eid *et al.*, in 2009 showed that IFN- γ and IL-17 were produced in high amounts by T cells within the coronary arteries, suggesting both these cytokines act synergistically to induce a pro-inflammatory response. However, the presence or absence of CMV-specific T cells in patients was not addressed by both research groups. Simon *et al.*, in 2012 found that acute MI patients with low amounts of IL-17 in serum were strongly linked to worse prognosis and increased levels of IL-17 was associated with better outcomes in MI patients. We, however, did not find any difference between CMV-seropositive patients and CMV-seronegative patients in their production of IL-17 by T cells in

response to stimulation with PHA. A more comprehensive investigation is required to identify a role for Th17 cells in myocardial infarction.

Release of cytokines in patient serum supernatants

Subsequent to the elevated levels of IFN- γ before PPCI detected by ELISpot, there was also an increase in serum IFN- γ levels in CMV-seropositive patients after PPCI. The levels of IFN- γ in serum samples of patients 30 minutes after reperfusion were significantly higher in CMV seropositive patients than in CMV seronegative patients. Although not significant, the levels of IFN- γ in CMV-seropositive patients at 15 minutes and 90 minutes after reperfusion/PPCI were also higher than the levels in CMV-seronegative patients. Although increase in IFN- γ levels in patients is in agreement with most research groups and is one of the main focus in the thesis, it will also be interesting to study the levels of other pro-inflammatory cytokines released in much more detail.

Haider *et al.*, in 2015 showed that IFN- γ levels in CMV seropositive MI patients was much higher than the levels in CMV seronegative MI patients. Furthermore, Liang *et al.*, in 2017 found that patients with coronary artery disease had significantly higher serum levels of CD40L, monokine induced IFN- γ /CXCL9 (MIG) and IFN- γ than the control group. Moreover, severe Coronary Artery Disease (CAD) patients had higher levels of MIG than moderately affected CAD patients indicating that MIG might play a role in the development of atherosclerosis as well as promote the increase in size of coronary artery lesions.

Expression of other cytokines such as TNF- α and IL-1 β are also associated with myocardial infarction. The role of TNF- α in myocardial infarction is pleotropic. Despite being associated with promoting cardiac injury and chemokine synthesis in the myocardium, they also protect cardiomyocytes from apoptosis. IL-1 β is actively involved the recruitment of leukocytes into the myocardium (Frangogiannis, 2004).

Relationship between Total ischemic time and CMV serology in patients

Total ischemic time is the total time from the onset of ischemia to medical contact, otherwise known as door to balloon time. We tried to find any relationship between total ischemic time and CMV serology of patients (**Figure 3.3.1**). We found CMV-seropositivity or CMV-seronegativity of patients had no impact on the total ischemic time. This clearly indicates that ‘speed of treatment’ rather than CMV seropositivity determines the extent of damage in the myocardium after MI. Thus, longer ischemic time equals more damage to the heart tissue.

IFN- γ spot forming cells/M in CMV-seronegative patients in response to PHA stimulation was lower and positively correlated with total ischemic time whereas in CMV-seropositive patients, IFN- γ spot forming cells/M was much higher and did not correlate with total ischemic time. A possible explanation for this phenomenon would be that in CMV-seronegative patients, the homing of T cells away from the myocardium will ease the extent of damage to the heart tissue. In CMV-seropositive patients, the homing of T cells into the heart tissue causes the increase in the release of IFN- γ by CMV-specific T cells which causes more damage to the heart tissue. This might explain the high production of IFN- γ in CMV-seropositive patients.

Tetramer analysis of CD8⁺ T cell population before and after PPCI/Reperfusion

We analysed monocyte, CD3⁺ T cell, CD4⁺ T cell and CD8⁺ T cell population in CMV-seronegative and CMV-seropositive patients before PPCI/Reperfusion. Only CD8⁺ T cells were increased in CMV-seropositive patients whereas other cell populations showed no difference between CMV-seropositive and CMV-seronegative groups, suggesting increased circulation of CD8⁺ T cells with ability to circulate into and out of the myocardium prior to and during ischemic attack. CMV-specific CD8⁺ T cells as a percentage of total CD8⁺ T cells, determined through HLA type 1 specific tetramer analysis ranged from 0.2 to 18%. The next step was to check the levels of CD8⁺ T cell levels after reperfusion. Flow cytometric analysis of HLA specific tetramers (See chapter 2, section 2.5.2) revealed a significant drop in CD8⁺ T cell count

at 90 minutes after reperfusion. Although CD8⁺ T cell count increased at 24 hours after reperfusion, they were still significantly lower than the levels before reperfusion. Forteza *et al.*, 2018 noticed a significant drop in both CD4⁺ and CD8⁺ T cell count 24 hours after PPCI and this level was restored to pre-PPCI levels at day 4. Interestingly, Th1 cells and not Th2 showed the drop which is yet another strong indicator of T cell homing to the myocardium.

Hoffmann *et al.*, 2015 suggested that CD8⁺ T cells were temporarily directed away from circulation to the myocardium through either the sphingosine pathway or the fractalkine pathway. Sphingosine-1-phosphate is a lipid messenger that uses G-protein coupled receptor for signalling purposes (Matloubian *et al.*, 2004). Matloubian research group generated S1P1^{-/-} mouse chimeras by transferring fetal liver cells from S1P1 knockout mice. These mouse chimeras showed a near complete absence of peripheral T cells. Moreover, T cells were also absent from the spleen, lymph nodes as well as Peyer's patches. There were however, increased numbers of single positive CD4⁺ and single positive CD8⁺ thymocytes. The presence of these cells in the thymus but absence in secondary lymphoid organs and in circulation show the lack of egress of T cells from the thymus in the absence of S1P1. To investigate the role and importance of S1P1 in the circulation of T cells in the peripheral blood, thymocytes from the S1P1^{-/-} and control thymocytes were transferred into wild-type recipient mice. It was found, unlike control thymocytes which were present in peripheral blood, lymph and secondary lymphoid organs, the S1P1^{-/-} thymocytes were absent from the blood and lymph but were present in the secondary lymphoid organs suggesting S1P1 is vital for the egress of T cells from peripheral blood and lymph to the secondary lymphoid organs. In addition, only highly matured single positive thymocytes (CD4⁺ and CD8⁺) are capable of strong chemotactic responses to S1P1. Thus, S1P1R plays an important role in lymphocyte migration. The role of S1P in Myocardial infarction and ischemic injury was investigated by Means *et al.*, (2007). S1P₃ and S1P₂ deficient mice had a larger infarct size than wild-type mice in artificially induced

I/R injury which suggests that S1P has a protective function in ischemic reperfusion injury. Knapp *et al.*, in 2009 found that the levels of S1P in plasma with myocardial infarction was lower immediately after reperfusion. They suggested that the loss of S1P from plasma causes a reduction in the protective effect of S1P in myocardial infarction patients. So, it is possible that at 90 minutes after reperfusion, coupled with permanent loss of S1P1r⁺ CD8⁺ T cells, there is also migration of anergic S1P1r⁺ CD8⁺ T cells to the myocardium. The second pathway mentioned by Hoffmann *et al.*, (2015) was the pathway that involves fractalkine (CX3CL1) (Please refer to ‘Flow cytometric Analysis of tetramer and non-tetramer CD8⁺ T cells section’).

Depletion of PD-1⁺ terminally differentiated CD8⁺ effector memory T cells (T_{EMRA})

The drop in percentage of CD8⁺ T cells at 90 minutes after PPCI/Reperfusion was intriguing. We wanted to find out which subset of CD8⁺ T cells was responsible for the drop. Prior to reperfusion, the percentages of activated terminally differentiated effector memory CD8⁺ T cells (T_{EMRA}) was significantly higher than the percentage of naïve CD8⁺ T cells (**Figure 3.5.1A**). Thus, it made sense to focus on T_{EMRA} cells (after PPCI/Reperfusion) which expressed high levels of PD-1. Coinciding with the drop in CD8⁺ T cells at 90 minutes, we found there was a permanent drop in PD-1 expressing T_{EMRA} cells at 90 minutes post reperfusion compared to baseline levels (**Figure 3.5.1B**).

Zajac *et al.*, in 1998 reported that persistent antigenic presence decreases the responsiveness of anti-viral CD8⁺ T cells of mice chronically infected with lymphocytic choriomeningitis virus (LCMV) and suggested that the unresponsiveness of CD8⁺ T cells could be further exacerbated in the absence of CD4⁺ T cells and professional antigen presenting cells. Barber *et al.*, in 2006 using genome wide microarray analysis, found the presence of PD-1 mRNA in exhausted CD8⁺ T cells of mice infected with LCMV whereas functional CD8⁺ T cells expressed very low levels of PD-1. Kinetic studies on PD-1 expression by the Barber research group also showed that

PD-1 expression during acute infection is rapidly downregulated whereas in a chronic infectious setting, the expression of PD-1 is increased and sustained for a long period of time.

In humans, Cytomegalovirus (CMV) causes low grade persistent infection and elicits very strong memory T cell responses in CMV-seropositive healthy individuals. However, as time progresses, CMV slowly dominates the entire T cell repertoire and thus impairs T cell functioning against other pathogenic antigens. Most CMV-specific CD8⁺ T cells have effector memory T cell phenotype (T_{EM}) (Klenerman & Hill, 2005). Interestingly, we found that the percentage of PD-1 expressing T_{EMRA} cells in CMV-seropositive patients is significantly lower than the percentage in CMV-seronegative patients at 24 hours after PPCI/Reperfusion (figure 3.5.3). This might indicate that the presence of CMV causes a persistent activation of T_{EMRA} cells leading to their eventual depletion. In addition, in CMV-seropositive patients, the percentages of PD-1⁺ T_{EMRA} cells were significantly lower than effector memory T cells (T_{EM}) suggesting that these cells are the most affected after reperfusion.

The high expression of PD-1 on T cells makes them more sensitive to apoptosis and thus we wanted to determine early and late apoptosis in PD-1⁺ T cells before reperfusion. The presence of annexin V only indicated early or spontaneous apoptosis whereas the presence of both annexin V and 7-AAD indicated late apoptosis. As expected CD8⁺ T cells expressing high levels of PD-1 and medium levels of PD-1 were more numerous than PD-1 negative cells. PD-1 negative T cells showed only early or spontaneous apoptosis whereas PD-1^{dim} and PD-1^{high} cells showed both early and late apoptosis. The percentages of early apoptosis were higher in PD-1 negative and PD-1^{dim} cells whereas the percentage of late apoptosis was much higher in PD-1^{high} cells. This suggests that T cells expressing medium and high levels of PD-1 are more prone to apoptosis and cell death. This was corroborated by the fact that PD-1^{high} cells were present in least numbers compared to PD-1^{negative} and PD-1^{dim} cells among the total percentage

of live cells. Thus, the loss of CD8⁺ T cell due to high expression of PD-1 diminishes CD8⁺ T cell mediated immunity.

Barber *et al.*, in 2006 also used PD-L1 blocking antibody in LCMV specific CD8⁺ T cells in mice. The virus-specific T cells showed improvement qualitatively (functionality) as well as quantitatively. Petrovas *et al.*, in 2006 also reported the increase in proliferative capacity of HIV- and CMV-specific CD8⁺ T cells obtained from PBMCs of HIV positive and HIV negative volunteers. So, we decided to find out if the loss of cells could be salvaged. To achieve this, we wanted to see the difference in the percentage of late apoptosis in T cells stimulated with CD3, a polyclonal activator of T cells, and the addition of PD-1 blocking antibody. We found the percentage of late apoptosis was significantly lower in CMV-specific T cells with PD-1 blocking antibody than with T cells with no blocking antibody. Our results indicate a strong connection between increased expression of PD-1 and the exhaustion/ loss of CMV-specific T cells and T_{EMRA} cells.

Cytokine production by CD8⁺ and CD4⁺ T cells in CMV and EBV seropositive AMI patients

In the next study group (group B), by carrying out *ex-vivo* ELISpot experiments, we found CMV-specific CD4⁺ T cells show a drop in production of IFN- γ at 90 minutes after PPCI/reperfusion and recover by 24 hours. However, there were no changes in the CD8⁺ T cell compartment, i.e. the production of IFN- γ remained stable throughout all time points. Cultured ELISpot responses, in contrast, did not show any significant changes across any time point for the CD4⁺ T cell compartment. The cultured CD8⁺ T cell responses showed a significant increase at 24 hours compared to pre-reperfusion levels. The sustained production of IFN- γ by effector CD8⁺ T cells (*ex-vivo* ELISpot), memory CD8⁺ T cells (Cultured ELISpot) and memory CD4⁺ T cell response, might suggest a lack of self-termination mechanisms and might lead to exacerbation of inflammation in CMV-seropositive MI patients. This result also indicates that

the memory T cells seem to be the primary cell type behind the sustained release of IFN- γ which could eventually lead to increased apoptosis of these cells.

The focus then shifted to *ex-vivo* ELISpot and cultured ELISpot EBV-specific CD4⁺ T cell and CD8⁺ T cell responses. *Ex-vivo* ELISpot EBV-specific CD4⁺ T cell response showed a drop at 90 minutes after PPCI which recovered at 24 hours. EBV-specific CD4⁺ T cell Cultured ELISpot response, however showed no changes before PPCI and 90 minutes after PPCI and production of IFN- γ remained stable. There was however, significant increase in production of IFN- γ at 24 hours after PPCI. *Ex-vivo* ELISpot EBV-specific CD8⁺ T cell response showed no changes before PPCI and 90 minutes after PPCI and production of IFN- γ remained stable. There was however, significant increase in production of IFN- γ at 24 hours after PPCI. EBV-specific CD8⁺ T cell cultured ELISpot response, showed a dip at 90 minutes (non-significant) before recovering at 24 hours. It was noted that T cell responses to CMV in *ex-vivo* ELISpot were much higher compared to T cell responses to EBV.

Non-specific activation of patients' PBMCs with PHA also gave interesting results. Cytokines such as IL-5 and IL-17 which represent Th2 and Th17 cells, respectively, showed no difference across all time points in CMV-seropositive and CMV-seronegative patients. However, there was decline in production of IFN- γ and IL-2 at 90 minutes after reperfusion. This indicates that Th1 but not Th2 and Th17 cells are predominantly in AMI patients and Th1 cells could home to the myocardium. The current understanding is that the immune system limits the magnitude of the effector functions of T cells to make sure excessive inflammatory reactions and autoimmune reactions are minimized. This exhaustion or anergy of T cells occurs due to the chronic exposure of T cells to viral antigens (in this study CMV and EBV) (Vigano *et al.*, 2012). Thus, the apparent loss in effector T cell responses is most probably due to functional anergy and their subsequent trafficking from circulation and into inflamed tissues, including

the heart tissue. Research by Pasqui *et al.*, in 2005 found increased levels of IFN- γ in AMI and patients with unstable angina. They isolated lymphocytes from peripheral blood and stimulated them with PHA and LPS to test the release of pro-inflammatory cytokines. Liuzzo research group in 2007 reported on the expansion of a CD4⁺CD28^{null} T cell subset in unstable angina patients. These cells were also present in the plaque regions and associated with increased levels of C-reactive protein and myocardial infarction. This subset is considered to have Th1 functionality and is strongly associated with the exacerbation of chronic inflammatory diseases. These cells are resistant to apoptosis as well as resistant to the influence of Tregs and secrete high amounts of IFN- γ and TNF- α (Dumitriu, 2015). In the previous section, we described the permanent loss of the PD-1 expressing CD8⁺ T_{EMRA}. We believe the constant activation of CMV-specific T cells will eventually lead to PD-1 mediated apoptosis as well as functional anergy and exhaustion. Thus, it is possible that in AMI patients the combination of T_{EMRA} cells and CD4⁺CD28^{null} T cells account for the production of high amounts of IFN- γ production. However, while terminally differentiated effector memory CD8⁺ are prone to PD-1 mediated apoptosis, the CD4⁺CD28^{null} T cells continue their potent inflammatory action due to them being resistant to apoptosis. This could explain the sustained and stable IFN- γ secretion as shown in **figure 3.7.2** and **figure 3.7.4**.

Among the 11 cytokines tested by meso scale discovery, we found levels of IL-16 were decreased in CMV-seropositive patients and increased in CMV-seronegative patients. In addition, we also found that the drop in IL-16 levels at 90 minutes after PCCI correlated with the CD4⁺ T cell count at the same time point (**Figure 3.7.6 C**). IL-16 is a chemoattractant for CD4⁺ T cells and is involved in the migration of Th1 cells in the presence of CCR5. Through the action of IL-16 on its receptor, CD4 elicits a greater migratory response in Th1 subset compared to Th2 subset. Also, the presence of CCR5 strengthens the binding capacity of IL-16 to CD4 receptor. The presence of CCR5 could lead to the recruitment of T cells into sites

of inflammation (Lynch *et al.*, 2003). This could explain the temporary drop in T cell numbers at 90 minutes after PPCI.

Analysis of the relationship between Microvascular obstruction with *Ex-Vivo* and Cultured ELISpot

To our best knowledge, we are the first group to identify correlations between microvascular obstruction and ELISpot responses. Microvascular obstruction (MVO), or no-reflow phenomenon, occurs in patients after PPCI. MVO is characterised by the structural and functional changes to the myocardium such as endothelial destruction, emboli of neutrophils and platelets, and release of free radicals. The extent of MVO is a good indicator of reperfusion injury. Only CMV-specific cultured ELISpot CD8⁺ T cell response at 24 hour time point and cultured EBV-specific CD4⁺ T cells at 24 hour time point showed a positive association with MVO. No *ex-vivo* ELISpot response correlated with MVO. Thus, memory T cell responses rather than effector T cell responses seem to influence the extent of MVO in STEMI patients. IFN- γ production by cultured CMV-specific CD8⁺ T cells and by cultured EBV-specific CD4⁺ T cells at 24 hours after PPCI was the highest compared to all other time points. The extent of MVO was also highest at this time point. Our hypothesis suggests that increased production of Th1 cytokine, IFN- γ , by memory T cells causes increased inflammation which might lead to increased microvascular obstruction. While MVO is a good indicator of post-MI reperfusion injury, peak troponin levels is a good indicator of infarct size and pre-MI diseased condition. Cultured CMV-specific CD4⁺ T cell response before PPCI (0 minute time point) negatively correlated with peak troponin levels as did Cultured CMV-specific CD8⁺ T cell response at 24 hours after PPCI. There were no other correlations between troponin levels and ELISpot responses.

We also found no meaningful differences between *ex-vivo* CMV-specific CD4⁺ and CD8⁺ T cell responses and MVO levels (high, low and none) i.e. there was no difference in MVO levels

before and after reperfusion. In contrast, *ex-vivo* EBV-specific CD8⁺ T cell responses were higher in the ‘none’ MVO group compared to ‘high’ and ‘low’ MVO group at all-time points. *Ex-vivo* EBV-specific CD4⁺ T cell responses were also higher in the ‘none’ MVO group compared to ‘high’ and ‘low’ MVO groups. Among cultured ELIspot responses, the release of IFN- γ from CMV-specific CD4⁺ T cells was higher in ‘none’ MVO group. There were no differences among the MVO groups for CMV-specific CD8⁺ T cell responses. There were no meaningful correlations between MVO groups and cultured EBV ELIspot responses. Release of IFN- γ by non-specific activation of T cells with PHA also did not show any significant results. IL-5 and IL-17 production at 24 hours after PPCI were significantly higher in the ‘none’ MVO group compared to the ‘low’ group. The production of IL-2 before PPCI and at 90 minutes after PPCI was higher in the ‘none’ MVO group. It is plausible that unequal numbers of patient samples 12 for ‘high’ MVO, 10 for ‘low’ MVO and 15 for ‘none’ MVO may be the reason for lack of differences between T cell response and cytokine production with the extent of MVO in patients.

Flow cytometric Analysis of tetramer⁺ and non-tetramer⁺ CD8⁺ T cells

Within CD8⁺ tetramer⁺ cells, only CXCR3⁺ cells showed a significant drop at 90 minutes after PPCI. Levels of other receptors such as CX3CR1, S1PR1, C-met, CCR5 and CCR4 remained unchanged. In contrast, within non-tetramer CD8⁺ T cells, levels of CX3CR1, CCR5, CCR4 and CXCR3 showed a significant drop at 90 minutes before recovering at 24 hours after PPCI. The difference between the tetramer⁺ and non-tetramer⁺ cells might be due to limited number of MHC complexes tested in tetrameric T cells and also due to the low cell count in tetramer samples. CXCR3, is a chemokine receptor that plays an important role in antiviral immunity. CD8⁺ T cells upregulate this receptor in responses to viral infections and CXCR3 signalling is involved in T cell recruitment into virus-infected and inflamed tissues. In a vaccinia virus model, it was found that CD8 T cells in *Cxcr3*^{-/-} mice were characterized by reduced capacity

to locate and destroy virus infected cells. The adoptive transfer of wildtype CD8⁺ T cells restored viral clearance in *Cxcr3*^{-/-} mice (Hickman *et al.*, 2015).

The presence of other cell surface receptors within CX3CR1⁺ non-tetrameric CD8⁺ T cells was also carried out. Only CX3CR1 and S1PR1 showed a significant drop at 90 minutes after PPCI. This might indicate the combined action of S1PR1 and CX3CR1 in the migration of CD8⁺ T cells away from circulation. The role of CX3CR1 or fractalkine in the depletion or trafficking of T cells was investigated by Boag *et al.*, (2015). They found a strong correlation between the level of expression of fractalkine on T cells and the drop in T cell count. The expression of CX3CR1 on effector CD8⁺ T cells lacking in CCR7 was the highest. Gene transcription studies also showed the varied changes in fractalkine levels before PPCI, at 24 hours and at 3 months after reperfusion. Moreover, the increase in serum fractalkine levels coincided with the drop in CD8⁺ T cells at 90 minutes after PPCI. Boag's research suggested that the drop in CX3CR1 expressing T cells could be due the downregulation of fractalkine at 90 minutes, or the trafficking of CD8⁺ T cells from the blood stream into the heart tissue or the binding of serum fractalkine with T cells. Ikejima *et al.*, (2010) showed that patients with coronary artery disease (stable and unstable angina) had significantly higher levels of serum fractalkine levels as well as CD3⁺ CX3CR1⁺ T lymphocytes compared to healthy controls. They concluded that the elevated levels of serum fractalkine combined with CD3⁺ CX3CR1⁺ T lymphocytes and CD3⁻ CX3CR1⁺ monocytes contribute to plaque rupture and the eventual MI. Damas *et al.*, in 2005 showed significantly elevated levels of serum CX3CR1 and CX3CR1⁺ CD8⁺ T cells in patients with myocardial infarction than in healthy controls. The percentage of CX3CR1⁺ CD4⁺ T cells, however, showed no changes in patient group compared to healthy controls. The elevated levels of fractalkine and CX3CR1⁺ CD8⁺ T cells dropped 6 months after treatment with atorvastatin. Thus, they suggested that the use of atorvastatin may help in the reduction of fractalkine-mediated T cell recruitment and reduce inflammation. The administration of atorvastatin led to

the decrease in lipid levels in plasma. Atorvastatin also reduced the levels of CX3CR1 and CX3CL1, reducing fractalkine related inflammatory response in coronary artery disease patients. In AMI patients who died, immunohistochemistry analysis was performed. It was found that T cells in the lesion expressed high levels of CX3CR1 indicating infiltration of these cells via a fractalkine-mediated mechanism. In addition, it was also shown how CX3CR1 genotypes affect Th1 cell recruitment. The I249 allele of CX3CR1 correlated with decreased recruitment of Th1 cells and lower IFN- γ production. Thus, the authors suggested the use of fractalkine genotype as a marker to stratify population suffering from AMI (Pucci *et al.*, 2013).

CONCLUSION

In overall conclusion, CMV⁺ MI patients have higher Th1-mediated chronic inflammatory responses (IFN γ) than CMV⁻ patients. CD8⁺ T cells with T_{EMRA} PD-1⁺ phenotype, in which CMV-specific T cells reside, are reduced during myocardial reperfusion, likely via fractalkine-mediated homing to the myocardium. This reveals mechanistic insight and suggests a treatment option in MI+R injury of targeting these cells and associated molecules, possibly with monoclonal antibodies that block or neutralize IFN γ and fractalkine.

CHAPTER 4

Immune Responses in Chronic Fatigue Syndrome (CFS)

Abstract

Background

CFS is a disease that affects the central nervous system and the immune system and is characterised by other comorbidities such as gastrointestinal infection and cognitive impairment. Although CFS can affect people of all ages and genders, women are most affected. Despite being an idiopathic disease, dysfunction of the immune system is widely reported in CFS patients, with changes in the T cell, B cell and NK cell compartments in particular. Herpes viruses such as EBV are considered to be one of the viral triggers that leads to the onset of CFS. Apart from EBV, immune responses against various microbes have been proposed as a causative link. This study aims to utilize various immunological phenotypic assays to compare the immune responses of CFS patients to healthy controls, and potentially identify changes that could serve as a biomarker of pathogenesis and offer therapeutic insights.

Aims

The aim of this study was to measure and compare immune responses, particularly those direct against microbes, in patients suffering from CFS, and in healthy controls. The identification of immune parameters and any immune abnormalities in CFS patients will help in better understanding of the CFS disease aetiology and suggest diagnostics and treatments.

Methods

CFS patients were recruited by our clinical collaborators at the Royal Victoria Infirmary (RVI) as well as the CRESTA clinic at the old General Hospital site. The Fatigue Impact Scale (FIS) was used by our clinical collaborators to measure the fatigue levels in patients. ELISpot was used to test cytokine production by stimulating PBMCs with antigenic stimuli including CMV and EBV. ELISA was performed to test for antigen-specific antibodies in serum samples of patients and healthy controls. Multiplex cytokine analysis was performed to test for the release of cytokines in cell culture supernatants of patient samples. Flow cytometry was used to analyse T cell, B cell and NK cell populations and their subsets including mucosal associated invariant T cells (MAIT), natural killer T cells (NKT) and gamma delta T cells (TCR $\gamma\delta$).

Results

There was a negative correlation between percentages of NK cells and FIS score ($p=0.04$), and severely affected patients had lower NK cell numbers than mild or moderately affected CFS patients. Moreover, the percentage of NK cells, and CD57⁺ NK cells, were significantly lower in CFS patients than in healthy controls. Furthermore, the percentages of CD3⁺ MAIT and CD4⁺ T cells were significantly higher in CFS patients than in healthy controls whereas the percentages of CD8⁺ T cells were significantly lower in CFS patients compared to healthy controls. No correlations were found in antibody and cytokine analyses with FIS scores. Surprisingly only PMAi, a positive control used in ELISpot, positively correlated with FIS score.

Conclusion

The results suggest abnormalities in the immune system in individuals with chronic fatigue, including raised non-specific IFN γ secretion. The NK cells seem to be the most affected, as reported widely in research reports on CFS.

Introduction

CFS is defined as the onset of debilitating fatigue for a period of 6 months or more, characterized by at least short-term memory loss, sore throat, tender cervical and auxiliary lymph nodes, headaches, joint pain and lack of proper sleep. Conditions such as bipolar disorder, eating disorders, substance abuses, depression and psychosis are typically excluded from patient diagnosis of CFS (Brenu *et al.*, 2010). CFS is considered a nervous system disorder caused by changes in the immune system due to microbial infections, and heterogeneity among patient groups suggests CFS is a multifactorial condition. Unfortunately, CFS still lacks defined biomarkers or clinical signs that would confirm the condition. It is believed that through the study of the immune system in CFS patients, suitable biomarkers may be discovered (Lorusso *et al.*, 2009). CFS is categorized by the FIS which had recommendations made by Fukuda *et al.*, in 1994. Some of the important recommendations that were made were diagnosis of people with prolonged fatigue (longer than 1 month) accompanied by clinical and laboratory evaluation to include or exclude other ailments that a fatigued patient might have. In addition, a thorough psychiatric and physical examination was also included as a recommendation to ensure all physical and mental information about patients are known.

Numerous research studies have shown discrepancies in immune responses in CFS patients. Fluge *et al.*, (2011) showed that the depletion of B cells using rituximab (anti-CD20) in patients with CFS decreased the chronic fatigue in 3 patients who were all able to return to normal life.

Thus, they suggested that CFS might be an autoimmune-related disease. Curriu *et al.*, (2013) in contrast found no changes in the B cell compartment of CFS and healthy control group. Although the expression of CD69 and NKp46 was higher in NK cells of CFS patients, no significant correlations were found in the NK cell compartment. However, the level of regulatory T cells in CFS patients was increased. Furthermore, increased expression levels of CD5 and decreased levels of CD38 were also reported. Brenu *et al.*, in 2010 showed a decreased ability of NK cells of CFS patients to lyse K562 target cells in addition to reporting dysfunction of neutrophil function. Brenu *et al.*, (2013) showed significant changes in the immune cells of CFS patients. Elevated levels of regulatory T cells were shown in agreement with Curriu *et al.*, (2013). In addition, decrease in Plasmacytoid DCs, immature B cells, but increase in memory B cells was also reported. They also reported that reduced NK cell cytotoxic function, as the ability of NK cells of CFS patients to lyse K562 cells, was significantly lower. Maes *et al.*, (2012) showed that plasma of CFS patients showed elevated levels of interleukin-1, TNF- α , neopterin, lysozyme and PMN-elastase. This might suggest the existence of low grade inflammation in CFS patients. It is to be noted that the Maes group used Fibromyalgia and Chronic fatigue syndrome rating scale (FF) which is different from the FIS. Navaneetharaja *et al.*, in 2016, in their review, suggested that CFS might be an autoimmune disease. Germ-free animal models have been shown to have minimal or no sign of autoimmune disease suggesting that intestinal microbiota might be a contributing factor or trigger in CFS. The co-existence of CFS along with gastrointestinal conditions such as irritable bowel syndrome also point to the role of intestinal microbiota in the exacerbation of CFS. Apart from intestinal microbiota, Carding group also suggested that viruses, including bacteriophages, might also play a role in the onset of CFS in people. The gut microbiota could possibly also influence the central nervous system (CNS), thus creating an immune system-nervous system axis in CFS. Cameron *et al.*, (2010) studied the role of human herpes virus 6, cytomegalovirus

(CMV) and Epstein-Barr virus (EBV). Although, they did not find any significant results, it is still an interesting prospect to study the role of these viruses in CFS patients. The research of this study into CFS, hopes to find consistencies with the data obtained from other prominent research groups as well as identify novel immunological changes that will help us better understand CFS.

In our experiments, we tested CFS patient PBMCs and serum for immune reactivity of lymphocytes and antibodies, respectively, against a wide variety of pathogens to identify links between the Fatigue Impact Scale and immune measurements. The comparison of patient data with healthy control data may give new insights into the immune dysfunction in CFS patients. The identification of suitable diagnostic markers or biomarkers is the prime aim of researchers in chronic fatigue. To achieve this, it was vital to establish assays and set up parameters for experiments to ensure optimum and measurable immune responses from patient and control groups, with a main focus on T cell responses to bacterial and viral pathogens. T cell epitope peptides of selected pathogens were obtained to produce antigen panels. Appropriate concentrations of pathogenic antigenic peptides, and positive controls, were prepared to ensure optimal and measurable T cell responses. The ELISpot plate reader was set up in such a way so as to capture all 'spots' that represents all cytokine secreting cells and remove false positives. Experiments with blackout were repeated after cell concentration was diluted. The K562 cell line was used to determine natural killer cell response. Using different volumes and cell concentrations of PBMCs allows for the determination of the maximum NK cell response. The coating concentrations of in-house ELISA was determined as the coating concentrations of pathogens is vital to obtain optimum antibody response. In order to remove false positive results, the pathogenic antigens were also endotoxin tested. Finally, gating strategies and spectral overlap (Flow Cytometry) for cell phenotypes was determined.

The aims of this study were as follows:

- 1) To study antimicrobial T cell responses in chronic fatigue syndrome patients and compare them with immune responses of healthy controls, including testing cell culture supernatants for the release of cytokines using cytokine multiplex assays. Furthermore, FIS scores and immune parameters were investigated for association.
- 2) To test the production of antibodies for CFS patients and healthy controls against CMV and EBV by propriety ELISA, as marker of exposure.
- 3) To perform flow cytometric analysis to measure the levels of T cells, B cells and NK cells in patients and healthy controls, to identify immune dysregulation.

4.1 Patient demographics for the CFS study

A total of 50 CFS patients were recruited by consultants Prof. Julia Newton, Prof. Fai Ng and Dr Gavin Spickett, and clinician Victoria Hindmarsh at the CRESTA (Clinics for Research and Service in Themed Assessments) and at the Royal Victoria Infirmary (RVI). Under the NHS guidelines, apart from Age and Sex, all other information about patients have been classified, considering the sensitive nature of chronic fatigue in patients. FIS scores were generated using the Fukuda criteria. FIS questionnaire, used to determine the FIS score by doctors, is shown in appendix 2. Female CFS patients far exceeded male CFS patients. Only 5 patients were male and the remaining 45 were females. Patients belong to a wide age group ranging from 17 to 70. Based on the FIS score, patients were further divided into two sub groups: patients with FIS scores below 100 have been classified mild or moderately affected, and patients with FIS scores above 100 have been classified severely affected. For statistical analysis, the mild/moderately affected patients are designated as group 0 and severely affected patients as group 1. The age and sex of patients along with their FIS scores is shown in **table 4.1**.

CFS patient identification Number	Age	Sex	FIS score (maximum score 160)
61	39	F	50
63	17	F	75
64	47	F	122
66	57	F	88
73	53	F	140
80	45	F	60
82	49	F	117
83	26	F	110
84	21	F	111
86	44	F	73
89	35	F	111
90	42	F	85
92	27	F	74
93	30	F	140
94	21	F	106
95	45	F	80
96	67	M	52
101	20	M	121
104	24	F	90
106	48	F	125
108	20	F	130
110	61	M	101
111	61	F	149
112	62	M	146
113	70	F	108
116	33	F	93
118	46	F	113
119	43	F	102
123	52	F	60
124	65	F	139
130	60	F	71
131	26	F	57
132	34	F	98
133	56	F	143
134	61	F	153
135	47	F	96
136	38	M	102
138	38	F	70
139	43	F	132
143	21	F	91
144	40	F	120
145	36	F	110
149	42	F	101
152	21	F	94
153	65	F	113
154	29	F	92
155	42	F	126
156	28	F	99
157	50	F	128
158	25	F	123

Table 4.1 Age, Sex and FIS score of patients with Chronic Fatigue Syndrome. The details on the age and sex of CFS patients were obtained from our clinical collaborators at Newcastle upon Tyne Hospital Trust and at Newcastle University. CFS patients were given a unique identification code which were allocated by our clinical collaborators. The FIS score was calculated by the clinicians.

We first decided to establish the relationship between FIS score and age to see if age played a role in the onset of chronic fatigue in patients. There was however no correlation between FIS scores of patients and their age. (**Figure 4.1.1**)

Association between FIS score and age of CFS patients

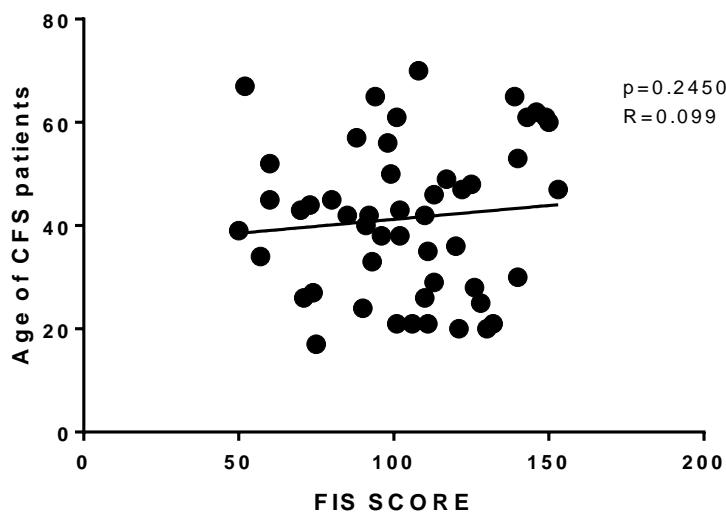


Figure 4.1.1 Relationship between Age of CFS patients and FIS score. Pearson correlation analysis revealed no significant association between age of CFS patients and the fatigue impact scale score. $R=0.099$, $p=0.2450$. $n=50$.

4.2 Bacterial Growth Curve & Lipopolysaccharide assay

Maes research group in 2007, found IgA ($p=0.01$) levels against LPS of *Pseudomonas aeruginosa* in chronic fatigue syndrome patients was significantly higher than in patients with partial CFS and healthy controls, whereas the IgM response was outside significance. The diagnosis of the CFS patients were made using the Fibro Fatigue Scale. Thus, we decided to use *Pseudomonas aeruginosa* for our ELIspot and ELISA experiments. We proceeded to

produce antigen from the bacterium. In order to achieve this, the bacterial cells were grown in the appropriate growth medium and harvested at the middle-log growth phase where the bacterial cell growth is without any cell death.

The LPS assay was used to identify the presence of endotoxins in antigenic solutions. The protocol for the LPS assay as well as the generation of standard curve is described in chapter 2 (Methods). It was important to test some of the antigens for endotoxin presence before further use. The results are shown in **Table 4.2**.

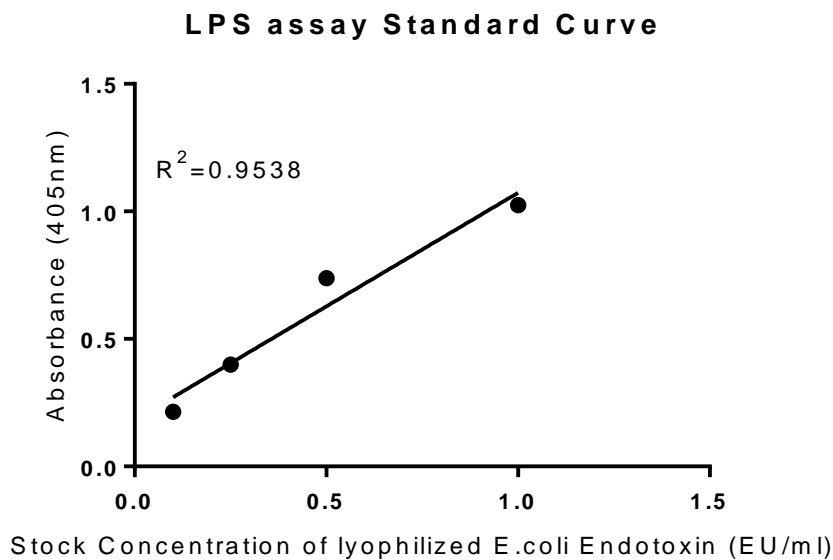


Figure 4.2.1 Standard Curve for the LPS assay. The endotoxin was added to the endotoxin free water to make a stock solution of 1EU/ml, a series of four dilutions were made from the stock solution. The Y axis indicates absorbance. The X axis indicates stock concentration of endotoxin. $R^2=0.9538$. The endotoxin levels in antigen samples are shown in table 4.2.

Table 4.2 Endotoxin Levels in antigen samples

Identification Code	Pathogen tested	LPS Concentration (ng/ml)	Protein Concentration (mg/ml)
PSA	<i>Pseudomonas aeruginosa</i> (NCTC)	0	16.2
STY	<i>Salmonella typhimurium</i>	0.2	29.44
MYCO	<i>Mycoplasma</i>	0	1.0

4.2.3 Bradford Assay

The protein concentration of each bacterial antigen was measured by Bradford assay as described in section 2.7 of chapter 2 (Methods). **Figure 4.2.2** shows the standard curve used to obtain the protein concentrations. The amount of protein (in $\mu\text{g/ml}$) in unknown samples was calculated using the equation shown in the standard curve. The concentration obtained from the curve was multiplied by the dilution factors of the samples. The results are shown in **Table 4.2**.

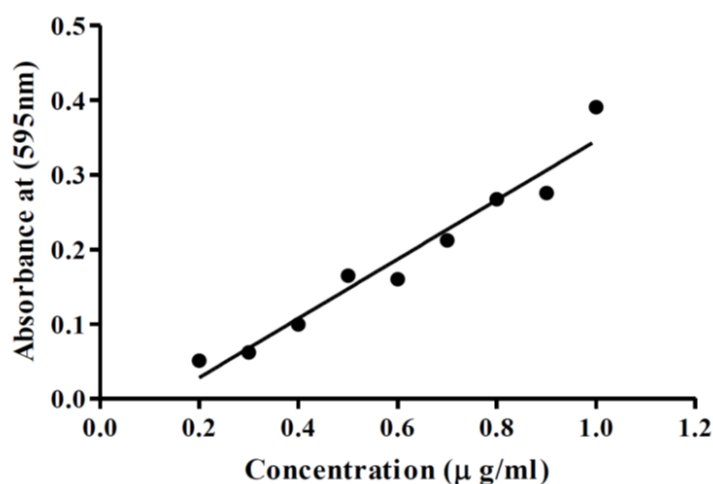


Figure 4.2.2 Standard curve for the Bio-Rad Bradford protein assay. The curve is produced by the addition of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/ml BSA in 500 μl of PBS to 250 μl of Bradford dye reagents.

4.2.4 Determination of protein content of bacterial lysates using SDS-PAGE

SDS-PAGE was performed to separate proteins from lysates from different species of bacteria: *Moraxella catarrhalis*, *Bordetella pertussis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Pseudomonas intermedia*, all of which were considered candidate microbes to be studied.

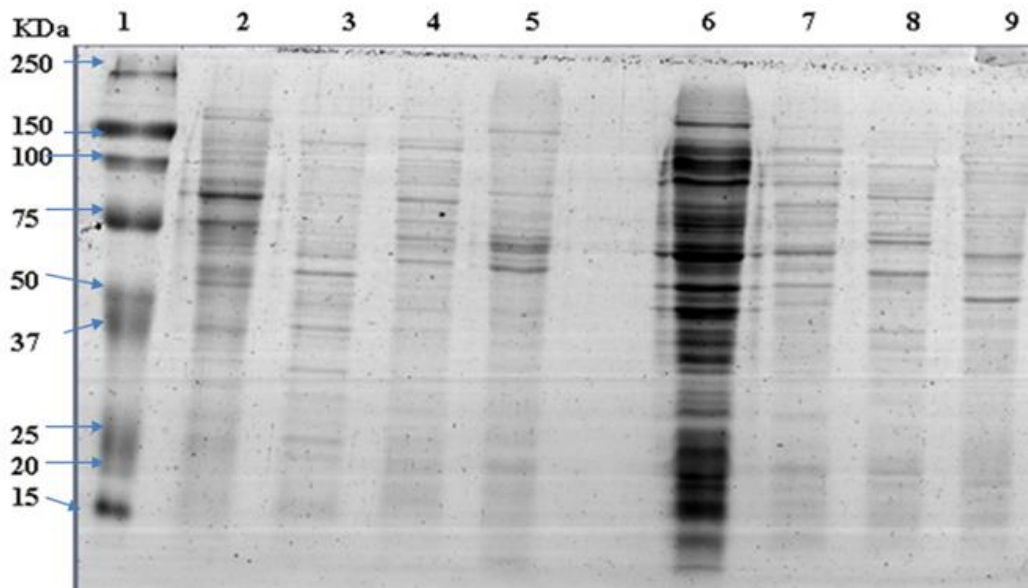


Figure 4.2.3 Visualization of protein bands of bacterial lysates. 12% SDS-PAGE was performed to separate proteins from the lysates of various bacteria. Lane 1-molecular weight ladder, Lane 2- *Moraxella catarrhalis*, Lane 3- *Pseudomonas intermedia*, Lane 4- *Pseudomonas aeruginosa*, Lane 5- *Pseudomonas aeruginosa*, Lane6- *Pseudomonas aeruginosa* (NCTC), Lane 7- *Salmonella typhimurium* control, Lane 8- *Staphylococcus aureus*, Lane 9- *Streptococcus pneumoniae*. 20 μ l of samples were loaded into the wells. The gels were stained with Coomassie blue solution and then de-stained with de-staining buffer.

We found that all samples showed clear bands indicating that they are made up of proteins and were thus suitable to be included in our research experiments. **Table 4.3** shows the antigens that were finally selected for use in the study.

4.2.5 Construction of peptide panels for microbes (CFS)

Numerous peptides belonging to a wide variety of chosen microbes were chosen and used in our experimental study. These peptide panels were obtained through an epitope database (refer appendix 8). We decided to choose pathogens that were persistent and pathogens that were implicated or tested in CFS in other studies. Furthermore, we looked for T cell epitopes that could be synthesised. We tested the peptides of the following microorganisms:

1. Cytomegalovirus (Cameron *et al.*, 2010; Beqaj *et al.*, 2008))
2. Epstein-Barr Virus (Loebel *et al.*, 2014 ; Fremont *et al.*, 2009))
3. Coxsackie virus (Schutzer *et al.*, 2011)
4. Human herpes virus 6,7 (Fremont *et al.*, 2009 ; Chapenko *et al.*, 2012))
5. Respiratory Syncytial virus + Rhinovirus
6. Parvovirus (Fremont *et al.*, 2009)
7. Adenovirus (Buchwald *et al.*, 1996)
8. *Pseudomonas aeruginosa* (Maes *et al.*, 2007, 2012)
9. Purified protein derivative (PPD) of *Mycobacterium tuberculosis*
10. Chlamydia (Komaroff *et al.*, 1992 ; Chia & Chia, 1999)
11. *Salmonella typhimurium*
12. Mycoplasma (Nijs *et al.*, 2002)
13. Influenza (Prinsen *et al.*, 2012 ; Magnus *et al.*, 2015)

Apart from testing peptides from the above chosen microorganisms, we included positive controls such as anti-CD3, phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and Phorbol 12-myristate 13-acetate (PMA) with ionomycin.

4.3 Antibody Responses in CFS

4.3.1 Antibody responses to CMV and EBV by propriety ELISA

We used ELISA kits from Euroimmun to test IgG, IgM responses against CMV, and IgG and IgA responses against EBV. The immunoglobulin responses did not correlate with FIS scores. However, CMV-specific IgG response was just outside significance ($p=0.057$).

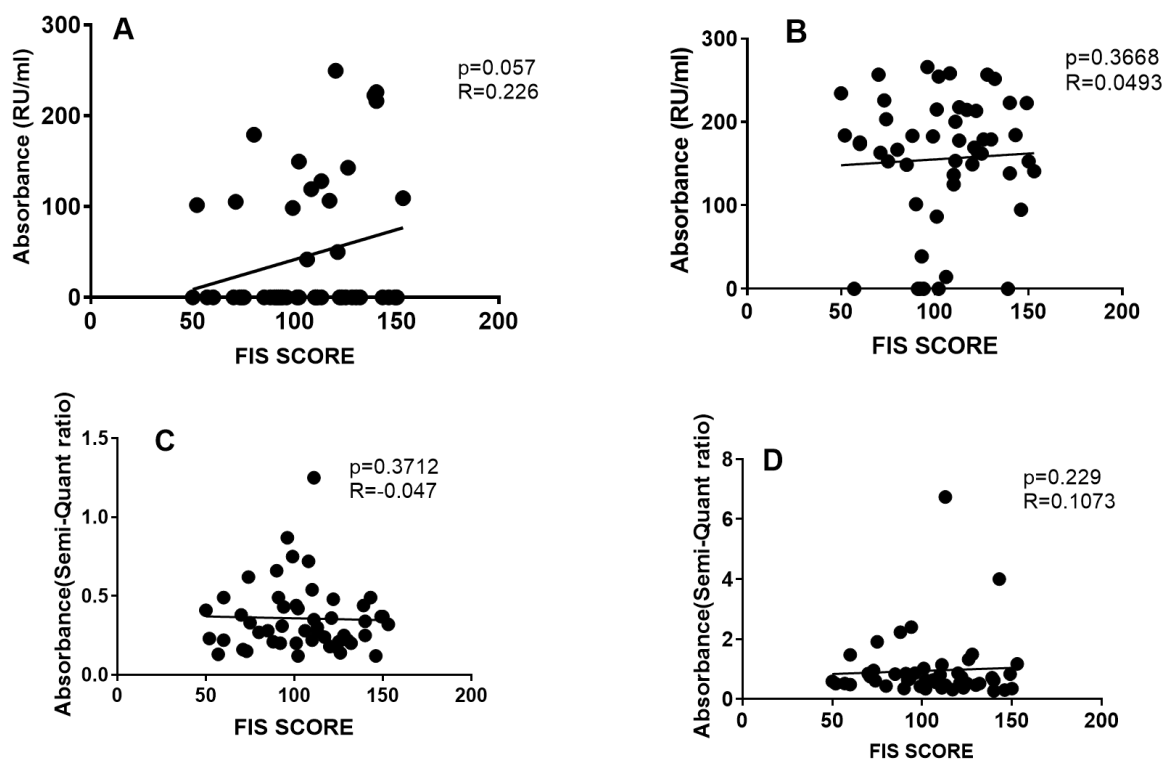


Figure 4.3.1 Immunoglobulin responses to CMV and EBV. ELISA kits obtained from Euroimmun were used to measure CMV and EBV specific immunoglobulin responses. There were however, no correlations between Ig responses and FIS scores. A. CMV-specific IgG response ($R=0.226$, $p=0.057$); B. EBV-specific IgG response ($R=0.049$, $p=0.366$); C. CMV-specific IgM response ($R=-0.047$, $p=0.371$); D. EBV-specific IgA response ($R=0.107$, $p=0.229$). Spearman or Pearson analysis was performed to obtain the above results. $n=50$ for all groups.

4.3.2 Comparison of Antibody responses to CMV and EBV between CFS patients and healthy controls

Next, we compared the IgG, IgM and IgA responses to CMV and EBV in CFS patients and healthy controls. Persistent pathogens such as CMV and EBV are the main focus of study and so we tested CMV and EBV specific antibody responses.

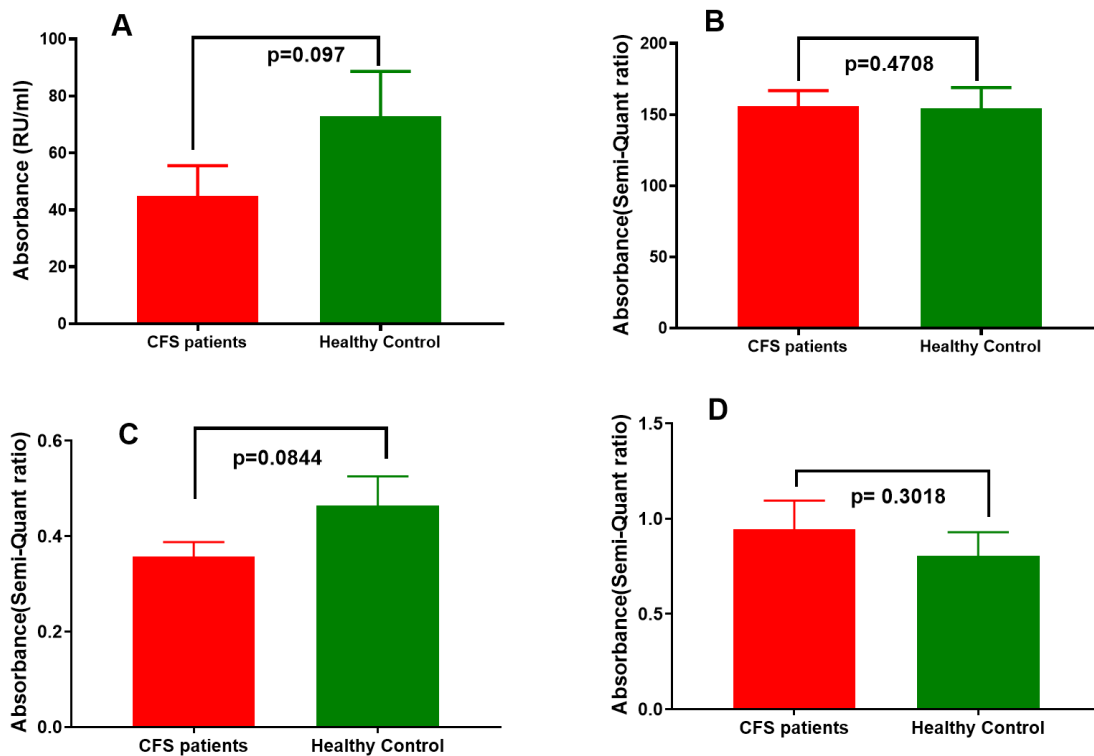


Figure 4.3.2 Comparison of CMV and EBV specific antibody responses in CFS patients and healthy controls. A. CMV-specific IgG response in CFS patients and healthy controls ($p=0.097$); B. EBV-specific IgG response in CFS patients and healthy controls ($p=0.4708$); C. CMV-specific IgM response ($p=0.0844$); D. EBV-specific IgA response ($p=0.3018$). $p<0.05$ was considered significant. The comparison of two unrelated groups was performed using the non-parametric Mann-Whitney test or the parametric unpaired t test. $n= 34$ for healthy controls and $n=50$ for CFS patients.

We found no difference between CMV and EBV specific immunoglobulin responses in CFS patients and healthy control. Only CMV-specific IgG and IgM response were approaching significance.

4.4 NK cell assay

Furthermore, keeping in mind the frequent research reports on the connection between NK cells and CFS, it was decided to perform the Natural Killer cell assay to establish optimal PBMC response to K562 stimulation. This was achieved by stimulating different volumes (and therefore cell numbers) of PBMCs with different volumes of K562 cells. For example, 5µl of PBMC was stimulated with 5µl, 10µl, 25µl and 50µl of K562 cells. The volume in a single ELISpot well was made up to 100µl using RPMI-1640 medium with 10% FCS (R10). A similar process was carried to stimulate a fixed volume of K562 cells with varying volumes of PBMCs. The results of the assay are shown in **Table 4.4.1**.

PBMC/K562 (µl)	5	10	25	50
50	22970	33630	38300	34630
25	3936	7608	6172	3724
12.5	1466	2626	2732	2482
5	495	558	789	772

Table 4.4.1 NK cell assay. ELISpot analysis involving PBMCs and K562 cell line at different volumes revealed different IFN-γ spot forming cells/M and thus an optimal combination of the two would help in establishing ideal NK cell responses in future experiments.

For our experiments, we chose 50µl of PBMC and 50µl of K562 cells as we felt this combination gave the optimum response without causing a blackout in the ELISpot experiment.

4.5 Anti-microbial T cell Responses in CFS patients

4.5.1 Composition of stimuli used for T cell assays including pathogen peptides and lysates

To perform ELISpot to test for anti-microbial recall responses and to obtain measurable spot forming cells/M PBMCs, generating suitable concentrations of pathogenic peptides is essential.

Table 4.3 shows the list of antigens used for ELISpot analysis.

Identification code used in experiment	Stimuli tested	Composition of antigenic stimulus
F1	R10 Medium	RPMI-1640 medium + 10% fetal calf serum+ 2.5ml each of penicillin and streptomycin + 2.5 ml of L-glutamine
F4	<i>P. aeruginosa</i> (NCTC)	Lysate + Peptides
F6	Mycobacterium tuberculosis (TB)	Purified protein derivative (PPD) + 6 peptides
CMV ALL	Cytomegalovirus (CMV)	CD4 ⁺ and CD8 ⁺ peptides + Lysate.
EBV ALL	Epstein-Barr Virus (EBV)	CD4 ⁺ and CD8 ⁺ peptides + Lysate
Mycoplasma	Mycoplasma pneumoniae	Lysate (obtained from Native Antigen, Oxford, UK)
RSV(Rh)	Respiratory syncytial virus + Rhinovirus	Lysates + 4 peptides for RSV and Lysates for Rhinovirus
Adeno	Adenovirus	Peptides
F9	Influenza	55 peptides
Salmonella	Salmonella typhimurium	1 in 500 of the original stock solution. (R10 is the medium used).
BCHP	Bkt Rotavirus, Coxsackie Virus, Human Herpes virus 6,7 and Parvovirus	Peptides and lysates
PWM	Pokeweed mitogen (B cell mitogen)	5µg/ml

PMAi	Phorbol 12-myristate 13-acetate with ionomycin	50 µl of Phorbol myristate acetate, 1 µl of Ionomycin in 2 ml of R10 = 20 ng/ml and 1µg/ml, respectively
F2	α-CD3	Anti-CD3 monoclonal antibody (mAb) 1 in 200 (Mabtech)
PHA	Phytohaemagglutinin	5 ml of R10, 50 µl of PHA = 5 µl/ml

Table 4.3 Composition of stimuli used in T cells assays including peptides and lysates. This table shows all the solutions used to stimulate PBMCs of CFS patients and healthy controls. Some antigenic solutions were diluted from a pre-existing stock solution. Antigenic solutions of pathogens thought to be implicated in CFS were used in this study.

Next, we decided to focus on T cell responses of CFS patients in response to antigenic stimulus. This was achieved by performing ELISpot. The analysis of T cell responses along with FIS scores would give an initial insight into the functioning of the immune system under diseased conditions. Among all the antigens and positive controls tested, only PHA (p=0.0316) and PMAi (p=0.008), positively correlated with FIS scores of patients' PBMCs. However, patient PBMC responses to cytomegalovirus (p=0.123), a mixture of Rota virus, Coxsackie virus, human herpes virus and parvo virus (p=0.333), Influenza (p=0.181), *Salmonella typhimurium* (0.293), purified protein derivative of tuberculosis (p=0.225), *Pseudomonas aeruginosa* (p=0.185), Epstein-Barr virus (p=0.463), Mycoplasma (p=0.337) and K562 cell line (p=0.160) did not show any correlation with patients' FIS scores. Production of IL-5 and IL-17 by patient PBMCs in response to positive control α-CD3 also did not show any significant correlations.

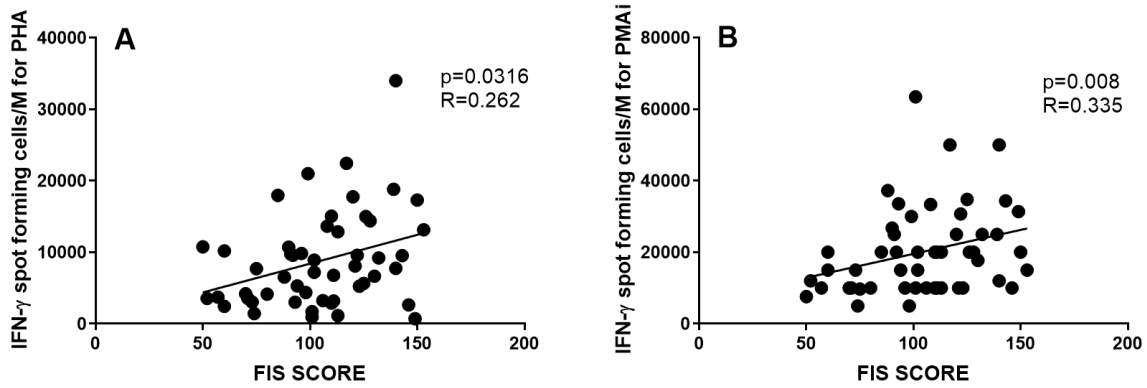


Figure 4.5.1 T cell responses to antigenic stimulus measured by ELISpot. CFS patient samples were stimulated with a wide variety of stimuli to measure T cell responses. A. Correlation between FIS score and spot forming cell/M for PHA; B. Correlation between FIS and PMAi; $n=51$, Spearman analysis or Pearson analysis was performed to test for associations between two groups.

We, then proceeded to compare the ELISpot responses between severely affected CFS patients, mild/moderately affected CFS patients and healthy controls. We compared the immune responses to cytomegalovirus (CMV), Epstein-Barr virus (EBV), *Salmonella typhi*, Adenovirus, Influenza, Respiratory syncytial virus +Rhino virus (RSVRh), *Pseudomonas aeruginosa*, purified protein derivative (PPD) and K562 cell line. We also tested the production of IL-5 and IL-17 in response to the positive control α -CD3.

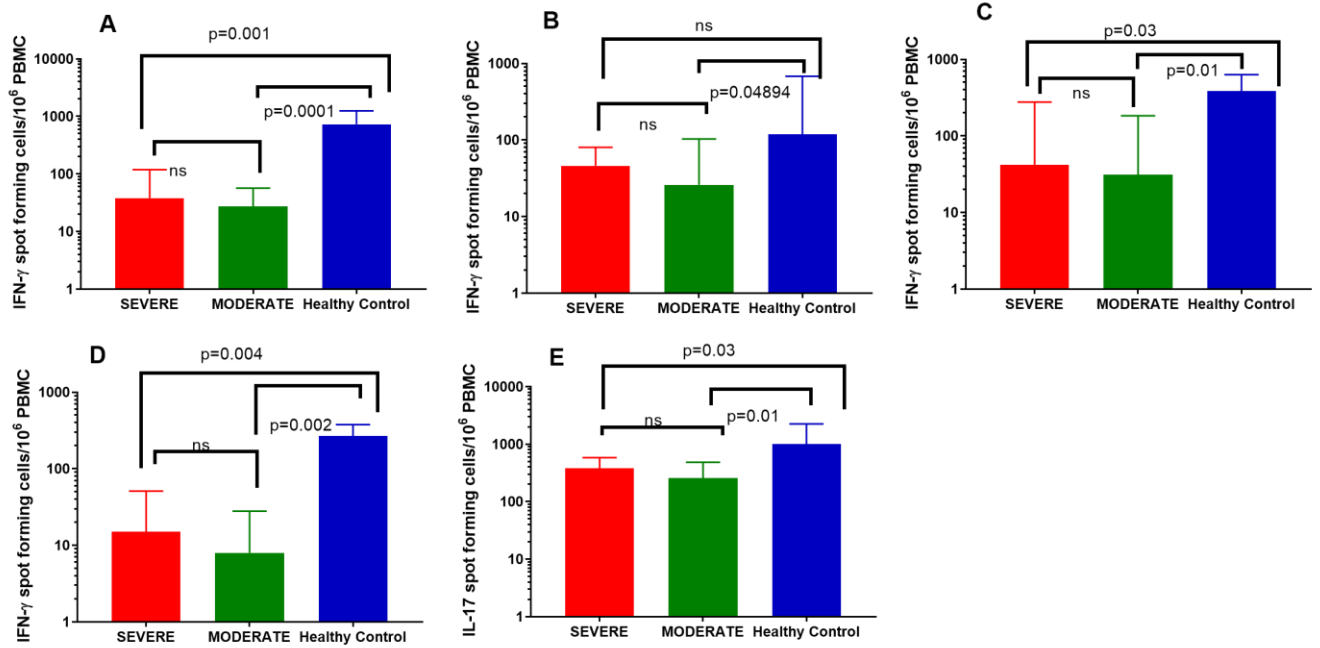


Figure 4.5.2 Comparison of ELISpot responses in severely affected, moderately affected CFS patients and healthy controls. A, B, C, D, E). Immune responses to purified protein derivative of *Mycobacterium tuberculosis*, Epstein-Barr Virus (EBV), K562 cell line, A mixture of respiratory syncytial virus and Rhino virus (RSVRh) respectively and Polyclonal secretion of IL-17 by PBMCs in response to stimulation with α -CD3 respectively. $p < 0.05$ was considered as significant. Kruskal-Wallis test with Dunn's multiple comparison test was used to analyse the patient groups and healthy controls. $n=29$ for severely affected CFS patients, $n=21$ for mild/moderately affected CFS patients, $n=7$ for healthy controls.

4.6 Multiplex Cytokine analysis

Multiplex cytokine analysis was carried out to test the different cytokines secreted by mild/moderate and severely affected CFS patients. PBMCs of CFS patients were stimulated with R10 medium and Epstein-Barr virus. The cell culture supernatants were cultured, and the release of cytokines was measured. 19 EBV seropositive patients (including mild and severely affected patients) were investigated. The production of IFN- γ , IL-2, IL-4, IL-5, IL-10, IL-12, IL-13 and IL-17 was analysed by Meso Scale Discovery. We found no significant correlations between mild/moderately affected CFS and severely affected CFS patients.

Apart from the above analysis, we also found some significant differences between EBV stimulated production of cytokines and cytokines released with no stimulation (negative control), the results of IL-5 and IL-17 is not shown here since the concentration (ng/ml) of both these cytokines in both groups were zero.

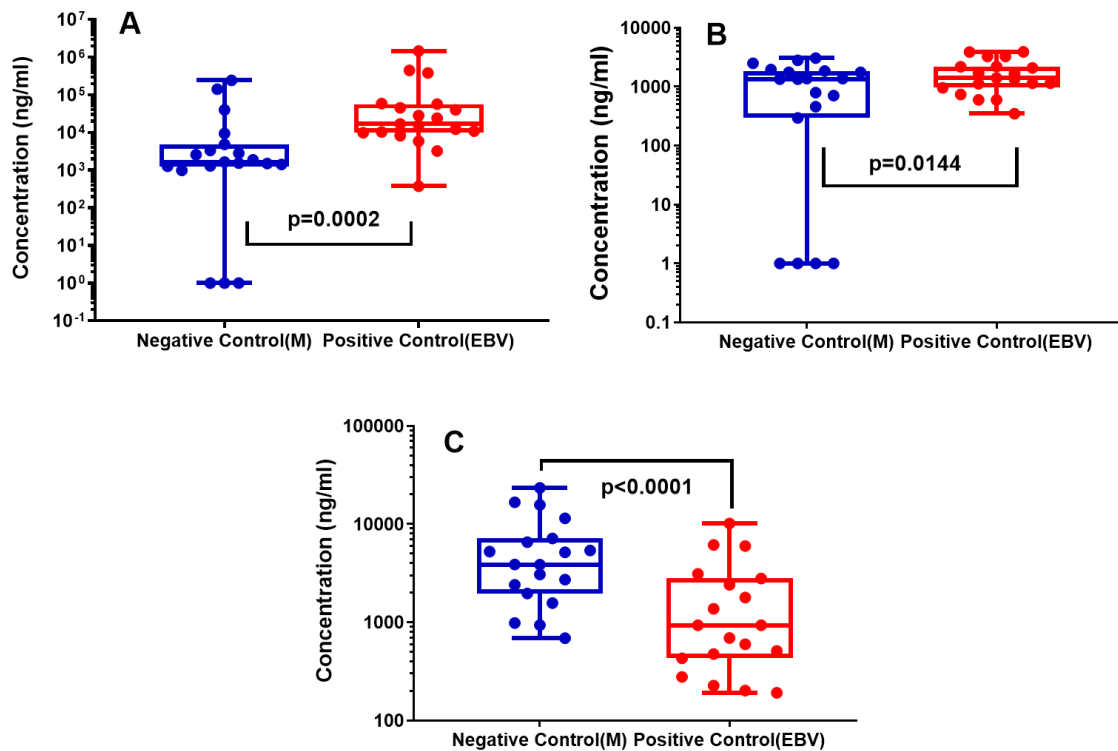


Figure 4.6.1 Comparing the release to cytokines in steady state and EBV stimulated CFS patient PBMCs by Meso Scale Discovery platform. A. Release of IFN- γ in steady state condition and EBV stimulated CFS patients ($p=0.0002$); B. Release of IL-4 in steady state condition and EBV stimulated CFS patients ($p=0.0144$); C. Release of IL-10 in steady state condition and EBV stimulated CFS patients ($p<0.0001$). Either the non-parametric Wilcoxon signed rank test or the paired t test was performed to compare the two paired groups. $p < 0.05$ was considered significant. $n=19$ (including both severely and moderately affected CFS patients. M-negative control, EBV- positive control.

4.7 Flow cytometric analyses of patient samples

4.7.1 Flow cytometric gating strategy for CFS

Identification of lymphocytes and their different subsets, according to expression of various cell surface receptors using multiple fluorescently-labelled antibodies, is the essence of flow cytometry, and we used it to analyse T cells, NK cells and B cells along with unconventional lymphocytes such as mucosal invariant T cells (MAIT) and $\gamma\delta$ T cells (Gamma Delta T cells). A typical gating strategy to identify, measure and depict the levels of expression of cell surface receptors of CD4⁺ T cells, CD8⁺ T cells and B cells is shown below and depicted in **Figure 4.7.1**.

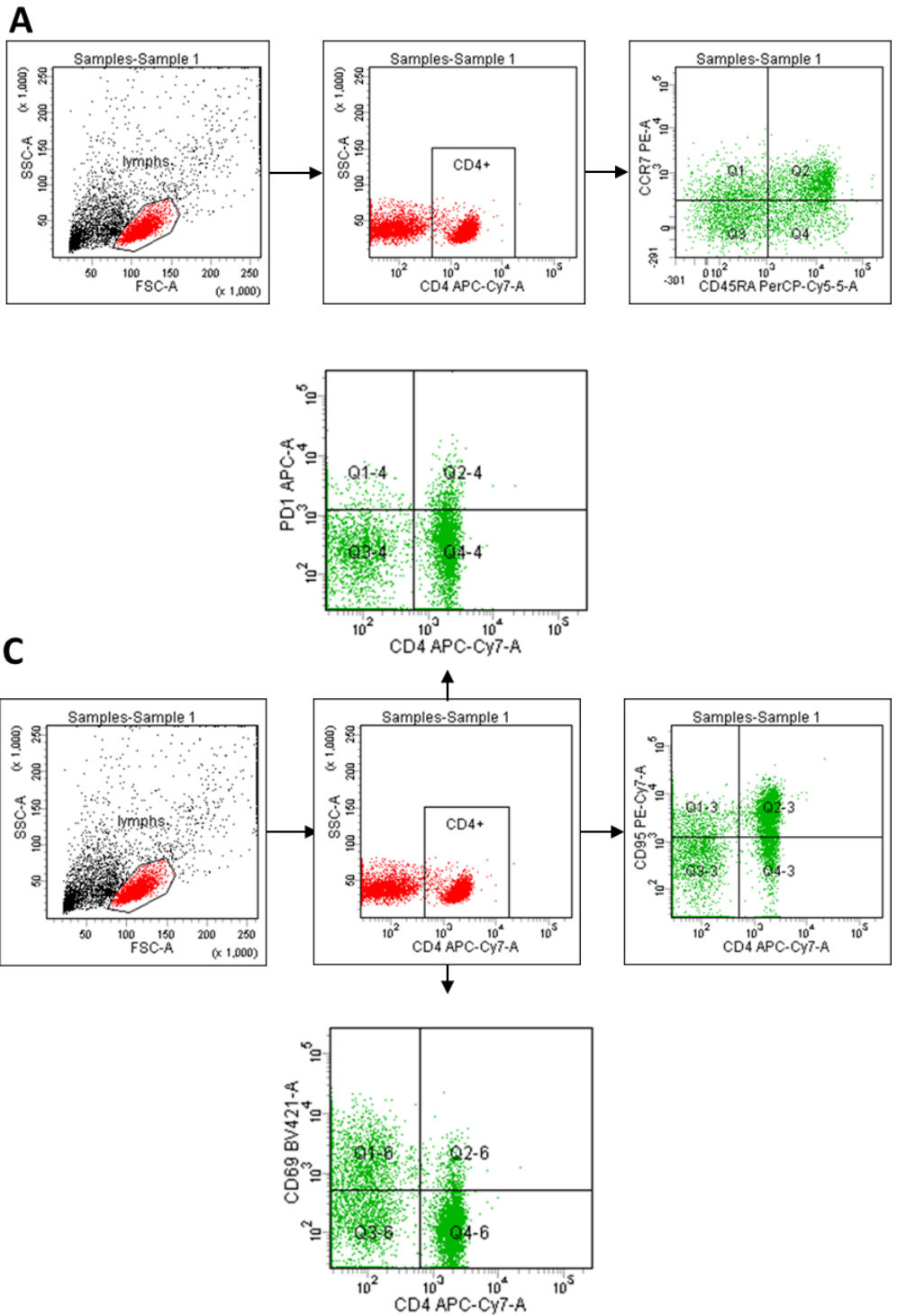
4.7.1.1 Gating strategy

Each sample was analysed on a BD FACS Canto II cytometer with BD FACSDiva acquisition software. The samples were run until 10000 T cell events had been acquired, or until just before the sample ran dry in cases with low T cell numbers.

1. Lymphocytes were gated according to their typical forward and side scatter characteristics.
2. Lymphocytes were plotted by CD4 APC-Cy7 against side scatter as well as CD8 BV510 against side scatter. CD4⁺ T cells were plotted by CCR7 PE against CD45RA PerCP-Cy5.5 to obtain the naïve, effector, effector memory as well as terminally differentiated effector memory CD4 sub populations (**Figure 4.7.1**). Similar process was carried out for CD8⁺ T cells (**Figure 4.7.1**).
3. To measure levels of CD95, CD69 as well as PD-1 levels in CD4⁺ T cells, CD4⁺ T cells (APC-Cy7) were plotted against CD95 (PE-Cy7), CD69 (BV-421) and PD-1 (APC). Only double positive cells were taken into consideration (**Figure 4.7.1**). Similarly,

CD8⁺ T cells (BV-510) were plotted against CD95 (PE-Cy7), CD69 (BV-421) and PD-1 (APC) (**Figure 4.7.1**). To correct issues such as non-specific interactions and incorrect compensation between the BV521 and BV421, brilliant stain buffer was added to optimize the interactions.

4. Lymphocytes were also plotted by CD3 BV510 fluorescence against side scatter and classified into T cells (CD3⁺) and CD3⁻ lymphocytes.
5. CD3⁺ lymphocytes were also plotted by CD161 PE against TCR V α 72 APC. Cells positive for both markers were classified as mucosal invariant T cells (MAIT). Furthermore, CD3⁺ lymphocytes were plotted by side scatter against TCR V α 24-J α 18 PE-Cy7 to identify natural killer T cells (NKT) (**Figure 4.7.2**).
6. CD3⁺ lymphocytes were plotted by CD25 PE against CD127 APC. Cells positive for CD25 and CD127 were classified as regulatory T cells. In addition, TCR $\gamma\delta$ was plotted against side scatter to obtain the $\gamma\delta$ T cell population (**Figure 4.7.2**).
7. CD3⁻ lymphocytes were then plotted by CD16 APC-Cy7 against CD56 BV421. Cells positive for both markers were classified as NK cells (**Figure 4.7.3**).
8. CD19 PerCP-Cy5.5 was plotted against side scatter and CD19⁺ B lymphocytes were plotted by CD27 PE-Cy7 against CD38 BV421. Cells were then classified as CD27⁺CD38⁺, CD27⁻CD38⁺, CD27⁻CD38⁻ and CD27⁺CD38⁻ B cells (**Figure 4.7.3**).



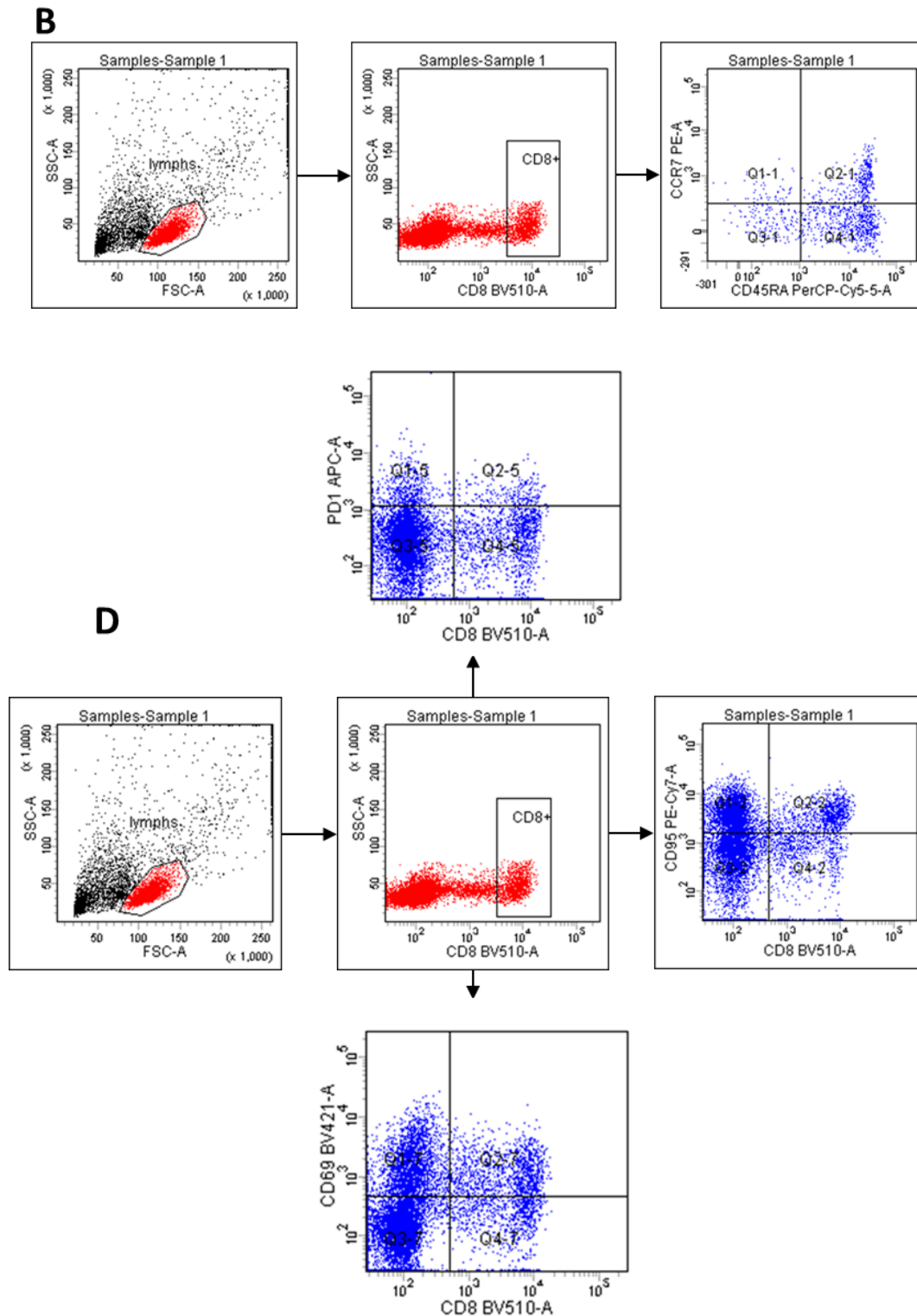


Figure 4.7.1 Flow cytometry gating strategy and expression of cell surface markers on CD4 and CD8 T cells. A, B. The expression levels of naïve (CD45RA⁺ CCR7⁺) central memory (CD45RA⁻ CCR7⁺), effector memory (CD45RA⁻ CCR7⁻) and TEMRA (CD45RA⁺ CCR7⁻) populations in CD4⁺ and CD8⁺ T cells respectively. C, D. The expression levels of CD95, PD-1 and CD69 was investigated on

CD4⁺ and CD8⁺ T cells respectively. These flow images are representative for one patient and the same process was carried out for all CFS patients and healthy controls. BD FACS Diva software was used to analyse data after data acquisition.

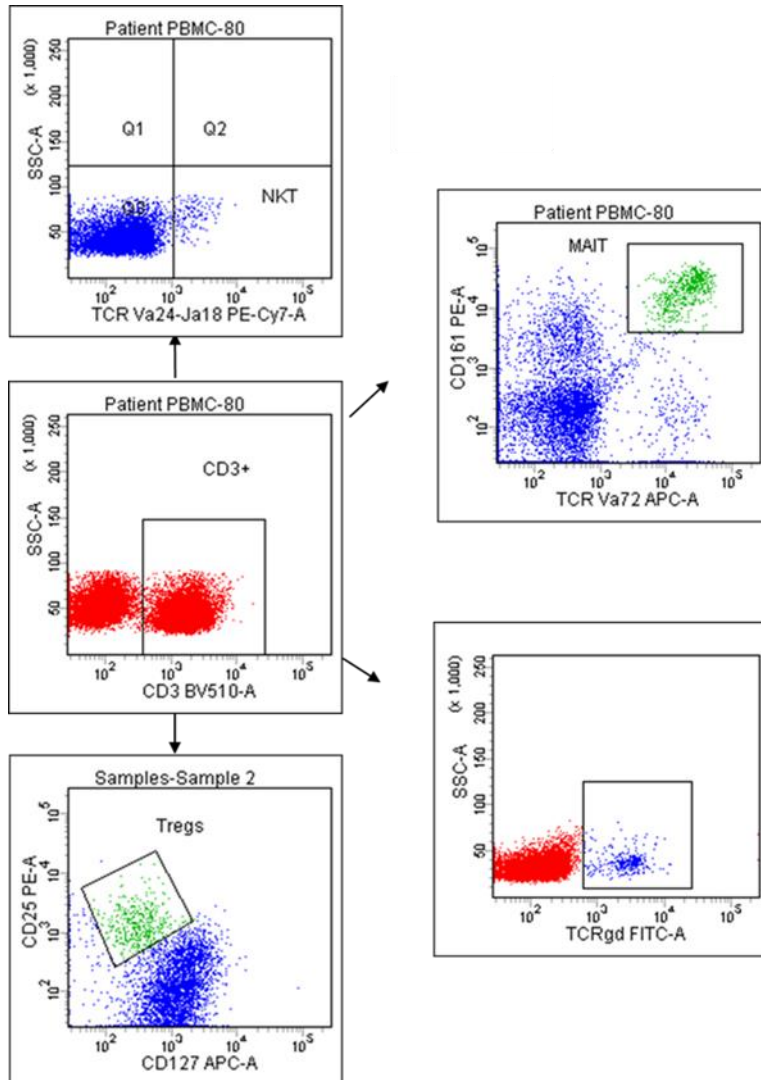


Figure 4.7.2 Flow cytometry gating strategy and expression of cell surface markers on NKT cells, MAIT cells, Regulatory T cells and $\gamma\delta$ T cells. $\gamma\delta$ T cells and regulatory T cell populations were also identified and in addition, mucosal invariant T cells (MAIT) and natural killer T cells (NKT) were gated on CD3 Cells. These flow images are representative for one patient and the same process was carried out for all CFS patients and healthy controls. BD FACS Diva software was used to analyse data after data acquisition.

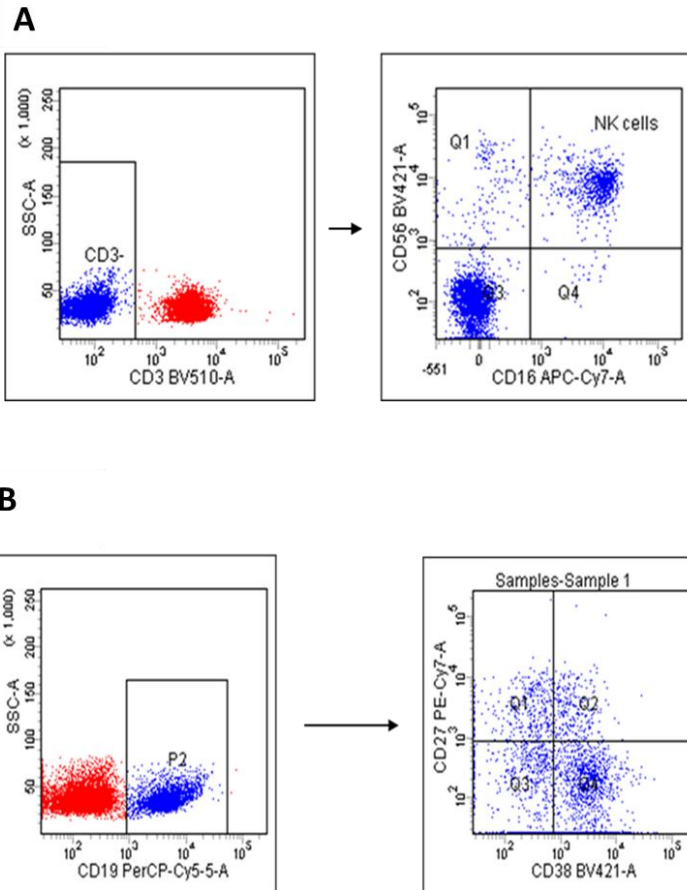


Figure 4.7.3 Flow cytometry gating strategy and expression of cell surface markers on B cells and NK cells. A. The expression levels of CD38 and CD27 on CD19⁺ B cells was investigated to identify naïve B cells (CD27⁻CD38⁻), plasma cells (CD27⁺CD38⁺), immature B cells (CD27⁻CD38⁺) and memory B cells (CD27⁺CD38⁻). B. To obtain NK cells, all CD3⁻ were chosen and gated on cell surface markers CD16 and CD56. In addition, mucosal invariant T cells (MAIT) and natural killer T cells (NKT) were gated on CD3 Cells. These flow images are representative for one patient and the same process was carried out for all CFS patients and healthy controls. BD FACS Diva software was used to analyse data after data acquisition.

Flow cytometric analyses of patient samples revealed the percentage of total NK cells negatively correlated with patient FIS scores ($p=0.0036$), however, the percentages of NK CD57⁺ cells and CD69⁺ NK cells showed no correlation. NKT cells also showed no correlation with FIS. Percentage of CD3⁺ mucosal invariant T cells also showed a negative correlation

with FIS scores ($p=0.0375$). Apart from NK cells and CD3 MAIT cells, there were no other associations between the FIS score and other flow cytometric measurements.

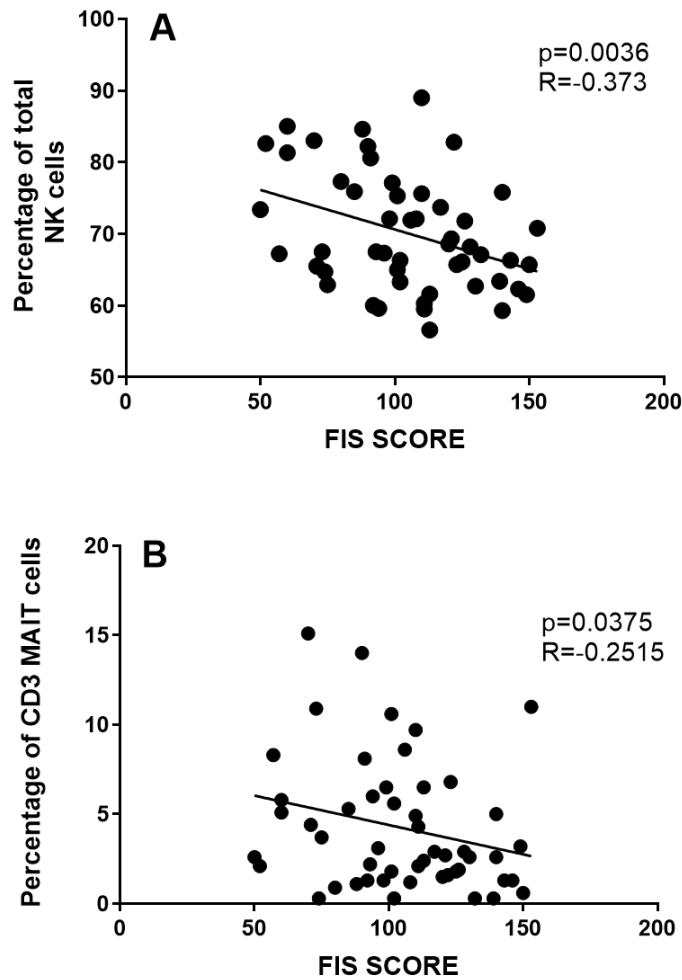


Figure 4.7.4 Association between FIS scores and Flow cytometric analyses in CFS patients. A. Association between the percentage of NK cells and FIS score, B. Association between CD3 MAIT cells and FIS score. Spearman or Pearson analysis was performed to test for correlations. p denotes value of significance. $P<0.05$ were considered significant. $n=51$.

4.8 Analyses of Severe and Mild/Moderate Chronic fatigue patients

Having established the correlation between FIS scores and immune measurements of CFS patients, we wanted to find out the differences in immune responses in High and Low chronic fatigue syndrome patients. Patients with a FIS score of less than 100 were considered to have mild/moderate fatigue whereas patients with a FIS score of above 100 were classified as severely affected chronic fatigue patients. IFN- γ spot forming cells/M specific to PMAi ($p < 0.0001$) was significantly higher in severely affected patients. In contrast, the percentage of NK cells ($p = 0.0129$) in severely affected patients were significantly lower than the NK cell levels in mild or moderate patients. Apart from NK cells and PMAi, we found no other significant differences between severe and moderately affected CFS patients.

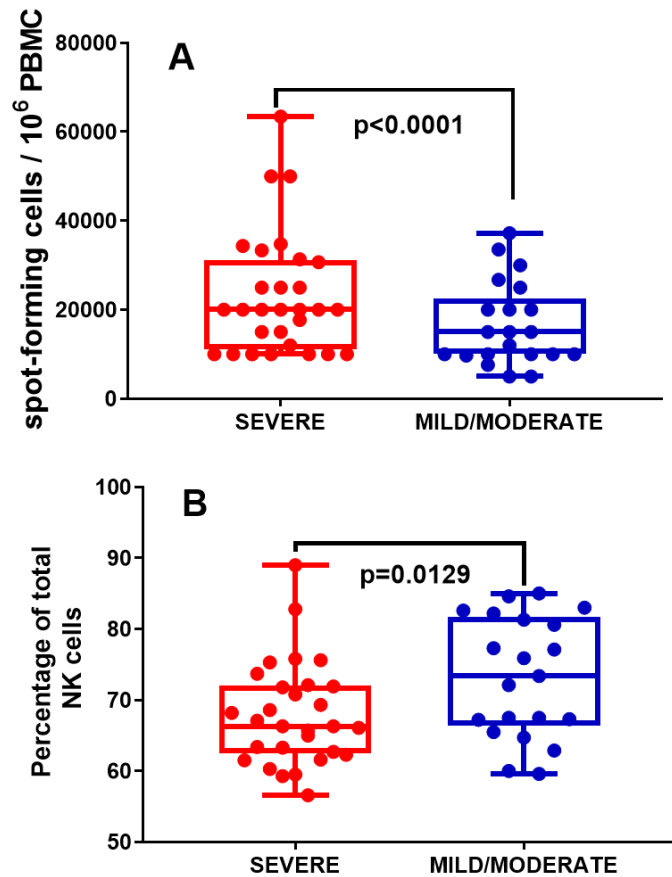
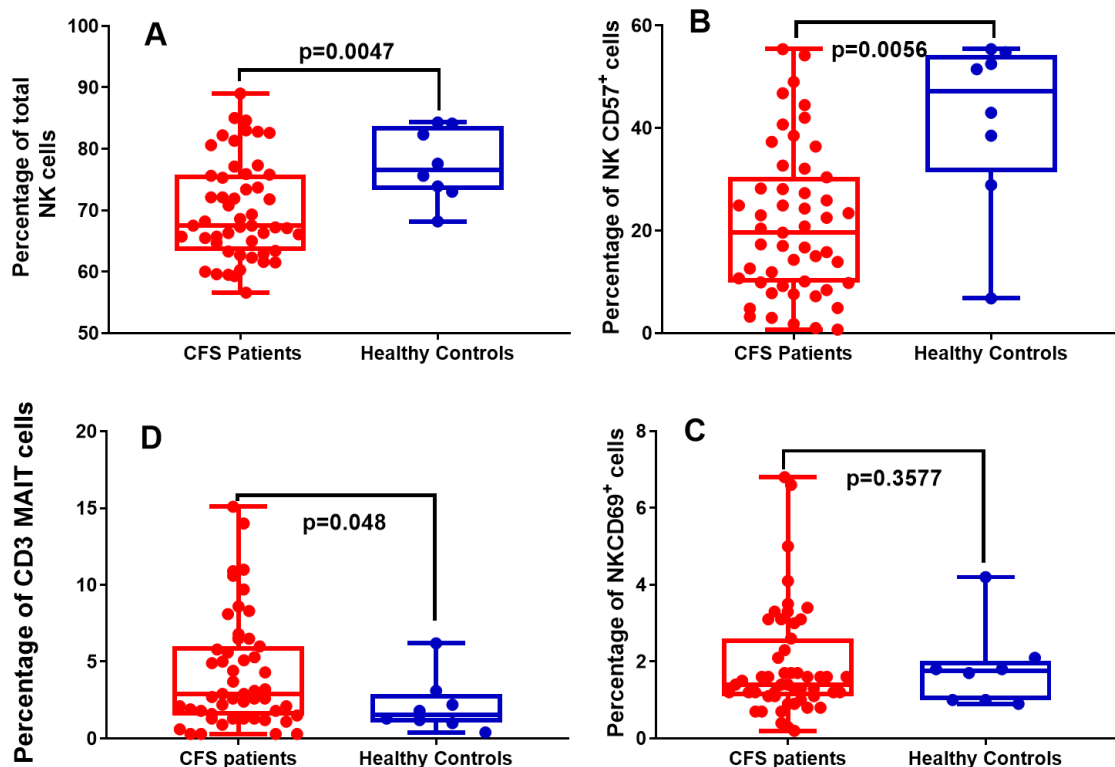


Figure 4.8.1 Analyses of High and Low chronic fatigue groups. A. Severely affected chronic fatigue patients have higher responses to PMAi (positive control), $p < 0.0001$); B. The percentages of NK cells in severely affected patients were significantly lower than in mild/moderately affected patients, $p = 0.0129$). Patients with a FIS score of below 100 were classified as mild/moderate and patients above the score of 100 were classified as severely affected patients. $n = 29$ for severely affected CFS patients and $n = 21$ for mild/moderately affected CFS patients. Mann-Whitney U test or the unpaired t test with welch correction was performed to test the relationship between the two group. $P < 0.05$ was considered significant.

4.9 Comparison of CFS patients and Healthy control data obtained by Flow cytometry

It is vital to compare the immune parameters of CFS patients to the immune measurements of the healthy controls. This will help in the identification of disturbances in the immune system of diseased individuals. Keeping that in mind, we decided to compare the percentages of NK cells, T cells and B cells in CFS patients to healthy controls, as measured by Flow cytometry. The NK cell compartment, being widely researched in patients with CFS, was analysed first. The percentage of total NK cells and CD57⁺ NK cells were significantly lower in CFS patients than in healthy controls. However, there were no changes in NKT cells or CD69⁺ NK cells. In addition, percentage of CD3⁺ MAIT cells were significantly higher in CFS patients than in healthy controls.



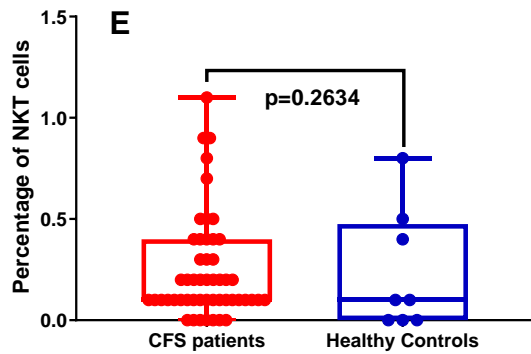
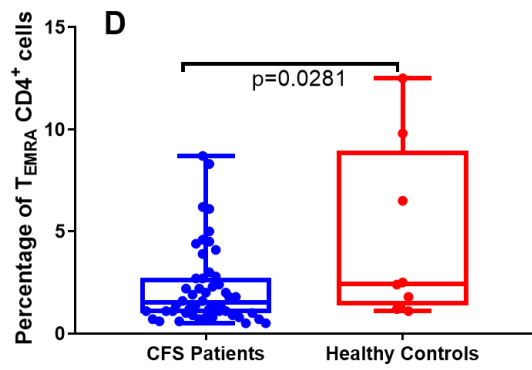
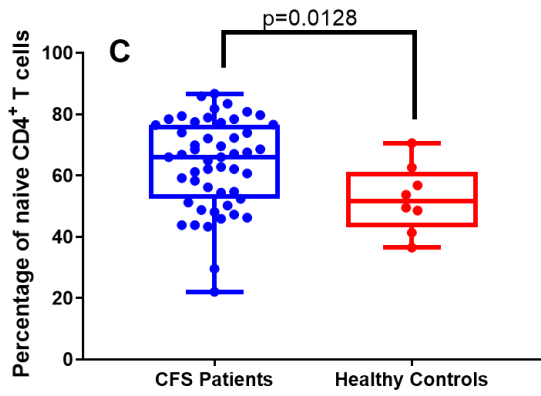
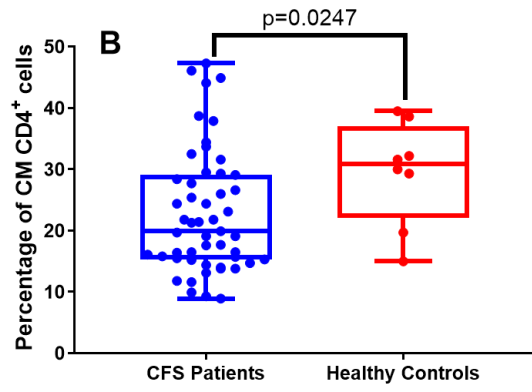
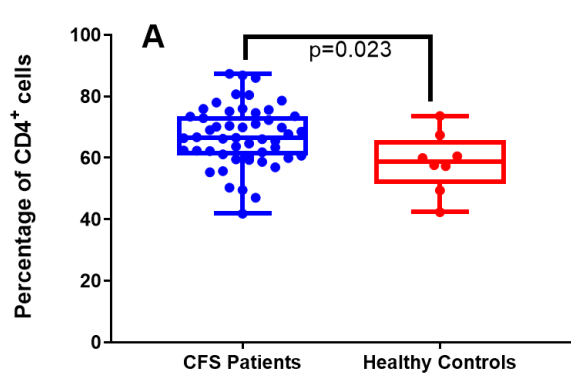


Figure 4.9 A Comparison of NK Cells in CFS patients and Healthy controls. A. Percentage of Total NK cells in CFS patients was significantly lower than in healthy controls ($p=0.0047$); B. Percentage of $CD57^+$ NK cells were significantly lower in CFS patients than in healthy controls ($p=0.0056$); C. There was no significant difference between the two groups in the NK $CD69^+$ cell compartment ($p=0.3577$); D. Percentage of $CD3^+$ MAIT cells were significantly higher in CFS patients than in healthy controls ($p=0.048$); E. There was no difference in NKT compartment between both groups ($p=0.2634$); For CFS group, $n=51$; for healthy control group, $n=8$. Non-parametric Mann-Whitney U test or the parametric unpaired t test with Welch correction was used to test the relationship between two unpaired groups.

Next, we decided to study the differences in $CD4^+$ and $CD8^+$ T cell compartment in CFS patients and healthy controls. Variations were seen in the $CD4^+$ T cell compartment. Total percentage of $CD4^+$ T cells ($p=0.023$) was higher in CFS patients than in healthy controls. In addition, CFS patients also had higher numbers of naïve $CD4^+$ T cells ($p=0.028$), but lower levels of central memory cells ($p=0.0247$), $PD-1^+$ $CD4^+$ T cells ($p=0.0045$) and T_{EMRA} $CD4^+$ T cells ($p=0.0281$). However, activated $CD4^+$ T cells, evidenced by the expression of $CD69$ ($p=0.4467$), $CD95^+$ cells ($p=0.3233$) and effector memory $CD4^+$ T cells ($p=0.0517$) did not differ among both groups. In the $CD8^+$ T cell compartment, only the total percentage of $CD8^+$ T cells was significantly lower than in healthy controls ($p=0.004$). In addition, $CD95^+CD8^+$ T cells were significantly higher in CFS patients. The other $CD8^+$ T cells subsets did not differ between groups.



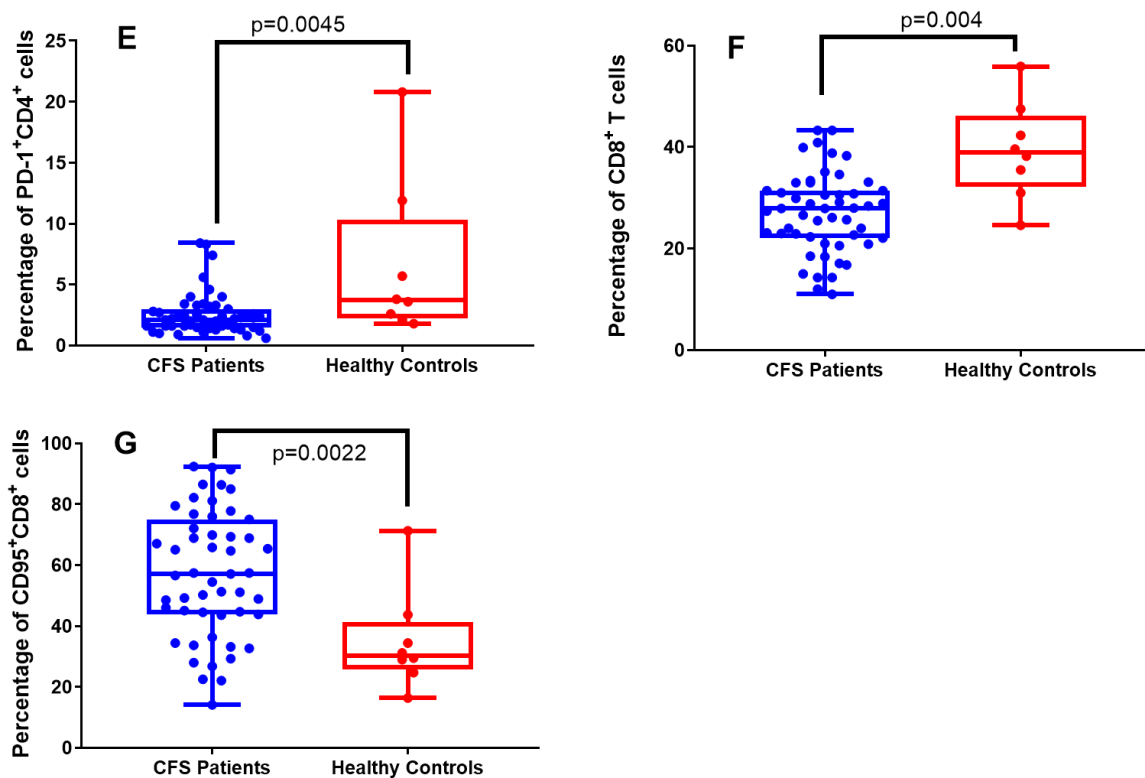


Figure 4.9 B Analysis of the CD4 and CD8 T cell compartment in CFS patients and Healthy controls. A. The percentage of total CD4⁺ T cells was significantly higher in CFS patients than healthy controls (p=0.023); B. Central memory CD4⁺ T cells was significantly lower in CFS patients (p=0.0247); C. Naïve CD4⁺ T cells were significantly higher in CFS patient (p=0.0128); D. Terminally differentiated effector memory T cells (T_{EMRA}) was also significantly lower in CFS patients group (p=0.0281); E. CD4⁺ T cells expressing PD-1 were also lower in CFS patients (p=0.0045); F. Percentage of total CD8⁺ T cells was significantly lower in the CFS patient group (p=0.004); G. CD95⁺CD8⁺ T cells were higher in the CFS patient group compared to healthy controls (p=0.022). For CFS patients group, n=51 and for the healthy control group, n=8. Mann-Whitney U test or the unpaired t test with welch corrections was performed to analyse both groups.

We then analysed the B cell compartment to identify more immune differences between the CFS patient group and healthy control group. It was found that CD19⁺ B cells were significantly elevated in the patients' group (p=0.0065), however, CD27⁻CD38⁻ (p=0.4675), CD27⁻CD38⁺ (p=0.281), CD27⁺CD38⁻ (p=0.4632) and CD27⁺CD38⁺ B cells (p=0.2299) did not

differ between patient and control groups. Percentage of $\gamma\delta$ T cells ($p=0.2122$) and regulatory T cells ($p=0.2891$) also did not differ between the two groups.

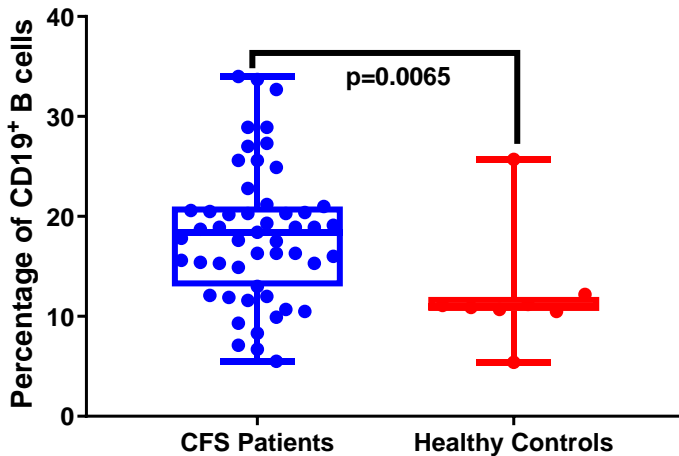


Figure 4.9 C Comparison of B cells/Tregs in patients and healthy controls. CFS patients had a significantly higher number of CD19⁺ B cells than in healthy controls ($p=0.0065$). For CFS patients group, $n=51$ and for the healthy control group, $n=8$. Un-paired Mann-Whitney U test was performed to analyse both groups.

DISCUSSION

The use of Fatigue Impact Scale to diagnose chronic fatigue syndrome patients

In our research, our clinical collaborators used the FIS to measure the extent of chronic fatigue in patients. FIS score has been translated and validated in over 30 languages which shows its universality (Frith & Newton, 2010). Fisk *et al.*, in 1994 conducted a study to test the efficacy of FIS. Rather than just ask patients to rate their fatigue levels, it was decided that measuring the fatigue on functional activities of patients would lead to a much better understanding of chronic fatigue in patients. Thus, the Fisk group concluded that patients' perception of functional limitations attributed to fatigue must be examined. The FIS consisted of three major domains, the cognitive functioning domain, the psychological functioning domain and the physical functioning domain. The internal consistency of three sub domains was determined

by Cronbach's α score, which was very high. The Cronbach's α score measures the extent to which items in the test relate to each other and thus ensures validity of the test (Tavakol & Dennick, 2011). Fisk *et al.*, (1994) found that the FIS had a high Cronbach's α value of 0.98 and the three sub domains showed a value of 0.87 thereby ensuring that FIS scale is a valid test to use to diagnose CFS patients. Another important aspect of the FIS was its ability to efficiently distinguish between chronic fatigue patients and patients with multiple sclerosis (fatigue is an important component of MS). Furthermore, other fatigue scales such as modified fatigue impact scale (MFIS), and daily FIS all derive from the FIS (Frith & Newton, 2010).

Antibody Responses in CFS patients

IgG, IgM and IgA responses to CMV and EBV were identified by Euroimmun ELISA kits and did not correlate with FIS scores although IgM responses to CMV was just outside significance. Maes *et al.*, (2006) showed that CFS patients had higher antibody titres of IgM against oxidized fatty acids such as oleic acid, palmitic acid and myristic acids than healthy controls. It was also found that IgM responses positively correlated with muscular pain, fatigue etc. It is well known that IgM responses are abundant during primary infection (Ehrenstein & Notley, 2010) and it is possible that recent CMV infection triggered CFS in patients and resulted in the production of CMV-specific IgM. There is growing consensus suggesting that CFS might originate from the dysfunctions of the gut mucosa (Navaneetharaja *et al.*, 2016). IgA responses are predominant at the mucosal surfaces and act as an indicator of primary EBV infection (Mcintosh-Bhaduri *et al.*, 2007). Giloteaux *et al.*, in 2016 showed that the gut microbiota of CFS patients had a proinflammatory profile and this led to the microbial migration from the gut and thus causing an immune response. CFS patients had significantly higher levels of serum LPS. Along with LPS, CD14 (receptor for LBP), and LPS binding protein (LBP) were also significantly higher in CFS patients compared to healthy controls. These results suggest that the high levels of serum LPS in CFS patients could be due to the presence of high levels of

endotoxins in the gut, which in turn could be due to the changes in the gut. Thus, changes to the gut microenvironment could lead to microbial translocation and eventually lead to the dysregulation of the normal immune functioning. Shukla *et al.*, in 2015 used exercise as a factor in determining changes in the gut microenvironment in CFS patients. It was found that at 72 hours post maximal exercise, CFS patients had significantly higher levels of bacteria than healthy control. In addition, the clearance of bacterial from systemic circulation was significantly lower in CFS patients compared to healthy controls. Both these results indicate increased bacterial translocation from the gut and into systemic circulation.

Relationship between ELISpot responses and FIS scores

Only the positive controls PHA and PMAi showed a positive correlation between IFN- γ spot forming cells and FIS scores in CFS patients. This indicates that as FIS scores increases the levels of pro-inflammatory cytokine IFN- γ also increases. Patients with severe fatigue may in fact have higher inflammatory T cell response than patients with mild fatigue or healthy people. Apart from positive controls, none of the other antigenic stimulus showed any association or correlation with the fatigue impact scale scores. We then proceeded to compare the relationship between ELISpot responses among severely affected, mild/moderately affected CFS patients and healthy controls. We found that ELISpot responses to purified protein derivative (PPD) of *Mycobacterium tuberculosis* were significantly higher in healthy controls than in severely affected and moderately CFS patients. This suggests that CFS patients have deficient 'IFN- γ spot forming cells' specific to PPD, which suggests that CFS patients have a defective immune response against *Mycobacterium tuberculosis*. Nunes-Alves *et al.*, (2014) in their review mentioned that although increased production of IFN- γ has been generally associated with protection from pathogens, increased IFN- γ is also associated with disease progression rather than protection in *Mycobacterium tuberculosis* infection. Thus, they concluded by mentioning that IFN- γ is not the sole determinant, but one of the many important factors (including TNF-

α , IL-1, IL-6) in the protection against TB. Thus, further investigation is required to understand the role mycobacterium plays in chronic fatigue syndrome. We also found that moderately affected CFS patients had significantly lower levels of EBV-specific IFN- γ spot forming cells than healthy controls. Loebel *et al.*, (2014) reported deficient B cell and T cell response to EBV in CFS patients. The IFN- γ producing EBV-specific T cells were significantly reduced in the CFS patients group than in the healthy controls. The low levels of IFN- γ may impair anti-EBV immune responses in CFS patients and may lead to the increased activation of EBV.

K562 cells lack the expression of MHC molecules on the cell surface and are killed by natural killer cells. Thus, testing the response of NK cells against the K562 cells is a good indicator of the cytotoxic capacity of NK cells. In our cohort, we found that severely affected and moderately affected CFS patients had significantly lower NK cell responses than healthy controls. Fletcher *et al.*, (2010) also showed significantly decreased natural killer cell cytotoxicity in CFS patients. Brenu *et al.*, (2012) conducted a longitudinal study to investigate the extent of natural killer cytotoxicity in CFS patients. They found CFS patients had significantly lower ability to kill K562 cells even at 12 months. Thus, deeper studies into the functioning of NK cells in CFS patients may be the key in the identification of a biomarker. Reduced function of NK cells may make CFS sufferers more susceptible to viral re-activation, thus enhancing the infection-inflammation vicious circle.

Interestingly, we also found that CFS patients, both severe and moderate had significantly lower ELISpot response to respiratory syncytial virus (with the addition of Rhinovirus). This indicates impaired immune responses and protection against respiratory syncytial virus. However, a definite role for RSV in the pathogenesis of CFS remains to be seen. IL-17 production by anti-CD3 (α -CD3) was significantly lower in the CFS patient groups than in the healthy control group. IL-17C confers pro-inflammatory protection against intestinal pathogens by binding to its receptor, IL-17RE (Song *et al.*, 2011). Thus, lower amount of IL-

17 may lead to decreased protection against mucosal pathogens and might be a trigger for the onset on CFS.

Multiplex Cytokine analysis (Meso-Scale Discovery)

Epstein-Barr virus has long been considered the initiating factor in CFS. Jones *et al.*, (1985) found EBV-specific antiviral capsid antigen and anti-early antigen titres of patients with myalgia, fatigue and depression was significantly higher than in healthy controls. Sairenji *et al.*, in 1995 also showed that antibody titres to ZEBRA, a product of the immediate early EBV gene BZLF1, was high in CFS patients compared to healthy controls. Loebel group in 2014 found significantly lower levels of EBV-specific antibody secreting memory cells as well as IFN- γ , IL-2 and TNF- α producing T cells in CFS patients. However, in contrast, Cameron *et al.*, 2010 compared the presence of viral DNA of EBV with healthy controls and found no significant differences. Furthermore, no changes were found in the antibody titres as well. So, to investigate the effects of EBV on CFS patients, we measured secretion of cytokines in cell culture supernatant in severely and moderately affected chronic fatigue patients. We found no correlations in the secretion of Th1 (IFN- γ , IL-2, IL-12), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17) and the anti-inflammatory cytokine IL-10. We next compared the cytokine levels of cell culture supernatants stimulated by EBV and negative control (R10 medium). We found cells stimulated with EBV released significantly higher levels of IFN- γ and significantly lower amounts of IL-10 compared to PMBCs stimulated with medium only (negative control). In addition, we also found significantly higher levels (slightly) of IL-4 in cells stimulated with EBV. Apart from these cytokines, we found no other differences between the other cytokines. Increased levels of IFN- γ and decreased levels of IL-10 might indicate increased pro-inflammatory mediators in EBV-seropositive CFS patients.

Analyses of Severe and Mild/Moderate Chronic fatigue patients

Firstly, using flow cytometric analysis we found that both NK cells and CD3⁺ MAIT are negatively associated with FIS scores. i.e. as the chronic fatigue of patients increase, the levels of NK cells and MAIT cells become lower. Apart from NK cells and non-specific antigenic stimulus PMAi, there were minimal other differences between severe and moderate immune measurements in other groups. NK cells were significantly lower than moderate patients whereas severe CFS patients had a higher level of IFN- γ than moderate CFS patients. These data point to an inflammatory component to CFS as well as the NK cell dysfunction.

Comparison of CFS patients and Healthy control data obtained by Flow cytometry

We compared the NK cell, T cell and B cell compartment of CFS patients and healthy controls and obtained some interesting results. In the NK cell compartment, we found the total percentage of NK cells and percentage of NKCD57⁺ to be significantly reduced in CFS patients compared to healthy controls. In addition, percentage of CD3⁺ mucosal invariant T cells was significantly higher in CFS patients. Discrepancies in the NK cells in CFS has been widely reported. Brenu *et al.*, in 2010 showed that CFS patients had significantly lower CD56^{bright} cells than healthy controls. They also reported the decreased ability of NK cells to lyse K562 tumour cells. Brenu *et al.*, in 2013, again, showed that NK cells of CFS patients displayed significantly lower cytotoxicity when cultured with K562 tumour cells along with lower levels of Granzyme B, an important cytolytic protein. Surprisingly, the reduction in cytotoxic or lytic capacity of NK cells was associated with increased degranulation in NK cells cultured with K562 and PMAi. CD56⁺ NK cells also produced high amounts of IFN- γ . Thus, reduced levels of NK cell cytotoxicity combined with increased degranulation and production of IFN- γ suggested the presence of highly activated but non-functional NK cells. We mentioned earlier that apart from total NK cells, NK CD57⁺ cells were also significantly reduced. This has important ramifications for the functioning of the immune system. Lopez-Verges *et al.*, (2010) surmised

that CD57⁺ NK cells represent a more mature, terminally differentiated phenotype than other NK cell subsets. CD57 was highly expressed on CD56^{dim} mature NK cells but much lower in CD56^{bright} cells. CD57⁺ NK cells also proliferated poorly when stimulated with mitogens. CD57⁺ NK cells have increased cytolytic capacity and are upregulated during viral infections. A reduction in CD57⁻ NK cells could severely impact anti-microbial immune responses. Decreased levels of CD57⁻ NK cells have also been associated with autoimmune diseases such as Sjogren's syndrome and psoriasis (Nielsen *et al.*, 2013). Thus, is it also possible that CFS has an autoimmune component to it. CD3⁺ mucosal associated invariant T (MAIT) cells were significantly increased in CFS patients compared to healthy controls. MAIT cells are abundantly present in peripheral blood, mucosa of the lung, and intestine and liver. MAIT recognise an MHC class I-like protein called MR1 which captures antigenic peptides and displays them on antigen presenting cells (Serriari *et al.*, 2014). MAIT cells specialize in anti-bacterial and anti-yeast immune response. It is suggested that MAIT cells interact with commensal bacterial flora and initiate an immune response against pathogens (Bourhis *et al.*, 2010). In CFS, therefore, it is possible that activated MAIT cells produce pro-inflammatory cytokines to altered microbiota in the gut and add to the exacerbation of chronic inflammation.

The CD4⁺ T cell compartment showed variations between CFS patients and healthy controls. The total percentage of CD4⁺ T cells was significantly higher in the CFS patients than healthy controls. It is also interesting to note that central memory CD4⁺ T cells but not effector memory CD4⁺ T cells showed a significant drop. The increase in CD4⁺ T cells indicates increased propensity to release IFN- γ (Zhu & Paul, 2008). The low numbers of central memory CD4⁺ T cells could affect the numbers of effector memory CD4⁺ T cells and impair anti-microbial immune responses. The levels of naïve CD4⁺ T cells was significantly higher in the CFS patient group compared to the healthy control group. This could indicate that these cells still retain the ability to interact with novel pathogenic peptides so that a robust immune response could be

mounted (Roederer *et al.*, 1995; Hardcastle *et al.*, 2015). The increase in the naïve CD4⁺ T cells compartment was accompanied by the significant decreases in the PD-1 and TEMRA compartments. The CD8⁺ T cell compartment showed a significant drop in CFS patients compared to healthy controls. Curriu group in 2013 also showed a drop in CD8⁺ T cell levels in CFS patients. This could indicate a decrease in cytotoxic functioning against persistent pathogenic infections such as CMV and EBV. Among all subsets of CD8⁺ T cells, only CD95⁺ CD8⁺ T cells showed a significant increase compared to healthy controls. It is well known that CD95 is a marker for apoptosis (Paulsen & Janssen, 2011) and thus the elevated levels in CFS patients may indicate that they are marked for cell death through excessive antigenic stimulation. This could severely impair anti-pathogenic functions of CD8⁺ T cells. Brenu *et al.*, 2010 also reported a significant decrease in the cytotoxic capacity of CD8⁺ T cells in CFS patients.

In the B cell compartment CD19⁺ B cells were significantly elevated in CFS patient group compared to healthy controls whereas the other B cell subsets showed no differences between the two groups. Fluge *et al.*, (2011) investigated the effects of B cell depletion using rituximab (anti-CD20) on CFS patients. Although rituximab treatment started to show effects at different times for different patients, the general consensus was the quality of life was greatly increased after B cell depletion. They also suggested that CFS could be an autoimmune disease caused by the production of auto-antibodies by B cells and thus the depletion of auto-antibody producing B cells from patients could help in improving health conditions in chronic fatigue syndrome patients.

Conclusion

Chronic fatigue syndrome appears to be a chronic inflammatory disease that is characterized by dysfunctions in the immune system. The hallmark of CFS seem to be the low cytotoxic potential of NK cells as well as increased levels of IFN γ secretion. However, apart from NK

cells, B cells and T cells are also dysfunctional. There is growing evidence to show CFS resembles an autoimmune disease, and it that might be influenced by changes to the gut integrity and the microbiota. Further extensive research into associations of immune phenotypes with disease presence, and degree of disease, will give valuable insights into CFS and will hopefully translate into the identification of an elusive biomarker. Komaroff in 2017 commented on dysfunctions of the nervous system, energy metabolism, oxidative and nitrosative stress along with immune imbalances that have all been implicated in the onset and pathology of CFS. CFS patients have been reported to have higher levels of protein, white blood cells, lactic acid, abnormalities in their white matter, abnormalities in the secretion of growth hormone etc. CFS patients have also been reported to have impaired oxidative phosphorylation owing to increased lactate levels in the cerebrospinal fluid, reduced levels of antioxidants and increased levels of nitric oxide. Imbalances in the immune system includes the well documented dysfunction of NK cells as well as cytotoxic CD8 T cells, presence of autoantibodies and increased levels of proinflammatory cytokines. The pathophysiology of CFS is extremely complex. The point of origin of disease is still unclear. Infections could very well arise from inside as well as outside the brain. It is also highly unlikely for CFS to be a single pathogen disease and it is more likely that a combination of various infections acts as a trigger and possibly even contribute to the exacerbation of CFS. Thus, as mentioned earlier, extensive multi-disciplinary research is of absolute necessity, particularly longitudinal studies beginning at early onset and continuing through the evolution of the disease.

CHAPTER 5

General Discussion

In the face of physical injury and microbial infection, the maintenance of a balance between pro-inflammatory and anti-inflammatory actions of the immune system is vital to maintaining the steady-state condition and for the normal functioning of organs of the human body. Any imbalance either in favour of pro-inflammatory reaction or anti-inflammatory reaction may lead to the formation of a disease state. In this thesis, the focus is on chronic inflammation, i.e. continual pro-inflammatory reaction, in response to persistent infectious microbes (particularly herpes virus family) and the regulation of these responses in two major disease states: AMI+R, characterized by the presence of injury to the heart and CFS, characterized by debilitating fatigue mediated in the central nervous system. The constant activation of the immune system, skewing towards a pro-inflammatory profile in the above-mentioned conditions, appears to be influenced by the presence of persistent pathogens such as CMV and EBV, and other pathogens which chronically activate the immune system. T and B cell mediated immunity is implicated in many disease states including MI and CFS and thus it made sense to measure T cell and antibody responses to a panel of pathogen antigens, in addition to the careful recruitment, clinical characterization, and sampling of the contributing patients. The measurement of immune responses by ELISpot, Flow cytometry, MSD, and ELISA gave an insight into chronic activation of the immune system in AMI and CFS and that may translate into therapeutic interventions in the near future. To make this thesis a coherent whole, it is vital to highlight the similarities and differences between these two conditions (**Table 5.1**).

Both AMI and CFS are associated with dysfunction of the immune system. T cells, B cells and NK cells have all been implicated in both AMI and CFS, and this thesis has confirmed much of these findings. In addition, active CMV replication is a major contributor for AMI whereas CFS has been more associated with EBV. Thus, herpes viruses seem to be a common link

between the two conditions. A main difference between these two conditions is the site of inflammation. AMI occurs in the heart muscle, whereas CFS occurs in the brain/central nervous system. AMI is associated with CMV reactivation in the elderly whereas CFS is mainly pan-age with focus on the younger population.

Table 5.1 Similarities and Difference between AMI+R & CFS

	<u>AMI+R</u>	<u>CFS</u>
Immune dysregulation	√	√
Lymphocyte involvement	√	√
Involvement of Herpes viruses	√ (CMV)	√(EBV)
Location	Heart Muscle	Brain & CNS
Treated by B cell depletion	Not treated yet	√
Treated by T cell inhibition	√	Not treated yet

Role of CMV-specific T cells in Acute Myocardial Infarction and reperfusion injury (AMI+R)

In patients with ST segment elevated myocardial infarction (STEMI), before PPCI was performed to restore blood supply, the CMV-specific Th1 response was significantly stronger compared to Th2 or Th17 responses. This was reflected in an increased production of IFN- γ by T cells shown in patient groups, which continued even 30 minutes after reperfusion was performed to restore blood flow. In addition, CD8⁺ T cells were present in high numbers before reperfusion which lead to focussing on the role of CD8⁺ T cells. After reperfusion, the CD8⁺ T cells showed a drop in circulation and the terminally differentiated effector memory T cells (T_{EMRA}) subset of CD8⁺ T cells showed the highest drop in cell number. It was likely that the drop in CD8⁺ T_{EMRA} was facilitated by the high expression levels of programmed cell death

ligand-1(PD-1). PD-1 is an indicator of hyper-activation of T cells and marks the cell for apoptosis. Thus, there was a permanent loss of PD-1⁺ CD8⁺ T_{EMRA} from circulation. In addition, T_{EMRA} population in CMV-seropositive patients was significantly higher than the T_{EMRA} population in CMV-seronegative patients which suggests that active CMV causes increased activation of T cells which are a reservoir of pathogenic T cells ready to home to areas of inflammation (Hoffman & Frantz, 2015).

In our patient sub-group B cohort, we found significantly lower production of IFN- γ at 90 minutes post reperfusion in CD4⁺ T cells which could be attributed to the migration or trafficking of T cells away from circulation and into the myocardium. In contrast, the levels of CD8⁺ T cells did not change at any time point. The cultured ELISpot response which measures memory T cell response and functionality was however different for CD4⁺ T cells. There was no association between IFN- γ spot forming cells before and after reperfusion. In contrast, IFN- γ production by cultured CD8⁺ T cells also showed a significant increase at 24 hours compared to pre-PPCI levels suggesting stimulation by the myocardial inflammatory process.

Similar to our results, Forteza *et al.*, (2018) also showed in their translational study involving a porcine model, significantly reduced lymphocyte count attributed to apoptosis as well as to the homing of T cells to the myocardium. Both of CD8⁺ T cells and Tregs showed reduced counts 2 hours after reperfusion. Regulatory T cell count, however, recovered 1 month after reperfusion therapy. To ascertain the presence of T cells in the myocardium, the authors used immunohistochemical analysis. mRNA levels of transcription factor T-bet significantly increased in the heart samples strongly indicating the presence of Th1 subset which further indicates the presence of IFN- γ . The authors suggested that apoptosis of T cells coupled with the increase in regulatory T cells was an inhibitory response to curb excess inflammation. Such a response may however promote viral-reactivation and an inflammatory vicious circle.

Furthermore, they also suggested that the presence of IFN- γ could severely impact TGF- β mediated wound healing process. The migration of T cells into the myocardium at 90 minutes post reperfusion in the present human study may cause an increase in inflammation which exacerbates the prevailing condition and might lead to the formation of microvascular obstruction (MVO) and hence a poorer outcome for patients.

Although T cells are heavily implicated for causing increased inflammation, they also quench inflammation and promote cardiac wound healing. Borg *et al.*, in (2017) showed that CD73 on T cells was involved in promoting wound healing. CD4⁺ T cells lacking CD73 showed impaired myocardial wound healing and trigger the release the proinflammatory cytokines. Xia *et al.*, (2015) reported regulatory T cells attenuate wound healing of the heart through a CD39-dependent mechanism. Reperfusion injury in mice was aggravated by regulatory T cells lacking CD39. Moreover, it was also shown that PBMCs of patients who have undergone PPCI treatment also show an increased level of CD39⁺ regulatory T cells. Thus, targeting CD39 and CD73 with neutralizing monoclonal antibodies could have beneficial therapeutic potential in treating reperfusion injury.

Immune responses in CFS

Differences in immune parameters of CFS patients as compared to healthy controls were observed in this study. Also, immune parameters showed significant relationships with the FIS score. Most research groups studying CFS have uniformly reported the dysfunctional or abnormal levels of NK cells in CFS patients. In this work, the NK cell compartment of CFS patients was measured. The percentage of total NK cells and CD57⁺ NK cells (mature phenotype) were lower in CFS patients which could severely impact anti-viral immune responses allowing viral reactivation and increased systemic inflammation. The levels of CD3⁺ MAIT cells was significantly increased in CFS patients as were the levels of CD4⁺ T cells. This

indicates increased inflammation in CFS patients possibly via increased microbial translocation through the damaged gut. The lower numbers of CD8⁺ T cells suggest a deficit in the capacity of these cells to exert their anti-viral cytotoxic function. Furthermore, increased levels of CD19⁺ B cells suggest that CFS has an autoimmune component where excessive self-reactive antibodies are produced. The successful treatment of CFS patients with rituximab (anti-CD20) therapy, which depletes mature B cells suggests this might be the case (Fluge *et al.*, 2015).

Secretion of cytokines in cell culture supernatants also did not reveal any associations between severely affected and mild/moderately affected CFS patients. However, EBV-specific production of IFN- γ was significantly higher than the baseline levels (stimulation using negative control). This was accompanied by a significant drop in IL-10 levels in CFS patients. This indicates a skew towards a pro-inflammatory profile in chronic fatigue syndrome in response to EBV. Montoya *et al.*, (2017), using the Luminex platform, studied the cytokine profiles in CFS patients. They found a cytokine profile that was overwhelmingly proinflammatory. Cytokines such as IFN- γ , GM-CSF, IL-12, IL-5, IL-4 were all significantly higher in CFS patients.

Only positive control PMAi correlated with FIS scores in this thesis, showing that as the FIS scores increase the production of IFN- γ increases. This suggests that IFN- γ secreted from multiple cell sources and specificities mediates disease rather than a single or a few antigens. Since no other antigenic stimulus correlated with FIS scores, we compared the ELISpot responses of severely affected, mild/moderately affected and healthy controls since their grouping could reveal different significances. We found significantly decreased IFN- γ production in CFS patient PBMCs stimulated with *Mycobacterium tuberculosis*, Respiratory syncytial virus (RSV) and EBV. Previous work suggests that EBV might be directly involved in the pathogenesis of CFS (Loebel *et al.*, 2014; Fremont *et al.*, 2009). The roles of RSV and *Mycobacterium tuberculosis* in the pathogenesis of CFS has not yet been described.

CFS patients also showed decreased capacity to respond to K562 cells. The severely and moderately affected patients had significantly lower responses to K562 cells than healthy controls. Dysfunctional NK cells in CFS patients have been widely reported, and this makes NK cells a promising target to identify as biomarkers for fatigue which could help in the identification of ideal therapeutic interventions. Bielekova *et al.*, (2006) showed that the administration of daclizumab, a monoclonal antibody against the IL-2R α chain in multiple sclerosis patients reduced brain inflammation in a phase II clinical trial. They found that the administration of this monoclonal antibody led to the gradual reduction in the level of CD4⁺ and CD8⁺ T cells while the numbers of CD56^{bright} NK cells increased significantly. The percentage of these NK cells positively correlated with inhibition of contrast enhancing brain lesions (brain MRI). Perhaps such stimulation of NK cells could be used as a therapeutic approach for CFS.

In addition to the cells of the immune system involved in CFS, a genetic predisposition has also been implicated. A genome wide association analysis was conducted by the Schlauch group in 2016. They performed a large SNP survey to identify genetic factors that could explain CFS pathogenesis. This group identified 442 SNPs that could be involved in the pathogenesis of CFS. Some of the genes were RECK (a negative regulator of matrix metalloproteinases) and GRIK3 (a transmembrane subunit of neuroexcitatory receptors) and two SNPs in the T cell receptor alpha locus and the alpha/delta locus. Schlauch also developed a bioinformatic tool, Genotype Plotter which allowed to identify genotypic differences near SNPs of interest. This tool allowed the authors to identify genotypic signatures that are prominent in CFS patients but not in the healthy control cohort. Further research into the role of these genes, in conjunction with extended research on immunity such as our own could highlight them as important therapeutic targets.

Another research study using random peptide microarrays to screen serum antibodies of CFS patients and healthy controls was performed. A BLAST search identified the peptides specific to CFS patients belonging to human self-antigens and endogenous retroviruses and to a minor extent bacterial and viral sequences. Some of the human self-antigens that were identified are CD274, cutaneous T cell lymphoma-associated antigen 1, Legumain and electron transfer flavoprotein dehydrogenase. Some of the endogenous retroviruses components involved are endogenous retrovirus group K members 21 and 25, group 3 member 1 etc. Thus, the use of random peptide microarray could be useful in differentiating antibody profiles in CFS patients and healthy controls. Such antibodies may be simply markers of CFS rather than being involved in immunopathology (Singh *et al.*, 2018).

Overall Conclusion

In chronic inflammatory diseases it is potentially of great value to measure and use immune responses to diagnose and monitor disease (for identification of biomarkers). Both AMI+R and CFS showed increase of Th1 type cytokine IFN- γ associated with disease. Thus, both diseases are candidates for therapies with neutralizing IFN- γ monoclonal Ab. Indeed, Cui *et al.*, (2013) used anti-interferon gamma therapy to treat Crohn's disease, a chronic Th1 inflammatory disease. Having similar features to AMI+R, dilated cardiomyopathy is the inflammation of the heart muscle caused by either bacterial or viral infection. In addition to being an inflammatory disease, dilated cardiomyopathy also has an autoimmune component. Boldizsar *et al.*, (2016) used FTY720, a SIP antagonist, to arrest the migration of lymphocytes within the lymphoid tissue, instead of homing to the heart, and inhibited the activation of auto-reactive cells. Thus, it will be interesting to use therapies like the ones mentioned above on chronic inflammatory diseases such as ischemic reperfusion injury and chronic fatigue syndrome.

Limitations of Work

With respect to the MI part of the project, investigating patient samples for evolution of immune responses and patients, long term clinical outcome beyond the 3-month time frame would have been ideal, which unfortunately was not possible due to time and ethical constraints. Another drawback of the MI project was the lack of availability of equal numbers of patient samples across all points. In addition, immunohistochemical analysis of culprit lesions looking for T cell presence would have been extremely beneficial to our research but was unfortunately not possible.

In the CFS part of the project, we had a difficulty trying to recruit sufficient healthy controls who were required to be sedentary, female and middle aged. We also received a poor response to our advertisements. Thus, a more focussed approach is required to recruit such healthy controls. More data on the patients with regard to clinical parameters such as inflammatory marker CRP, would have been highly beneficial to investigate associations with T cell populations.

Future Work

A multi-faceted approach could be taken for further work, in order to further solidify and build upon the findings of this thesis, and to translate the findings to approaches that could benefit patients. Novel developments in ELISpot, such as the multi-parametric Fluorospot, would allow the investigation of the T cells that simultaneously secrete pro-and anti-inflammatory cytokines. This balance is likely to dictate immunopathology. For the MI patients it would be very useful to repeat the findings of the thesis showing the role of homing of herpes viral-specific T cells, particularly T_{EM} PD-1⁺ cells; but on larger cohorts of patients to reveal subtler phenotypic differences (e.g. CX3CR1) with sufficient statistical power. At least 100 subjects would be needed for further tetramer studies of T cells specific for CMV and EBV. Being able to sample T cells homing to the heart via the coronary artery would be highly valuable as would

the immunohistological examination of heart muscle undergoing reperfusion injury. Such approaches are most appropriate to be carried out on animal models of disease and on tissues from deceased patients, respectively. Furthermore, several trials are currently being undertaken or planned, to modulate T cell migration and activity during MI+R; and so being able to obtain PBMC samples from patients in such trials would be important for testing T cell phenotype and function (as in this thesis) during this clinical trial process. The hypothesis would be that T cells implicated in this study would be regulated by those T cell-targeting therapeutics that reduce disease severity (e.g. Atorvastatin).

In CFS, extended investigation of the immune responses should be carried out. The dysregulations in the natural killer cell compartment that were found in our CFS patients, and which agreed with the findings from other CFS research groups across the world, need to be probed deeper by investigating the activity of key receptors and signalling pathways such as KIRs and Transient Receptor Potential Melastatin 3 (TRPM3) ion channel e.g. through targeted knockdown with siRNA. Indeed, this molecule could be a target for gene SNP analysis of CFS patients. Such a study would require the recruitment of well-characterized CFS patients in the 100's, together with matched controls, for sufficient power to observe statistical differences. Therapeutic approaches for CFS using molecules such as TLR agonists that specifically activate NK cells, thus reversing their dysfunction in CFS, have been suggested (Navaneetharaja *et al.*, 2006). Gowen *et al.*, in 2007 showed that in mice deficient in TLR3, the introduction of dsRNA analog poly (I:C₁₂U) did not have any protective effect against the punta toro virus (PTV) and there was no production of interferon- α , interferon- β and IL-6 whereas the wild type mice were conferred protective immunity due to the presence of the TLR3 receptor. A double blind, placebo controlled, randomized clinical trial to test the efficacy of Rintatolimod treatment (poly (I:C₁₂U)) in severe chronic fatigue syndrome patients was carried out by Strayer *et al.*, in 2012. They found that treatment with Rintatolimod led to

improvement in the cardiopulmonary exercise tolerance as well as decrease in concomitant medicine usage. It is possible that the administration of this drug might stimulate NK cells and cause increased release of interferons. Trials with such molecules would necessitate the measurement of NK numbers and function as carried out in this thesis and suggested in further work.

A recent study of CFS patients showed the use of immunoadsorption to remove IgG against β 2 adrenergic receptor. This led to the significant reduction in autoantibodies as well as memory B cell numbers and resulted in the reduction of CFS symptoms in patients (Scheibenbogen *et al.*, 2018). This suggests that the T cell responses are likely directed against self-antigens, and their measurement would provide a useful biomarker of disease.

In conclusion, since both diseases (AMI+R and CFS) appear to involve dysregulated immune responses, ways of altering these (such as by cell depletion or neutralization of cell products like $\text{IFN}\gamma$) could be attempted, and their further measurement is warranted.

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APPENDIX -1

Ethical permission for MI research



Health Research Authority

NRES Committee North East - Newcastle & North Tyneside 2

Room 002
TEDCO Business Centre
Rolling Mill Road
Jarrow
NE32 4BW

Tel: 0191 428 3565
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21 December 2012

Professor Ioakim Spyridopoulos
Chair of Cardiovascular Gerontology
Newcastle University
Central Parkway
Newcastle upon Tyne
NE1 3BZ

Dear Professor, Spyridopoulos

Study title: Effect of cytotoxic T-lymphocytes in mediating myocardial ischemia/reperfusion injury following primary PCI-Role of cytomegalovirus seropositivity

REC reference: 12/NE/0322

Amendment number: Minor Amendment

Amendment date: December 2012

IRAS project ID: 108247

Thank you for your notification of 28 November 2012, informing the Committee of the above amendment.

The amendment has been considered by the Chair. The Committee does not consider this

WREHD³VXEVDQWLDODPHQGPHQW³DVGHILQHGLQWKH6WDQGDUG2SHUD
WLQJ3URFHGXUHVIRU Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

Document	Version	Date
Notification of a Minor Amendment	Stephen Boag	20 November 2012
Protocol	v 3	20 November 2012
Participant Information Sheet	v 3	20 November 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

A Research Ethics Committee established by the Health Research Authority

12/NE/0322:

Please quote this number on all correspondence

Yours sincerely



Gillian Mayer Committee Co-ordinator

E-mail: nrescommittee.northeast-newcastleandnorthtyneside2@nhs.net

Copy to: *Sean Scott ± Joint Research Office, Newcastle upon Tyne Hospitals
NHS Foundation Trust*

A Research Ethics Committee established by the Health Research Authority

Ethical Permission for CFS research



Health Research Authority

NRES Committee North East - Sunderland

Room 002
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10 July 2012

Dr Wan-Fai Ng
Clinical Senior Lecturer and Honorary Consultant in Rheumatology
Newcastle University
Clinical Research Facilities Level 6, Leazes Wing
Royal Victoria Infirmary Newcastle upon Tyne
NE1 4LP

Dear Dr Ng

Telephone: 0191 4283563
Facsimile: 0191 4283432

Study title: Identifying the biological fingerprints of fatigue

REC reference: 12/NE/0225

Protocol number: 6020

Thank you for your letter of 04 July 2012, responding to the Committee's request for further information on the above research [and submitting revised documentation].

The further information has been considered on behalf of the Committee by myself as Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a **favourable ethical opinion** for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

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Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Covering Letter		04 May 2012
Covering Letter		04 July 2012
Evidence of insurance or indemnity	Zurich Municipal	11 July 2011
GP/Consultant Information Sheets	Version 1.0	09 April 2012
Investigator CV	Dr Wan-Fai Ng	
Letter of invitation to participant	Version 1.0, PSS and CFS	09 April 2012
Letter of invitation to participant	Version 1.0, Health Volunteers	09 April 2012
Other: Letter from funder		22 December 2011
Other: Confirmation of insurance for design of study		13 April 2012
Other: reply slip	version 1.0	09 April 2012
Other: Confirmation of Caldicott approval		13 April 2012
Other: Withdrawal form	Version 1.0	09 April 2012
Other: Protocol - tracked changes	Version 1.1	04 July 2012
Participant Consent Form: PSS and CFS	Version 1.1	04 July 2012
Participant Consent Form: PSS and CFS - tracked changes	Version 1.1	04 July 2012
Participant Consent Form: Healthy Volunteers	Version 1.1	04 July 2012
Participant Consent Form: Healthy Volunteers - tracked changes	Version 1.1	04 July 2012
Participant Information Sheet: PSS and CFS	Version 1.1	04 July 2012
Participant Information Sheet: PSS and CFS - tracked changes	Version 1.1	04 July 2012
Participant Information Sheet: Healthy Volunteers	Version 1.1	04 July 2012

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Participant Information Sheet: Healthy Volunteers - tracked changes	Version 1.1	04 July 2012
Protocol	Version 1.1	04 July 2012
REC application	Version 3.4	16 April 2012
Response to Request for Further Information		04 July 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document “*After ethical review – guidance for researchers*” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/NE/0225 Please quote this number on all correspondence

With the Committee’s best wishes for the success of this project.

Yours
sincerely
pp



**Mr Paddy
Stevenson
Chair**

Email: Helen.Wilson@suntpct.nhs.uk

Enclosures: “After ethical review – guidance for researchers”
Copy to: Ms Amanda Tortice, Newcastle upon Tyne Hospitals NHS Foundation
Trust

12/NE/0225 Please quote this number on all correspondence

12/NE/0322: Please quote this number on all correspondence

Yours sincerely



Gillian Mayer
Committee Co-
ordinator

E-mail: nrescommittee.northeast-newcastleandnorthtyneside2@nhs.net

12/NE/0322: Please quote this number on all correspondence

*Copy to: Sean Scott - Joint Research Office, Newcastle upon Tyne Hospitals
NHS Foundation Trust*

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FIS Questionnaire

Fatigue Impact Scale

	No problem	small problem	Moderate	Big problem	Extreme
1. I feel less alert					
2. I feel that I am more isolated from social contact					
3. I have to reduce my workload or responsibilities					
4. I am more moody					
5. I have difficulty paying attention for a long period					
6. I feel I cannot think clearly					
7. I work less effectively (this applies to work inside or outside the home)					
8. I have to rely more on others to help me or do things for me					
9. I have difficulty planning activities ahead of time					
10. I am more clumsy and uncoordinated					
11. I find that I am more forgetful					
12. I am more irritable and more easily angered					
13. I have to be careful about pacing my physical activities					
14. I am less motivated to do anything that requires physical effort					
15. I am less motivated to engage in social activities					
16. My ability to travel outside my home is limited					
17. I have trouble maintaining physical effort for long periods					
18. I find it difficult to make decisions					

19. I have few social contacts outside my own home					
20. Normal day to day events are stressful for me					
21. I am less motivated to do anything which requires thinking					
22. I avoid situations that are stressful for me					
23. My muscles feel much weaker than they should do					
24. My physical discomfort is increased					
25. I have difficulty dealing with anything new					
26. I am less able to finish tasks that require thinking					
27. I feel unable to meet the demands that people place on me					
28. I am less able to provide financial support for myself and my family					
29. I engage in less sexual activity					
30. I find it difficult to organise my thoughts when I am doing things at home or at work					
31. I am less able to complete tasks that require physical effort					
32. I worry about how I look to other people					
33. I am less able to deal with emotional issues					
34. I feel slowed down in my thinking					
35. I feel it hard to concentrate					
36. I have difficulty participating fully in family activities					
37. I have to limit my physical activities					
38. I require more frequent or longer periods of rest					

39. I am not able to provide as much emotional support to my family as I should

40. Minor difficulties seem like major difficulties

APPENDIX-2

SDS-PAGE for protein analysis

SDS-PAGE running buffer X10

Running buffer was made up to 10X concentration and (diluted to 1X before use).

Tris-HCl 30.3 g

Glycine 144.0g

SDS 10.0 g

12% (W/V) SDS Acrylamide resolving gel components

40% (w/v) solution (37.5:1 acrylamide:biscrylamide) 3.0 ml

Solution B* 2.5ml

Deionised water 4.50 ml

10% (w/v) ammonium persulphate (ASP) 50 µl

TEMED 10 µl

***Solution B**

2M Tri-HCL, PH 8.8 75ml per 100 ml

10% (w/v) SDS 4ml per 100 ml

4% (W/V) Acrylamide stacking gel components

40% Acrylamide 250 ml

10% ammonium persulphate (APS) 30µl

Deionised water 1.2 ml

Solution C* 500 µl

TEMED 10 µl

*** Solution C**

1M Tris-HCL, PH 6.8 50 ml per 100 ml

10 % (w/v) SDS 4 ml per 100 ml

SDS-PAGE sample buffer

60mM Tris-HCL pH 6.8 0.6ml per 10 ml

10 % (w/v) SDS 2.0 ml per 10 ml 237

50% (v/v) glycerol 5.0ml per 10 ml

14.4 mM β - Mercaptoethanol 0.5 ml per 10 ml

0.1% (w/v) Bromophenol Blue 1.0 ml per 10 ml

Deionised water 0.9ml per 10ml

Coomassie blue gel stain solution

Coomassie Brilliant Blue R250 1.0g per L

Glacial acetic acid 100.0 ml per L

Methanol 450.0 ml per L

Coomassie gel destaining solution

Glacial acetic acid 100.0 ml per L

Methanol 100.0ml per L

APPENDIX-3

LAL Chromogenic Endotoxin Quantitation Kit

Endotoxin-free-water 1 vial, 30mL

Limulus Amebocyte Lysate (LAL), lyophilized, 2vial, 1.4mL

E. coli Endotoxin standard (011: B4), lyophilized, 1 vial, 15-40 endotoxin unit (EU)/mL

Upon reconstitution 28EU/ml

Chromogenic substrate, lyophilized, 1 vial, 6.5mL/vial upon reconstitution (7mg of lyophilized substrate was reconstituted in 9 ml of in endotoxin-free water).

APPENDIX-4

Reagents used for ELISA and ELISpot

Phosphate buffer saline

Sodium Chloride (NaCl) -8.17g

Disodium Hydrogen phosphate (Na_2HPO_4) -1.12g

Mono sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) -0.22g

Then add distilled water till the 1 litre mark.

For **10 X PBS**, add 1 litre of water to 10 times the mass of the above ingredients.

PBS TWEEN (washing buffer for in-between steps)

PBS- 1 Litre

Tween-0.5 ml

PBS TWEEN + SKIMMED MILK (blocking buffer for ELISA)

2.5g of milk powder in 50ml of PBST (5%).

For **DILUENT solution**, add 5ml of blocking buffer in 50 ml of PBST.

Citrate phosphate buffer (for substrate solution)

Citric acid- 5.1g

Na_2HPO_4 -7.31g

Distilled Water-1 litre

Substrate Solution

1) 1 tablet (20mg) - add 25ml of citrate buffer and 20 μl H_2O_2 .

2) 200 μl of OPD + 5 μl of H_2O_2 + 12.5ml of citrate buffer.

Freezing solution for storing PBMCs in -150°C

For 2 vials: 1.8ml of FCS/FBS & 0.2ml of DMSO.

For 5 vials: 4.5ml of FCS/FBS & 0.5ml of DMSO.

Substrate solution for ELIspot

The solution for 1 plate

AP Conjugate substrate kit 50 µl of each (A&B)

AP development buffer 200 µl

Deionised water 4700 µl

APPENDIX-5

Reagents used for Meso scale discovery (Multiplex Cytokine Analysis)

Cytokines panel1 (human) multi-spot 96 well (10 spot plates)

Pro-inflammatory panel 1(human) multi-spot 96well (10 spot plates)

MSD Blocker B- 1g

MSD Diluent1- 50ml

Diluent 3- 5ml

MSD Read Buffer (4X) with surfactant- 50ml

Detector antibody pro-inflammatory panel 1(human) kit (calibrator blend):

Anti-human IL-2 antibody

Anti-human IL-4 antibody

Anti-human IL-10 antibody

Anti-human IL-13 antibody

Anti-human IFN- γ antibody

Anti-human IL-12P70 antibody

Cytokines panel 1 human kit (calibrator blend)

Anti-human IL-5 antibody

Anti-human IL-17A antibody

List of Capture antibodies

IFN- γ : Mouse Monoclonal ; IL-1 β : Mouse Monoclonal ; IL-2: Mouse Monoclonal ; IL-4: Mouse Monoclonal ; IL-5: Mouse Monoclonal ; IL-8: Mouse Monoclonal ; IL-10: Rat Monoclonal ; IL-12p70: Mouse Monoclonal ; IL-13: Rat Monoclonal ; TNF- α : Mouse Monoclonal.

APPENDIX-6

Isotype controls used in Flow cytometric analysis

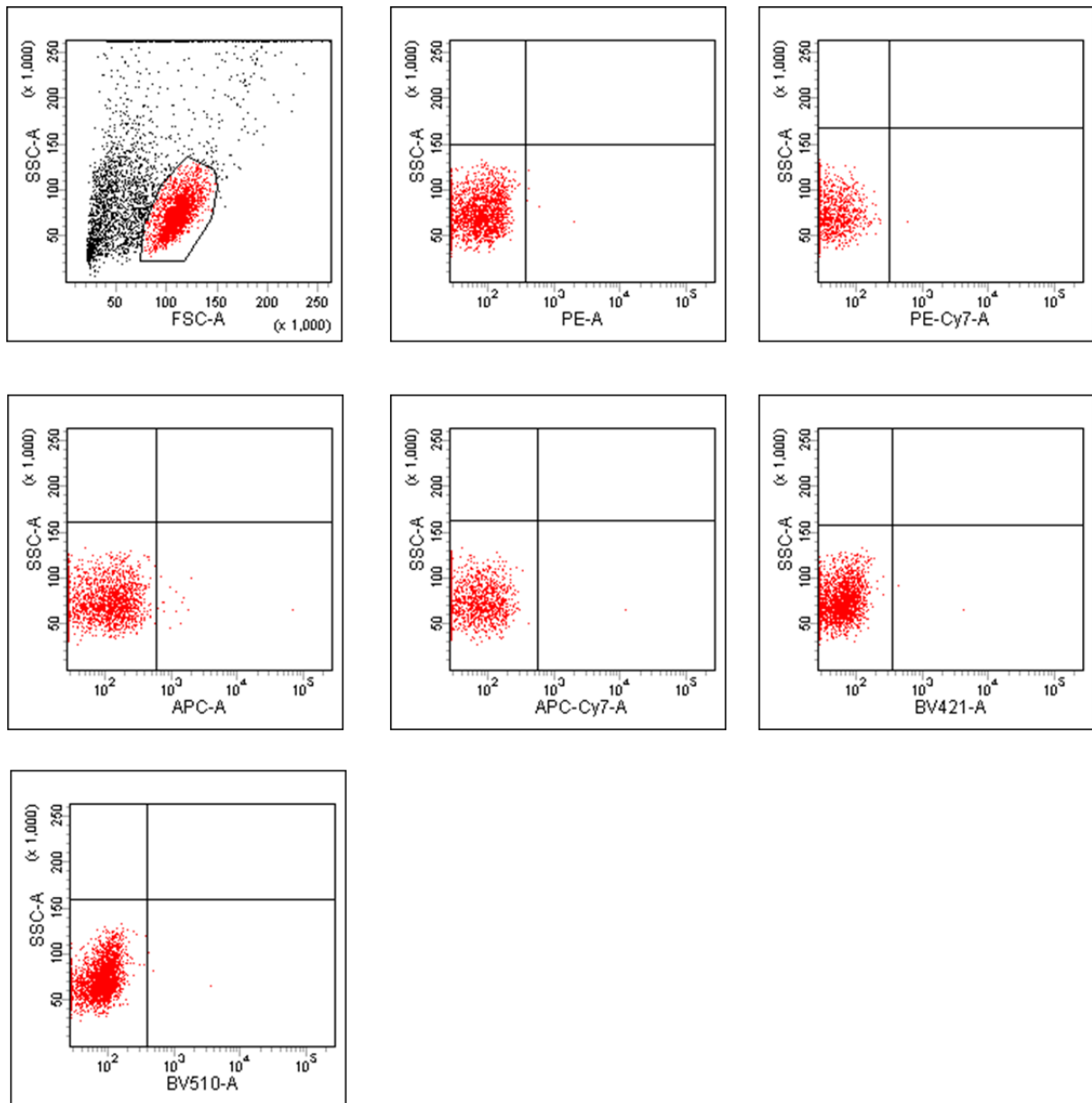


Figure A6: Isotype controls for Flow experiments. For both Myocardial Infarction and Chronic Fatigue syndrome, we first carried out a run with negative controls to measure non-specific background to primary antibodies. FSC-forward scatter, SSC- Side scatter, PE- Phycoerythrin, APC-Allophycocyanin, BV-Brilliant violet.

APPENDIX-7

List of other Cytokines tested by MSD

Three MSD panels were tested, 1). Proinflammatory panel, 2). Cytokine panel and 3). Chemokine panel. Of the panels tested, only IP-10, IL-10 and IL-16 showed an association between CMV seropositive and CMV seronegative patients. The complete list of panels tested is shown below:

<u>Proinflammatory panel</u>	<u>Cytokine panel</u>	<u>Chemokine panel</u>
IFN- γ	GM-CSF	Eotaxin
IL-1 β	IL-1 α	MIP-1 β
IL-2	IL-5	Eotaxin-3
IL-4	IL-7	TARC
IL-6	IL-12/IL-23p40	IP-10
IL-8	IL-15	MIP-1 α
IL-10	IL-16	IL-8(HA)
IL-12p70	IL-17A	MCP-1
IL-13	TNF- β	MDC
TNF- α	VEGF	MCP-4

APPENDIX-8

Peptide sequences of pathogens used for ELIspot

Peptides sequences used for the AMI and CFS study were obtained following detailed investigation from the immune epitope database and analysis resource, <http://www.iedb.org/> and some of the peptide sequences are shown below:

CMV epitopes

<u>CD8 T cell epitopes</u>	
<u>Peptide identifier</u>	<u>Peptide sequence</u>
Cpp65.495	NLVPMVATV
Cpp65.512	EFFWDANDIY
Cpp65.415	TPRVTGGGAM
HCMV3	SDEEEAIVAYTL
Cpp65.508	KYQEFFWDANDIYRI
Cpp65.363	YSEHPTFTSQY
Cpp65.341	QYDPVAALF
Cpp65.265	RIPHERNGFTVL
Cpp65.113	VYALPLKML
Cpp65.123	IPSINVHHY
Cie1.184	KLGGALOAK
<u>CD4 T cell Epitopes</u>	
Cpp65.508	KYQEFFWDANDIYRI
Cpp65.281	IIKPGKISHIMLDVAFTSHE
Cpp65.37	HETRLQLTGIHVRVS
Cpp65.41	LLQTGIHVRVSQPSL
Cul86.494	RIPHFYRVRREVPRTVN
Cpp65.415	RKTPRVTGGGAMAGA
Cpp65.115	LPLKMLNIPSINVH
Cpp65.363	YSEHPTFTTSQYRIQ GKLEYR

EBV Epitopes

<u>CD8 T cell epitopes</u>	
<u>Peptide identifier</u>	<u>Peptide sequence</u>
EBV LMP2.426	CLGGLTMV
EBV BMLF1.259	GLCTLVAML
EBV BRLF1	RVRAYTYSK
EBNA3A.603	RLRAEAQVK
EBV EBNA 4NP	AVFDRKSDAK
EBNA4.416	IVTDFSVIK
EBV	ATIGTAMYK
EBV RTA/ LRF1.28	DYCNVLNKEF
EBNA3A.247	RPPIFIRRL
EBV BZLF-1	RAKFKQLL
EBV EBNA 3A	FLRGRAYGL
<u>CD4 T cell epitopes</u>	
EBV LMP2	ICLTWRIEDPPFNSILFALL
EBNA 3C	PHDITYPYTARNIR
EBNA2	PRSTVFYNIPPMPLPPSQL
EBNA1	NPKFENIAEGLRALLARSHV
LMP2	TYGPVFMSLGGLTMVA
EBNA1.515	TSLYNLRRGTALA
EBV	PYYVVDLSVRGM

Influenza

<u>CD8 T cell Epitopes</u>	
<u>Peptide identifier</u>	<u>Peptide Sequences</u>
INFLUENZA RNA polymerase catalytic subunit	VSDGGPNLY
INFLUENZA NP	CTELKLSDY
INFLUENZA M/MATRIX 1	GILGFVFTL
INFLUENZA NP	KTGGPIYKR
INFLUENZA NP	ILRGVAHK
INFLUENZA NP	ELRSRYWAI
INFLUENZA NP	SRYWAIRTR
INFLUENZA MP1	ASCMGLIY
INFLUENZA NP	LPFDKTTVM
INFLUENZA MP1	SIIPSGPLK
INFLUENZA NP	RVLSFIKGTK
<u>CD4 T cell epitopes</u>	

FLU(NP)	RIAYERMCNILKGKF
FLU(NP)	IRPNENPAHKSQVM
FLU(NP)	RASVGKMIGGIGRFY
FLU(MATRIX 1)	AVKLYRKLKREITFH
FLU(MATRIX 1)	TKGILGFVFTLTVPS

Chlamydia

<u>T cell epitopes</u>	
<u>Peptide identifier</u>	<u>Peptide sequences</u>
ChlO.258	RLNMFTPVI
ChlP.179	ALAYGIDKV
ChlT.367	DADKYAVTV
ChlT.121	NAACMALNI

BCHP (Bkt Rota virus, Coxsackie virus, Human herpes virus 6,7 and Parvovirus)

<u>T cell epitopes</u>	
<u>Peptide identifier</u>	<u>Peptide sequence</u>
ParvoB19	GRMTENIVEVAKA
ParvoB19	LASEESAFYVLEHSSFQLLG
ParvoB19	FYTPLADQF
ParvoB19.NS1.613	GLCPHCINVG
ParvoB19.NS1.276	LLHTDFEQV
HHV6.IEAT.57	FESLLFPEL
HHV6.U14.602	TEMMNDARL
HHV6.IEAT.39	VEESIKEIL
HHV7.RdIpR.544	ENIYYSSVRT
HHV7.Gp	KEKNGDVVEL
COXB3.PP.1505	GIIYIIYKL
COXB3.PP.1587	ILMNDQEVGV
COXB3.PP.2050	YGDDVIASY
COXB4.PP.1137	EVKEKHEFL
bkT.406	VIFDFLHCIVFNVPKRR
bkVP1.108	LLMWEAVTV
bkT.604	RLDSEISMY
rota1	IIVILSPLLNAQN
rota2	TLLANVTAV

Adenovirus

<u>CD8 T cell epitopes</u>	
<u>Peptide identifier</u>	<u>Peptide sequence</u>
Adp99.492	SADNNNSEY
Ad5H37	TYFSLNKF
Ad5Ha	IPYLDGTFY
AdHb	GLRYRSMML
AdHc	LPGSYTYEW
Ad5H114	KPYSGTAYNAL
<u>CD4 T cell epitopes</u>	
Ad5H917	DEPTLLYVLFVFDV
Ad5i	IIRFDENGVLLNNSF
Ad5ii	SIALMFVCLIMWLICCLKR
Ad5iii	FQRPTISSNSHAIFR
Ad5H317	QQSMPNRPNYIAFRDNFIGL
Ad5H886	TDLGQNLLYANSAHAL

Respiratory Syncytial virus

<u>T cell epitopes</u>	
<u>Peptide identifier</u>	<u>Peptide sequence</u>
rsvG.163	FHFEVFNFVPC
rsvM.238	TTNWKHTATRFAIKPMED
rsvNP.137	KMLKEMGEV

Mycobacterium tuberculosis

<u>T cell epitopes</u>	
<u>Peptide identifier</u>	<u>Peptide sequence</u>
TB85A.200	KLIANNTRV
TB85A.12	VPSPSMGRDIKVQFQ
TB85A.27	GKAGCQTYKWETFLTSELPG
TB85A.37	YHPQQFVYAGAMSGLLDPSQ
tbMP.59	WDINTPAFEWYYPSGLSIVM
TB cons	VLVATNFFGINTIPIALN