INVESTIGATION OF T CELL RESPONSES IN MULTIPLE SCLEROSIS

BY

MUNEERA ABDULMOHSEN AL MULHIM



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Abstract

Multiple myelin proteins, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and myelin proteolipid protein (PLP), are critical T cell autoantigens in multiple sclerosis (MS). However, the contribution of these autoreactive T cells to disease pathology remains unknown.

We identified CCR6+ CD4+ myelin-reactive T cells in the peripheral blood of healthy controls where they produced low levels of inflammatory and regulatory cytokines. CCR6+ myelin reactive T cells from patients with untreated MS exhibited significantly enhanced production of interferon-γ (IFN-γ), interleukin-17 (IL-17), granulocyte-macrophage colony- stimulating factor (GM-CSF) and Tumor necrosis factor-α (TNF-α) compared to healthy controls. However, IL-10 expression was less abundant in healthy controls when compared to untreated MS patients, suggesting a role for IL-10 in regulating the pathology of MS when there is inflammation. Interestingly, the pro-inflammatory profile of CCR6+ myelin reactive T cells including GM-CSF, IFN-γ, IL-17 and TNF-α were higher in MS patients treated with IFN-β.

Our data suggest that despite the impact of IFN-β on reducing relapse rates in MS, there is little impact on the frequency of pro-inflammatory cytokine secreting autoantigen-specific CCR6+ CD4+ T cells. It remains to be established if the increased pro-inflammatory cytokine production is present early during the disease.

Dedication

To my lovely Grandmother (Muneera) and all my grandparents,

your inspiration and love will guide and surround me through my life until we meet in heaven.

My wonderful family, I am grateful for this bless. My Great parents (Abdulmohsen and Basma), my sunshine, without your love and prayers, I couldn't be here. My lovely brothers and sisters (Fatma, Abdullah, Haitham, Latifah and Sara), you were always with me.

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Abbreviations

ADCC Antibody-dependent cellular cytotoxicity

APC Antigen presenting cell

BAB Blood-arachnoid barrier

BAFF B cell activating factor

BBB Blood-brain barrier

BCR B cell receptor

BCSFB Blood-cerebrospinal fluid barrier

CCR/L CC chemokine receptor/ligand

CD Cluster of differentiation

CDR Complementarity-determining region

CIS Clinically isolated syndrome

CLP Common lymphoid progenitor

CLR C-type lectin receptor

CMP Common myeloid progenitor

CNS Central nervous system

CSF Cerebrospinal fluid

CXCL Chemokine CXC ligand

CXCR Chemokine CXC receptor

DC Dendritic cell

DNA Deoxyribonucleic acid

EAE Experimental autoimmune encephalomyelitis

EBNA-1 Epstein-Barr nuclear antigen-1

EBV Epstein-Barr virus

EDSS Expanded disability status scale

EMRA Revertant effector memory T cell

FACS Fluorescence-activated cell sorting

G-CSF Granulocyte-colony stimulating factor

GM-CSF Granulocyte macrophage colony-stimulating factor

GWAS Genome-wide association study

HLA Human leucocyte antigen

HSC Haematopoietic stem cell

ICAM Intercellular adhesion molecule

IFN-α Interferon alpha

IFN-β Interferon beta

IFN-γ Interferon gamma

Ig Immunoglobulin

IL Interleukin

MBP Myelin basic protein

M-CSF Macrophage-colony stimulating factor

MHC Major histocompatibility complex

MMP Matrix metalloproteinase

MOG Myelin oligodendrocyte protein

MRI Magnetic resonance imaging

MS Multiple sclerosis

NET Neutrophil extracellular trap

NF-kB nuclear factor kappa light chain enhancer of activated B cell

PAMP Pathogen-associated molecular pattern

PBMC Peripheral blood mononuclear cell

PLP Myelin proteolipid protein

PML Progressive multifocal encephalopathy

PPMS Primary progressive multiple sclerosis

PRR Pattern recognition receptor

RAG Recombination-activating gene

ROS Reactive oxygen species

RRMS Relapsing-remitting multiple sclerosis

SCF Stem cell factor

S1P Sphingosine-1-phosphate

SNP Single nucleotide polymorphism

SPMS Secondary progressive multiple sclerosis

TCM Central memory T cell

TCR T cell receptor

TEM Effector memory T cell

TGFβ Transforming growth factor beta

Th T helper cell

TLR Toll-like receptor

TNFα Tumour necrosis factor alpha

Treg Regulatory T cell

VCAM-1 Vascular cell adhesion molecule-1

VLA-4 Very late antigen-4

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1: General Introduction

1.1 The Immune system

The host immune system plays a fundamental role in the elimination of pathogens relying on immune surveillance and this is maintained by innate and adaptive immune systems. Different pathogens that can infect our body and cause damage, starting from very small molecules as tiny viruses, bacteria, fungal and helminthes that can be a very big and long. For all of these, there should be protection to prevent any infection and homeostasis, which means to maintain a balance. In some conditions, our immune system can attack itself and this can lead to autoimmune diseases causing harm to the body.

The ability of the immune response to avoid damaging self-tissues is referred to as self-tolerance. Long lasting damage to the body can be caused from self-antigens.

To discover effective treatments for the autoimmune diseases and other immune related diseases we have to understand the cellular and molecular foundation of an immune response.

1.1.1 The components of the Immune system

The first line of defense is the physical barrier, which includes the skin and the mucosal tissue (Gallo and Huttner, 1998). This includes soluble proteins and bioactive small molecules that are either constitutively present in biological fluids such as the complement proteins and defensins that are released from cells as they are activated including 1-cytokines that regulate the function of other cells 2- chemokines that attract inflammatory leukocytes 3- lipid mediators of inflammation, reactive free radical species, and 4- bioactive amines and enzymes that also contribute to tissue inflammation (Sjoberg et al., 2009; Lacy, 2015). Also, the innate immune system includes membrane bound receptors and cytoplasmic proteins that bind molecular patterns expressed. The complement is a primary element of the innate system. The adaptive immune system is composed of specialized cells releasing molecules such as antibodies and cytokines and retaining memory of infection that can lead the fight against re-infections (Bonillaand Oettgen, 2010).

Basically, all immune cells originate from a common precursor in the bone marrow, the pluripotent hematopoietic stem cell and mature in the bone marrow and differentiate into either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP) (Kondo, 2010). Common myeloid progenitors can differentiate to granulocytes, megakaryocytes and platelets, and to erythrocytes or dendritic cells. Cells of the granulocyte lineage that play prominent immune functions include neutrophils, monocytes, macrophages, eosinophils, basophils, and mast cells. Common lymphoid progenitors include T, B, or natural killer (NK) cells that are the adaptive immune cells (Weiskopf et al., 2016).

Some of the important myeloid cells of the innate immunity and their function are summarized in Table 1.1.

Lineage precursor	Cell	Niche signal	Function
Common myeloid progenitor	Neutrophil	GM-CSF G-CSF IL-3 IL-4	Phagocytic activity Produce large quantities of reactive oxygen species (ROS) that are cytotoxic to bacterial pathogens Degranulation and release of NET's to trap and kill microbes Production of TNF and IL-12
	Basophil	GM-CSF IL-3 IL-4	Induction of allergic response Protection from parasitic infection through histamine production Production of IL-4
	Eosinophil	GM-CSF IL-3 IL-5	Protection from parasitic infection Degranulation to release cytotoxic inflammatory mediators including cytokines and leukotrienes,
	Macrophages	GM-CSF M-CSF	Highly phagocytic Antigen presentation Production of monokines (CSF, IL-1, IL-6, IL-12, IFN-α, IFN-β, IFN-γ,TNF-α)
	Dendritic cells	GM-CSF IL-4	Most potent Antigen presenting cell (APC)

Table 1.1 Summary of function of remarkable innate immune cells

Table includes some important cytokines and growth factors involved in the differentiation of each lineage. G-CSF; granulocyte colony stimulating factor, GM-CSF; granulocyte macrophage-colony stimulating factor, M-CSF; macrophage-colony stimulating factor, SCF; stem cell factor, IL-1, IL-3, IL-4, IL-5, IL-6; Interleukin family members, TNF-α; Tumour necrosis factor alpha, IFN-α and IFN-β; IFN-γ, Interferon alpha, beta and gamma, ROS; Reactive oxygen species, NET; Neutrophil extracellular trap.

1.1.2 Innate immunity

Innate immune responses are relatively non-specific defense mechanisms, which are rapid and independent of specific antigen. One of the characteristic features of innate immune cells is the ability to recognize molecular motifs, which lead to an appropriate molecular response. This is done by pattern recognition receptors (PRRs), which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs) (Iwasaki and Medzhitov, 2015). Some classes of PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and cytosolic deoxyribonucleic acid (DNA) receptors (CDRs) (Thompson et al., 2011). PAMP's include double stranded RNA and unmethylated CpG DNA motifs (viral), lipopolysaccharide (LPS) for gram negative and lipoteichoic acid for gram positive (bacterial) and β-glycan and mannan (fungal). When PRR signalling is triggered, an intracellular signalling cascade is initiated which results in the release of several inflammatory mediators which are recognised by receptors expressed by various immune cell types to clear the pathogen (Takeuchi and Akira, 2010). Complement system play an essential role by the binding of acute phase response proteins (C-reactive protein or mannose-binding lectin) directly to the bacterial surfaces. Proteins are elevated in inflammation and the resulting proteolytic cascade of complement products which enhance phagocytosis (C3b protein), act as a chemo attractant for other innate immune cells, activate eosinophils and basophil degranulation to form a membrane attack (Arumugam et al., 2006, Nesdargikar et al., 2012).

Natural killer (NK) cells are a type of cytotoxic lymphocytes that differentiate from a common lymphoid progenitor; however, they are classified as innate cells as they

respond to infections. When activated, they have the morphology of a large granular lymphocyte. They develop in the bone marrow under the influence of IL-2, IL-15, and bone marrow stromal cells (Iannello and Raulet, 2013; Sharma et al., 2017). They represent a small fraction of peripheral blood cells and a small fraction of lymphoid cells in the spleen and other secondary lymphoid tissues and they don't have an antigen-specific receptors (Vivier et al., 2011).

NK cells secrete interferon gamma (IFN-γ), tumor necrosis factor (TNF-α) and release lytic granules to kill the infected cells (Wang et al., 2012). They express activator and inhibitory receptors, such as NKG2D (activator) or killer-cell immunoglobulin-like receptors (KIRs-inhibitor) (Germain et al., 2011). The CD161 (cluster of differentiation 161) is an inhibitory NK cell receptor (also known as killer cell lectin-like receptor, B1) expressed on the majority of NK cells, when activated by its ligand, lectin like transcript 1 (LLT1), inhibits NK cell cytotoxicity and IFN-γ secretion (Cosmi et al., 2008).

1.1.3 Adaptive immunity

In contrast to innate immunity, adaptive immunity, also called acquired immunity, is specific and provides long lasting protection. It is mediated by B and T lymphocytes following exposure to specific antigen and characterized by immunological memory. Innate immune cells in some cases cannot clear the infection, so the adaptive immune cells will be vital to remove it and protect from future infection.

Recombinase enzymes are mandatory for the adaptive immune cells to rearrange gene segments in their pathogen recognition receptors.

Cells of the adaptive immune system express an antigen receptor with a single specificity which is produced through a process of rearrangement of a variety of

different gene segments that code for different parts of the receptor. This allows it to code for millions of different combinations of the same receptor, which gives the specificity for the adaptive immune system by ability to recognise different pathogens and a great repertoire of antigens. Proliferation and effector function will happen when only cells meet an antigen that their receptor recognises with adequate affinity.

Adaptive immunity includes humoral immunity that is mediated by antibodies produced by B lymphocytes, and cell-mediated immunity which is mediated by T lymphocytes (Chaplin, 2010). B and T cells are two major cell types in the immune system. T cells will be discussed in the following chapters with a focus on their differentiation.

B cells constitute approximately 15% of peripheral blood leukocytes and they are defined by their production of immunoglobulin (Ig). Ig molecules are composed of two identical 50 kDa heavy chains and two identical 25 kDa κ or λ light chains.

B cells differentiate from hematopoietic stem cells in the bone marrow. Their antigen receptors (surface Ig) B cell receptor (BCR) are assembled from genetic building blocks in a recombination-activating gene (RAG1/ RAG2) mediated process similar to TCR production. When B cell is activated, it proliferates and differentiates into plasma cells, which produce high quantities of the soluble form of the BcR, known as an antibody (Ab). Abs will bind the antigen and act either as a neutralising agent or will opsonise the pathogen (Merle et al., 2015; Market and Papavasiliou, 2003).

Beside plasma cells, there are memory B cells that are activated cells and they are long lived and are rapidly activated when they encounter the specific antigen, similar to memory T cells. Immature B cells express CD19, CD20, CD34, CD38 and CD45RA but not IgM. For most mature B cells, they express IgM and CD19 (Nutt et al., 2015).

B cells also include regulatory B cells as immunosuppressive cells that stop the expansion of pathogenic pro-inflammatory lymphocytes through secretion of IL-10, IL-35 and transforming growth factor beta (TGFβ) (Sokolov et al., 2018).

1.2 General view of multiple sclerosis (MS)

Multiple sclerosis (MS) is a chronic neurodegenerative disease driven by autoimmune responses and inflammation of the central nervous system (CNS) (McFarland and Martin, 2007; Nylander and Hafler, 2012).

In 1868, the relation of demyelinated lesions in the brain to neurological dysfunction was noticed (Komar et al., 2011). Autoreactive T-cells that recognize and react against autoantigens, including myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocytic glycoprotein (MOG) and myelin-associated glycoprotein (Hemmer et al., 2002).

The disease process is influenced by multiple factors, including environmental effects such as UV light from sun exposure, which contributes to vitamin D synthesis; genetic factors such as human leukocyte antigen (HLA) genes; and infectious agents including viruses. There are other factors that can also be correlated with MS, such as microbial factors. The EBV virus is one virus that may contribute to MS (Dendrou et al., 2015)

Diagnosis of MS depends on taking a patient's medical history, performing magnetic resonance imaging (MRI) of the brain and taking a histopathological picture, if available (Agrawal, 2007). Blood and CSF samples are also taken to investigate the cells and the markers that are characteristics of MS (Hohlfeld et al., 2016).

1.2.1 MS prevalence

According to the MS Society in the United Kingdom, MS has a significant effect on the lives of 2.5 million people in the world and 100,000 in the United Kingdom. Most people are diagnosed between the ages of 20 and 40, but it can affect younger and older people, too. Females are more susceptible than males (a ratio of 3 to 1), which could be due to hormonal effects, although the exact reasons are still unclear (Bostrom et al., 2013).

The incidence of MS varies with geography and with ethnicity. It is more frequent in high latitude regions and northern European populations, but it is relatively low in black Africans. That might be related to the protective role of Vit D produced by the skin in response to sunlight and in diet (Goldberg, 1974; Gelfand et al., 2011; O'Gorman et al., 2012).

1.2.2 Clinical symptoms and disease phenotypes

Symptoms vary from one individual to another depending on the location of their lesions, but they generally include visual impairment, muscle weakness, fatigue and coordination problems (McFarland and Martin, 2007). There are different phenotypes of MS, each of which represents clinical features that vary among patients (Fig. 1.1)

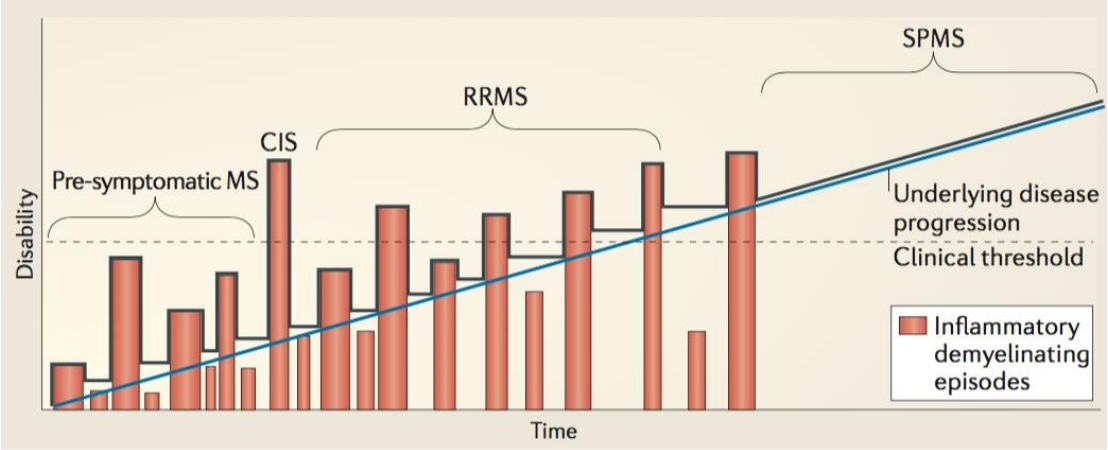


Figure 1.1 Clinical phenotypes of MS. Disease activity that can occur over time, MS can be variable in nature. Disability gradually increases over time. Disease can be divided into subtypes. Adapted from (Stys et al., 2012).

The disease usually starts with an acute episode, which lasts for few weeks and is known as clinically isolated syndrome (CIS) according to the revised McDonald criteria (Thompson et al., 2018). This could be the first sign of MS but does not necessarily proceed to clinically definite MS. Brain MRI is useful to detect if CNS lesions are

disseminated in space which rare affecting separate regions in the CNS and in time which persist for long period in the brain (Inglese, 2006; Polman et al., 2011).

The majority of CIS patients have immunoglobulin (IgG) oligoclonal bands in the CSF and a high IgG index, indicating the presence of plasma cells or plasmablasts within the CNS. Most of MS patients will develop relapsing-remitting multiple sclerosis (RRMS) when the symptoms largely resolve between episodes and the CNS goes back towards its normal baseline. New lesions often appear on the brain after a relapse (Compston and Coles, 2008). RR-MS frequently develops into secondary progressive (SPMS), which is characterized by progressive neurological impairment due to a loss of neurons (Disanto et al., 2010; Nylander and Hafler, 2012).

A low percentage of MS patients may develop primary progressive disease (PP-MS), where there will be a weakness in neurological functions without a relapsing and remitting disease course (Miller and Leary, 2007). Benign MS, which is characterised by minimal disability accumulation as measured by the expanded disability status scale (EDSS) can be found in some MS patients (Hawkins and McDonnell, 1999; Pittock et al., 2004).

Imaging studies have revealed differences between RR-MS and PP-MS. In patients that suffer from RR-MS, acute CNS lesions with spontaneous resolution are frequently detected, even in the absence of clinical attacks. These lesions are usually located in areas of white matter and are often characterized by a disturbance of the blood–brain barrier and demyelination. Severity of the symptoms can vary depending on the extent of location of the nerve damage (Dutta and Trapp, 2015)

Radiologically isolated (RIS) syndrome is defined where incidental imaging findings suggest inflammatory demyelination in the absence of clinical signs or symptoms

(Lublin et al., 2014). RIS was defined by (Okuda et al., 2009). It is not an MS subtype since there is by definition no clinical evidence of demyelination detected in patients. Around two-thirds of patients show radiological progression and one-third develop neurological symptoms within 5 years (Yamout and Al Khawajah, 2017).

1.2.3 Risk factors

The underlying mechanisms leading to MS are not clear, but there are many factors related to MS as genetics, infectious and environmental factors (Fig. 1.2).

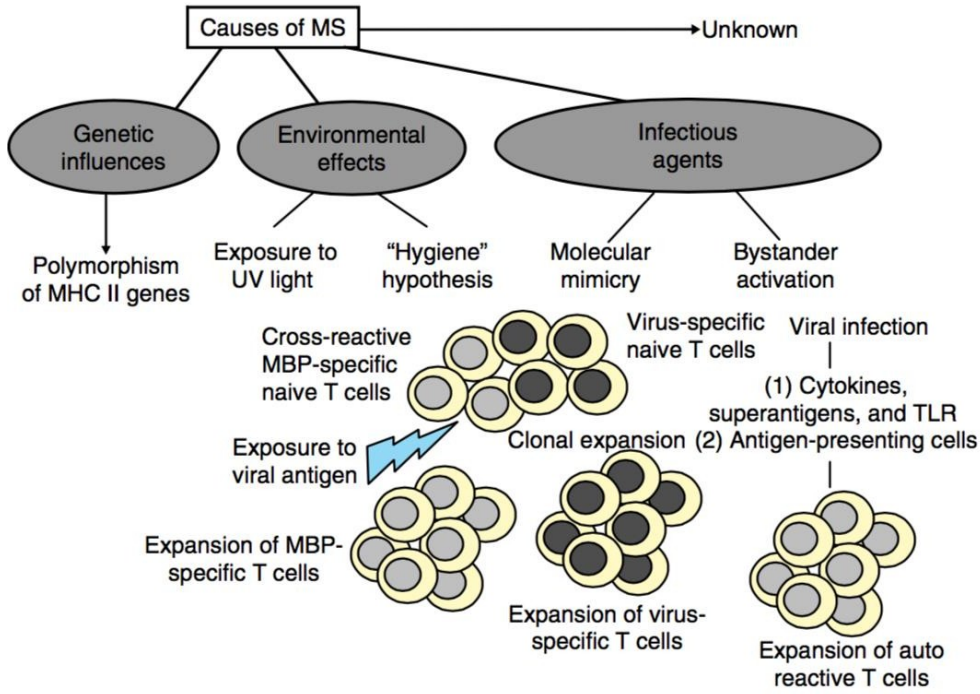


Figure 1.2. Etiology and mechanism of MS. Several factors influence the onset of MS. Genetic, environmental and infectious agents all contribute to the causes of MS. Adapted from (Agrawal, 2007).

1.2.3.1 Genetics

Although MS is not an inherited disease, there are genetic associations with MS (Baranzini and Oksenberg, 2017).

The concordance rate of 15–25% in monozygotic twins suggests that non-genetic factors can also affect disease susceptibility (Hollenbach and Oksenberg, 2015; Sawcer et al., 2014). Only 2% chance of a child to develop the disease with parent affected with MS (Compston and Coles, 2002). Usually MS results from gene polymorphism rather than gene mutation (Agrawal, 2007).

Genome wide association studies (GWAS) are essential to understand the pathogenesis of MS where they analyse the whole genome of patients for small nuclear polymorphisms (SNP), that is a change in the nucleotide sequence of genes, which are common in MS patients. They have identified more than 200 common genetic variants SNPs associated with MS that are linked to several HLA genes, HLA is a complex of genes on the short arm of chromosome 6 (Lincoln et al., 2005; Alcina et al., 2012; Schmidt et al., 2007). Some HLA polymorphisms increase susceptibility to MS, including DRB1*0401 and DRB1*1501 (Wallstrom et al., 1998; Rioux et al., 2009; Raddassi et al., 2011; Hollenbach and Oksenberg, 2015; Cao et al., 2015). Patients with DRB1*15:01 develop more lesions (T2 foci) on MRI and a decrease in brain volume (Okuda et al., 2009). It has been shown in some studies that DR15 is more common in females with MS and predisposes to a younger age at onset; this could be due to alterations in immune function (Hensiek et al., 2002).

It has previously been suggested that T cells from DR15 positive patients produce increased quantities of TNF-α that has a role in inflammation and demyelination (Kemanetzoglou and Andreaou, 2017).

HLA-A SNP rs6457110, which is major histocompatibility complex (MHC) class I

are also associated with MS susceptibility (Jafari et al., 2010).

HLA-DRB1*0401/MOG peptide tetramers can differentiate between MS patients and healthy patients by detecting antigen-reactive CD4+ T cells (Raddassi et al., 2011).

There are also other gene polymorphisms that are related to MS susceptibility, including HLA A, HLA B, IL2RA, IL7R, CD58, CD226, CD6, CD25, CD40 and IFR8 (Sawcer et al., 2011; Beecham et al., 2013; Didonna and Oksenberg, 2015; Miyazaki and Niino, 2015; Hafler et al., 2007; Kofler et al., 2011).

In 2011, a study by GWAS and following meta- analysis comprising and GO analysis on the 79 MS risk genes highlighted the categories "leukocyte activation," "apoptosis," and "positive regulation of macromolecule metabolic process" as well as the KEGG pathways the Janus Kinase/Signal Transducer and Activator of Transcription "JAK-STAT signaling pathway," "acute myeloid leukemia," and "T cell receptor (TCR) signaling" (International Multiple Sclerosis Genetics Consortium, 2013). (JAK/STAT) signaling pathway mediates the biological activities of different cytokines including interleukin-12IL-12, IL-6, IL-17, IL-21, IL-23, granulocyte macrophage-colony stimulating factor (GM-CSF) and interferon-gamma (IFN-γ) and is essential for the development and regulation of immune responses. Dysregulation of the JAK/STAT pathway contributes to numerous autoimmune diseases, including MS/Experimental autoimmune encephalomyelitis (EAE) (Benveniste, 2014).

Extending pathway analysis to all the 110 non-*MHC* variants identified after the ImmunoChip study detected the (nuclear factor kappa light chain enhancer of activated B cell) NF-kB cascade to be significantly associated with MS risk genes (International Multiple Sclerosis Genetics Consortium, 2013; Hussman et al., 2016). In MS, the pro-inflammatory NF-κB signaling pathways are out of balance and it contributes to MS pathogenesis and could represent a target for therapeutic intervention (Leibowtis and Yan, 2016)

In a recent paper, a gene network candidate approach has highlighted the putative role of cellular adhesion molecules (CAMs) in MS pathology (Damotte et al., 2014). By using eight

GWAS datasets and considering all the genes interacting in the CAM pathway, five sub-networks were found associated with MS susceptibility, possibly connecting the risk to the regulation of blood–brain barrier (BBB) crossing by T cells.

More research is needed to understand how these genes can impact the pathogenesis of MS.

1.2.5 Infectious agent

It has been frequently suggested that viral infections are triggers of MS (Agrawal and Yong, 2007; Goverman, 2009). In (EAE) models, animals under germ-free conditions were resistant to disease, while those exposed to commensal bacteria developed EAE (Berer et al., 2011). Different viruses as rabies, herpes simplex virus, measles, virus, Epstein-Barr virus (EBV) and others could be related to MS (Virtanen and Jacobson, 2012). EBV is one of the viruses that have the greater risk of developing MS if the person had late onset infectious mononucleosis, the evidence for prior EBV infection in adult onset MS is essentially 100% (Goodin, 2009).

Many studies implicate EBV in the pathogenesis of MS. This include High anti-EBV antibody levels, alterations in EBV-specific CD8+ T-cell immunity, increased spontaneous EBV- induced transformation of peripheral blood B cells, increased shedding of EBV from saliva and accumulation of EBV-infected B cells and plasma cells in the brain (Pender and Burrows, 2014).

Several mechanisms have been hypothesized to explain the role of EBV in the development of MS including 1- cross-reactivity between EBV and CNS antigens, 2- bystander damage to the CNS by EBV-specific CD8+ T cells, 3- activation of innate immunity by EBV-encoded small RNA molecules in the CNS, infection of autoreactive B cells, which produce pathogenic autoantibodies and provide co-stimulatory survival signals to autoreactive T cells in the CNS (Pender, 2011). This could help to prevent and cure MS by controlling EBV infection through vaccination, antiviral drugs or treatment with EBV-specific cytotoxic CD8+ T cells.

DRB5*0101 and DRB1*1501, which present the EBV peptide, have been recognized in the T cells of MS patients (Hohlfeld et al., 2016; Starautins; 2014).

DRB1*1501 presented peptide (MVFLQTHIFAEVLKD) was identified by specific T-cell clones; that was $CD4^+$ T-cell clone killing EBV- infected T cells positive for DR51 and EBV-infected NK cells transduced with DRB5*0101 (Okamura et al., 2008).

Variances between CD4+ and CD8+ responses to EBV Ags have been found by applying T-cell clones to Ags. CD8+ T cells usually recognise HLA-class I–specific epitopes, while, on other hand, $CD4^+$ T cells recognize HLA-class II epitopes (Long et al., 2011).

One of the tetramers (DRB1*04:01) that I examined along with the myelin peptides was an epstein-barr nuclear antigen-1 (EBNA-1). In our study, the specificity of these tetramers was investigated and approved in MHC-II–matched alleles by detecting antigen-specific CD4+ T-cell.

1.2.6 Environment

Epidemiological studies suggest that environmental factors significantly contribute to the development of MS. Increased prevalence of MS are associated to low sunlight exposure and the defeciency of vitamin D and melatonin levels in countries far from the equator (Ascherio and Munger, 2007; Farez et al., 2015). This could be by its ability to promote T-regs and suppress Th1 and B cells (Nishikawa et al., 2006). Increased vitamin D levels, especially before the age of 20, are associated with a reduced risk of MS in later life (Munger et al., 2006). Cigarette smoking could be linked to both MS risk and disease by driving T-helper 17 (Th17)-type responses in the lung (Wingerchuk, 2012; Hedstrom et al., 2016; Hedstrom et al., 2011).

1.2.7 Hormones

It has been proven that female is more susceptible to MS than male. Sex hormones may play a role in MS. Women with MS tend to have fewer symptoms during pregnancy, a time when estrogen levels spike, which suggest the effect of sex hormones. Mechanisms of action include both immunomodulatory and neuroprotective pathways (Nicot, 2009).

More studies are needed to understand the exact cause of the higher incidence of MS in females.

In summary, all these findings suggest that the risk of developing MS is mainly due to genetic factors and that disease initiation is driven by CNS extrinsic factors.

1.3 Immunopathology of MS

1.3.1 Pathogenesis

Immune cells, specifically T cells (CD4+) are considered central to MS, CD8+ T cells and other immune cells are involved in the pathogenesis of the MS process by infiltrating through the blood-brain barrier and leading, via activation of macrophages, to demyelination and destruction of axons in the brain (Friese and Fugger, 2005; Salou et al; 2015; Dendrou et al., 2015; Filippi et al., 2018).

The mechanisms by which astrocytes and macrophages/microglia drive the neurodegenerative process are summarized in Fig. 1.3.

Autoreactive T cells against myelin components exist in normal individuals and in these individuals, do not cause disease and may even have brain-protecting properties

MS is triggered when pathogenic Th17- and Th1-type and CD8 myelin autoreactive T cells are induced (Allan et al., 2018).

CNS lesions are a hallmark of MS and represent sites of demyelination, which are associated with inflammatory immune cell infiltrates. It is well known that MS affect the white matter in the brain entirely (Tillema and Pirko, 2013).

In progressive MS, the lesions gradually expand overtime with macrophage only at the periphery of the lesion (Zrzafy et al., 2017)

EAE is an animal model for MS. CNS antigens including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) are all strong encephalogenic antigens.

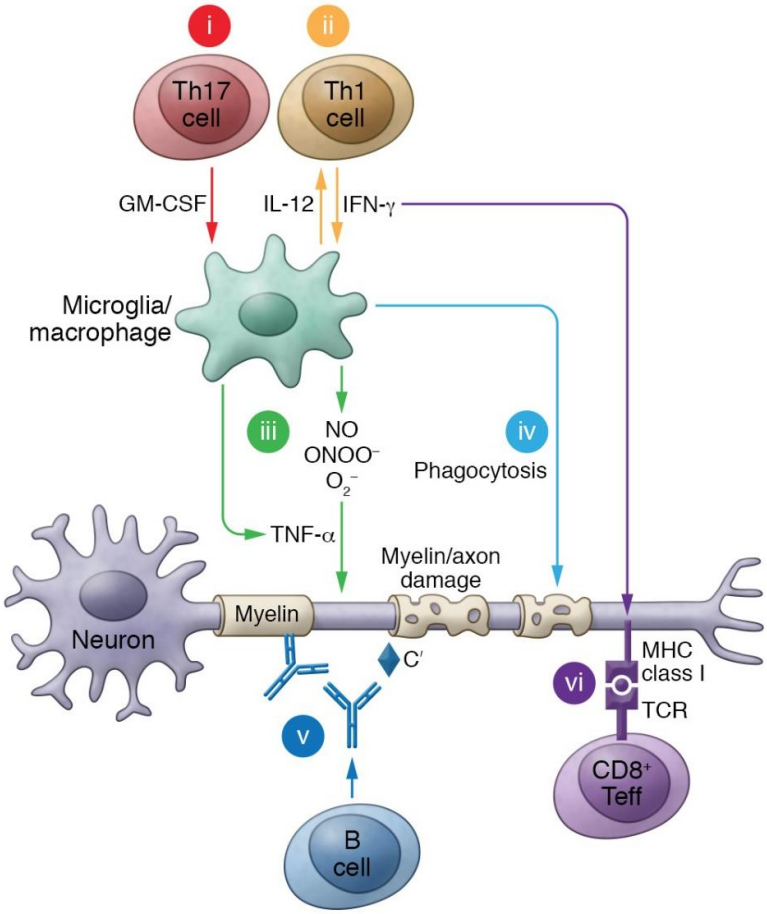


Figure 1.3. Demyelination mechanism in MS. During MS and EAE, axonal damage and demyelination are initially mediated by the inflammatory response within the CNS. (i) CD4+ Th17 cells produce GM-CSF, which activates macrophages and microglia. (ii) CD4+ Th1 cells invade the CNS and produce IFN-γ, which activates macrophages and microglia to produce the cytokine IL-12 (the major promoter of Th1 cytokine production). (iii) Macrophages and microglia also produce nitric oxide (NO), peroxynitrite (ONOO-), and superoxide (O_{2}-), which are each capable of mediating cellular damage. This capability is enhanced by microglia- and macrophage-derived TNF-α production. (iv) Activated macrophages and microglia may also consume damaged myelin sheaths and axons. (v) B cells produce antibodies that bind to myelin sheaths and may promote complement-mediated damage (C). (vi) IFN-γ upregulates the expression of MHC class I by resident CNS cells, potentially inciting a CD8+ cytotoxic T cell response. Adapted from (Chitnis and Weiner, 2017).

1.2.4 Lymphocyte migration into the CNS

Cells have limited access to the CNS due to the tight junction between the endothelial cells of the blood-brain barrier and the epithelial cells of the blood CSF barrier. The CNS is considered an immune-privileged environment. Immune surveillance can be carried out by memory and activated T cells as they express adhesion molecules and chemokine receptors. Chemokines expressed on T cells and they are crucial for cell migration, they are able to bind to several receptor, chemokine receptor signaling results in cell chemotaxis (Wilson et al., 2010).

Three major access sites for leucocytes have been identified for CNS entry; the blood-arachnoid barrier (BAB), the (BBB) and the blood-CSF barrier (BCSFB) (Fig. 1.4)

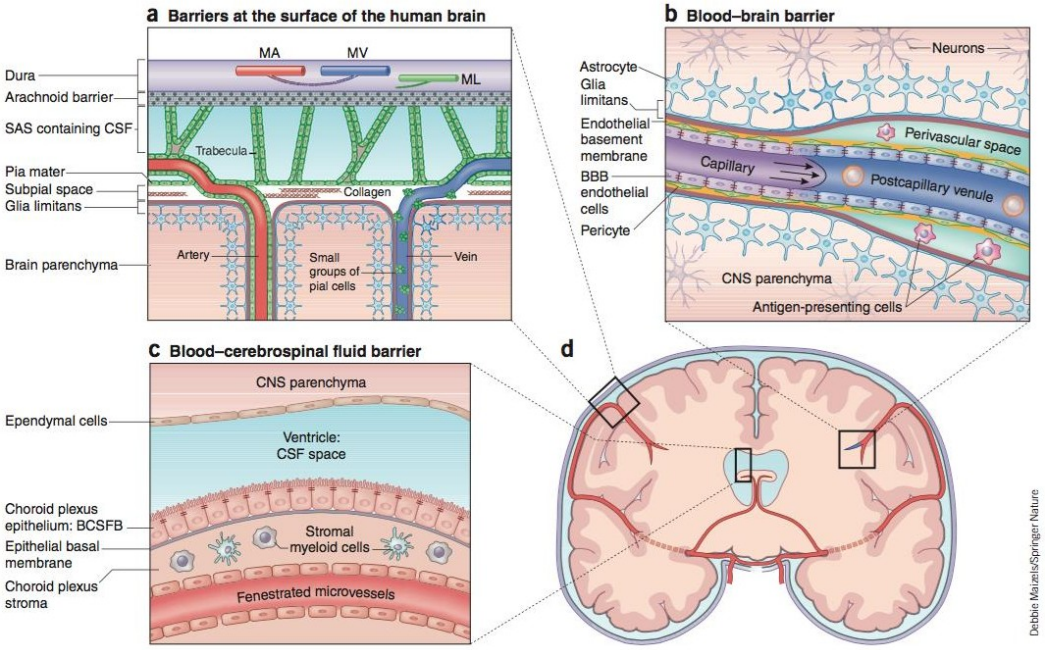


Figure 1.4 Anatomy of CNS barriers. a) Barriers at the surface of the human brain. Dura mater is supplied by meningeal arteries (MA) and veins (MV), and dural microvessels lack a BBB; the dura mater also contains lymphatics (ML). The dura is immediately adjacent to the arachnoid mater, which forms the outer BCSFB of the SAS. Sheet-like trabeculae cross the SAS to the pia mater and to the leptomeningeal arteries and veins. The subpial space contains bundles of collagen. The pia mater coats veins and arteries in the SAS and separates the SAS from the subpial space, and a sheath of pia mater surrounds arteries entering the brain. The glia limitans forms a barrier at the surface of the CNS parenchyma and around blood vessels. (**b**) The blood–brain barrier. The capillary BBB is the site of controlled transport of fluids and solutes into the CNS, whereas extravasation of immune cells into the CNS parenchyma occurs at the level of postcapillary venules, the vascular segments into which blood flows after passing through the capillaries. Highly specialized BBB endothelial cells are coated by a basement membrane formed by the fusion of endothelial and glia limitans basement membranes. Pericytes are embedded in the endothelial basement membrane. CNS microvessels are surrounded by the glia limitans, which is composed of the parenchymal basement membrane and astrocyte end-feet. At the capillary BBB, the endothelial and parenchmyal basement mebranes fuse; at the level of the postcapillary venules, the adjacent basement membranes leave a virtual perivascular space in which occasional APCs are embedded. (**c**) The blood–CSF barrier of the choroid plexus. Choroid plexus epithelial cells establish the BCSFB. The choroid plexuses are localized in the ventricles of the brain and produce CSF. The choroid plexus stroma harbors DCs and macrophages and is supplied by a dense microvascular network that does not establish a BBB; instead the epithelial cells of the choroid plexus are connected by unique parallel running tight junctions and express efflux pumps. (**d**) Schematic coronal brain section depicting the localization of the brain barriers. Adapted from (Engelehardt et al., 2017).

Endothelial cells of the brain do not express the adhesion molecules that are required for the adherence of activated T cells. When there is inflammation, activated T cells enter the CNS, as T cells can cross the blood-brain barrier (Ransohoff et al, 2003). It has been shown that in EAE models, CCR6 facilitates the entry of T cells to CNS. It is in the subarachnoid space (SAS), which is the area between the arachnoid and pial membranes where CSF circulates, $CD4^+$ T cells have been activated previously are reactivated by antigen presenting cell (APC) MHC-II, which lead to T-cell proliferation in the SAS (Reboldi et al., 2009; Kivisakk et al., 2009). The mechanism involves adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 (CD106) and intercellular adhesion molecule (ICAM)-1 (CD54), which are expressed on the apical surface of the choroid plexus and are not available to T cells (Figure 1.5). CCR6-expressing Th17 cells may cross the BCSFB through binding CCL20 in animal models that is expressed by the choroid plexus epithelium (Vajkoczy et al., 2001; Alt et al., 2002; Reboldi et al., 2009). CCR6+ Th17 and CCR6+ Th1 cells were absent from the CNS in the initial stage. Very late antigen-4 (VLA-4)-α4β1 is involved in T cell migration into the brain parenchyma, which led to the discovery of natalizumab, a monoclonal antibody against VLA-4 intergrin (Wilson et al., 2010).

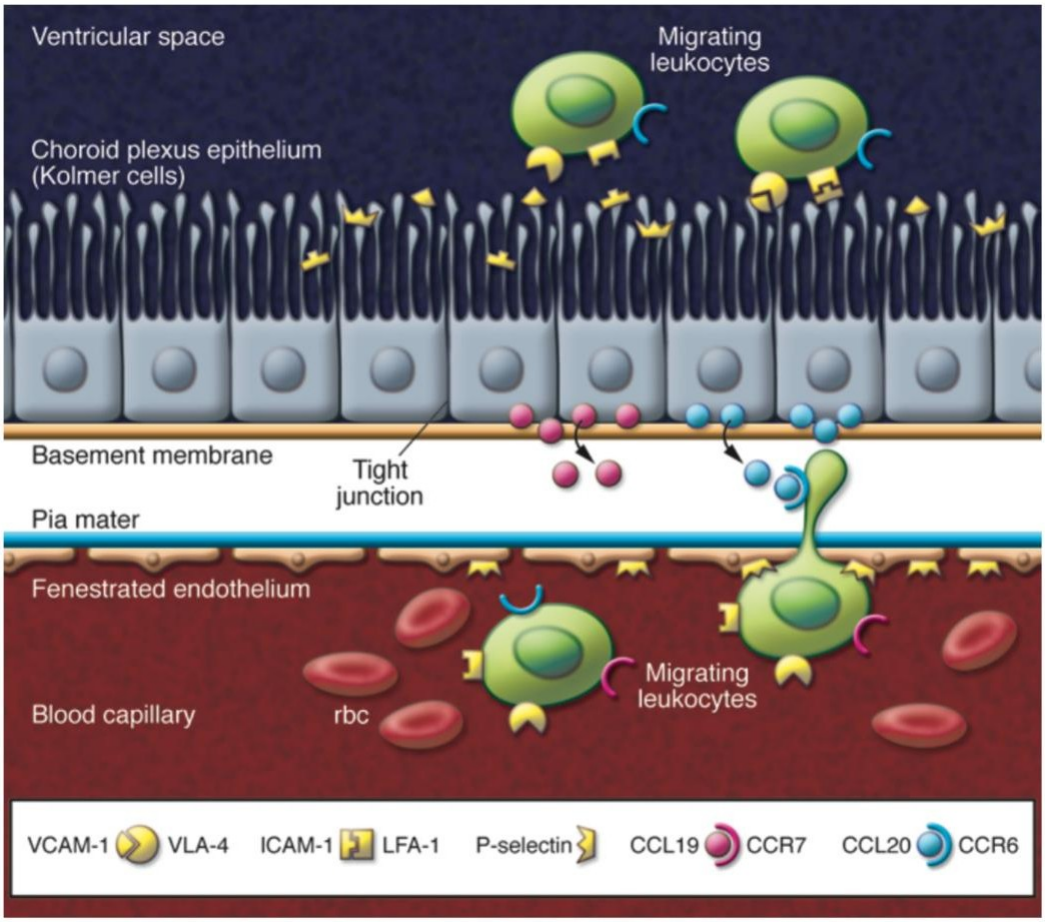


Figure 1.5 Adhesion molecules facilitate leukocyte migration. Leukocyte from the blood under the influence of chemokine undergoes adhesion, rolling and diapedesis across the fenestrated capillary endothelium and pia matter of choroid plexus. Infiltrating leukocytes migrating through epithelial cells enter the ventricles that contain CSF and circulate around CNS. The chemokine CCL20 is expressed on the basolateral side of the choroid plexus epithelia cells attracting CCR6 expressing CD4+ T cells. Adapted from (Wilson et al., 2010).

The CNS is protected from cellular infiltration by the blood-brain barrier, which surrounds the parenchymal venules, and to a lesser degree by the blood CSF barrier, which surrounds the choroid plexus, therefore there has been a focus on understanding the mechanisms by which inflammatory cells enter the CNS in EAE and MS. Although MS is considered to be a T-cell-mediated disease, the dramatic effects produced by anti-CD20 therapy (rituximab and ocrelizumab) in MS demonstrate a central role for B cells in its pathogenesis (Greenfield and Hauser, 2018).

The mechanisms of antigen recognition differ between B cells and T cells: APCs cells usually recognise antigens by direct binding, while T cells recognise HLA-peptide protein antigens, which are processed by antigen-presenting cells (Hohlfild, 2016). Antigen-presenting cells that present myelin antigens reactivate the T cells, resulting in inflammation. Long-lasting immune protection requires the memory T cells which evolved from the naive T cells that differentiate to the central memory, effector memory (TEM) and more recently, stem cell memory (Oliveira et al., 2015). Upon secondary infection or vaccination, memory T cells come into existence and persist for several years.

1.3 T cell involvement in MS

The bone marrow in the human body contains pluripotent hematopoietic stem cells (HSCs), which differentiate into lymphoid or myeloid progenitor cells depending on cytokine stimulation. After cytokine stimulation, the myeloid progenitors differentiate to become one of the following cell types; granulocytes (neutrophil, eosinophil or basophil), monocytes, red blood cells or platelets. Lymphoid progenitors differentiate to T cells or B cells or NK cells (Kondo et al., 1997).

1.3.1 T cell development and differentiation

T cells derived from hematopoietic stem cells in the bone marrow. Progenitors migrate to the thymus, where they maturated and identified based on the expression of surface markers. Majority of cells in the thymus are αβTCR where 5% are γδTCR. T cell progenitors enter the thymus through blood vessels at the corticomedullary junction and then subsequently localize to the outer regions of the thymic cortex, where they undergo rearrangement of the TCR α and β loci (Kurd and Robey,

2016). The earliest developing thymocytes are double negative, which lack the expression of CD4 and CD8 co-receptor (Zuniga-Pflucker, 2004).

In the thymus, T cells develop following the gene segment recombination and produce a functional T-cell receptor. Thymocytes are then selected for those that with a TCR can bind to self-MHC, while removing any autoreactive T cells that bind self-peptide and self-MHC with a high affinity. Following successful TCRβ gene rearrangement and preTCR signaling, thymocytes progress to the CD4+CD8+ double positive (DP) stage, migrate to the thymic cortex, and rearrange their TCRα locus (Klein at al, 2014; Ladi et al, 2006; Takahama, 2006). Cortical DP thymocytes that experience αβTCR signals in response to self-peptides presented by MHC can undergo positive selection, resulting in maturation and commitment to either the CD4 or CD8 lineage, depending on whether the selecting MHC was class II or class I, respectively (Starr et al., 2003, Wieczorek et al., 2017).

Positive selection also leads to the relocalization of thymocytes from the cortex to the medulla, and the majority of medullary thymocytes exhibit a CD4+CD8- or CD4-CD8+ "single positive" SP phenotype. After several days of further maturation in the medulla, SP thymocyte become competent to leave the thymus as fully functional mature T cells (Hogquist et al., 2015). Naive T cells in the periphery can differentiate into a number of phenotypes. Upon recognition of their specific antigen through the T-cell receptor (TCR) with the appropriate co-stimulatory signals, T cells clonally expand and traffic to tissues where they perform effector functions as cytokine secretion and killing of infected T cells.

Most of the expanded T cells removed by apoptosis, except residual populations remains and become memory cells. Memory T cells include central memory (CM)

circulating lymph nodes and effector memory resident in tissues which have the capacity to respond to secondary antigen encounter (Pennock et al., 2013; Nikolich-Zugich, 2008).

In the parenchymal brain tissue of MS patients, CD8+ memory T cells are the dominant phenotype, whereas in EAE, it is CD4+ memory T cells. Memory T cells can be classified into central memory T cells (CD44hi CD62L hi CCR7+) (mouse) which are located in the lymphoid organs, whereas the non- lymphoid organs have the effector memory T cells (CD44hi CD62low CCR7-).

A similar distinction is found in humans; naive T cells are CD45RA+ CD45RO- CCR7+ CD62L+, central memory T cells are CD45RA- CD45RO+ CCR7+ CD62L+, effector memory T cells are CD45RA- CD45RO+ CCR7- CD62L- and effector memory RA cells are CD45RA+ CD45RO- CCR7- CD62L- (Hohlfeld et al., 2016).

T cell lineage include CD8+ T cells mediating direct T cell lysis and cytokine production, CD4+ T cells basically secrete cytokines to orchestrate the immune response. CD4+ T cells are divided to T helper 1 (Th1), Th2, Th17 performing different types of immune responses specific to the type of pathogen and regulatory T cells (Treg) responsible for suppressive functions to control and prevent excessive or inappropriate inflammatory response (Fig.1.6).

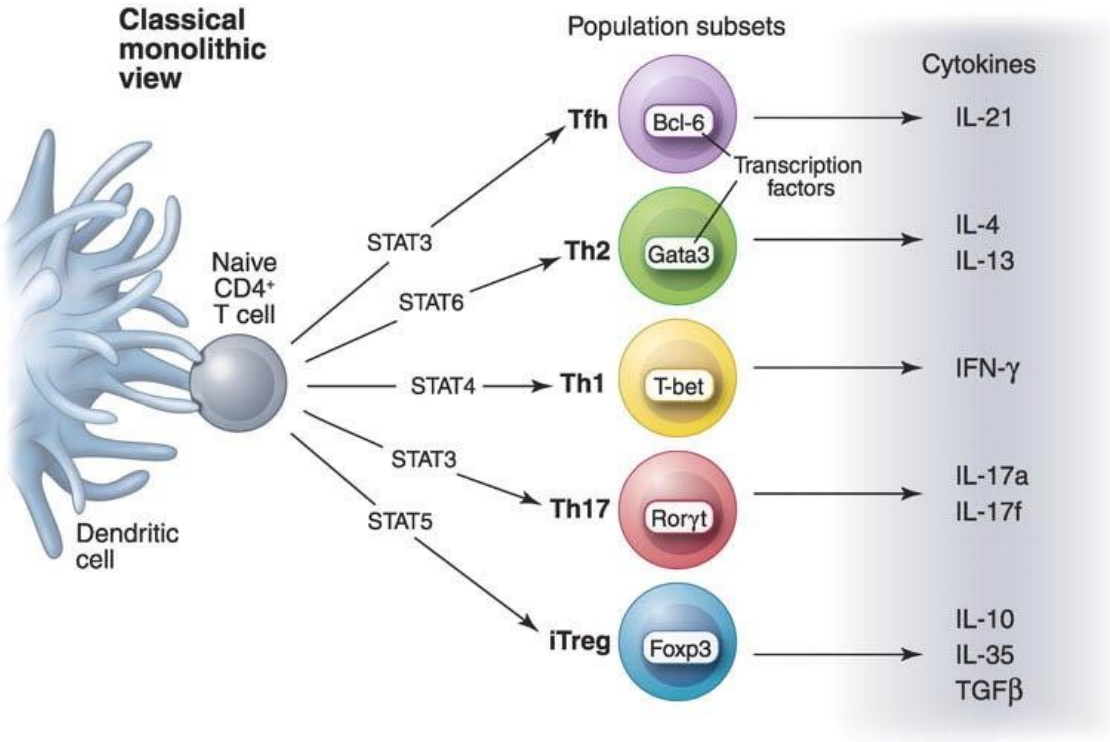


Figure 1.6 CD4+ T helper (Th) cell differentiation. Signals from innate immune cells, induced by pathogens and inflammatory stimuli, drive naive CD4+ T cells to differentiate into one of several effector fates, including T helper 1 (Th1), Th2, and Th17 cells. Adapted from (O'shea et al.,2010).

A naive T cell (CD45RA+ CCR7+) circulate through the peripheral lymphoid tissue until it recognizes antigen in the context of MHC on APCs as dendritic cells (DC) which is the most powerful APCs (Signal 1). In addition to TCR binding to Ag binding MHC, activated T cells require a number of secondary signals to become activated and respond to the threat. In the case of helper T cells, the first of these is provided by CD28. This molecule on the T cell binds to one of two molecules on the APC – B7.1 (CD80) or B7.2 (CD86**)** to initiates T-cell proliferation. Signal 3 include the release of the cytokines by DCs (Chen and Flies, 2013).

T helper become Th1, Th2 or Th17, each of these has a specific role. These cells move to the site of infection. Other cells such as neutrophils, mast cells can also release

cytokines, chemokines, short peptides and other molecules, which induce further activation and proliferation of the T cells.

Activated T cells will proliferate and become effector and express cytokine. CD45 will transform form the long isoform CD45RA to the shorter form CD45RO. Naive T cell will lose CCR7 expression. CCR7 allows T cells to enter high endothelial vessels and migrate into secondary lymphoid tissue. CCR7 expresssion on memory T cells can be used further divide the CD45RO+ cells based on their broad function as a memory cell. Central memory T cells (TCM) are CD45RO+ and express CCR7 (Berard and Tough 2002; Rosenblum et al., 2016).

1.3.2 The role of CD4+ T cells in MS

It has been suggested that MS is caused by activation of peripheral autoreactive CD4+ T cells that migrate to CNS and initiate the disease. CD4+ T cells were identified in the late 1980s as IFN-γ Th1 cells (Tada et al., 1987). CD4+ T cells recognize MHC class II molecules and the antigen (external and internal) presented in them. MHC II is restricted to APC (dendritic cells, macrophages and B cells. Specific immune response is modulated by CD4+ T cells which can respond to a wide range of pathogens and secrete cytokine. CD4+ T cells produce IFN-ψ and IL-17 cytokine that initiate the pathogenesis of MS.

It has been reported in the EAE model that myelin-specific CD4+ T cells are the primary mediator and that EAE is MHC-II restricted. MHC-II molecules have a strong association with MS through antigen presentation. In MS patients, it has been shown that CD4+ T cells activated by MBP were related to the deterioration of their status (Bielekova et al., 2000).

CD4+ T cells are mainly primed by APCs such as de that present the myelin epitope in the peripheral lymph nodes. Recently activated CD4+ T cells can then migrate from

the blood across the blood cerebrospinal fluid barrier formed by the choroid plexus. T cells are reactivated by dendritic cells, macrophages or B cells expressing MHC-II-restricted myelin epitopes. Microglial cells can also be activated by antigen-specific T cells, which lead to distal activation of blood vessels. T cells cross the blood-brain barrier, are reactivated by perivascular dendritic cells or macrophages and then enters the parenchyma. T cells secrete mediators that trigger demyelination in the brain (Brown and Sawchenko, 2007).

It has been shown that myelin-specific CD4+ is the primary mediator in EAE models, not CD8+, as the stimulation prefers MHC class II restricted T-cell activation (Goverman, 2009).

1.4.2.1 T helper 1 (Th1)

It has been known that the primary effector T cell in the pathologenesis of both EAE and MS is the CD4+ T helper 1 (Th1) cell, that requires interleukin-12 (IL-12) for its differentiation and is characterized by the secretion of interferon-γ (IFN-γ), IL-2 and TNF. IFN-γ activation of T-bet up- regulates IL-12Rβ2 expression that is not expressed on naïve CD4+ T cells. Binding of IL-12 to its receptor activates STAT-4 which lead to T-bet expression and consequently the Th1 phenotype. L-12 is a heterodimer protein composed of a p35 and a p40 subunit. IL-12 was found to have a common p40 subunit with the cytokine IL-23 (Lyakh et al., 2008).

In 1986, Mossman and Coffman described the CD4+ as Th1 and Th2. Cytokines play an important role; IFN-γ and TGFβ favor Th1 response while IL-4 induce Th2 response (Zhu and Paul, 2008). Th1 cells develop in response to intracellular pathogens. CXCR3 is rapidly induced on naïve cells following activation and preferentially remains highly expressed on Th1 CD4+ T cells and effector CD8+ T cells. Th1 have

long been associated with autoimmune diseases (Groom and Luster, 2011).

IFN-γ induces MHC class II expression in the CNS, triggers the production of chemokines that attract macrophages and monocytes and activates macrophage function, which is consistent with the idea that Th1 cell-mediated responses establish pro-inflammatory responses in the CNS (Goverman, 2009).

Th1 cells are detectable in brain tissue early in the development of MS. The effector cells that are capable of transferring disease appear to be IFN-γ and TNF-ß-producing Th1 cells with specificity for myelin basic protein or proteolipid protein related peptides (McDonald and Swanborg, 1988; Powell et al., 1990; Kuchroo et al., 1993; Van der veen and Stohlman, 1993).

Th1 cells may can tribute in two ways to the development and progression of disease; as IFN-γ cells that activate macrophages in the degradation of the myelin sheath and by directly damaging the myelin sheath and oligodendrocytes through the secretion of TNF-α and TNF-ß (Selmaj and Raine, 1988).

1.4.2.2 T helper 2 (Th2)

IL-4 is the cytokine which play a role in the differentiation of CD4 T cells to Th2. Th2 has a role in humoral immunity as Leishmaniasis. Th2 cells produce IL-4, IL-5, IL-6 and IL-13. These cytokines activate eosinophils and stimulate B cells to produce IgM, IgG1, IgA and mainly IgE which is a strictly Th2-dependent isotype. They express CCR3 and CCR4, to migrate towards chemokines including RANTES (CCL5) and CCL11. GATA-3 is the transcription factor that is essential for a Th2 phenotype which was discovered in 1997. GATA-3 is expressed in naive T cells, with decreased expression in Th1 committed cells and transgenic expression of GATA-3 induces a Th2 cytokine profile (Zheng and Flavell, 1997). Two pathways can induce Th2. IL-4 is

a cytokine that can activate STAT-6 which leads to GATA-3 activation. Also it can be induced via an IL- 4 independent pathway in the absence of STAT-6 (Agrawal, 2007; Ho et al., 2009).

1.4.2.3 Th 17

In the last decade, much attention has been focused on defining the role of Th17 cells in the pathogenic process of tissue inflammation. The importance of Th17 cells in the pathogenesis of organ-specific autoimmune inflammation has been demonstrated in different animal models. As IL-17 was recognized to be increased in human autoimmune diseases like MS, rheumatoid arthritis and psoriasis as well as in animal models of autoimmunity (Lock et al., 2002; Matusevicius et al., 1999; Teunissen et al., 1998; Steinman, 2007).

IL-23 was first thought to be a key mediator in the induction of Th17 cells mainly because it was shown to expand Th17 cells in vitro. Resident microglial cells and infiltrating macrophages during an autoimmune reaction in the CNS produce IL-23 (Cua et al., 2003; Dardalhon et al., 2008).

Th17 cell is defined by secreting IL-17A and expressing the transcription factor RORC in human. Studies have suggested that Th17 is more potent than Th1 in inducing MS. IL-17 was first cloned in 1993 which is named as IL-17A (Rouvier, et al 1993; Chen and Kolls, 2017). Other members of the family are: IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F (Aggarwal and Gurney, 2002; Mosely et al., 2003). Both IL-17A and IL-17F are produced by a variety of cell types including subsets of CD4+ T cells, CD8+ T cells, γδ-T cells, NK cells and neutrophils (Aggarwal et al., 2003). IL-17 production has also been associated with memory CD4+ T cells. IL-17A and IL-17F have pro-inflammatory properties and act on a broad range of cell types to induce

cytokines (IL-6, IL-8, GM-CSF, G-CSF), chemokines (CXCL1, CXCL10). IL-17A and IL-17F are also key cytokines for the recruitment, activation and migration of neutrophils (Kolls and Linden, 2004).

1.4.2.4 Regulatory T cells (Tregs)

As well as subsets of inflammatory CD4+ T cells, there are a number of regulatory T cell subsets that are likely to influence T cell responses in MS.

It is a population of T cells that modulate and regulate or suppress the immune system and thereby maintain homeostasis and tolerance to self-antigen (Sakakuchi et al., 2008; Corthay, 2009). They express transcription factor Forkhead box protein 3 (FoxP3) which hmake less than 4% of circulating CD4 T cells, and other inhibitory immune checkpoint surface molecules (Lu and Rudensky, 2009; Kurd and Robey, 2016). FOXP3 is induced and sustained in Tregs by TGFβ signaling (Bommireddy and Doetschman, 2004). TGFβ can suppress T cell responses either through a direct or an indirect pathway. T regs secrete the anti-inflammatory cytokines IL-10, IL-35 and TGFβ. TGFβ's pivotal functions in suppressing T cell responses and maintaining immune tolerance is further supported by studies on TGFβ1-deficient mice (Gorelik and Flavell, 2000; Kulkarni et al., 1993). They are characterized by the high expression of CD25, as low or negative expression show defective immune regulation (Santis et al., 2010). Recently, the absence of CD127 has been identified as an additive more stringent surface marker for T regulatorycells detection (Michel et al., 2008). The role of T reg cells is to regulate effector Th1, Th2 and Th17 cells.

CD4+ regulatory (T-regs) are classified into mainly two populations: thymic- derived n T-reg (naturally occurring) and peripheral iT-reg (induced). T-regs (FOXP3+ CD127

low CD25 hi) can play a suppression role via a number of mechanisms, including IL-1 and depletion of T-regs (FOXP3 deletion) in humans or animals lead to autoimmune diseases. IFN-ψ enhances CD4+ regulatory T-cell function (Kofler et al., 2011; Larkin et al., 2013).

In the course of MS, it has been shown that CD4+CD25+high Tregs lose their immunosuppressive function, thereby favoring the activation of T cell clones active against CNS elements (Viglietta et al., 2004; Feger et al., 2007; Venken et al., 2006). It has been also demonstrated that in early stages of the disease FoxP3 is expressed at low levels in Tregs from MS patients compared to healthy controls (Venken et al., 2008). In addition to the presence of inflammatory T cells in the brain in MS, marked reductions in regulatory T cells have been shown in different studies (Gartner et al., 2006).

NK cells also have regulatory properties and suppress immune responses via their capacity to kill activated, and potentially pathogenic, CD4 T cells. Daclizu- mab has been shown to enhance CD56 hi NK regulatory activity and to be effective in relapsing forms of MS (Bielekova et al., 2006).

1.3.3 The role of CD8+ in MS

Although many studies have focused on CD4+ T cells role in the CNS for many years, there has been less research on CD8+ T cells. In MS lesions there are more CD8+ T cells than CD4+ T cells, and clonal expansion is also higher (Jacobson et al., 2002). These cells also exist in CSF and blood in MS patient. However, CD8+T cell responses to antigens are unclear, as they can be pathogenic and protective at the same time.

CD8+ T cell identify antigen presented on an MHC class I molecule which is expressed on all nucleated cells of the body. CD8+ T cells recognize cytosolic

proteins from

intracellular infections such as viruses or tumour antigens. Antigens are presented on the surface of an APC during an infection, MHC:peptide complex and co-stimulator molecules will be up regulated. CD8+ T cells can effectively kill them by apoptosis, but CD4+ T cells cannot. CD8+ T cells secrete IFN-γ, TNF-α and TGFβ that can help in defense. IFN-γ can initiate anti-viral responses in infected cells and activate macrophages. TNF-α and TGFβ also help in the killing of infected cells via their receptors and in the activation of macrophages.

The depletion of CD8+ T cells in mice has been found to lead to decreased mortality, but studies in EAE models have showed that CD8+ T cells are pathogenic. Some MHC-I molecules (i.e., HLA-A3) are susceptible to MS. Just like CD4+ T cells, CD8+ T cells must be activated in the peripheral lymph nodes. CD8+ T cells are activated by dendritic cells, macrophages, or microglial cells in the brain only by cross-presentation; activated macrophages and CD8+ T cells secrete mediators that result in demyelination. CD8+ T cells can also directly lyse oligodendrocytes that express MHC-I and myelin peptides (Goverman, 2009).

In a model with peripheral infection, by evaluating T cell trafficking, antigen-specific CD8+ T cells in the brain were shown to exhibit a persistently activated phenotype, suggesting that CD8+ T cells may persist in the brain of patients with MS even following resolution of an acute inflammatory episode. For the homing of Ag-specific CD8+ T cells to the brain, CD49d is a crucial mediator α4β1 integrin (VLA-4) antibodies have been found to prevent EAE in mice, and it is used as a powerful drug for halting MS progress in humans (Young et al., 2011, Khare et al., 2003).

1.3.4 The role of CCR6 in MS

The chemokine receptor CCR6 is strongly associated with Th17 cells (Annunziato et al., 2007; Singh et al., 2008). All IL-17 secreting cells express CCR6, while not all CCR6 expressing cells make IL-17. Other chemokine receptors can bind to several different chemokines, however CCR6+ cells only migrate towards one chemokine - CCL20 (MIP1α) (Wang et al., 2009)

CCR6 has been used to identify cells that have characteristic of Th17 cells in other populations. Within peripheral blood there are CCR6+Tregs cells that are RORC+ FoxP3+ and express IL-17 but have suppressive potential (Voo et al., 2009).

CCR6 is also expressed on B cells and is important for B cell differentiation and maturation (Bowman et al., 2000).

CCR6, which is also known as CD196, is a chemokine receptor that is expressed in immature dendritic cells, B lymphocytes and memory T cells. CCR6 expression in Th17 cells is regulated by TGFβ and requires two nuclear receptors, RORα and RORψ. CCR6 binds CCL20, while members of the defensin family also bind CCR6 with a lower affinity. CCR6+ cells, and their ligand CCL20, have been detected in various organs, including the choroid plexus. CCL20 is selectively created by the follicle-associated epithelium overlying Peyers patches and isolated lymphoid follicles. CCL20 contributes to the recruitment of CCR6 expressing B cells to these structures. In humans, CCR6 can mediate the arrest of T cells on dermal endothelial cells and is highly expressed in T cells resident in both normal and psoriatic skin. CCR6 and/or CCL20 have been implicated in the pathogenesis of rheumatoid arthritis and inflammatory bowel disease. Human T cells that are able to produce IL-17 express CCR6. It is therefore suggested that CCL20 and CCR6 play a role in inflammatory

diseases by recruiting Th17 cells to target tissues. It has also been shown that CCR6 deficient mice have a normal Th17 response but do not develop EAE.

Migration of CCR6+ Th17 cells across the choroid plexus plays a role in initiating inflammation in MS (Agrawal et al., 2007; Yamazaki et al., 2008).

CCR6 positive myelin–reactive T-cell phenotypic analysis has been used to compare MS patients and healthy controls and memory CCR6+ T cells from myelin-reactive T cells from patients were found to secrete more pro-inflammatory cytokines than those of healthy patient (Cao et al., 2015). IL-17 is generally considered as the signature cytokine produced by CCR6+ Th17 cells. However, this greatly underestimates Th17 effector function, since subpopulations also produce IFN-ψ and granulocyte macrophage colony-stimulating factor (GM-CSF). Beside IL-17, also IFN-ψ and GM-CSF are strongly produced by myelin-specific CCR6+ T helper cells in MS (Cao et al., 2015).

GM-CSF was increased in both CCR6- and CCR6+ memory myelin-reactive T cells from MS compared with that from healthy controls.

It has been shown that CCR6, were enriched in the cerebrospinal fluid (CSF) and brain specimens of patients with MS.

In our group, CCR6+ Th cells in the CSF of MS patients were dominated by pathogenic non classical Th1 cells and GM-CSF secreting T cells (Restoric et al., 2017).

1.3.5 Cytokine responses in MS

Cytokines and their receptors are important in the evolution of MS lesions, and pro- and anti-inflammatory cytokine levels have been found to correlate with changes in MS disease activity.

Pathological mechanisms in MS include the release of cytokines and chemokines, such as TNF-α and IFN-γ. These cytokines induce expression of vascular cell adhesion molecule-1 (VCAM-1), which will bind T-cells to allow migration through the CNS endothelium, leading to inflammation and destruction of myelin and plaque formation in the brain. Auto-antigen stimulates T- helper-1 (Th-1) cells, which induce the release of the pro-inflammatory cytokines IFN-γ, TNF-α and interleukins (ILs) such as IL-1β. As a consequence, this activates monocytes and macrophages if these cells are presenting antigen to the T cells (Agrawal and Yong, 2007).

The increased production of either IFN-γ or IL-17 is associated with human MS (Lock et al., 2002). A pathogenic role for IFN-γ in MS is supported by a trial in 1987 in which the administration of IFN-γ to MS patients exacerbated their disease (Panitch et al., 1987).

Carbajal and others have shown that Th1/ Th17 myelin-reactive T cells, which secrete IL-17, granulocyte macrophage colony-stimulating factor (GM-CSF) and IFN-γ, are inflammatory mediators (Carbajal et al., 2015); on the other hand, IL-10 may play a protective role in MS (Cao et al., 2015; Bettlli et al., 2004; Lee et al., 2011). IL-10 also has a suppressive role in other autoimmune diseases such as diabetes mellitus (Ng et al., 2013).

IFN-γ and in IL-12 are increased in brain, (CSF) and peripheral blood of MS patients, specifically during acute phase (Kallaur et al., 2013). By contrast, IL-10 which

downregulate Th1 cells and exert anti-inflammatory functions, are increased during the remission phases of MS (Imitola et al., 2005).

Navikas et al reported that MS patients who have high numbers of MBP-reactive cells in their blood which after culturing with MBP and myelin PLP, the produced high cytokines including IFN-γ, IL-4, TGFβ, IL-10 and TNF-α. Also, they have demonstrated that MS patients presented elevated MBP- and PLP-reactive IL-6 mRNA expressing cells in the peripheral blood (Navikas et al., 1995).

1.4.5.1 Granulocyte Monocyte Colony Stimulating Factor (GM-CSF)

GM-CSF was discovered 40 years ago and its essential role in EAE was demonstrated 14 years ago, but little is known about MS (Burgress and Metcalf, 1980; Rasouli et al., 2015). In recent study, IFN-γ and GM-CSF expressing CD4+ T cells (CXCR3 and CCR6, RORt and T-bet) were enriched in the CSF and brain specimens of patients with MS (Langelaar et al., 2018).

GM-CSF is now best viewed as a major regulator governing the functions of granulocyte and macrophage lineage populations at all stages of maturation (Hamilton, 2002).

GM-CSF also known as colony-stimulating factor 2 (CSF2) is a monomeric glycoprotein secreted by macrophages, T cells, mast cells, NK cells, endothelial cells and fibroblasts that functions as a cytokine. GM-CSF signals via signal transducer and activator of transcription STAT5 (Sheng et al., 2014).

GM- CSF from myelin-specific CD4+ T cells is essential to EAE development

GM-CSF is classified as powerful inflammatory mediator released by Th17 cell (Shiomi et al., 2016).

Studies have shown that these myelin reactive CD4 cells are more frequent in MS patients as compared to healthy controls (Raddassi et al., 2011; Housley et al., 2015). Beside this, cytokine production and RNA profiles of these myelin reactive T cells had shown an increase in IFN-γ, GM-CSF, and IL-17 production in myelin reactive CD4 cells from MS patients (Cao et al., 2015).

Autoreactive T cells facilitate CNS inflammation by secreting a variety of pro-inflammatory cytokines, such as GM-CSF in MS patients and EAE models (Ponomarve et al., 2007).

Increased production of GM-CSF from CD4+ and CD8+ T cells were detected in peripheral blood of untreated MS patients compared with patients treated with IFN-丿 healthy controls. Ex vivo analysis also showed that IFN-丿 suppressed GM-CSF production by T cells in vitro. T cells in MS lesions also expressed GM-CSF, confirming that GM-CSF is strongly contributes to MS pathogenesis in the CNS (Rasouli et al., 2015).

1.4.5.2 Interferon gamma (IFN-γ)

IFN-γ has been implicated in the pathogenesis of MS and its animal model (EAE) (Zamvil and Steinman, 1990) and it is the hallmark of Th1-type immune responses, it has been strongly linked to disease pathogenesis through several findings.

immunity against viral, some bacterial and protozoal infections. The effects of IFN-γ on APCs are pleiotropic and encompass up-regulation of MHC molecules, induction of reactive oxygen species (ROS) production, phagocytic activity, and increased pro-duction of other pro-inflammatory cytokines including TNF (Mittal et al., 2014). CNS APCs, such as resident microglia and infiltrating peripheral macrophages and dendritic cells (DCs), have been shown to express MHC class II molecules as well as costimulatory molecules, including CD80 and CD86 (Chastain et al., 2011).

Increased production of IFN-ψ precedes clinical attacks (Beck et al., 1988; Lu et al., 1993) and treatment of MS patients with rIFNg-induced exacerbations (Panitch et al., 1987).

Kallaur et al have shown that increased IFN-γ levels obtained among MS patients compared with controls were also consistent with previous studies (Kallaur et al., 2013).

IFN-γ or type II interferon, is a cytokine that is critical for innate and adaptive immunity. Hue at al has reported that IFN-ψ/IL-17-co-expressing CD4+ T cells are abundant in post-mortem MS lesions, and that lymphocytes obtained from relapsing MS patients have a propensity to expand into IFN/IL-17 co-producers (Kebir et al., 2009). Transcriptional analyses have shown increased levels of mRNA-encoding CXCR3 and reduced levels of IFN, CCL3, CCL4, Granzyme B, and IL-10, in IFN/IL-17-co-producing CD4+ T cells sorted from the peripheral blood mononuclear cells (PBMC) of untreated, relapsing MS patients versus age- and sex-matched healthy controls (Hu et al., 2017).

Begolka and Miller observed that IFN-γ expression increases during the peak of the disease and decreases during the remission phase (Begolka et al., 1998)

Clinical attacks correlate with increased IFN-γ production and patients experiencing relapses have significantly increased IFN-γ production by PBMC following stimulation with PH (Beck et al., 1988).

Y. Cao et al (2015) have used libraries of CD4+ T cell clones from MS patients to show that myelin-specific CD4+ T cells which express CCR6 (a chemokine receptor associated with Th17 subset) produce higher amounts of IL-17, IFN-γ and GM-CSF than analogous auto-reactive cells from healthy controls (Cao et al., 2015).

1.4.5.3 Interlukin 10 (IL-10)

IL-10 that was initially considered as a Th2 cytokine may also be produced by Th1 cells (Del-Prete et al., 1993). IL-10 also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. In humans, IL-10 is encoded by the IL10 gene (Eskdale et al., 1997). IL-10 signals through a receptor complex consisting of two IL-10 receptor-1 and two IL-10 receptor-2 proteins. Consequently, the functional receptor consists of four IL-10 receptor molecules. IL-10 binding induces STAT3 signalling via the phosphorylation of the cytoplasmic tails of IL-10 receptor 1 + IL-10 receptor 2 by JAK1 and Tyk2 respectively (Mosser and Zhang, 2008; Walter et al., 2014). It is primarily produced by dendritic cells, macrophages, B cells, CD4+ and CD8+ T cells (Couper et al., 2008).

A T-cell library technique used in humans revealed that single cell clones isolated by MHC peptide tetramers from myelin specific CCR6+ T cells secrete more regulatory cytokines (IL-10) cytokines than MS clones (Cao et al., 2015).

It has also been reported that during the relapsing stage of MS, anti-inflammatory and Th2 cytokines (IL-4, IL-5 and IL-10) are predominate (Agrawal and Young, 2007). It was previously suggested that IL-10 is important for recovery of EAE (Mann et al., 2007). In contrast to other Th2 cytokines (IL-4), the absence of IL-10 results in a more severe EAE (Lyer and Cheng, 2012).

1.4.5.4 Interlukin 17 (IL-17)

IL-17 or IL-17A is a pro-inflammatory cytokine and it is produced by a group of T helper cell known as Th 17 cell in response to their stimulation with IL-23 (Jin et al., 2013). Originally, Th17 was identified in 1993 isolated IL17 transcript from a rodent T-cell hybridoma (Rouvier et al., 1993).

IL-17 is considered a pathogenic cytokine as its role in many autoimmune diseases.

However, it can play a protective role in against fungal and bacterial infections by recruitment and activation of neutrophils.

IL-17 production by CD4+ T cells in response to an infectious agent was first discovered in response to *Borrelia burgdorferi,* identifying IL-6 as a potential inducer for IL-17 (Zambrano-Zaragoza et al., 2014).

IL-17 expression has been determined to produce a highly pro-inflammatory environment and to induce severe pathological diseases as MS (Gold and Luhder, 2008). Th17 is critical for the development of MS as Th1(Iwakura and Ishigame, 2006). Higher levels of IL-17 were found in RR-MS patients with inactive disease (Kallaur et al., 2013). Th17 cell differentiation is orchestrated by multiple cytokines, including TGFβ and IL-6, -1-β, -21 and -23 (stimulating) and IFN-γ and IL-4, -12, -10 and -27 (inhibiting).

Studies from MS patients indicated that IL-17 mRNA is elevated in active MS brain lesions (Lock et al., 2002).

Y. Cao et al 2015 have used libraries of CD4+ T cell clones from MS patients to show that myelin-specific CD4+T cells that express CCR6 (a chemokine receptor associated with Th17 subset) produce higher amounts of IL-17, IFN-γ and GM-CSF than analogous auto-reactive cells from healthy controls.

1.4.5.5 Tumor Necrosis Factor α (TNF-α)

TNF-α is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signalling events within cells, leading to necrosis or apoptosis. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons.

CD8+ T cells also release IFN-γ, TNF-α and TGFβ, which contribute to the defence in several ways. TNF-α and TGFβ also aid in the killing of infected cells via their receptors and in the activation of macrophages (Idriss and Naismith, 2000).

TNF-α levels were lower in RR-MS patients than the healthy controls (Kallaur et al., 2013; Duddy et al., 1999; Trenova et al., 2011; Dhib-Jalbut et al., 2006).

Although low levels of TNF-α have been reported in specific MS patients, another study showed that TNF-α is the primary mediator of inflammatory response and is increased among MS patients, which means that TNF-α is important in the pathogenesis and progression of MS (Brandão et al., 2005).

Cytokines only function when bound to membrane receptors and the TNF-α receptor is present in almost all cells (Imitola et al., 2005; Begolka and Miller, 1998; Ehling et al., 2004). In MS patients, TNF-α is produced and consumed at sites of inflammation, including lesions in brain white matter, spinal cord and optic nerve (Benvenuto et al., 1991; Selmaj et al., 1991)

TNF-α is present in the CSF of subjects with MS and its level correlates with severity and progression of the disease (Maimone et al., 1991; Hauser et al., 1990; Sharief and Hentges, 1991).

1.4.5.6 Interlukin 4 (IL-4)

The balance between Th2- and Th1-related cytokines plays a key role in MS. A shift from a Th1 towards a Th2 cytokine profile could have a beneficial effect on the clinical course of the disease. It is well known that IL-4 is a strong inducer of Th2 responses (Oreja-Guevara et al., 2012; Chen et al., 2004).

IL-4 is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4 in a positive feedback loop. The cell that initially produces IL-4, thus inducing Th2 differentiation, has not been identified, but recent studies suggest that basophils may be the effector cell. IL-4 has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of B cells into plasma cells. It is a key regulator in humoral and adaptive immunity. IL-4 induces B-cell class switching to IgE, and up-regulates MHC class II production (Murphy and Reiner, 2002).

In contrast, in some studies in comparison to control Tcell clone, MBP-specific TCC from MS patients secreted increased amount of IFN-γ, IL-4 and IL-10 and decreased quantities of TGFβ (Hemmer et al., 1996; Kallaur et al., 2013).

1.3.6 Peripheral blood responses in MS

In CSF, T-cell and B-cell MOG antibodies have also been detected, as well as the oligoclonal bands (Khare et al., 2003) For many years, T-cell responses to different epitopes of MOG, PLP and MBP have been detected in the peripheral blood cells of MS patients. In some studies, there have been no differences in the number of myelin-reactive T cells between MS patients and healthy patients, possibly because the number of the antigen- specific T cells was too low (Cao et al., 2015). The detection and frequency of MOG- specific T cells were measured using DRB1*0401/MOG97–109(107E-S) tetramers in MS subjects, and the percentage of CD4+ CD25 intermediate DRB1*0401/MOG97– 109(107E-S) tetramer–positive T cells was significantly higher in patients with RR-MS as compared with healthy control subjects (Raddassi et al., 2011). In some studies, HLA-DRB1* 1501 has been

considered the most powerful susceptibility gene for MS (Hohlfild et al., 2016).

Th1 and Th17 are involved in the pathological mechanism of MS, and recent studies have reported the phenotype of these cells (Kofler et al., 2011). Th17 cells are more significant, although Th1 can mediate inflammation, and proinflammatory cytokines, such as IFN-γ and TNF-α, are secreted by Th-1 cells. It has also been reported that during the relapsing stage of MS, anti- inflammatory cytokines IL-4, IL-5 and IL-10 are predominating (Agrawal and Young, 2007).

Studies have shown that Th1/Th17 myelin-reactive T cells, which secrete IL-17, GM-CSF and IFN-α, are inflammatory mediators; on the other hand, IL-10 and IL-17 play a protective role in MSI (Cao et al., 2015). IL-10 also has a suppressive role in other autoimmune diseases such as diabetes mellitus.

1.3.7 CSF reactivity in MS

CNS is protected from cellular infiltration by the blood-brain barrier, which surrounds the parenchymal venules, and by the blood CSF barrier, that surrounds the choroid plexus. Cells have limited to access to the CNS due to the tight junction between the endothelial cells of the blood-brain barrier and the epithelial cells of the blood CSF barrier. Immune surveillance can be carried out by memory and activated T cells as they express adhesion molecules and chemokine receptors. Endothelial cells of the brain do not express the adhesion molecules that are required for the adherence of activated T cells. When there is inflammation, activated T cells enter the CNS, as T cells can cross the blood-CSF barrier. It has been shown that in EAE models, CCR6 facilitates the entry of T cells to CNS. It is in the subarachnoid space (SAS), which is the area between the arachnoid and pial membranes where CSF circulates, that CD4+

T cells that have been activated previously are reactivated by APC MHC-II, which lead to T-cell proliferation in the SAS. Also, T-regs and Th17 express the chemokine receptor (Goverman, 2009).

In CSF that is collected through lumbar puncture, B cells and Igs can enter the CNS in MS patients. The oligoclonal immunoglobulin bands, which have been considered to be a diagnostic marker for MS for a long time, can be detected in MRI diagnosis that reveals white matter lesions in the brain (Hohlfild et al., 2065).

Besides CCR6 and CXCR3, also the presence of the α4⌡1 integrin very late antigen-4 (VLA-4), which is abundant on Th17 cells in multiple sclerosis CSF (Brucklacher-Waldert et al., 2009) determines T cell transmigration capacities.

1.5 Treatment of MS

Over 15 disease-modifying agents are currently in clinical use that significantly reduces annualized relapse rates. Many of these drugs specifically target lymphocytes, but they do not distinguish between beneficial and pathogenic subsets, thereby increasing the risk of opportunistic infection. The desire to treat patients with MS early and to target the correct therapy to patients is hampered by a lack of useful prognostic bio- markers that can predict disease progression, severity, and therapeutic response.

An increased understanding of the pathways by which encephalitogenic (pathological-autoreactive) T cells migrate across the blood–brain barrier, expand in the CNS microenvironment, and inflict damage to CNS tissues will inevitably lead to the discovery of distinguishing biomarkers and therapeutic targets. However, this endeavor will be further complicated by the diversity of myelin-reactive T cells with encephalitogenic potential, indicating that therapeutic approaches will have to be customized for clinical subsets of patients.

Treatment with neutralizing antibodies to IL-17 or GM-CSF inhibited EAE induced by the transfer of IL-23-polarized, but not IL-12-polarized cells. In contrast, neutralizing antibodies to TNF-α suppressed, while neutralizing antibodies to IFN-γ exacerbated, both forms of EAE. Axtell et al. subsequently reported that IFN-β therapy ameliorates EAE mediated by C57BL/6, $MOG_{35–55}$-reactive Th1 cells, but exacerbates disease mediated by their Th17 counterparts (Axtell et al., 2010).

However, most of the drugs are not effective to treat the progression of MS. The major challenge to develop the effective treatment is to understand the mechanism of MS

1.5.1 Interferon-beta (IFN-β)

Interferons α and β were discovered about 50 years ago to have antiviral properties. IFN-α is clinically used as an antiviral agent, whereas IFN-β (fibroblast IFN) results in anti-inflammatory effects and constitutes a cytokine used in the treatment of MS. Although IFN -β is extensively used in MS, its exact mechanism of disease modulation remains unclear. IFN-β is a polypeptide, normally produced by fibroblasts, that has antiviral and antiproliferative effects and is known commercially as Rebif, Avonex or Plegridy (Stetson and Medzhitof, 2006).

Potential mechanism of its actions includes, upregulation HLA class I expression and down-regulation expression of HLA class II, therefore inhibiting Th cell activation (Keskinen et al., 1997). CD73 that produce adenosine which reduces Th17 and T cell activation through binding to IFN receptor (Airas et al., 2007).

IFN-β treatment upregulated the signal transducer and activator of transcription 1/2 (STAT 1/2) signaling pathway, GDNF family receptor alpha-2 (GFRα2), nucleotide-binding oligomerization domain-containing protein 1 (NOD1), caspases 1/12, tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10), and GDNF which, possibly playing a neuroprotective role in the CNS by modulating neurotrophic factors in addition to the peripheral immunomodulatory effect (Hirsch et al., 2009).

In vitro studies show that it is effectively decreased T-cell migration through reduction of matrix metalloproteinase (MMP-9) levels, adhesion molecules (ICAM and VCAM) and chemokines, also decreased BBB cerebral endothelial cell permeability which is seen as a rapid decrease in gadolinium enhancing lesions (Stuve et al., 1996).

Binding of IFN-β to its receptor induces a complex transcriptional response. In immune cells (the most likely target of IFN-β therapeutic effect in MS), IFN-β reduces antigen presentation and T-cell proliferation, alters cytokine and MMP expression, and restores suppressor

function, in addition it can modulates expression of pro-inflammatory cytokines and of molecules that facilitate extravasation, such as MMP and VLA-4 (Markowitz, 2007). However, IFN-β can ameliorate EAE driven by Th1 cells but exacerbated disease induced by Th17 cells (Axtell et al., 2010). It has been shown in studies that IFN-β can shift to Th2 response (IL-4, IL-5 and IL-13), increase T-regs (CD4 CD25) and IL-10. Also, it can reduce IL-17, IFN-γ and GM-CSF (Bermel and Rudick, 2007; Noronha et al., 1993; Karp et al., 2001; Ransohoff RM, 2003).

Lymphopenia which is reduction in lymphocyte number is reported as a side effect of IFN-β therapy.

IFN-β treatment in MS patients decreases the expression of Th1 inflammatory cytokines such as IFN-γ and TNF-α in their mRNA serum transcript and increases IL-10 production (Dhib-Jalbut et al, 2006).

A novel mechanism of IFN-β therapy in MS was suggested, where IFN-β enhances development of FoxA+ regulatory T cells (Liu et al., 2014).

1.5.2 Natalizumab

Natalizumab Tysabri is a humanized monoclonal antibody with an IgG4 framework (Rispesn et al., 2011). It was specifically designed for the treatment of MS and was FDA approved on 2004. Its target molecule is CD49, the α4 subunit of (VLA-4) receptor. VLA-4 interacts with vascular cell adhesion molecule-1 so that immune cells can migrate through the blood brain barrier. By binding to CD49, natalizumab prevents the adhesion between the endothelial cell and the immune cell, thus migration of leukocytes into the central nervous system is blocked (Loma and Hyeman., 2011; Hohendorf et al., 2014; Coisne et al., 2009).

It is abrogating the development of EAE in mice, consistent with this integrin being important in the trafficking of T cells into the CNS by interacting with its ligand on endothelial cells, vascular cell adhesion molecule-1 (Yednock et al., 1992). In the CSF of MS patients treated with Natalizumab, there is a prolonged reduction in the number of T lymphocytes (Stuve et al., 2006).

It has a robust benefit on relapse rate, disability progression, and MRI activity.

Despite the side effect progressive multifocal leukoencephalopathy (PML) that has been reported in 2005, it is widely believed that targeting leukocyte trafficking using Natalizumab may still be a promising treatment of t MS (Stuve al., 2006).

1.5.3 Glatiramer acetate (GA)

GA is known as copolymer 1 is a random polymer of glutamic acid, lysine, alanine and tyrosine, the most common amino acids in myelin basic protein. Copaxone is one of the marketed drugs and is currently approved for the treatment of RRMS. It has proven effects on exacerbation rate and MRI clinical assessment (Loma and Heyman, 2011).

Different immune-mechanisms have been suggested to contribute to the beneficial effects, including its ability to affect the antigen-binding groove of MHC class II molecules, thereby potentially competing with other autoreactive epitopes for antigen binding (Farina et al., 2005; Yong, 2002).

Other activities include the generation of Th2 cytokine reactivity with anti- inflammatory effects (Aharoni et al., 2000; Neuhaus et al., 2000) the increase in activity of CD4+CD25+ regulatory cells (Hong et al., 2005), the modulation of macrophage activity (Karandikar et al., 2002) and the increase in CD8+ cells with suppressor activity. Finally, the GA-reactive Th2 cells may also enter into the CNS to confer neuroprotection (Yong, 2002).

GA increases cytokine levels such as IL-10, TNF-α, and IL- 4 and decrease IL-17, therefore regulating the disease and consequently it shifts the pro-inflammatory Th1 state to an anti-inflammatory Th2 state.

1.5.4 Fingolimod

Fingolimod (Gilynea), is a structural analogue of sphingosine. It blocks receptor of sphingosine-1-phosphate (S1P) which is an important signalling lipid involved in the migration of lymphocytes from secondary lymphoid organs to the periphery. More lymphocytes released from secondary lymphoid organs with SIP (Brunkhorst et al., 2014). It inhibits mature dendritic cells migration to secondary lymphoid organs and also improves myelination in animal models of EAE. Patients treated with fingolimod showed lower annualized relapse rate compared to patients treated with interferon (Derfuss et al., 2015).

1.5.5 Alemtuzumab

It is also known as Lemtrada as a market drug**.** (*Campath-1H*) is a humanized antibody that was initially approved for the treatment of B-cell chronic lymphocytic leukemia. CD52 the target molecule, is a glycoprotein expressed widely on T and B cells, NK cells, dendritic cells, monocytes, macrophages and granulocytes. Depletion of CD52 expressing cells and NK cells are caused by Alemtuzumab (Hu et al., 2009). EDSS were reduced in MS patients treated with Alemtuzumab than IFN-丿. However, Alemtuzumab can be given with caution as there could be side effects such as idiopathic thrombocytopenic purpura, Graves' disease and Goodpasture syndrome (Loma and Hyeman, 2011).

1.5.6 Dimethyl Fumurate (DMF)

Marked as Tecfidera, it was postulated that it plays both antioxidant and immunomodulatory effects, including a reduction in cytokine production, in the migratory capacity of the immune cells by inhibition of nuclear factor kappa B (NF-κB) and the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) transcriptional pathway. It was also suggested that it affects predominantly memory T and B cells and induces a shift toward anti- inflammatory (Th2) response (Gill and Kolson, 2013)

Clinical trials of DMF-treated RRMS patients showed significant reductions in the proportion of patients with relapse, disability progression, and numbers of new magnetic resonance imaging (MRI) lesions (Gold et al., 2015).

RRMS patients treated with DMF show a decrease in CD4+ cells producing IFN-ψ, TNF-α, and GM-CSF after 6 months of treatment, also a reduction in the total number of B lymphocytes and in memory B cells which is very efficacious in producing the pro-inflammatory cytokine

as TNF-α and GM-CSF. Wu et al, has recently reported that RRMS patients treated with DMF had reduced levels of naive and memory B cells, whereas there was an increase in B cells with phenotypes reported to produce IL-10 and to have regulatory capacity (Wu et al., 2017). Also, an increase in IL-4 and decrease in IFN-ψ and IL-17–expressing CD4+ T cells were observed. Rare cases of progressive multifocal leukoencephalopathy have been reported to be associated with long-term of treatment.

1.5.7 Teriflunomide

It is known as Aubagio, it is the second oral drug to be approved by the FDA. Teriflunomide is an active metabolite of leflunomide that inhibits the proliferation of blasting B and T cells (Ingwersen et al., 2016).

It is selectively and reversibly inhibiting dihydroorotate dehydrogenase, a key mitochondrial enzyme in the de novo pyrimidine synthesis pathway, leading to a reduction in proliferation of activated T and B lymphocytes without causing cell death. Results from animal experiments modelling the immune activation implicated in MS demonstrate reductions in disease symptoms with teriflunomide treatment. It impairs formation of immunological synapse (Bar-Or et al., 2014).

It reduces proinflammatory cytokines as IL-6 and IL-8, inhibition of tyrosine kinase activity and inhibition of interaction between APCs and T cells ¨This decrease in pro inflammatory cytokine release is consistent with earlier reports of impaired Th1 differentiation and enhanced Th2 differentiation in the presence of teriflunomide.

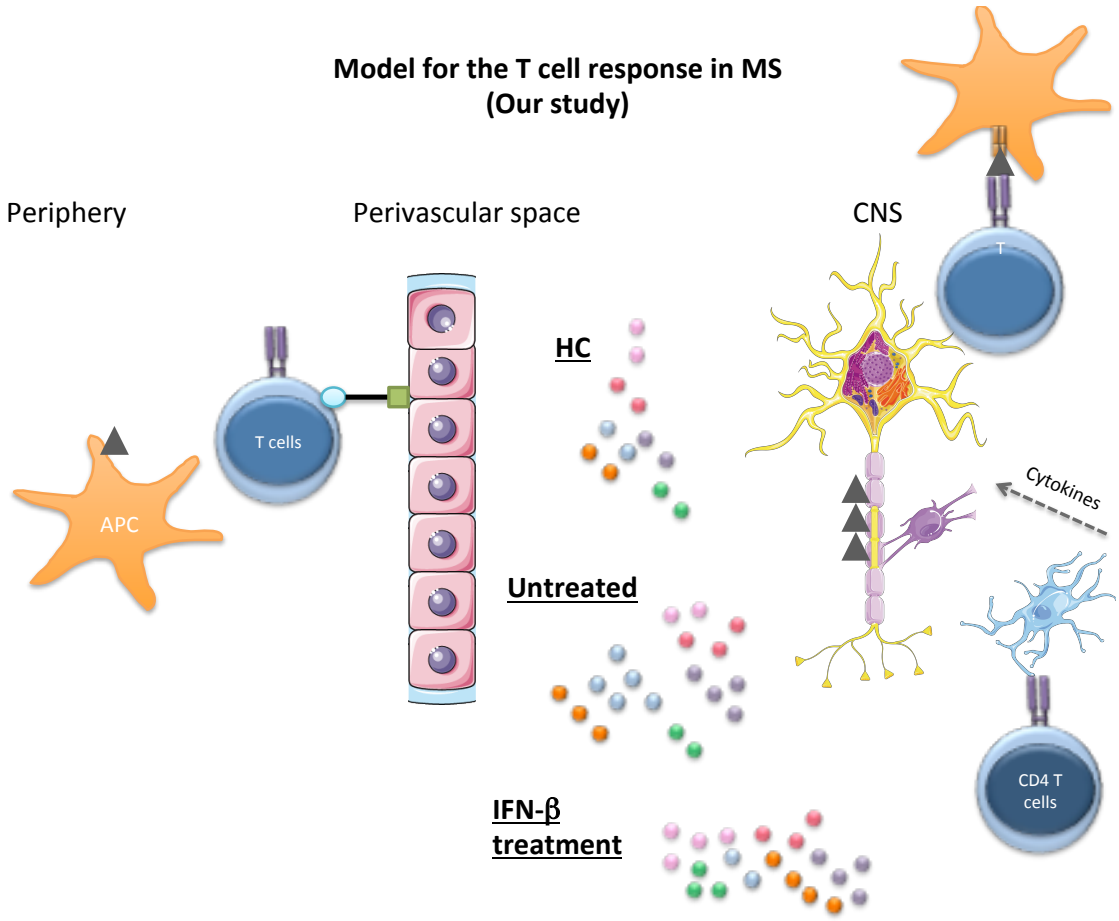
The side effect may include a reduction in the number of lymphocytes in the peripheral blood.

Cao et al., 2015 findings and relation to our work

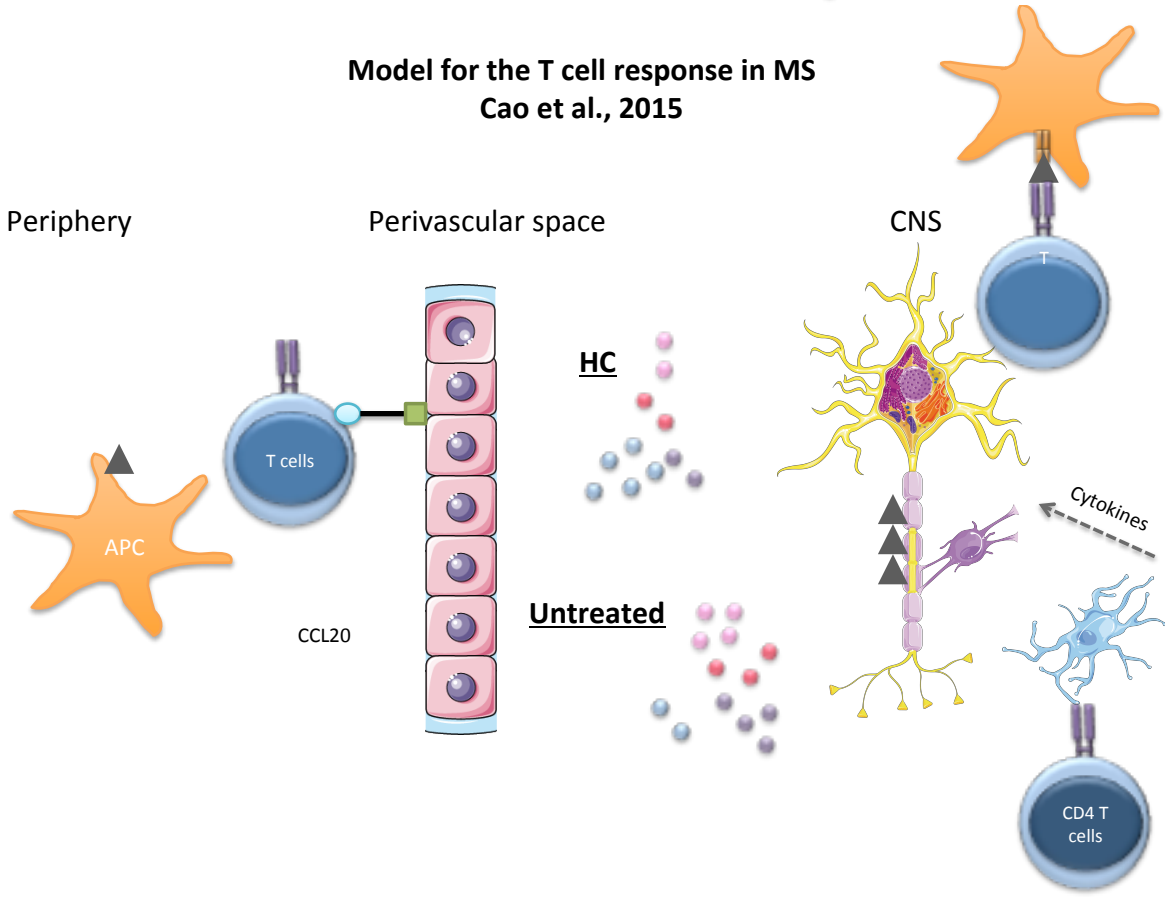
One study identified antigen-specific T cells in MS patients, with enrichment in the CCR6+ fraction by Cao et al., 2015. They demonstrated functional and transcriptional differences in myelin-reactive CD4+ T cells in untreated MS patients as compared to healthy controls, identifying secretion of IL-17, GM-CSF, and IFN-γ in MS as compared to IL-10 secretion in healthy controls. Further, these autoreactive T cells are CCR6+ memory CD4+ T cells in both MS patients and healthy controls. In our study, we extended this approach by examining the antigen specific T cells in patients treated with IFN-b, which is a common treatment for MS beside healthy controls and untreated MS patients. In addition, we measured TNF-a which is a pro-inflammatory cytokines involved in the pathogenesis of MS. Autopsy of MS patients have elevated TNF levels at the site of active MS lesions; CSF and serum TNF levels in individuals with MS are elevated compared to unaffected individuals and TNF levels correlate to the severity of the lesions; and evidence that PBMC from MS patients just prior to symptom exacerbation have increased TNF secretion after stimulation compared to cells form the same patients during remission. IL-4 also were measured, a Th2 cytokine is a well-established role in the pathogenesis of allergy and other immunological diseases, where it is secreted by T cells to induce B cell activation and IgE class switching. Less well studied, but nevertheless also important, is its influence in other diseases, including neurological ones such as MS.

MHC Class II tetramers have emerged as an important tool for characterization of the specificity and phenotype of CD4 T cell immune responses. Therefore, we measured myelin MHC II tetramer-specific CD4+ T cells in the blood of MS patients and healthy controls. In addition, DR4 EBV (AEG) and (PRS) tetramers were measured.

Model for the T cell response in MS
(Our study)



Model for the T cell response in MS
Cao et al., 2015



Hypothesis and aims

It is appreciated that T cells play a role in MS pathogenesis, but their precise mechanism remains to be determined. Autoreactive T cells have an important role by responding to the myelin antigen as myelin basic protein (MBP), myelin oligodendrocyte protein (MOG) and proteolipid protein (PLP).

Studies have investigated the involvement of CD4+ and to a lesser extent CD8+ T cells in MS.

In this study we hypothesized that IFN-β treatment will alters the cytokine profile of CNS specific $CCR6^+$ $CD4^+$ T cells in MS patients.

The initial aims of this thesis were to:

1) **Confirm the published paper on CNS specific T cells of cytokine production (Cao et al., 2015).**

Th1 and Th17 are involved in the pathogenesis of MS, Th17 cells are more significant, although Th1 can mediate inflammation, and pro-inflammatory cytokines, such as IFN-γ and TNF-α, are secreted by Th-1 cells. It has also been reported that during the relapsing stage of MS, anti-inflammatory and Th2 cytokines (IL-4, IL-5 and IL-10) are predominate. Studies have shown that Th1/Th17 myelin-reactive T cells, which secrete inflammatory cytokines as IL-17, GM-CSF and IFN-γ were higher in myelin reactive CD4+ T cells from MS patients While marked reductions in regulatory T cells have been shown in different studies. Th17 which produce IL-17 express CCR6 that is suggested to play a role in inflammatory diseases as MS by secreting inflammatory cytokines. The majority of research has examined the peptide responses during RR-MS and compared them to the healthy stage.

Cao et al., 2015 is the only study identified antigen-specific T cells in untreated MS patients, with enrichment in the CCR6+ fraction, which secrete GM-CSF, IFN-γ, IL-17 and IL-10. In addition to the previous work, we also measure TNF-a and IL-4 cytokine responses.

We examined the response of the T cells in the RR-MS with/without IFN-β treatment.

Therefore, the next aim of this investigation was to:

2) **Study the effect of IFN-β treatment on the phenotype of CNS antigen- specific $CD4^+$ T cells in MS patients and compare it to healthy controls**

For many years, T-cell responses to different epitopes of MOG, PLP and MBP have been detected in the peripheral blood cells of MS patients and the frequency of myelin CD4+ T cells are higher in MS patients compared to healthy controls (Raddassi et al., 2011). The detection and frequency of MOG-specific T cells was measured using DRB1*0401/MOG97– 109(107E-S) tetramers in MS patients; CD4+CD25intermediate DRB1*0401/MOG97– 109(107E-S) tetramer–positive T cells percentage were significantly higher in (RR- MS) as compared to healthy controls (Kofler et al., 2011).

EBV is a very common virus associated with MS, so we tested 2 (DR4) tetramers for EBV in HC and MS patients. (Long et al, 2013) detected EBV (PRS) in healthy controls; we examined it also in MS patients. In addition, we measured EBV (AEG) tetramers which is the first study to examine it in healthy control and MS patients

The third aim of this study was to:

3) **Detect and quantify antigen specific CD4+ T cells in healthy controls and MS patients by staining with DR4 (myelin and EBV) and DR7 (EBV) tetramers.**

Many studies have been made in the treatment of relapsing forms of MS. Different treatments are currently in clinical use that significantly reduce annualized relapse rates. The next step in the evolution of MS therapeutics will be to develop drugs that preferentially deplete or inactivate pathogenic cells without curtailing protective immunity. It is important to investigate the effect of the treatment by measuring the physical and the immunological markers.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2: Materials and Methods

2.1 Reagents

Table 2.1 Culture medium and solutions

PBS	Phosphate Buffered Saline (8g/l NaCl, 0.26/l KCl, 1.15g/lNA_2HPO_4, 0.2g/ml KH_2PO_4); 1 PBS tablet added per 100ml distilled H_20 [Oxoid, Basinstoke,UK]
PRMI medium	RPMI 1640 medium (Sigma Aldrich) with 1% GPS (2mM L-glutamine, 100U/ml penicillin, 100ug/ml streptomycin; HyClone, Northumberland, UK) and 1% HEPES (Sigma Aldrich)
FACS buffer	PBS with 2% Bovine Serum Albumin (BSA; Sigma Aldrich, Dorset, UK)
MACS buffer	PBS, 0.5% BSA and 2mM ethylenediaminetetraacetic acid (EDTA; Sigma Aldrich)
RPMI + 10% FCS	RPMI-1640 medium with 10% heat-inactivated foetal calf serum (FCS; Biosera, Ringmer, UK)
Sort buffer	1:1 RPMI-1640 medium with heat-inactivated foetal calf serum

2.2 Antibodies

Specificity	Fluorochrome	Clone	Company	Cat No	Dilution
CD3	BV-421	OKT3	Biolegend	317332	1/100
CD4	PE-Vio-770	MT466	Miltenyi	130096552	1/80
CD25	BV-421	BC96	BioLegend	302629	1/20
CD45RA	PE-Texas Red	HI100	BD Bioscience	562298	1/800
	BV510	HI100	BD Bioscience	562298	1/800
CD127	FITC	eBioRDR5	e Bioscience	14127882	1/20
CCR6	APC	G034E3	BioLegend	353416	1/20
CCR7	Alexa Fluor 488	G043H7	Biolegend	353206	1/20
CD14	APC efluor 780	G043H7	BioLegend	14127883	1/20
CD19	APC efluor 780	G043H7	Biolegend	41278827	1/20
PD-1	BV786	G043H7	BioLegend	4127845	1/160
	PE	G04345	BioLegend	4127821	1/20

Table 2.2 Primary monoclonal antibodies used for flow cytometry-against surface molecules. Abbreviations used: Alexa Fluor (AF), Allophycocyanin (APC), Cyanine 5.5 (Cy5.5), Cyanine 7 (Cy7), Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Phycoerythrin Texas Red (PETR). All antibodies raised in mouse.

2.3 Patients and ethics

For this study, peripheral blood was drawn from healthy individuals and approved by the Institute of Inflammation and Ageing at Queen Elizabeth Hospital. Patients were recruited after obtaining informed consent forms and ethical approval was provided under study 'Molecular and Cellular Studies on Inflammation in Multiple Sclerosis' (UKCRN ID 13585). Healthy control donors were without a history of autoimmune or chronic disease. Donors were recruited at the University of Birmingham after obtaining informed written consent. Ethical approval for healthy controls was provided under study 'Immune mechanisms in the ocular microenvironment' (UKCRN 4654). Clinical information can be found within Appendices I-III.

2.4 Sample collection and PBMC isolation

Blood samples were collected by venipuncture in 10-ml EDTA vacutainer tubes (Greiner Bio-One, Gloucestershire, UK) according to the guidelines at the hospital. PBMC were isolated, and the blood was diluted 1:1 with serum-free RPMI-1640 media containing 5% L-glutamine (200 mM), penicillin (10,000 units), streptomycin (10 mg/ml) (GPS) and a 5% HEPES buffer (all from Sigma Aldrich, Poole, UK). Blood was layered onto Ficoll-Paque (GE Healthcare Bio-Sciences, Uppsala, Sweden) and centrifuged at 400 g for 30 min with no brake applied. Mononuclear cell layers were harvested and washed at 300 g for 8 min and then at 200 g for 8 min to remove platelets. The PBMC were resuspended in 10-ml serum-free RPMI-1640 media supplemented as described and counted using trypan blue dead cell exclusion dye (Sigma Aldrich). After being washed, the PBMC were resuspended in the suitable medium as required for each procedure. Trypan blue, DMSO was obtained from (Sigma Aldrich) and Ficoll-Paque was from (GE Healthcare Bio-Sciences, Uppsala, Sweden).

2.5 Monocyte isolation

A monocyte isolation kit was used to purify monocytes (MACS CD14 Microbeads; Miltenyi Biotec 130-050-201, UK). After counting the PBMC, they were centrifuged at 300 g for 10 min and removed from the supernatant completely. They were resuspended in 80 µl of buffer per 1 x 10^7 total cells; 20 µl of CD14 microbeads were added per 1 x 10^7 cells, and they were mixed well and then incubated for 15 min in the fridge. The cells were washed by adding 1 to 2 ml of buffer per 1 x 10^7 cells; they were centrifuged at 300 g for 10 min and then removed from the supernatant completely. The cells were resuspended in 500µl of buffer per 1x10^8 cells. The cell suspension was added to an MS column (Miltenyi Biotech, Surrey, UK) on a MiniMACS separator magnet (Miltenyi Biotech) that was rinsed with the buffer in a waste tube. The column was rinsed with buffer and collected in a waste tube, always waiting until the column reservoir was empty before adding the next solution. Cell suspension was applied to the column, and unlabelled cells that flowed through were collected. The column was washed three times with the buffer, and effluent was collected into the same tube as the unlabelled cells (this was the unlabelled non-monocyte fraction). The non-monocyte fraction was counted, and a small volume (50 µl) was kept for a purity check. The labelled monocyte fraction was removed from the column. The column was removed from the separator and placed in a collection tube; buffer was added, and the plunger was pushed into the column to flush out the labelled cells. A small volume was kept for a purity check.

2.6 CD4 memory T-cell isolation

By using the MACS memory $CD4^+$ isolation kit (Miltenyi 130-091-893, UK), unlabelled $CD4^+$ memory cells were isolated by depletion of non-$CD4^+$ cells and naive $CD4^+$ cells. After counting the PBMC, they were centrifuged at 300 g for 10 min and the supernatant was completely removed. The pellet was resuspended in 80µl of MACS buffer per 1x10^7 total cells.

The cell pellet was resuspended in 80µL of MACS buffer/10^7 total cells and 20µL of $CD4^+$ T-cell microbeads (Miltenyi Biotech)/10^7 total cells. The cells were mixed well and incubated for 15 min in the fridge (2 °C to 8 °C). The cell and bead mixture was washed in cold MACS buffer and resuspended in 500µL of MACS buffer. The cell suspension was added to an MS column (Miltenyi Biotech) on a MiniMACS separator magnet (Miltenyi Biotech) that was rinsed with the buffer in a waste tube. The column was rinsed with buffer and collected in a waste tube, always waiting until the column reservoir was empty before adding the next solution. Cell suspension was applied to the column, and unlabelled cells that flowed through were collected. The column was washed three times with the buffer, and effluent was collected in the same tube as the unlabelled cells (this was the unlabelled non-monocyte fraction). Then the non-monocyte fraction was counted. The labelled monocyte fraction was removed from the column, and $CD4^+$ microbeads were bound to the non-$CD4^+$ T-cells and $CD4^+$ naïve cells to retain in columns. The column was removed from the separator and placed in a collection tube; buffer was added and the plunger was pushed into the column to flush out the labelled cells. 50µl from all cell fractions were kept for a purity check.

2.7 T cell library assays

CCR6- and CCR6+ memory from all the samples were pre-sorted in 96-well round bottom plates (Costar) at $2X10^3$ per well in PRMI 1640 medium and human serum, and stimulated with PHA-L (1µg/ml) and IL-2 in the presence of irradiated (45Gy) allogenic feeder cells ($2x10^4$ per well), IL-2 was added in day 4, 7 and 10. After 2 weeks of expansion 10^6 T cells were stimulated with myelin peptides (10 µg/ml), peptide 1 that includes (MOG97-109 and PLP180- 199) and peptide 2 that includes (MBP85-99, MOG222-241, PLP30-49 and PLP129-148), or PHA, and 10^5 autologous CD14+ monocytes. **Peptide sequences are summarized in**

table 2.1.

Culture supernatants from these cultures were assayed for the presence of cytokines (TNF-α, IFN-γ, IL-4, IL-10, IL-17, GM-CSF) by using a multiplexed bead-based immunoassay (HSTCMAG- 28SK) (Merck, Millipore, UK) and run by Luminex (Bio-Plex Multiplex Immunoassay, UK) **(Figure 2.1).**

Peptide	Sequence
MOG97-109	RTELLKDAIGEGK
PLP30 39	ALFCGCGHEALTGTEKLIET
PLP180-199	TWTTCQSIAFPSKTSASIGS
PLP129-148	QHQAHSLERVCHCLGKWLGH
MBP85-99	NPVVHFFKNIVTPRT
MOG222-241	VALIICYNWLHRRLAGQFLE

Table 2.3 Myelin peptide sequences.

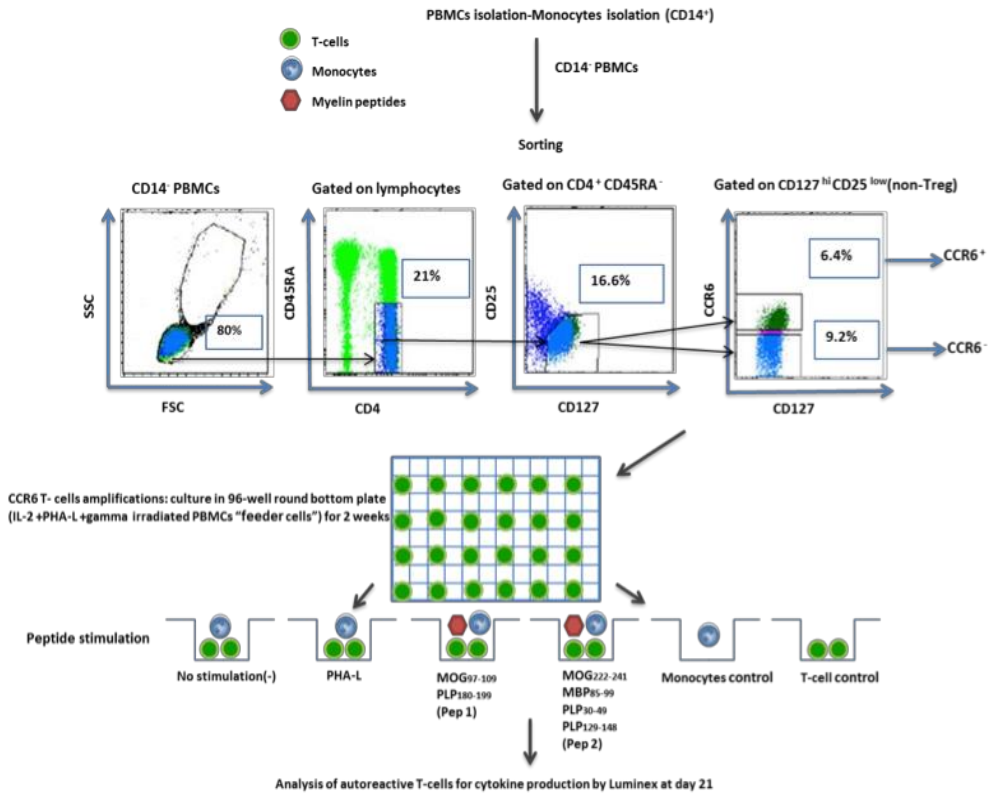


Figure 2.1. Amplified T cell library assay. Naïve, CCR6$^-$memory and CCR6$^+$ memory CD4 T cells from patients with MS or healthy controls (HC) were sorted and seeded at 2,000 cells per well in 96-well plates, and stimulated with PHA and IL-2 in the presence of irradiated allogeneic feeder cells for 2 weeks. After the stimulation cocktail was washed out, the cells from each well were re-stimulated by autologous monocytes and myelin (day7) were measured by ELISA assay.

2.8 HLA analysis

The QIAamp DNA Blood Midi Kit was used to extract DNA from 1-2 ml of blood. The blood was first lysed and the lysate then loaded onto a spin column. DNA binds to the column and impurities are washed away using 2 different wash buffers, the DNA is then eluted from the column. DNA is eluted in Buffer AE (10mM Tris-Cl, 0.5mM EDTA, pH 9.0) and is ready to use for PCR amplification Pipet 200µl of Protease into a 15ml centrifuge tube then add 2ml of blood and mix briefly. Add 2.4ml of Buffer AL and mix thoroughly by inverting the tube 15 times, followed by vigorous shaking for at least 1 min. Incubate sample at 70°C for 10 min.

Add 2ml of ethanol to the sample and mix by inverting 10 times, followed by additional vigorous shaking. Transfer half of the sample into the QIAamp midi column placed within a provided 15ml collection tube, taking care not to get sample on the rim of the column. Close the cap and centrifuge at 1850g for 3 min. Remove the column from the tube, discard the filtrate, and replace the column. Collect waste generated in waste container. Transfer the remaining sample into the column, close the cap and centrifuge at 1850g for 3 min. Remove the column from the tube, discard the filtrate, and replace the column. Wipe any spillage off the thread of the 15ml tube before re-inserting the column. Add 2ml of Buffer AW1 to the column, close the cap and centrifuge at 4500g for 1 min. Add 2ml of Buffer AW2 to the column, close the cap and centrifuge at 4500g for 15 min. Place the column in a clean 15ml centrifuge tube (provided in kit). Discard the filtrate as previously and dispose of collection tube in autoclave bag. Pipet 300µl of Buffer AE directly onto the membrane of the column and close the cap. Incubate at room temperature for 5 min, then centrifuge at 4500g for 2 min. Reload the eluted buffer containing the DNA onto the membrane of the column. Close the cap, incubate at room temperature for 5 min then centrifuge at 4500g for 2 min. Pipet 300µl of fresh Buffer AE onto the membrane of the column. Close the cap, incubate at room temperature for 5 min then centrifuge at 4500g for 2 min. The concentration and purity of extracted DNA can be measured using the Nano Drop Spectrophotometer.

DNA amplification by PCR: Prepare master mix 6µl, Taq Polymerase0.2µl (1U), Nuclease-free Water to 20µl per reaction. Pipette the appropriate amount of gDNA (or water for blank well) into 0.2ml PCR tubes. Each plate can include blank, and positive and negative control wells as desired. Add amplification mix to PCR tubes containing gDNA (giving a total volume of 20µl per reaction tube). Cap tubes tightly to prevent evaporation. Place tubes in thermal cycler and run program as directed.

DNA hybridisation: Warm probe mix in 55 - 60°C heat block for 5 – 10 minutes to thoroughly solubilise components. Sonicate briefly (15 sec) in sonicating water bath, then vortex for 15 seconds. Add 15µl of probe mix and 5µl of the locus specific PCR product to 96 well thermal cycler plates. Seal plate with PE sealing tape. Hybridise samples using the suitable program for hybridisation. Once hybridisation program is at the 56°C hold stage, with the plate still in the thermal cycler, dilute each sample with 170µl of the diluted SA-PE. It is critical to dilute all samples within 5 minutes (following the 8 minutes at 56°C step). Remove plate from thermal cycler, stop hybridisation program and turn off thermal cycler. Transfer samples from thermal cycler plate into 96 well flat bottom microtiter plates to run on Luminex, and wrap plate in foil. Samples must be read within 30 minutes of being diluted. Samples were analysed using Match IT DNA software.

2.9 T-cell expansion

$5x10^6$ PBMC were used per reactivation. Split the cells in half into 2 x 15ml tubes and pellet cells by centrifugation at 1600rpm for 5mins. Resuspend in 1ml sterile RPMI (no supplements) and add 5µg/ml of the relevant peptide to one tube of $2.5x10^6$ cells only (pulsing only half with peptide prevents all the antigen-specific T cells killing each other). Then Incubate for 1hr at 37°C, flicking every 15mins to resuspend the cells. Wash the cells x2 in RPMI. Put the peptide-pulsed and unpulsed cells back together after the final wash into 2ml RPMI containing L-glutamine, pen/strep and 5% Human serum. Transfer into one well of a 24-well plate. Re-stimulate the polyclonal on day 7 with $1x10^6$ γ-irradiated PBMC pulsed with peptide. You can add 50IU/ml IL-2 to the culture at this point.

2.10 Tetramer staining

Binding of each tetramer to antigen- specific $CD4^+$ T cell clones of the relevant MHC II restriction was optimized with respect to tetramer concentration and time. PBMC cells to be analyzed by flow cytometry were all cryopreserved before use. 10^6 PBMC were washed in ice-cold buffer (PBS containing 0.5% BSA and 2 mM EDTA, pH 7.2), before resuspending in 500µl human serum. After 5 min, a pre-titrated volume of MHC II tetramer (0.5–1.0 µl) was then added to relevant cells, or no tetramer to control tubes, which were incubated at 37°C for 2 h during which the cells were resuspended periodically by agitating the tubes. After wash, the cells were stained for surface markers by incubation for 30 min on ice with predetermined saturating amounts of various combinations of the following antibodies: BV421-conjugated CD3 mAb (Biolegend), PE-Vio770-conjugated CD4 mAb (Miltenyi), AF700-conjugated CD45RA mAb (BioLegend), BV510-conjugated CD45RA mAb (eBioscience), Alexafluor-conjugated CCR7 mAb (BD), APC- conjugated CCR6 mAb (BD), APCeFluor780-conjugated CD14 mAb (eBiosci- ence), Percp-Cy5.5-conjugated CXCR5 mAb (Beckman Coulter) and BV786-conjugated PD-1 mAb (eBioscience). After two subsequent washes, stained cells were either analyzed immediately or fixed in 2% paraformaldehyde for later analysis, on either an Epics flow cytometer (Beckman Coulter) or an LSRII flow cytometer (Beckman Coulter). $CD3^+CD4^+$ cells falling within the lymphocyte gate were analyzed for tetramer and surface antigen staining.

All data were processed using BD FACSDiva software and Kaluza (BD).

2.11 Cytokine analysis

Culture supernatants from the expansion T cell assay were assayed for the presence of cytokines by Luminex (Bio-Plex Multiplex Immunoassay) which is formatted on magnetic beads. Capture Abs detected against the desired biomarkers (IFN-γ, TNF-α, GM-CSF, IL-10,

IL-4 and IL-17) are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection Ab is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate, which serves as a fluorescent indicator.

The absorbance was measured and analysed on a microplate reader (Bio-Rad).

2.12 Flow cytometry

PBMC from peripheral blood (5 x 10^6 cells/200µl) were added to a round-bottom 96-well plate and washed in phosphate buffered saline (PBS) plus 2% bovine serum albumin (BSA; Sigma Aldrich) by centrifugation at 300 g for 4 min. Cells were stained with Zombie AquaTM Fixable Viability dye diluted in cold PBS (1/400; BioLegend, London, UK) on ice for 20 min and washed in PBS plus 2% BSA at 300 g for 4 min. Cells were stained with fluorescently labelled antibodies to detect surface markers for 20 min on ice and were washed in PBS plus 2% BSA and centrifuged at 300 g for 4 min. Cells were fixed in medium A (FIX & PERM® cell fixation kit, Life Technologies, Invitrogen, Paisley, UK) for 15 min at room temperature and washed in PBS plus 2% BSA by centrifugation at 300 g for 4 min. Cells for surface marker staining only were resuspended in 100µl PBS plus 2% BSA and stored at 2 °C to 8 °C for later analysis (eBioscience, Hatfield, UK). Cells were incubated in a fixation/permeabilisation buffer for 15 min at room temperature and washed in PBS plus 2% BSA by centrifugation at 300 g for 4 min. Cells were resuspended in PBS plus 2% BSA, and samples were run using the BD flow cytometer (BD Biosciences, Oxford, UK). Single colour compensation was achieved using OneComp eBeads (eBioscience).

Data were analysed using the Kaluza Flow Cytometry Analysis Software v1.2 (Beckman Coulter, High Wycombe, UK).

2.13 ELISpot

Pre-treat the plate using 50µl of filtered 70% ethanol per well for no longer than 30 seconds. Flick ethanol out and quickly wash x6 with 100µl filtered PBS (No tween). Tap the plate and dry it. Coat the plate with 50µl per well of 7.5µg/ml anti-IFN-γ mAb, 1-DIK (green top). For one plate, dilute 37.5µl of antibody in 5mls filtered PBS. Leave at RT for 3hrs. Wash plate x6 with 100µl RPMI (no serum). Block plate using 200µl RPMI/10% FCS for 30-60mins. Flick media out and tap the plate to dry it. Add PBMC to relevant wells in 100µl RPMI/10% FCS per well. Add 10µl of each of the following to relevant wells: 50µg/ml peptide (final conc 5µg/ml), 100µg/ml PHA (final conc 10µg/ml), DMSO diluted 1:2.5 in PBS. Leave in the incubator at 37°C, 5% CO_2 overnight. Flick cells out into virkon and wash plate x6 in PBS tween. Add 50µl per well of biotinylated anti-IFNγ mAb, 7-B6-1 diluted 1:1000 in filtered PBS (no tween). Incubate at room temperature for 2 hours. Flick antibody out and wash plate x6 in PBS tween. Add 50µl per well of streptavidin-conjugated alkaline phosphatase diluted 1:1000 in filtered PBS (no tween). Incubate at RT for 1 hour. Flick antibody out and wash plate x6 in PBS tween. Remove the plastic casing from the plate and carefully wash the backs of the membranes. Leave some PBS tween in the wells while making up the developing solution: For 1 plate (4.7mls sterile, filtered water, 200µl Developing buffer, 50µl solution A and 50µl solution B. Mix well, filter using a syringe and use immediately. Flick out the last PBS tween wash and tap the plate dry (front and back). Add 50µl of the developing solution and leave to develop in the dark for 20 mins to 1hr. Keep an eye on it. Flick out the developing solution and wash the plate (front and back) with tap water. Dry in warm room. Proceed to ELISpot reader.

2.14 Statistics

Statistical analyses were performed using GraphPad Prism v8.0 (GraphPad Software Inc., CA, USA). Median values were determined, and non- parametric testing was used, as these data were not normally distributed. Samples were analysed using a Kruskal-Wallis test and Dunn's multiple comparisons test.

Statistical comparisons with a P-value of $P<0.05$ or below were considered significant.

CHAPTER 3

T CELL RESPONSE TO CENTRAL NERVOUS PEPTIDES IN HEALTHY CONTROLS AND UNTREATED MULTIPLE SCLEROSIS PATIENTS

Chapter 3: T cell response to central nervous system in healthy controls and multiple sclerosis patients

3.1 Introduction

Different types of T cells play an important role in the pathogenesis of multiple sclerosis (MS) including CCR6+ CD4+ memory T cells. A characteristic function of CD4+ Th cells is the ability to produce cytokine in response to antigen stimulation (Agrawal and Yong 2007). Th1 cells secrete IFN-γ and GM-CSF, Th2 cells secrete IL-5, IL-4 and IL-13, and Th17 cells secrete IL- 17A, while some iTreg express IL-10. Actually, GM-CSF; which is usually classified as a pro- inflammatory cytokine, can be secreted by some Th17, Th1 and Th2 cells. Hence, Th1 and Th17 cells can be responsible for inflammation by production of pro-inflammatory cytokines, which can contribute to the pathogenesis of MS (Goverman, 2009, Harrington et al., 2005; Park et al., 2005). It has also been reported that Th2 cytokines as (IL-4, IL-5) and T regulatory (Treg) CD4+ T cell that inhibits cell proliferation primarily via the secretion of IL-10 are predominate during RRMS (Annunziato et al., 2007; Cheng et al., 2014; Gregori et al., 2012). Multiple myelin proteins, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and Myelin proteolipid protein (PLP), are important in the process of myelination of nerves in the CNS and they are critical T cell autoantigens in MS (Pittock et al., 2007; von Budingen et al., 2008; Menge et al., 2011).

Although Raddassi et al., 2011, has highlighted the presence of antigen specific T cells in RRMS, little is known about the T cell response in the early stages of MS. For many years, T cell responses to different epitopes of MOG, PLP and MBP have been detected in the peripheral blood cells of MS patients and the frequency of myelin specific CD4+ T cells are higher in MS patients compared to healthy controls.

In parallel with previous studies (Cao et al., 2015); we measured cytokines produced; including GM-CSF, IFN-ψ, IL-10 and IL-17, in response to myelin peptides in healthy controls and MS patients. In addition to their cytokines panel, we measured IL-4 and TNF-α.

3.1.1 Objectives

1. Examine if there is a specific secretion of several cytokines from $CCR6^-$ and $CCR6^+$ $CD4^+$ memory T cells in response to immunodominant CNS peptides in healthy controls.

2. Compare the production of a number of cytokines from $CCR6^-$ and $CCR6^+$ $CD4^+$ memory T cells, stimulating with a number of immunodominant CNS peptides in both healthy controls and untreated MS patients.

In this study, libraries derived from $CCR6^+$ myelin reactive T cells from healthy controls almost exclusively expressed IL-17 compared to $CCR6^-$. Higher production of inflammatory cytokines (Th1, Th2 and Th17) as IFN-ψ, GM-CSF, TNF-α, IL-17 and IL-4 were produced in untreated MS compared to healthy controls in both CCR6 fractions. By contrast, the regulatory cytokine (IL-10) was produced in lower amounts from untreated MS patients.

3.2 Methods

$CCR6^+$ and $CCR6^-$ $CD4^+$ memory T cells were isolated by enrichment followed by cell sorting, and cultured with IL-2 and PHA, and then stimulated with different myelin CNS peptide antigen and different cytokine were measured from the culture supernatant. Cytokine productions were determined using Luminex bead-based immunoassays. This method allowed the analysis of different cytokines expressed in T cells.

3.3 Results

3.3.1 MOG/ PLP and MBP induce Th1/Th2 and Th17 cytokines production from CCR6- and CCR6+ CD4+ memory T cell culture in healthy controls and MS patients

In general, cytokines were produced in response to myelin peptides (P1=MOG97-109/PLP180-199 and P2 (MOG222-241/MBP85-99/PLP30-49/PLP129-148), above the level of the unstimulated controls, in healthy controls and untreated MS culture supernatant from CCR6- **(Fig. 3. 1 A-F and 3.2 A-F).** In addition, cytokines were produced in healthy controls and untreated MS culture supernatant from CCR6+ **(Fig. 3.3 A-F and 3.4 A-F).** A significant difference was however observed with IL-4 production from CCR6- T cells in healthy controls **(Figure 3.1 F)** as well as IL-4 and GM-CSF in untreated MS **(Fig. 3.2 A and F).**

Although cytokines levels were not statistically significant, there were some differences after stimulation with myelin peptides.

Similarly, CCR6+ CD4+ memory T cells induced GM-CSF and IL-4 production in response to the same myelin peptides with statistical differences in healthy controls **(Figu.3.3 A, F)** as well as GM-CSF in untreated MS **(Figure 3.4 A).**

For the experimental controls, we used PHA as a positive control for all the samples, we also examined the differences between different conditions of stimulation in culture, after 2 weeks of expansion with IL-2 and feeder cells, CCR6- and CCR6+ CD4+ memory T cells were examined without stimulation, T cells only and monocytes only.

Phytohemagglutinin-L (PHA-L) consists of only L-type subunits (isolectin L, "leuko-agglutinin"), and is well suited for high-efficiency induction and the functional analysis of T-lymphocytes. It is highly purified and tested for the high-efficiency stimulation of human lymphocytes. It is used as positive controls for PBMC stimulation.

Within CCR6- fraction from both healthy controls and MS patients, there was almost no production of GM-CSF (10 pg/ml) without stimulation, variable proliferation with monocyte and T cell controls, some T cells could be stimulated previously in the culture, therefore some proliferation could be detected. It was significantly increased when CCR6- and CCR6+ stimulated with PHA control (median=1000 pg/ml) compared to negative control where there is no stimulation **(Figure 3.5 A, B)**.

Similar results with PHA were detected from CCR6- and CCR6+ fractions from all other cytokines measured.

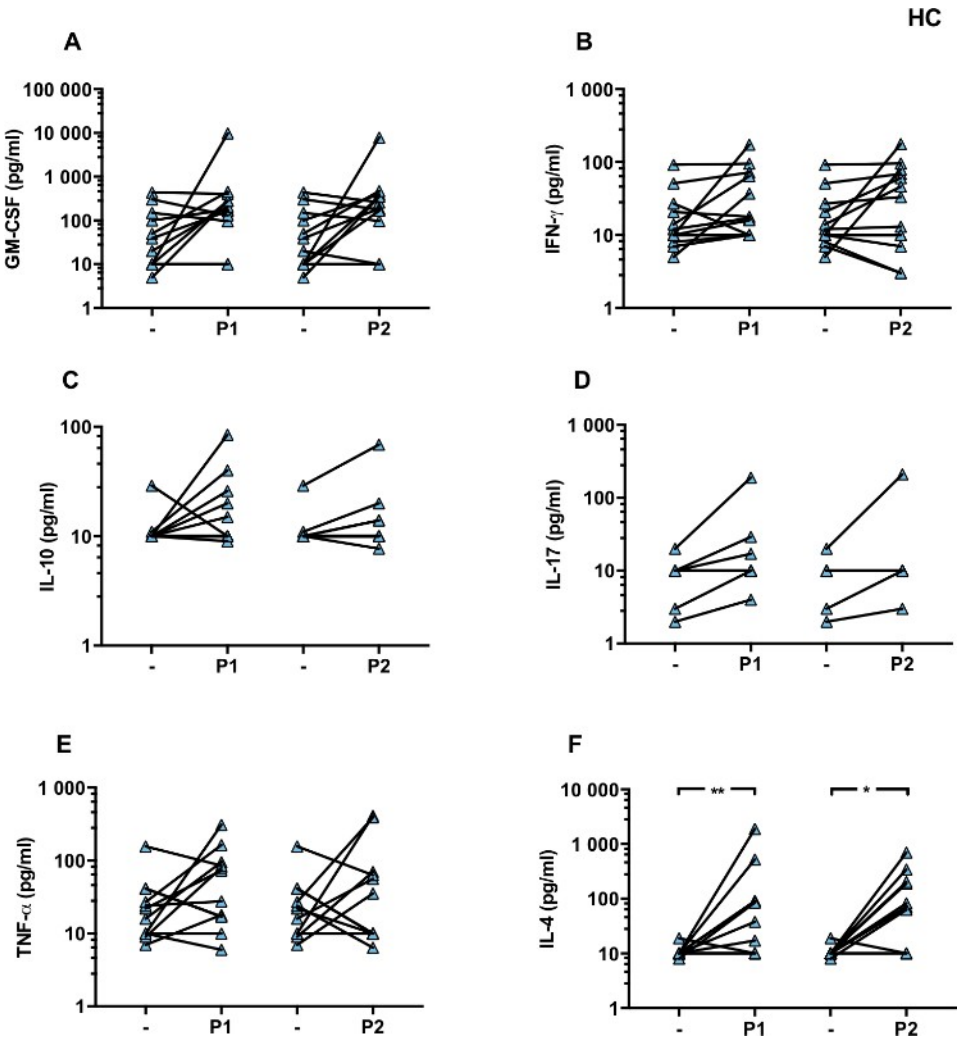


Figure 3.1 Specific cytokine production by CNS specific CCR6- CD4+ T cells in healthy controls. Cytokine levels of GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** without stimulation and after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6-CD4+ memory T cells. Data are significant to * ($P<0.05$) ** ($P<0.01$) using Dunn's multiple comparisons analysis (n=13). The data are represented as line graphs with each line representing a single individual.

- = No stimulation
P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

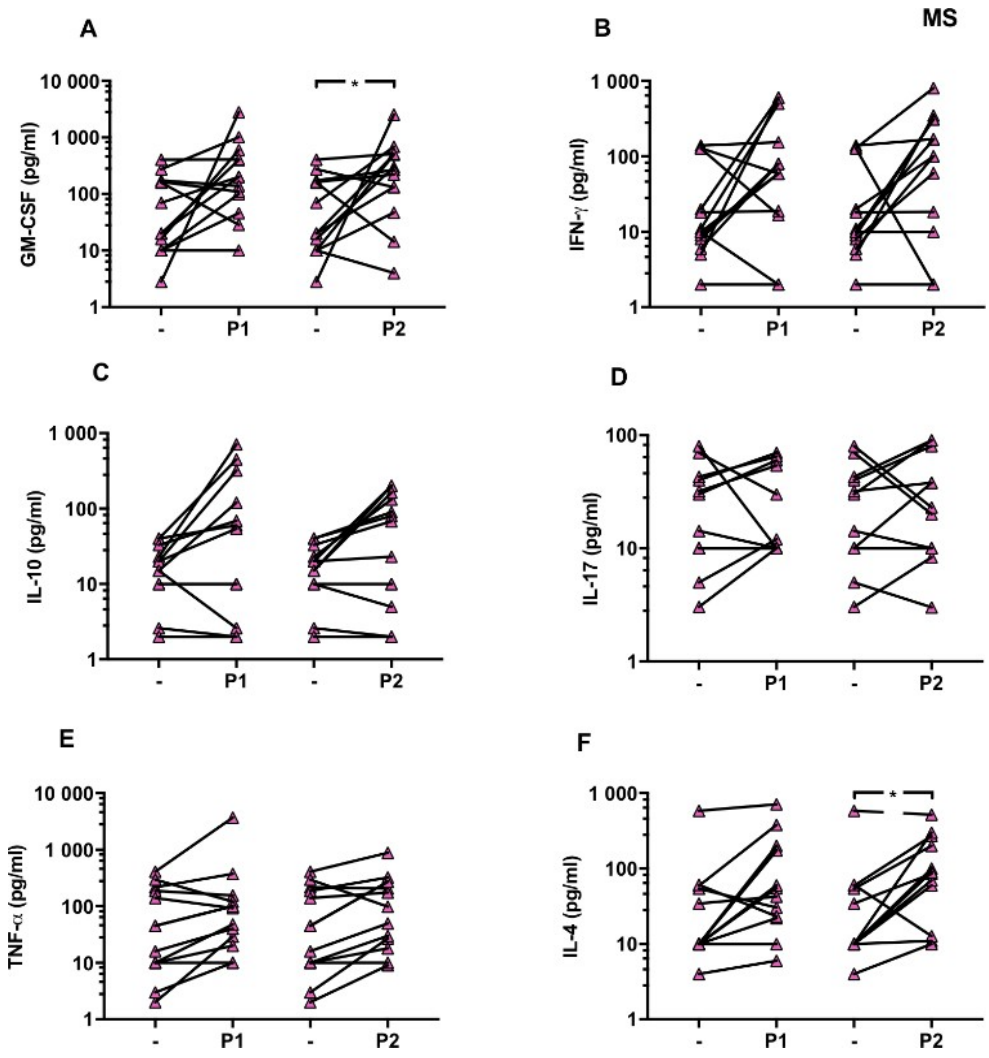


Figure 3.2 Specific cytokine production by CNS specific CCR6- CD4+ T cells in untreated MS. Cytokine levels of GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** without stimulation and after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- CD4+ memory T cells. Data are significant to * ($P<0.05$) ** ($P<0.01$) using Dunn's multiple comparisons analysis (n=13). The data are represented as line graphs with each line representing a single individual.

- = No stimulation
P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

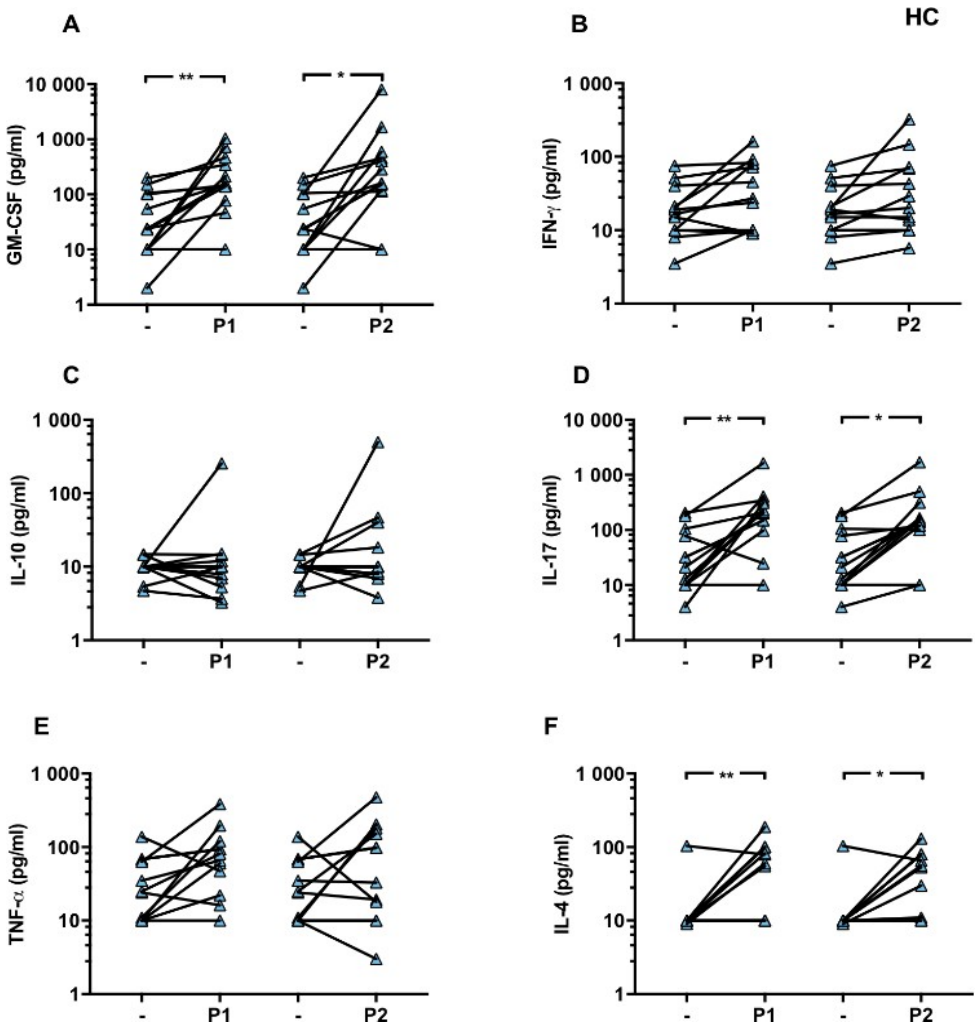


Figure 3.3 Specific cytokine production by CNS specific CCR6+ CD4+ T cells in healthy controls. Cytokine levels of GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** without stimulation and after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6+ CD4+ memory T cells. Data are significant to * ($P<0.05$) ** ($P<0.01$) using Dunn's multiple comparisons analysis (n=13). The data are represented as line graphs with each line representing a single individual.

- = No stimulation
P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

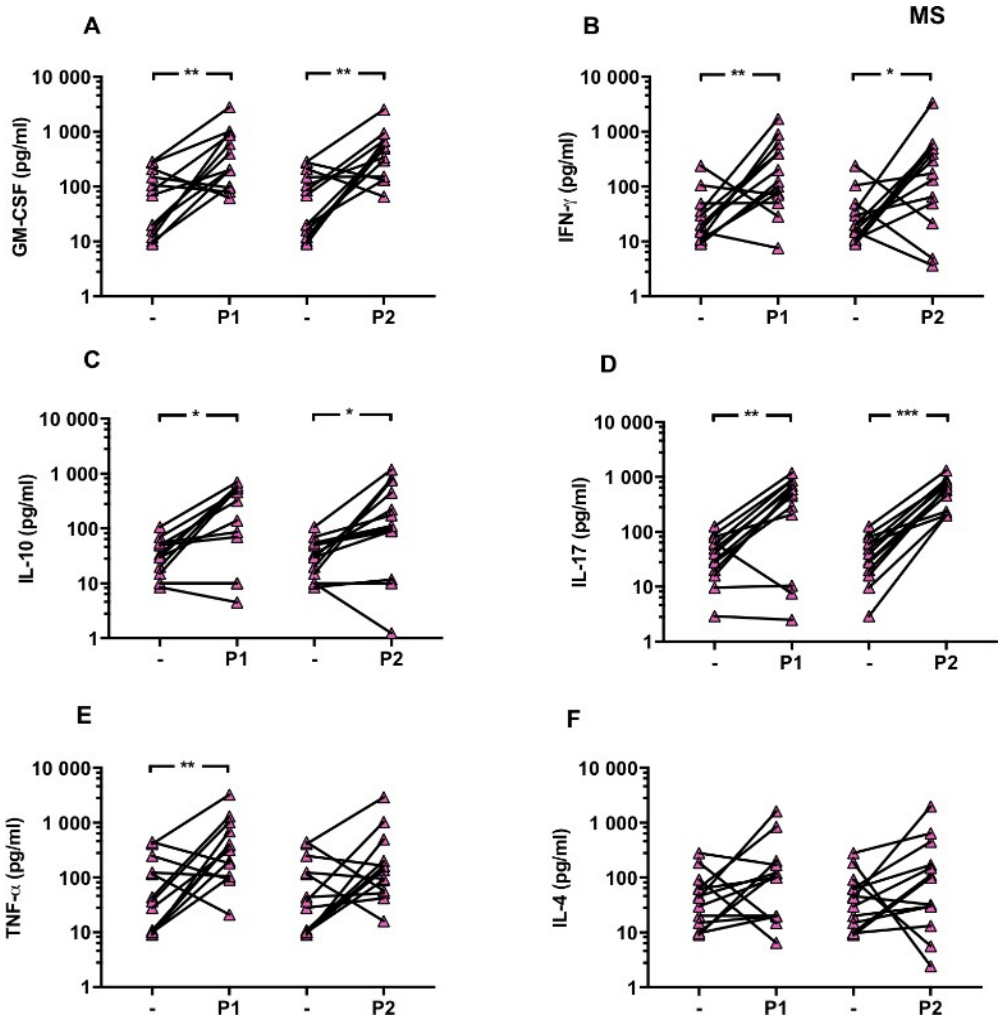


Figure 3.4 Specific cytokine production by CNS specific CCR6+ CD4+ T cells in untreated MS. Cytokine levels of GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** without stimulation and after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6+ CD4+ memory T cells. Data are significant to ** ($P<0.01$) using Dunn's multiple comparisons analysis (n=13). The data are represented as line graphs with each line representing a single individual.

- = No stimulation
P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

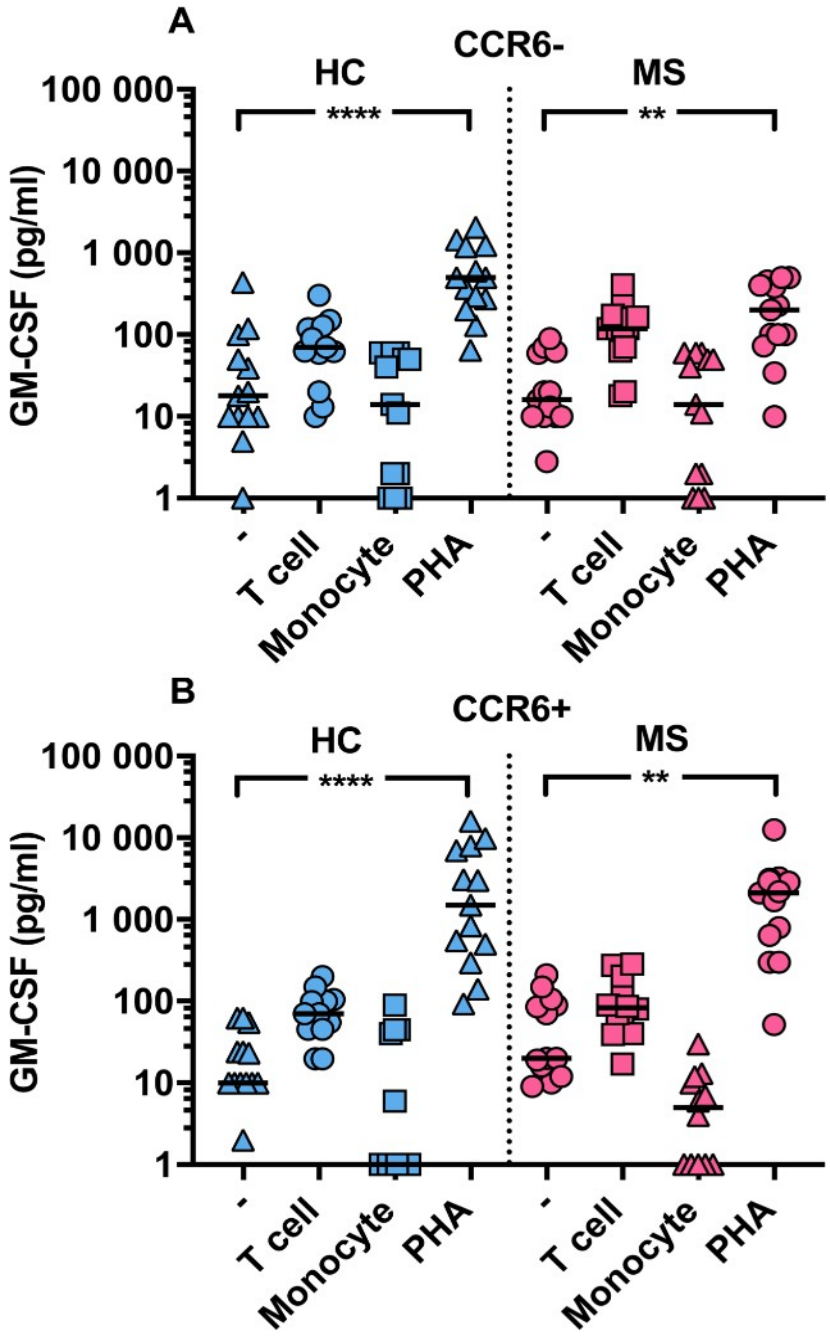


Figure 3.5 GM-CSF production from CCR6- and CCR6+ CD4$^+$ T cells is induced with PHA control in healthy control and untreated MS patients. GM-CSF levels in in culture supernatant of memory CCR6- (A) and CCR6+ (B) CD4+ memory T cells without stimulation, T cell only, monocyte only and after stimulation with PHA. Data are significant to ** ($P<0.01$), ***($P<0.001$) using Dunn's multiple comparisons analysis (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

- = No stimulation
PHA= Phytohemagglutinin

3.3.2 GM-CSF and IFN-ψ were produced in low levels from CCR6- and CCR6+ CD4+ memory T cell culture in healthy controls and MS patients

GM-CSF has been known as a hematopoietic growth factor and immune modulator (Shiomi et al., 2016). Recent studies showed that GM-CSF also had pro-inflammatory functions and contributed to the pathogenicity of Th17 cells in the development of Th17-mediated autoimmune diseases (Cook et al., 2016; Lotfi et al., 2019).

Th1 myelin-reactive T cells, which secrete GM-CSF and IFN-ψ, are inflammatory mediators (Behhi et a1., 2011; Codarri et al., 2011). IFN-ψ and GM-CSF are strongly produced by myelin specific CCR6+ T helper cells in MS (Cao et al., 2015).

In our study, in both healthy controls and MS patients, there were no statistical differences between CCR6- and CCR6+ fractions in GM-CSF (**Fig. 3.6 A, B**) and IFN-γ (**Fig 3.7 A, B**) with either P1 or P2 peptides.

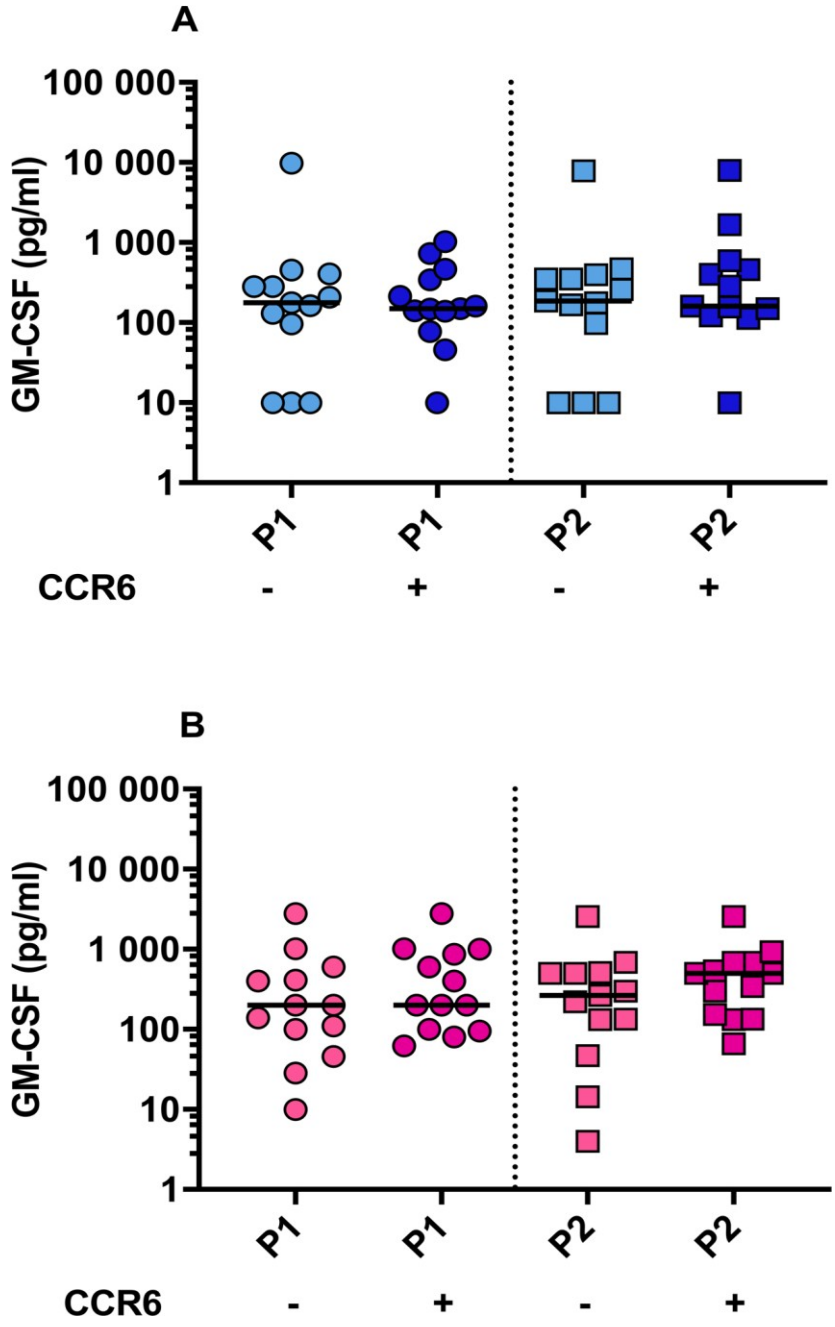


Figure 3.6 Comparison of GM-CSF production between CCR6- and CCR6+ CD4+ T cells in healthy controls and untreated MS groups. GM-CSF levels in healthy controls **(A)** and untreated MS **(B)** after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

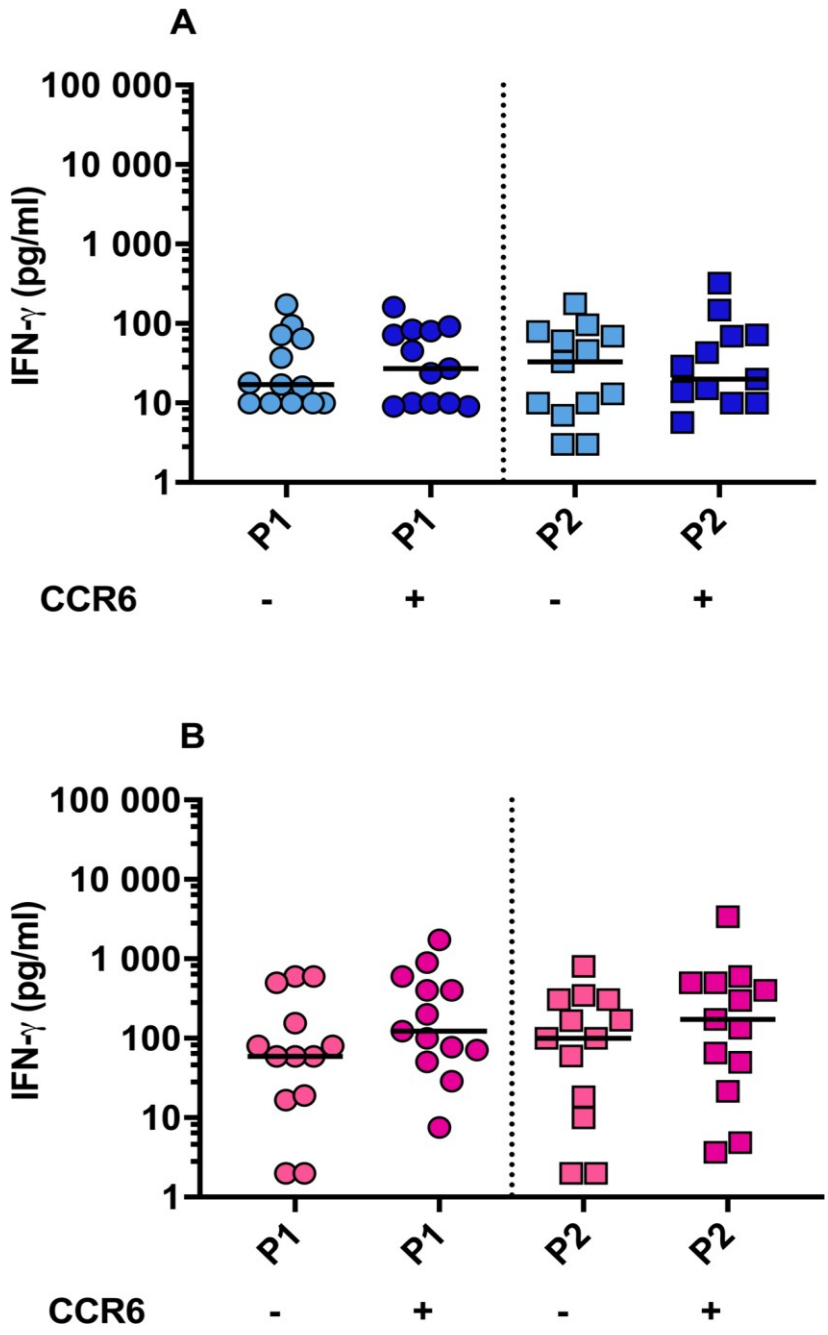


Figure 3.7 Production of IFN-γ in both CCR6- and CCR6+ CD4+ T cells in healthy controls and untreated MS groups. GM-CSF levels in healthy controls **(A)** and untreated MS **(B)** after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

3.3.3 IL-10 is produced from CCR6- and CCD6+ CD4+ memory T cells, while IL-17 is predominantly produced by CCR6+ but not CCR6- CD4+ memory T cells culture

IL-17 is produced by CD4+CCR6+ memory T cells when stimulated with myelin peptides (Cao et al., 2015) and IL-17 is mainly produced by CCR6+ CD4+ memory T cells in healthy controls and untreated MS patients.

IL-10 was detected in low levels in healthy controls **(Fig. 3.8 A)** from CCR6- and CCR6+ with no statistical differences. In MS, there was an increase in IL-10 from CCR6+ fraction response to myelin peptides, which has been shown before in previous research (Cao et al., 2015).

In our data, in healthy controls and MS cohort, very low levels of IL-17 (10 pg/ml) produced in CCR6- culture supernatant when stimulated with myelin peptides **(Fig 3.9 A, B)**. By contrast IL-17 where significantly increased (500pg/ml) from CCR6+ culture supernatant when stimulated with different myelin peptides **(Fig 3.9 A, B).**

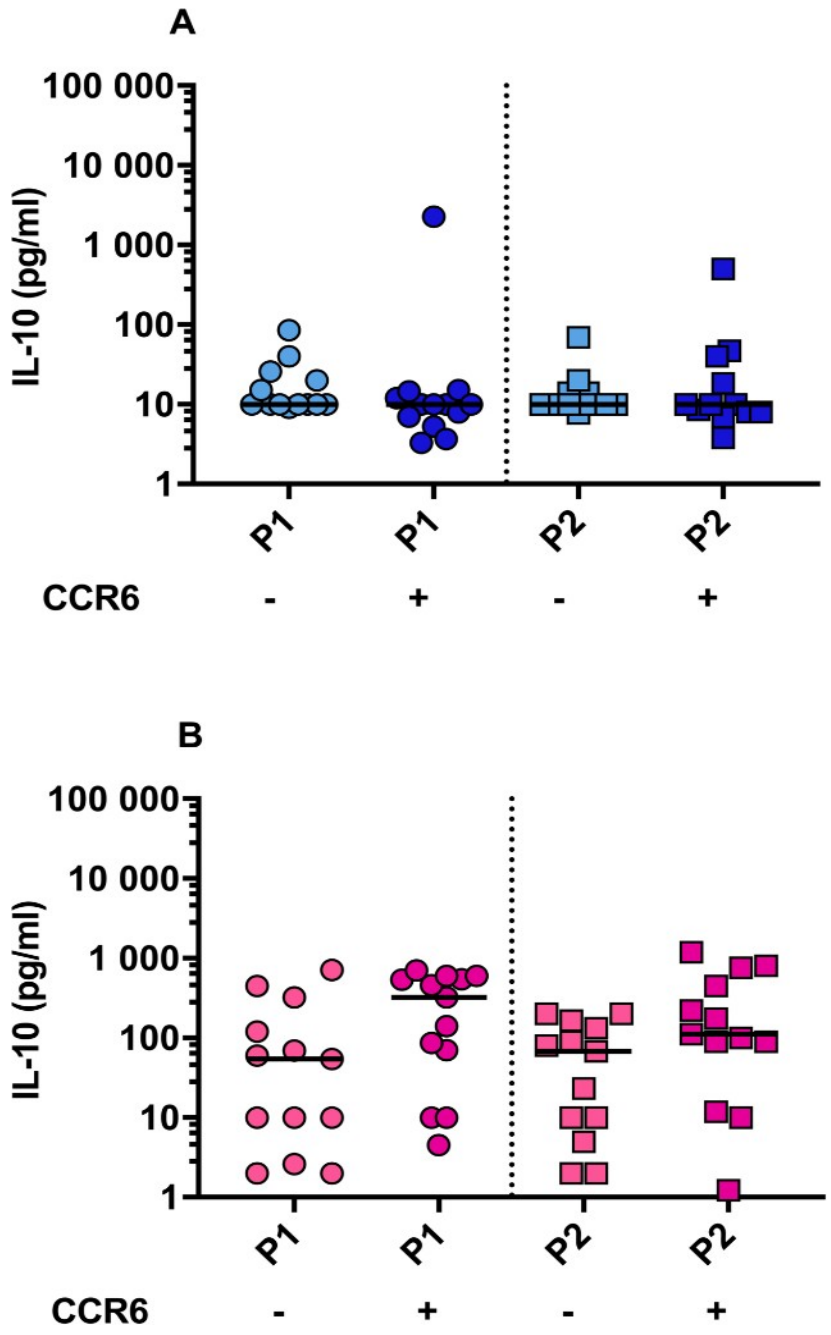


Figure 3.8 Production of regulatory cytokine in CCR6- and CCR6+ CD4+ T cells in untreated MS compared to healthy controls. IL-10 levels in healthy controls **(A)** and untreated MS **(B)** after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

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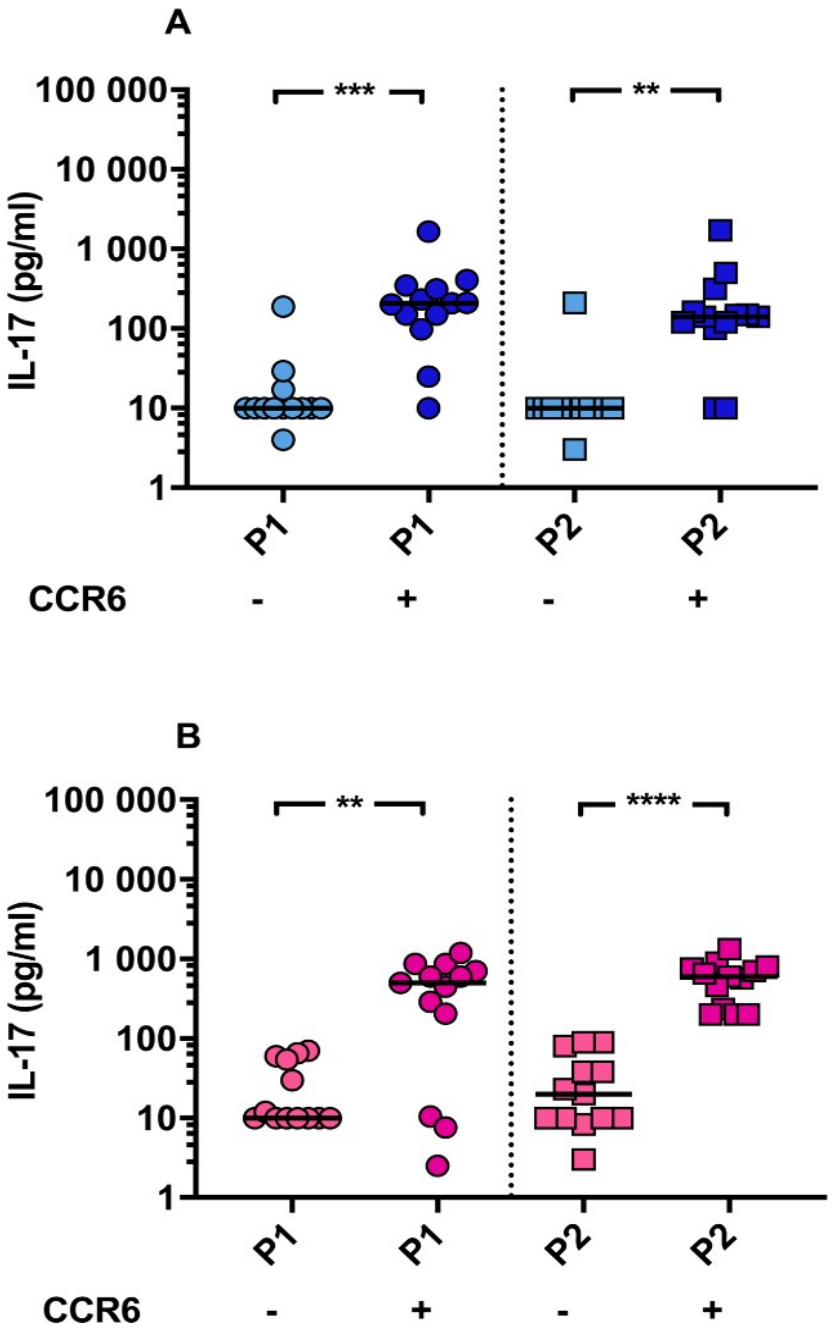


Figure 3.9 Increased levels of IL-17 in CCR6+, but not CCR6- CD4+ in untreated MS and healthy controls. IL-17 levels in healthy controls **(A)** and untreated MS **(B)** after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data are significant to ** ($P<0.01$), ***($P<0.001$) using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

3.3.4 TNF−α and IL-4 production from CCR6- and CCR6+ CD4+ memory T cells in healthy controls and MS patients

Furthermore, we measured TNF-α and IL-4, CCR6 were capable of secreting IL-4 and TNF-α. However, TNF-α was significantly higher from CCR6+ CD4+ T cells in MS patients in response to P1 myelin peptide, **(Fig. 3.10 B),** however there was no significant difference in healthy controls cohort **(Fig. 3.10 A).**

On the other hand, we did not observe any significant difference with IL-4 production between CCR6- and CCR6+ in healthy controls and MS patients **(Fig. 3.11 A, B).**

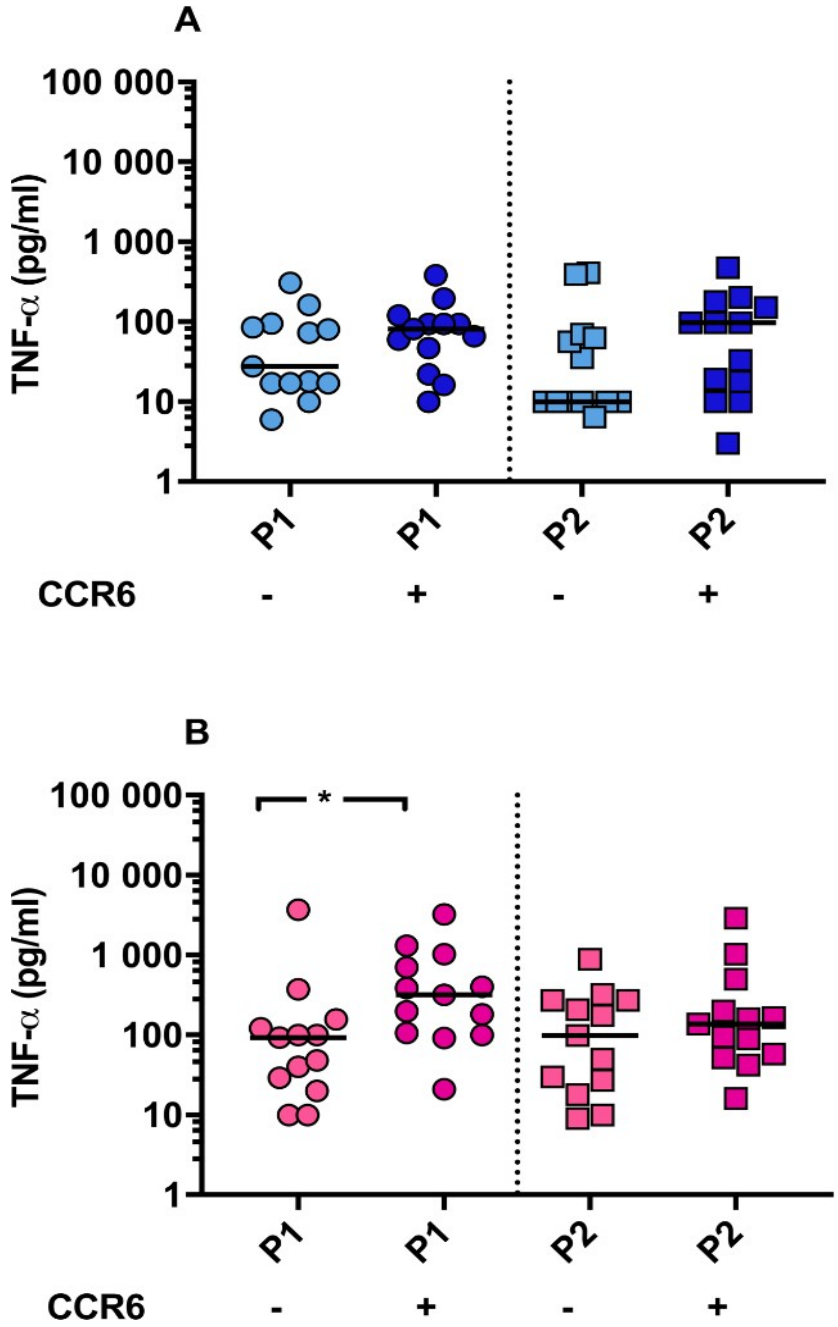


Figure 3.10 Increased production of TNF-α in CCR6+ CD4+ T cells in response to myelin peptide group in untreated MS group. TNF-α levels in healthy controls **(A)** and untreated MS **(B)** after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data are significant to * ($P<0.05$) using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

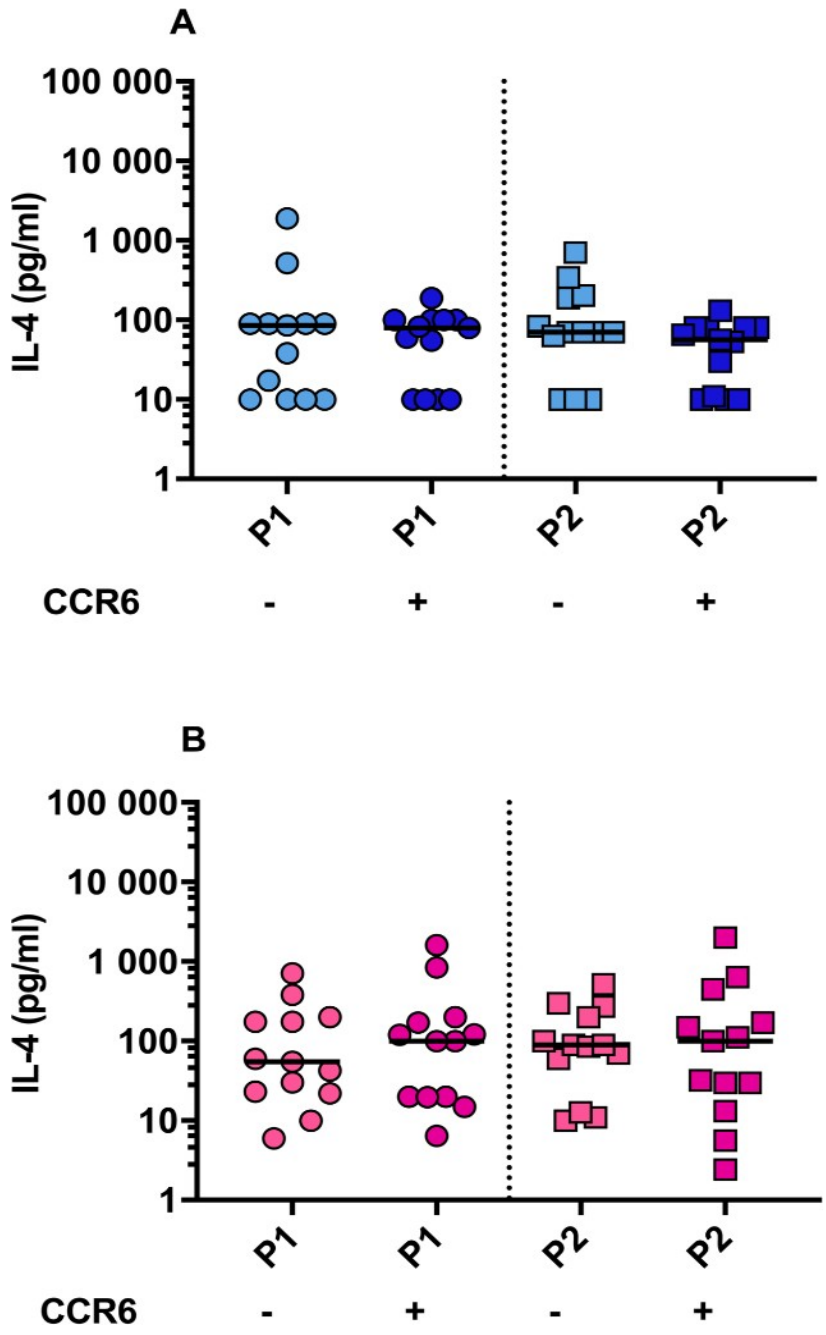


Figure 3.11 IL-4 production in similar levels in response to myelin peptides in CCR6- and CCR6+ CD4+ T cells in healthy controls and untreated MS groups. TNF-α levels in healthy controls **(A)** and untreated MS **(B)** after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

3.3.5 Ability of myelin peptides to induce similar production of GM-CSF and IFN-γ in healthy controls and untreated MS patients

Finally, we compared the healthy controls and untreated MS in response to different myelin peptides.

Several studies have shown the importance of the inflammatory cytokines as GM-CSF and IFN-ψ in MS patients and their higher production compared to healthy controls (Hirsch et al., 1985; Arellano et al., 2017; Cao et al., 2015; Shiomi et al; 2016).

We were able to detect both of the cytokines in healthy controls and MS, but with no significant differences **(Fig. 3.12 A, B)** and **(Fig. 3.13 A, B)**, although the levels of IFN-ψ were slightly elevated in MS as compared to healthy controls, from both CCR6- and CCR6+ fractions.

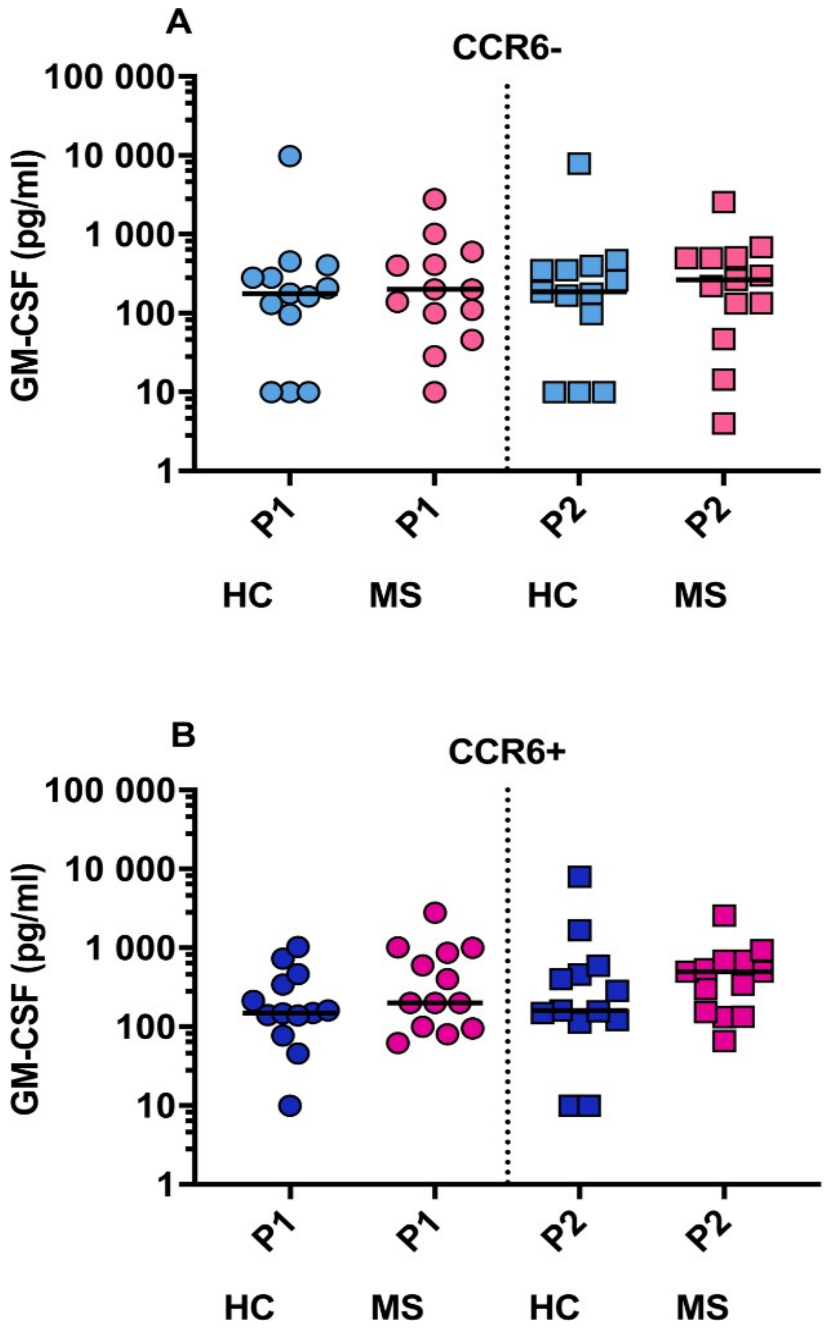


Figure 3.12 No differences in GM-CSF levels between healthy controls and untreated MS groups. GM-CSF levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

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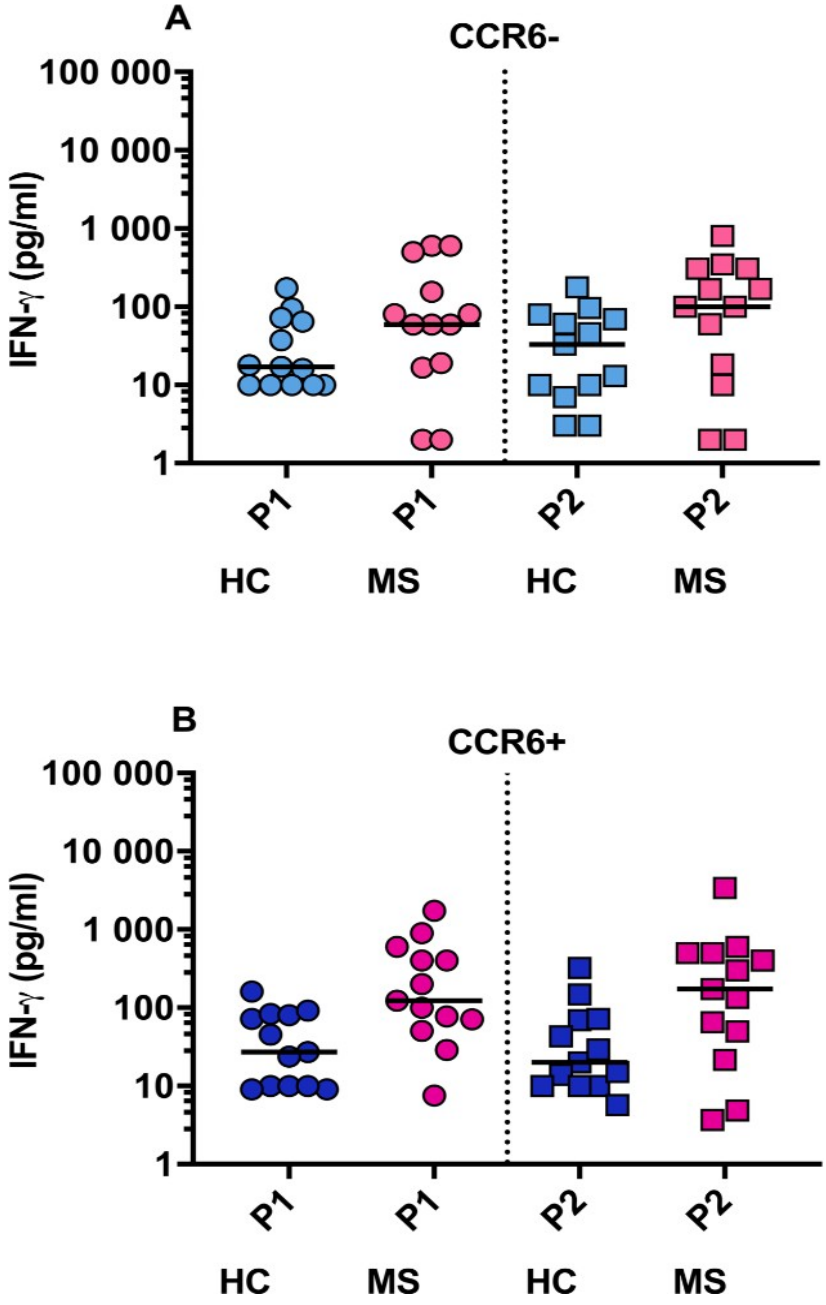


Figure 3.13 Comparable levels of IFN-γ are produced in healthy controls and untreated MS groups in response to myelin peptides. IFN-γ levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

3.3.6 IL-10 and IL-17 were more produced from CCR6+ CD4+ memory T cells in untreated MS than healthy controls

IL-17 has been studied before in the context of MS, where they produced more in MS than healthy controls (Ghaffari et al., 2017; Langellar et al., 2018; Cao et al., 2015; Arellano et al., 2017).

IL-10 was significantly higher in MS from CCR6+ fraction **(Fig 3.14 B)** than healthy controls in response to both P1 and P2 myelin peptides group, while no significant difference in CCR6- fraction **(Fig. 3.14 A)**. Similarly, IL-17 was increased significantly in response to P2 myelin peptides group in MS group than healthy controls **(Fig. 3.15 B),** but no differences in CCR6- fraction **(Fig. 3.15 A).**

It has been shown before that IL-10 is produced more in healthy controls than MS (Hedegaard et al., 2008; Penton-Rol et al., 2009; Cao et al., 2015). In contrast to this report, our data shows lower levels of IL-10 in healthy controls compared to untreated MS patients.

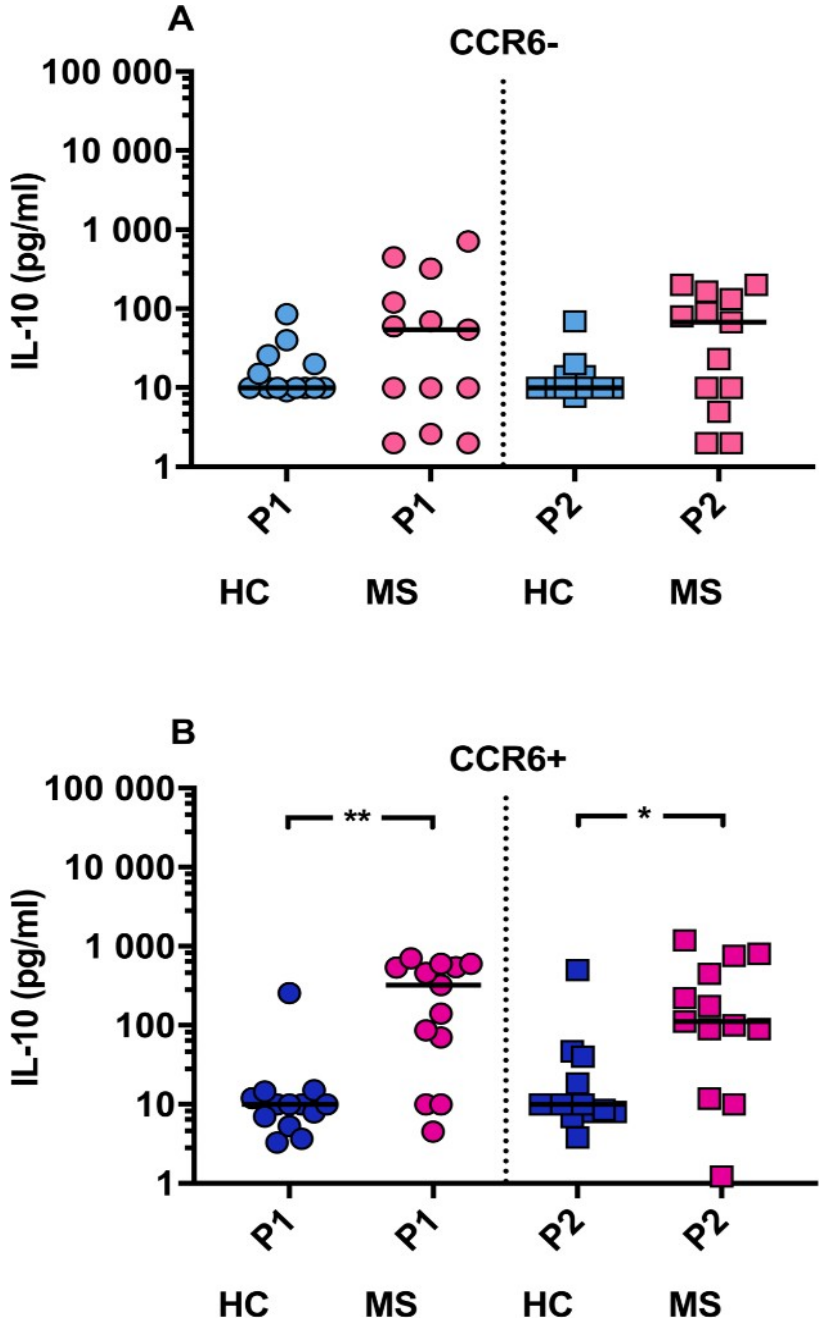


Figure 3.14 CCR6+ CD4+ memory T cells induce significant production of IL-10 in response to myelin peptides in untreated MS. IL-10 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data are significant to * ($P<0.05$), **($P<0.01$) using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

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P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

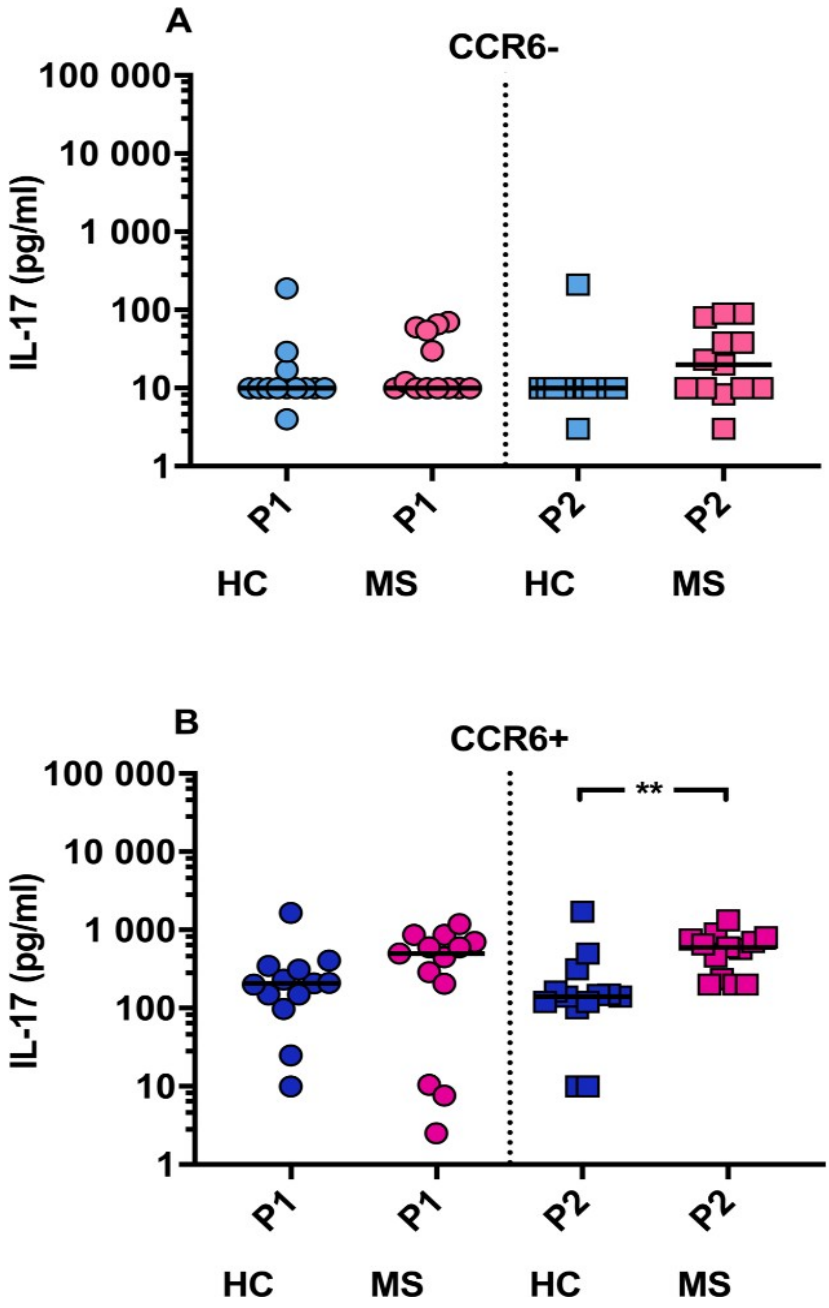


Figure 3.15 IL-17 is increased in CCR6+ CD4+ T cells in response to P2 myelin peptide in untreated MS. IL-17 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data are significant to **($P<0.01$) using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

3.3.7 TNF-α was detected more in MS than healthy controls, but IL-4 was detected in similar levels in healthy controls and MS

After measuring some cytokines in healthy controls and MS, we decided to measure TNF-α and IL-4 to detect if there is a difference between healthy controls and MS.

TNF-α was significantly higher in MS from CCR+ fraction **(Fig. 3.16 B)** than healthy controls in response to both P1 and P2 myelin peptides group, however no significant difference from CCR6- fraction **(Fig. 3.16 A)**. IL-4 was produced in response to both P1 and P2 myelin peptides from CCR6- and CCR6+ without any significant difference between healthy controls and MS **(Fig. 3.17 A, B).**

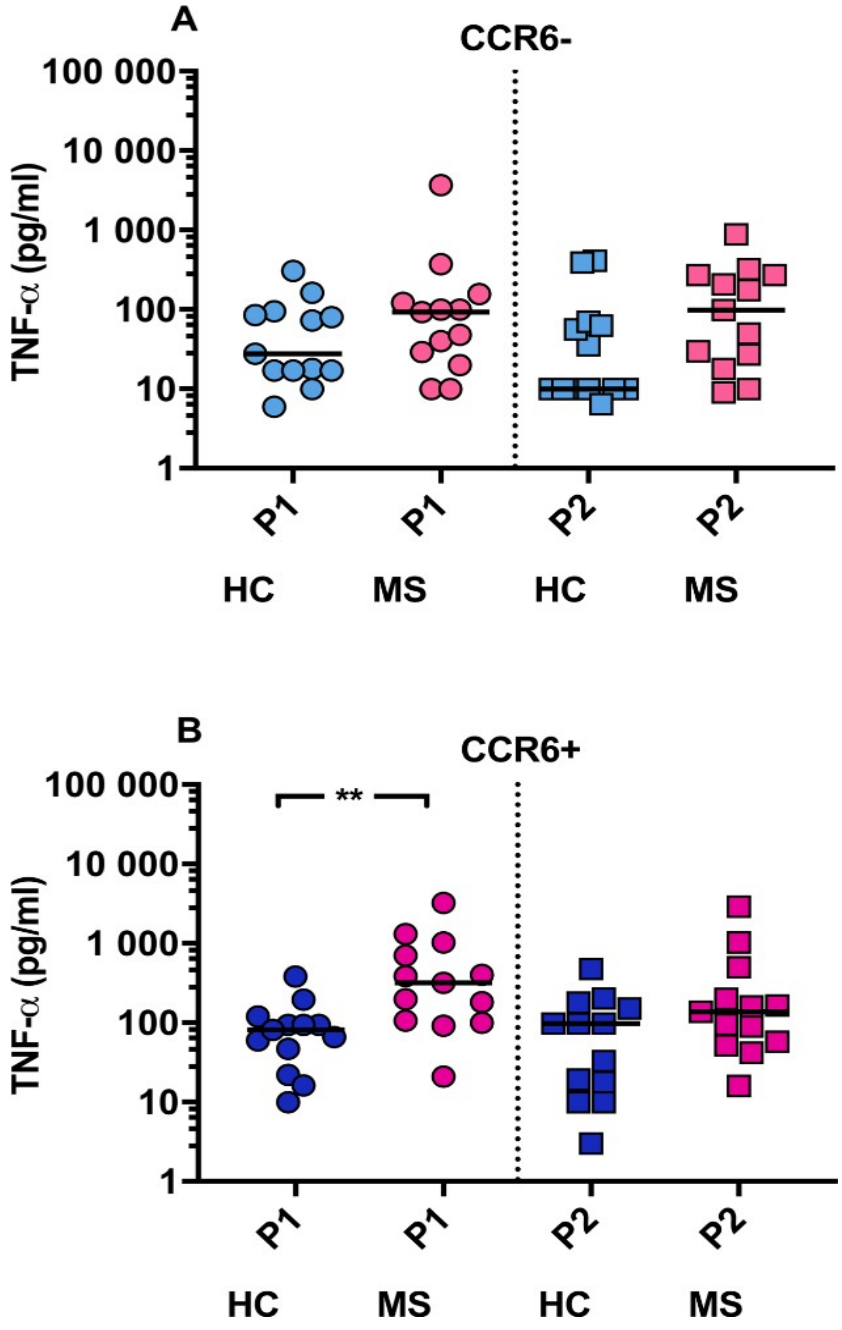


Figure 3.16 TNF-α is increased in CCR6+ CD4+ T cells in response to P1 myelin peptide in untreated MS. TNF-α levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data are significant to **(P<0.01) using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

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P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

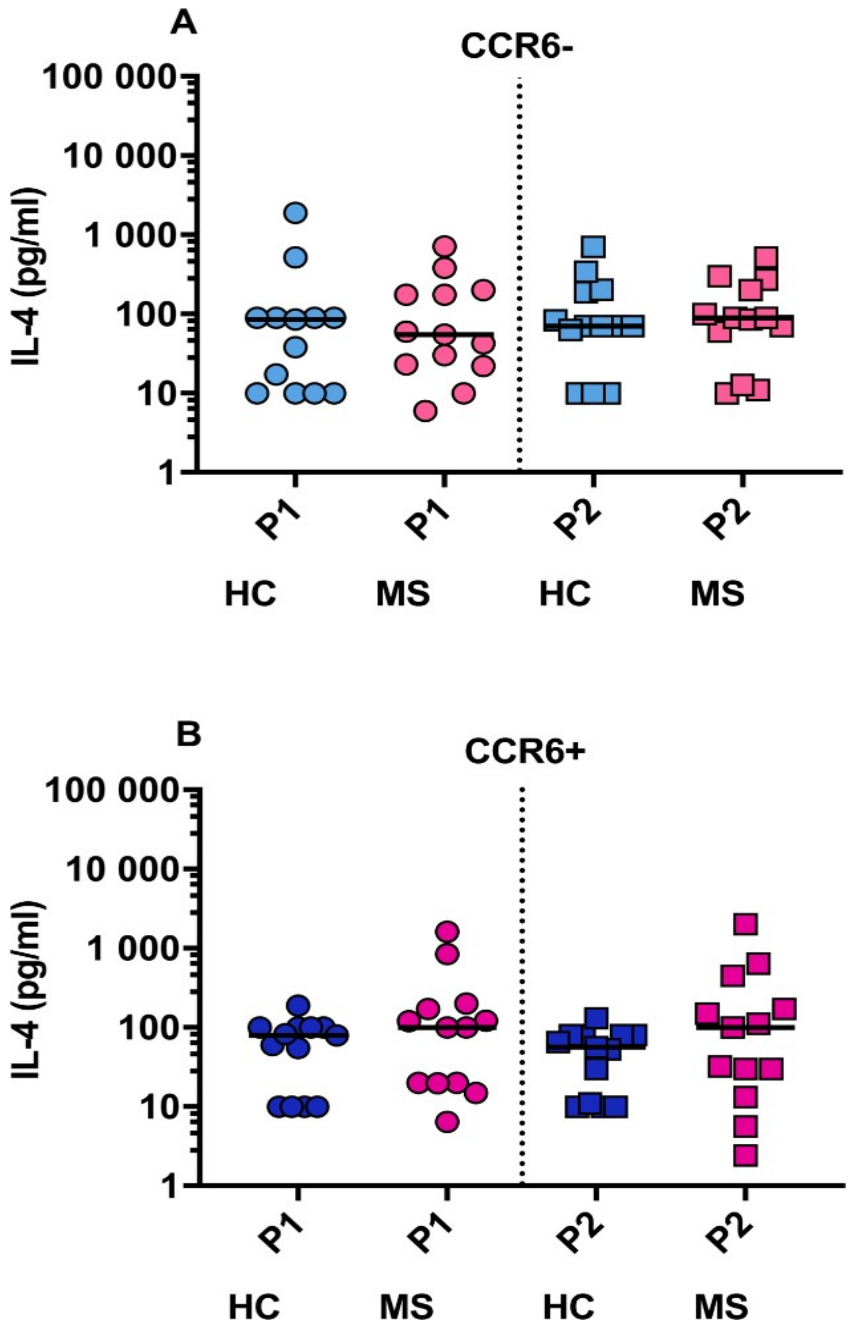


Figure 3.17 No significant IL-4 production between healthy controls and untreated MS patients in response to myelin peptides. IL-4 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2= MOG222-241/MBP85-99/PLP30-49/PLP129-148

3.4 Discussion

Several studies have investigated the main role of B cells and T cells, specifically CD4+ and CD8+ T cells, in the different stages of MS such as the RR and SP stages. Autoreactive T cells were detected in healthy individuals and patients with autoimmune diseases. Despite ongoing research, many challenges remain, and more research is required.

Most of the research has only observed a slight increase in the stimulation index when measuring T cell proliferation; there are few reports of cytokine production in response to myelin peptides including Cao et al., 2015. Therefore, more research is needed to discover the role of different cytokines in response to myelin antigens in healthy controls and MS.

Autoreactive T cells have been investigated in MS patients as well as healthy controls, as they are important in the pathogenesis of autoimmune diseases (Olsoon et al., 1993; Wucherpfenning et al., 1997). Th cells have been implicated in the development and regulation of the disease; e.g., Th1 and Th17 are responsible for inflammation by production of pro- inflammatory cytokines (Bettelli et al., 2006; O'Shea et al., 2010; Goverman 2013; Baecher-Allan et al., 2018).

In our study, CCR6 isolation was successful following cell sorting, after many attempts with different techniques including positive isolation and by using beads. CCR6+ memory T cells have been reported as an important phenotype of myelin reactive T cells in MS patients, and they will be useful for understanding the role of non-classical Th1 and Th17 CCR6+ cells in the mechanisms of MS.

Multiple myelin antigens have been considered putative autoantigens in MS, including myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG) (Diaz-Villoslada et al., 1990; Kaushansky 2010).

Previous studies have documented MBP, PLP, and MOG reactive T cells in MS patients as well as healthy controls within the normal T-cell repertoire (Bielekove et al., 2004). In addition, a higher frequency of myelin peptide-reactive T cells has been observed in MS patients (Cao et al., 2015).

In our healthy controls cohort, there was a specific secretion of cytokines in response to myelin peptides including GM-CSF, IFN-γ, TNF-α and IL-4 from both CCR6- and CCR6+ fractions. Cao et al., 2015 had reported IFN-ψ and IL-10 production in healthy controls mainly from the CCR6+ fraction.

Our data on production of IL-10 in the blood from healthy controls being lower than in MS, are contradictory to published data, with one study reporting equal production of IL-10 in healthy controls and MS in response to myelin antigenss (Cao et al., 2015). Our data suggests that IL-10 is not regulating the CNS-specific immune response in healthy controls as there is no pathological disease existing which requires regulation.

IL-17 was produced from CCR6+ T cells which could be related to the molecular mimicry hypothesis with microbial antigen in the gut, where the T cells are activated to induce Th17 and enhance autoimmunity (Zarate-Blades et al., 2016; Fleck et al., 2017; Pandiyan et al., 2019).

In MS cohort, also different cytokines were detected in response to CNS antigen. Although not all cytokines showed statistical differences, which could be the result of the technical sensitivity issues or a requirement for analysis of a larger cohort of patients, there were observational differences.

We demonstrated functional differences in myelin-reactive CCR6 CD4+ T cells in patients with MS when compared to healthy controls, identifying secretion of IL-17, IFN-ψ and TNF−α

and IL-10 in MS when compared to healthy controls. Further, these autoreactive T cells were CCR6+ memory CD4+ T cells in both MS patients and healthy controls. As expected, IL-17 was only produced in CCR6+ fractions. TNF-α was produced more in CCR6+ T cells from

MS patients than in healthy controls, emphasizing the pathogenic role of Th1 cytokines in the CNS. TNF-α is considered to be a pro-inflammatory cytokine that is promptly produced in response to stimuli, both systemically and locally in the affected tissues, predominantly by activated macrophages and monocytes (Zhang & An, 2007; Arango Duque & Descoteaux, 2014). Moreover, circulating TNF-α crosses the blood brain barrier (BBB) into the brain (Banks et al., 1995; Pan et al., 1999). Inflammatory stimuli in the CNS induce TNF-α production mainly by microglia, neurons, and infiltrating immune cells (Robert L, 2015).

The inflammatory states in MS and EAE are attributed to Th1 cell types.

During a relapse in MS, Th2 cells predominate and anti-inflammatory cytokines such as IL-5, IL-13, and IL-10 are produced. A shift from Th1 to Th2, T-cell response is

protective of autoimmune diseases (Agrawal & Yong, 2007). However, in our studies, IL-4 cytokine production showed no difference in production between healthy controls and MS patients suggesting that IL-4 is not exhibiting a protective role.

Our data indicate that there are myelin-reactive CCR6+ T cells that produce GM-CSF, IL-17, TNF-α, or IFN-ψ, which contribute to the inflammatory response in MS. Although CCR6+ T cells IL-10–secreting myelin-reactive T cells were produced higher in MS than healthy controls, it was reported in published reports that it has a protective role in the inflammation. The more reproducible response to myelin peptide was measured in IL-10, which has been higher in MS than healthy controls and could be a result of regulatory-inflammatory balance in the immune system.

Our data highlights the important role of CCR6+; the expression CCR6 by a subset of pathogenic T cells has been shown to have an important role in facilitating T cell entry into the CNS in EAE (Reboldi et al., 2009). Human T cells that are able to produce IL-17 express CCR6. It is therefore suggested that CCL20 and CCR6 play a role in inflammatory diseases by recruiting Th17 cells to target tissues (Agrawal & Yong, 2007; Yamazaki et al., 2008; Singh et

al., 2008). While these data strongly implicate IL-17 as being pathological in MS, it is important to note that IL-17 can also be secreted by non-T cells, such as NK cells and astrocytes (Cua and Tato, 2010; Tzartos et al., 2008).

IL-10 was produced from CCR6+ T cells mainly in our MS cohort. IL-10 can be produced from different T cell subsets including Th1, Th2 and T regs. IL-10 Tr1 regulatory $CD4^+$ T cell that inhibits cell proliferation primarily via the secretion of IL-10 (Gregori et al., 2012). It is suggested from our data that IL-10 is regulating the pathogenesis in MS patients. Further analysis of these cells in patients with active forms of disease would determine whether and how these populations shift during relapse.

3.5 Summary

Data in this chapter identify the CCR6+CD4+ in blood, where they produced inflammatory and regulatory cytokines in healthy controls in response to different myelin peptides

However, higher production of inflammatory cytokines (IFN-γ, IL-17, TNF-α) in response to myelin peptides were produced in untreated MS patients in myelin specific T cells than healthy controls cohort and that is consistent with published data (Cao et al., 2015).

In contrast to published data, IL-10 expression was less frequent in healthy controls when compared to MS patients (Cao et al., 2015).

In conclusion, there were functional differences between antigen specific T cells from untreated MS and healthy controls and these cytokines contribute to the pathogenesis of MS.

CHAPTER 4

CYTOKINE PRODUCTION IN MULTIPLE SCLEROSIS PATIENTS TREATED WITH INTERFERON BETA (IFN-β) TREATMENT

Chapter 4: Cytokine production in multiple sclerosis patients treated with Interferon beta (IFN-β) treatment

4.1 Introduction

Substantial efforts have been made over the last decades to understand the mechanism of MS and to find a treatment or cure.

Previous researchers have highlighted the response of antigen specific T cells to different myelin peptides in MS patient cohort with and without different types of treatment in MS patients, although very little is known about how these change in response to treatment.

IFN-β is a widely used treatment for MS, but its mechanisms of disease modulation are not well understood (Kasper and Reder, 2014; Yen et al., 2010). It is known, however, that IFN-β upregulates HLA class I expression and downregulates expression of HLA class II, thereby inhibiting Th cell activation.

In this study, we measured different cytokines from CD4+CCR6+/- T cells in culture supernatant stimulated with myelin peptides (GM-CSF, IFN-γ, IL-10, IL17) as previously described (Cao et al; 2015). In addition, we measured IL-4 and TNF-α form MS patients under different types of treatment at different stages of disease. The hypothesis of this chapter was that the IFN-β treatment decreases the inflammatory response, which includes Th1 and Th2 cytokines.

4.1.1. Objectives

1. Examine if there is a specific secretion of several cytokines from $CCR6^-$ and $CCR6^+$ $CD4^+$ memory T cells in response to immunodominant CNS peptides in MS patients under IFN-β

treatment.

2. Examine the production of $CCR6^+$ and $CCR6^-$ $CD4^+$ memory T cells, stimulated with a number of immunodominant CNS peptides in IFN-b treated MS cohort and compare it to untreated MS cohort.

3. Compare different parameters as age, gender, different stages of MS, duration of disease, expanded disability status scale (EDSS) and HLA typing between untreated MS patients and with different MS patients.

4.2 Methods

$CCR6^+$ and $CCR6^-$ $CD4^+$ memory T cells were isolated and cultured with IL-2 and PHA, after sorting and then stimulated with different myelin CNS peptide antigen and different cytokine were measured from the culture supernatant. Functional phenotype was investigated using surface marker staining and flow cytometry. Cytokine productions were determined using Luminex bead-based immunoassays. This method allowed the analysis of different cytokines expressed and provided the information about the frequency and the functional phenotype of memory cells. Additionally, the HLA-DR locus was typed from healthy controls and MS cohort.

4.3 Results

4.3.1 MOG/ PLP and MBP induce Th1/Th2 and Th17 cytokines from CCR6- and CCR6+ CD4+ memory T cells in MS patients treated with IFN-β

For many years, T-cell responses to different epitopes of MOG, PLP and MBP have been detected in the peripheral blood cells of MS patients and the frequency of myelin CD4 + T cells in MS patients under different treatment.

In this MS cohort which is treated with IFN-β, CCR6-/+ T cells were stimulated with different CNS myelin peptides as previously shown for healthy controls and untreated MS cohorts (Chapter 3).

In general, specific cytokines were produced in response to myelin peptides P1 (MOG97-109/PLP180-199) and P2 (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant from CCR6- **(Figure 4. 1 A-F),** as well as CCR6+ (**Figure 4. 2 A-F)**

Although not all cytokines levels were statistically significant, there were observational differences after stimulation with myelin peptides in all of them.

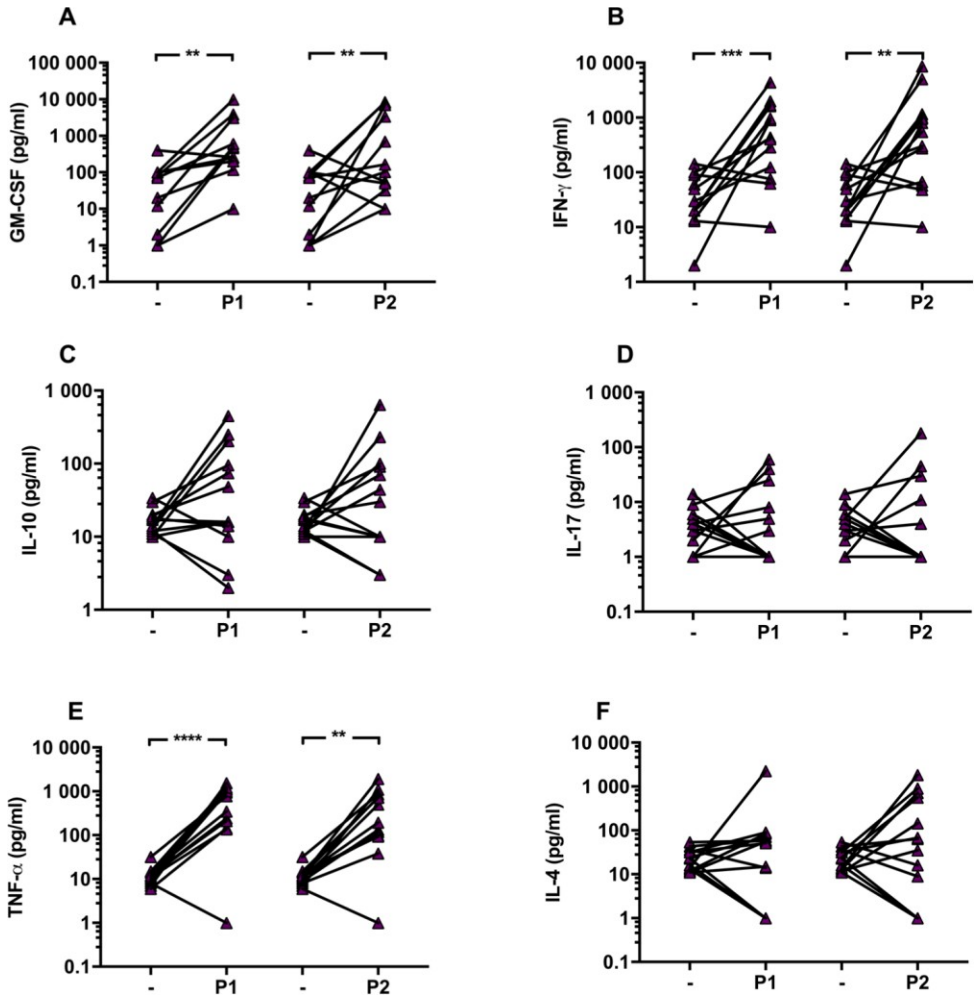


Figure 4.1 Specific cytokine production by CNS specific CCR6- CD4+ T cells in MS patients treated with IFN-β. Cytokine levels of GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** before stimulation and after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- CD4+ memory T cells. Data are significant to ** (P<0.01) *** (P<0.001) using Dunn's multiple comparisons analysis (n=13). The data are represented as line graphs with each line representing a single individual.

- = No stimulation
P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

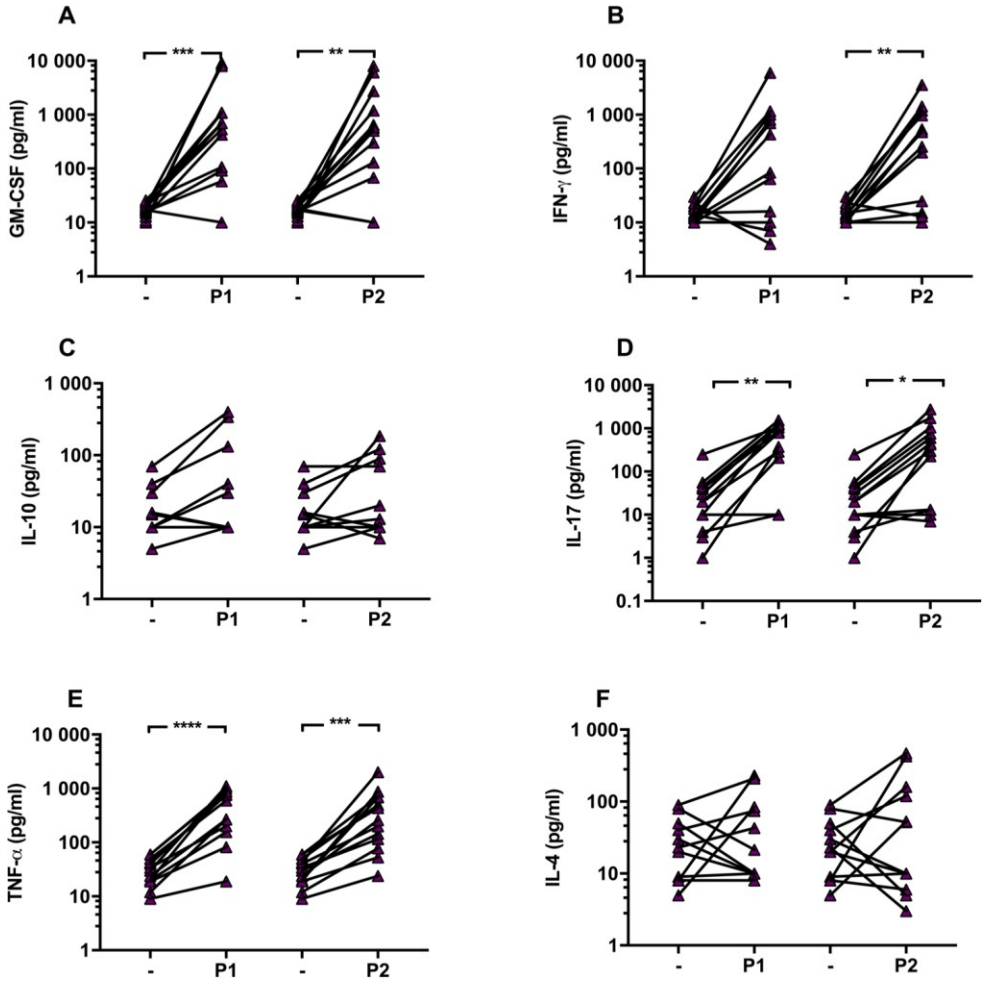


Figure 4.2 Specific cytokine production by CNS specific CCR6+ CD4+ T cells in MS patients treated with IFN-β. Cytokine levels of GM-CSF **(A)** IFN-ψ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** before stimulation and after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- CD4+ memory T cells. Data are significant to * (P<0.05) ** (P<0.01) *** (P<0.001) using Dunn's multiple comparisons analysis (n=13). The data are represented as line graphs with each line representing a single individual.

- = No stimulation
P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

4.3.2 Comparison of cytokine productions from CD4+CCR6- and CCR6+ memory T cell culture in MS patients treated with IFN-β

We examined if there is a difference in production of cytokine in response to different myelin antigens (P1 and P2) between CCR6- and CCR6+ fraction. Higher levels of GM-CSF were produced from CCR6+ than CCR6- in response to myelin antigens **(Figure 4.3 A).**

The second cytokine we measured is proved to have a role in inflammatory response is IFN-γ. In contrast to GM-CSF, CCR6- were more enriched with IFN-γ than CCR6+ culture supernatant in response to P1 and P2 **(Figure 4.3 B).**

IL-10 is believed to have a protective role in MS through suppression of the immune system when required as in the autoimmune disease as MS. Low levels of IL-10 (10 pg/ml) were produced in CCR6- and CCR6+ culture supernatant from IFN-β treated patients, with similar levels from CCR6- and CCR6+ fractions (**Figure 4.3 C).**

As expected and consistent with literature, IL-17 is exclusively produced by CD4+CCR6+ but not CD4+CCR6- memory T cells culture which shows statistical differences **(Figure 4.3 D).** IL-17 is mainly produced by CCR6+ CD4+ memory T cells and not by CCR6- in MS samples with and without treatment.

Similar levels of TNF-α were detected in MS patients from CCR6- and CCR6+ fraction in response to p1 and P2 peptides **(Figure 4.3 E).**

Finally, we measured one of the Th2 cytokines, which is IL-4.

IL-4 levels were slightly lower in CCR6+ than CCR6- fractions in response to P1 and P2 myelin antigens **(Figure 4.3 F).** Although not all of the comparison shows statistical significance, there were observational differences between CCR6- and CCR6+ fractions.

The treated patients tended to have a more uniform and low levels of cytokine production when unstimulated but a greater response to peptide stimulation **(Fig 4.2)** compared to the untreated patients **(Fig 3.4).**

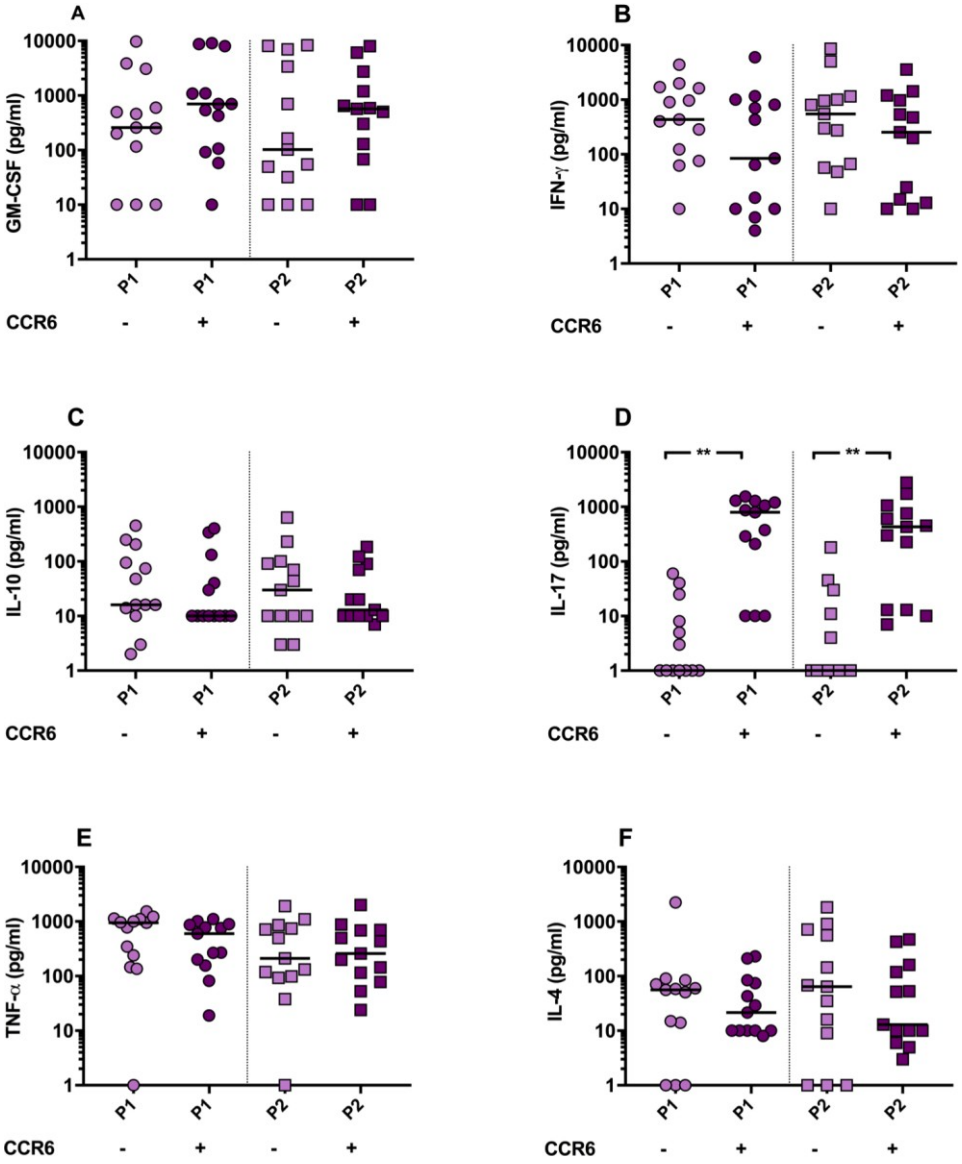


Figure 4.3 Th1/Th2 and Th17 production from CCR6- and CCR6+ CD4+ in MS patients treated with IFN-β. Cytokine levels of GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data are significant to ** ($P<0.01$), using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

4.3.3 MS patients treated with IFN-β may induce more inflammatory response after stimulation with different myelin peptides

We investigated the effect of IFN-b treatment with comparison to healthy control groups.

We were able to detect GM-CSF from CCR6- **(Figure 4.4 A)** and CCR6+ **(Figure 4.4 B)**, but with no significant differences between healthy controls and treated MS groups.

In contrast, IFN-g was higher in treated MS than healthy controls from CCR6- **(Figure 4.5 A)** and CCR6+ fractions in response to both P1 and P2 myelin peptides groups **(Figure 4.5 B).**

Low levels were both detected in healthy controls and treated MS groups in response to myelin peptides from CCR6- **(Figure 4.6 A)** and CCR6+ **(Figure 4.6 B)** cells, without any significant differences.

As expected, IL-17 was not produced from CCR6- fraction in both healthy controls and treated MS groups **(Figure 4.7 A),** but it was higher after treatment with IFN-⌡compared to healthy controls **(Figure 4.7 B).** Also, TNF-α was higher in treated MS than healthy controls from CCR6- **(Figure 4.8 A)** and CCR6+ fractions **(Figure 4.8 B)** in response to both P1 and P2 myelin peptides groups. We were able to detect IL-4 from CCR6- **(Figure 4.9 A)** and CCR6+ fractions **(Figure 4.9 B)** but with no significant differences between healthy controls and treated MS groups.

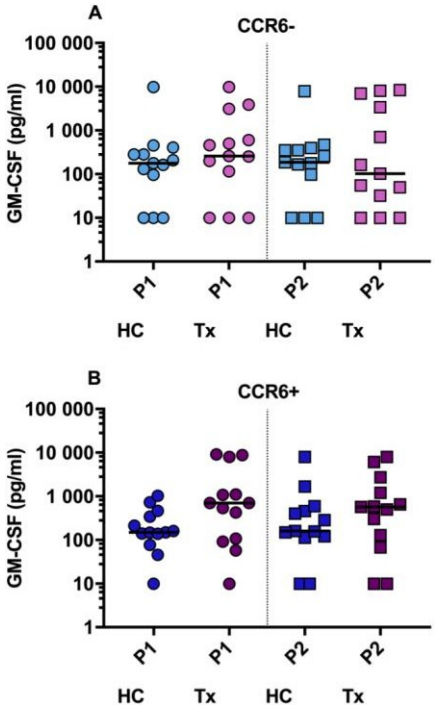


Figure 4.4 No differences in GM-CSF levels between healthy controls and IFN-丿 treated MS groups. GM-CSF levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.
P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

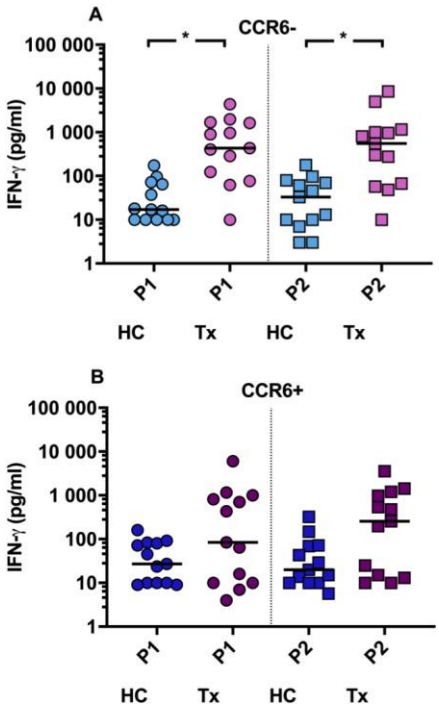


Figure 4.5 Increased levels of IFN-γ after treatment with IFN-β as compared to healthy control groups. IFN-γ levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) inculture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons are significant to * ($P<0.05$) using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

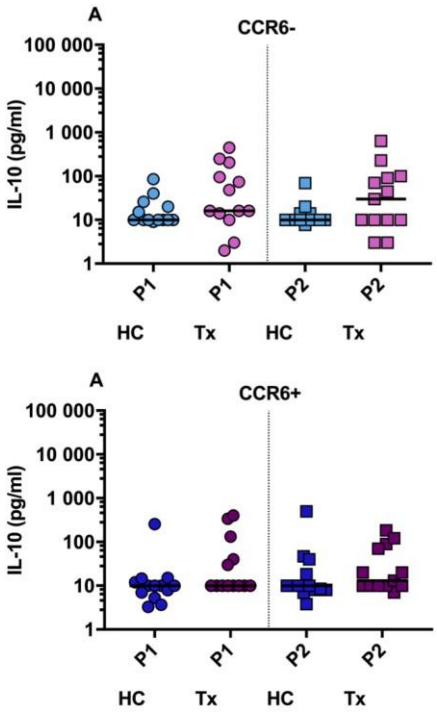


Figure 4.6 Low levels of IL-10 production in healthy controls and in MS groups treated with IFN-β. IL-10 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) inculture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

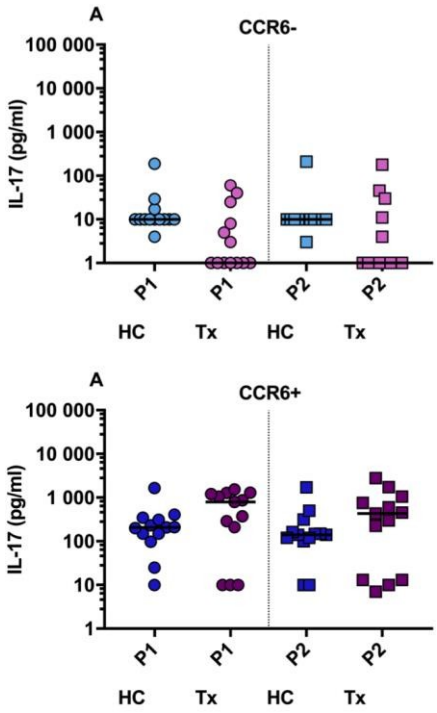


Figure 4.7 IL-17 is increased in CCR6+ CD4+ T cells in response to myelin in MS treated groups. IL-17 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

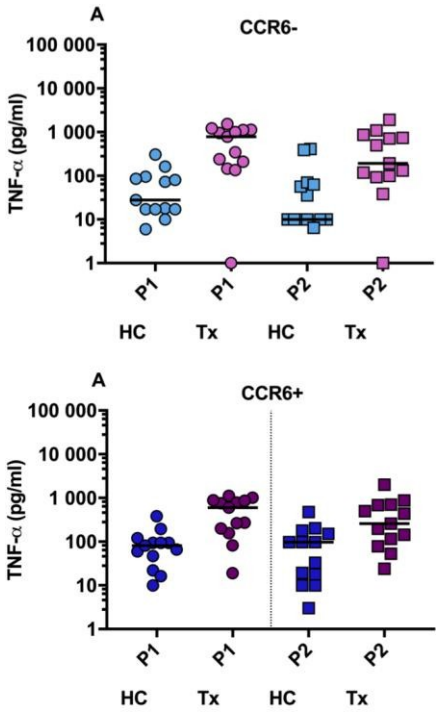


Figure 4.8 CCR6+ CD4+ T cells induce higher levels of TNF-α in response to myelin peptides. TNF-α levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) inculture supernatant of CCR6- and CCR6+ CD4+ T cells. Data were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

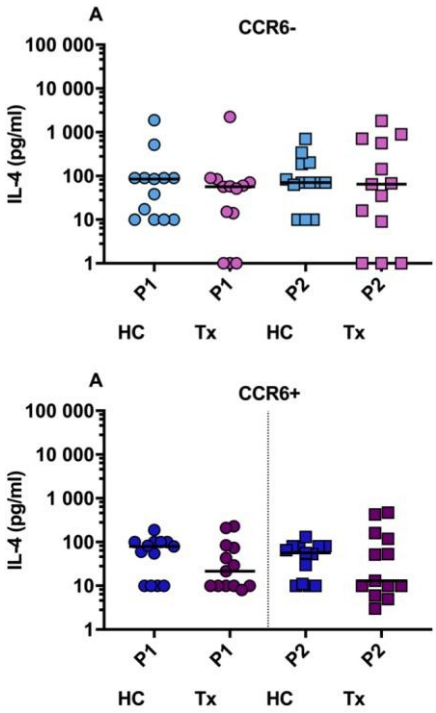


Figure 4.9 No significant IL-4 production between healthy controls and treated MS patients in response to myelin peptides. IL-4 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

4.3.4 Th1 response predominate in MS patients and sustained after treatment with IFN-β in MS

Next, we measured different cytokines in the culture supernatant of CD4+CCR6+ memory T cells stimulated with different myelin peptides; P-1 (MOG97-109/PLP180-199) and P-2 (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in untreated and treated MS patients with IFN-β as previously.

We were able to detect GM-CSF from CCR6- **(Figure 4.10 A)** and CCR6+ **(Figure 4.10 B)** cells, but with no significant differences between healthy controls and treated MS groups.

In contrast, IFN-ψ was elevated significantly after treatment with IFN-β from CCR6- **(Figure 4.11 A)** and CCR6+ fractions **(Figure 4.11 B)** f in response to both P1 and P2 myelin peptides groups.

The treatment with IFN-β reduced the level of IL-10 in MS groups from CCR6- **(Figure 4.12 A)** and CCR6+ **(Figure 4.12 B).** As expected, IL-17 was produced from CCR6- fraction in very low levels **(Figure 4.13 A),** and produced in higher levels from CCR6+ fraction **(Figure 4.13 B).** Although there was a statistical difference between untreated and treated MS groups from the CCR6- fraction, it was a very small difference.

TNF-α was also higher in MS treated with IFN-β from CCR6- **(Figure 4.14 A)** and CCR6+ fractions **(Figure 4.14 B)** in response to both P1 and P2 myelin peptides groups. We were also able to detect IL-4 from CCR6- **(Figure 4.15 A)** and CCR6+ fractions **(Figure 4.15 B)**, but with no significant differences between healthy controls and treated MS groups.

We observed higher levels of IFN-γ and TNF-α and lower IL-10 after treatment with IFN-β **(Figure 4.16 A, B).**

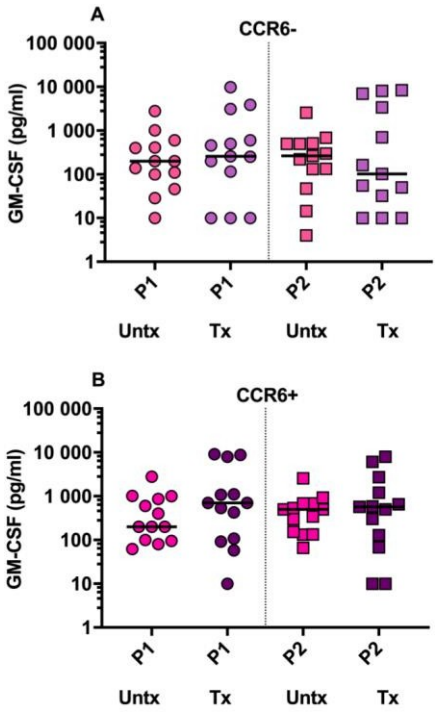


Figure 4.10 Similar levels of GM-CSF between untreated and IFN-β treated MS groups. GM-CSF levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

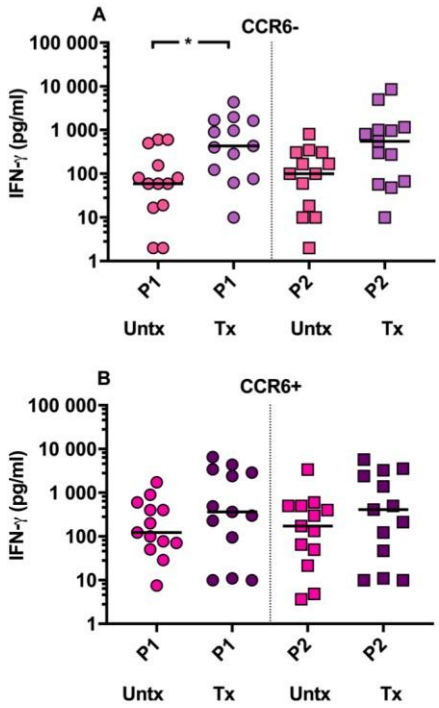


Figure 4.11 IFN-β induce higher levels of IFN-γ as compared to untreated MS groups. IFN-γ levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data are significant to * ($P<0.05$) using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

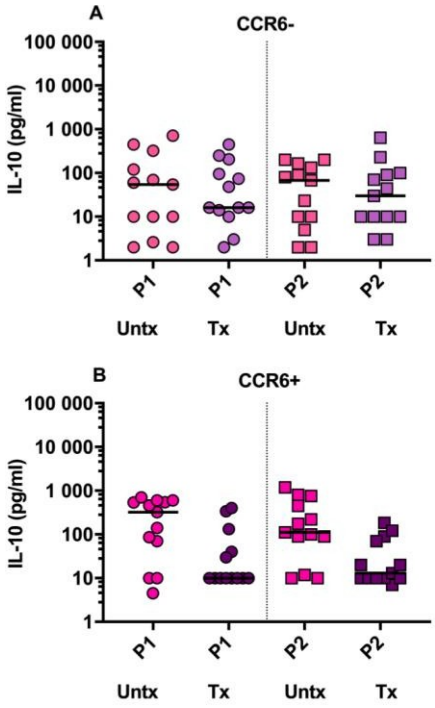


Figure 4.12 IFN-β treatment reduce the regulatory response. IL-10 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Comparisons were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

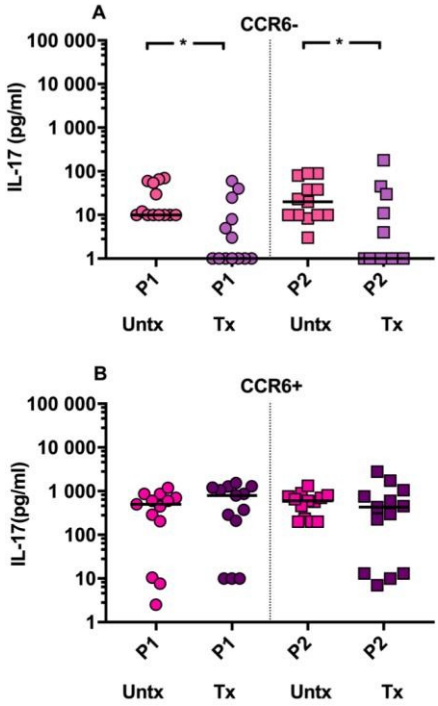


Figure 4.13 IL-17 is produced in CCR6+ CD4+ T cells in response to myelin in MS treated groups. IL-17 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.
P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

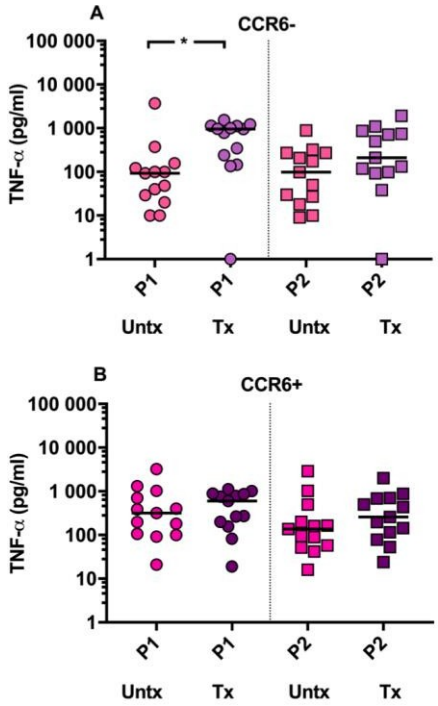


Figure 4.14 CCR6- CD4+ T cells induce higher levels of TNF-α in response to P1 myelin peptides. TNF-α levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data are significant to *($P<0.05$) using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

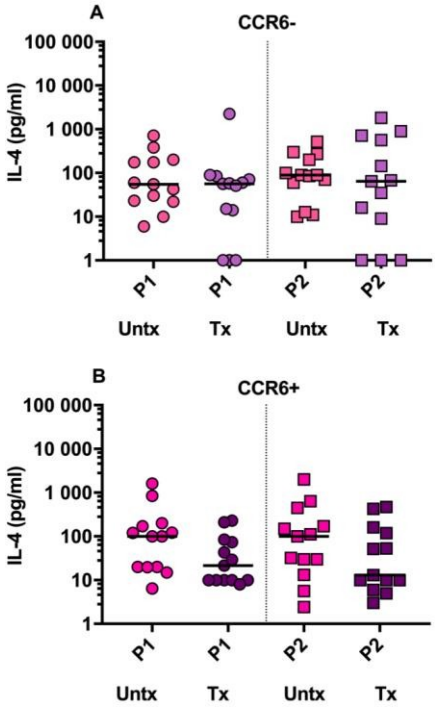


Figure 4.15 No significant IL-4 production between untreated and treated MS patients in response to myelin peptides. IL-4 levels in CCR6- **(A)** and CCR6+ **(B)** CD4+ memory T cells after stimulation with myelin peptide (MOG97-109/PLP180-199) and (MOG222-241/MBP85-99/PLP30- 49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

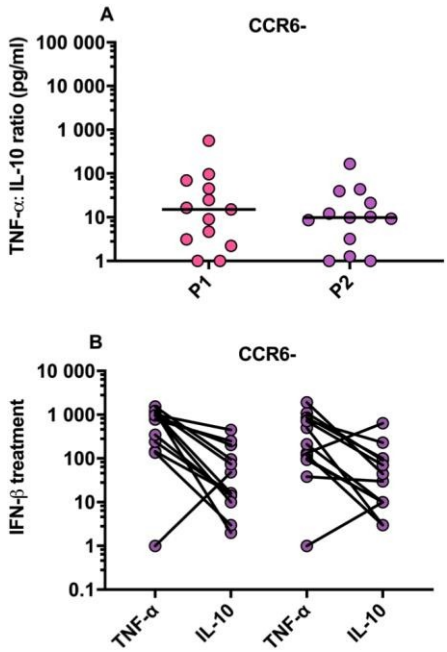


Figure 4.16 Increased TNF-α, but decreased IL-10 after treatment with IFN-β in MS cohort. Ratio of TNF-α to IL-10 production **(A)** and **(B)** after stimulation with myelin peptide (MOG97- 109/PLP180-199) and (MOG222-241/MBP85-99/PLP30-49/PLP129-148) in culture supernatant of CCR6- and CCR6+ CD4+ T cells. Data were not significant using Kruskal-Wallis test (n=13). The data are represented as scatter graphs with each scatter representing a single individual.

P1= MOG97-109/PLP180-199
P2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

4.3.5 Demographic of different parameters in healthy controls and MS patients

We then analysed the data for the gender and age and compared them in healthy controls and all MS groups. The average age for healthy controls ranged between 20-40 years and between 20-60 years in MS patients which is significantly different **(Figure 4.17 A).** It has been shown in previous studies that MS is affecting young people in Europe aged usually between 25-40 years.

Females are more susceptible to autoimmune diseases, such as MS, than males. The ratio in some studies of female to male is 3:1. In our MS group females (63%) were almost at double percentage when compared to male (37%) **(Figure 4.17 B, left).** There was not a significant difference between male (58.3%) and female (41.6%) in healthy controls cohort. **(Figure 4.17 B, right).**

In our study, most MS patients were RRMS (92%) and few were clinically isolated syndrome (CIS) and primary progressive multiple sclerosis (PPMS/SPMS) (2%) **(Figure 4.17 C).**

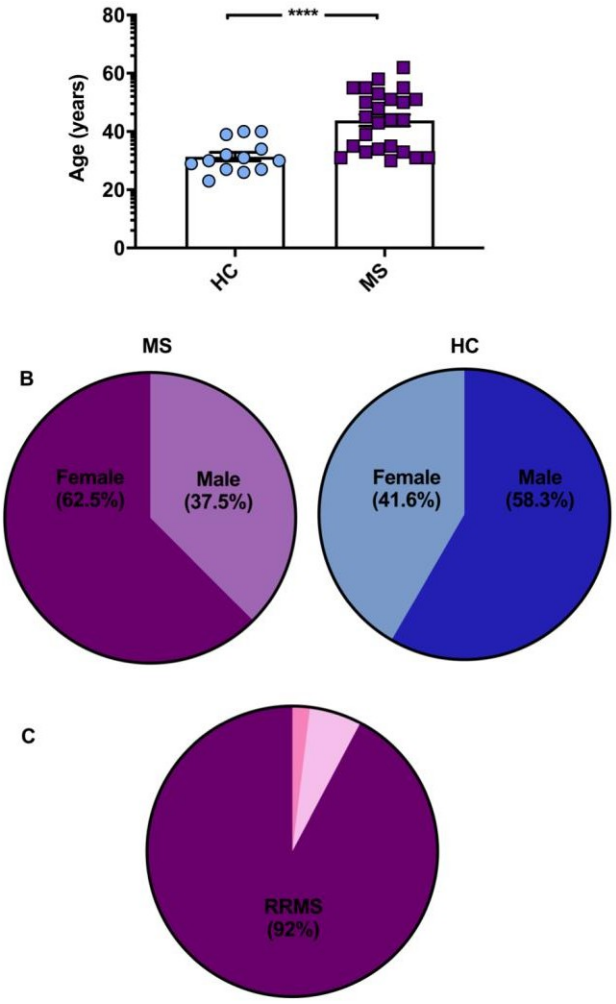


Figure 4.17 Age, gender and disease categories in healthy controls and MS groups. Mean ± SEM scatter plots of age in healthy controls and MS cohort **(A)** pie charts show the ratio of gender in MS (left) and healthy controls (right) **(B)** and categories of MS patients **(C).** Data are significant to ***($P<0.001$) using Mann-Whitney test (n=13). The data are represented as scatter graphs with each scatter representing a single individual

4.3.6 Cytokine production based on different ages in healthy controls and MS patients

People with early age could generate more regulatory cytokine and this would decrease with age.

In our cohort, healthy controls and MS were classified into two age groups; (20-35 years) and (36-60 years). When we measured the cytokine there was a rise GM-CSF production in young healthy **(Figure 4.18 A).**

IFN-ψ was produced in higher amounts in early young MS group **(Figure 4.18 B)** as well as IL-17 **(Figure 4.18 D)** and IL-4 **(Figure 4.18 F).** In contrast, IL-10 was produced more in older healthy controls and MS groups than younger groups **(Figure 4.18 C).** TNF-α was produced in comparable amounts in both groups in healthy controls and slightly increased in older groups in MS cohort **(Figure 4.18 E)**.

We didn't observe any significant correlations between cytokine levels and age in healthy controls and MS groups **(Figure 4.19 A-C).**

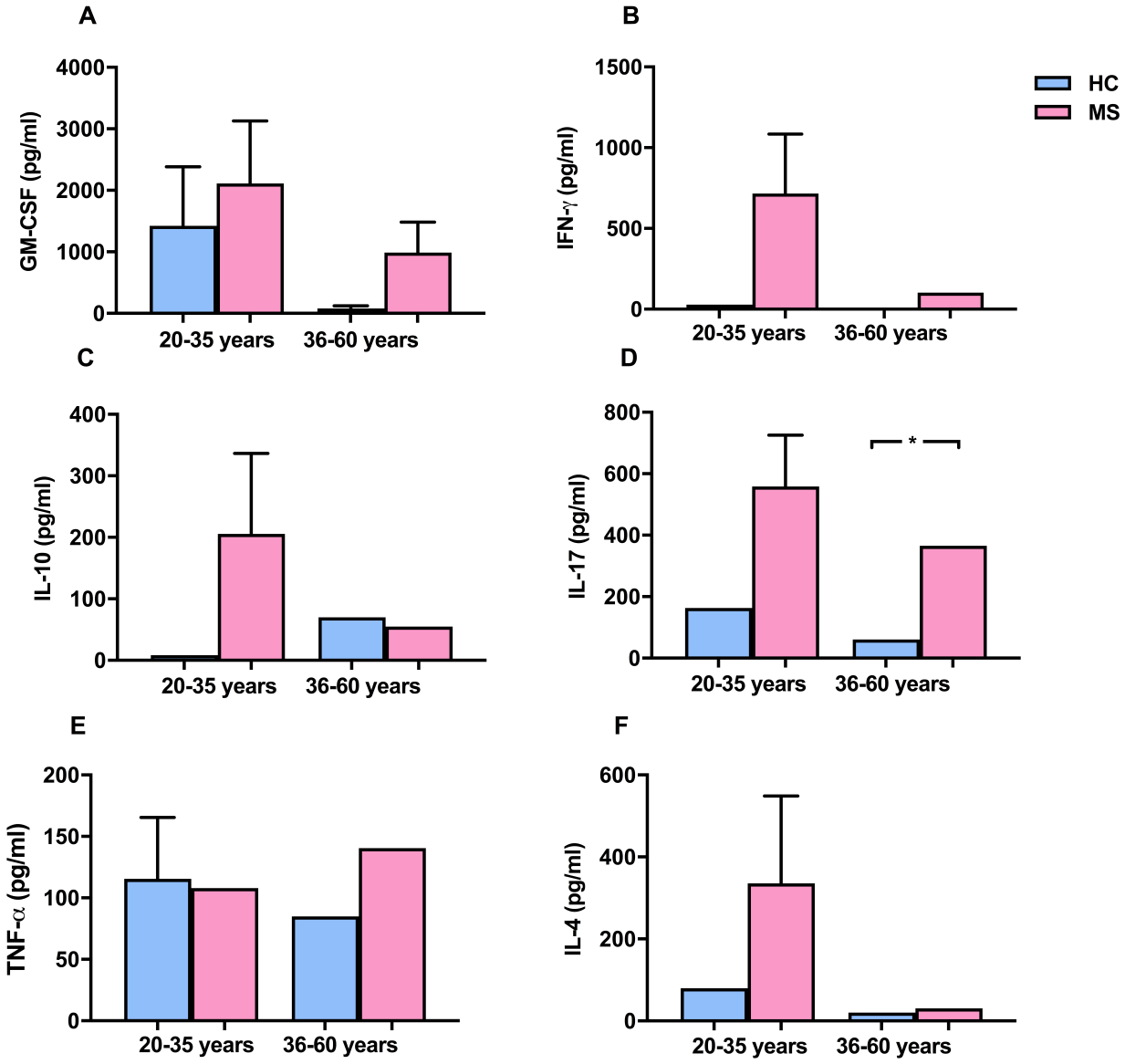


Figure 4.18 Impact of age on cytokine profile. Mean ± SEM Bar columns showing production of GM-CSF**(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** in culture supernatant of CD4+ CCR6+ memory T cells stimulated with myelin peptide P2 (MOG222-241/MBP85-99/PLP30- 49/PLP129-148. Data were not significant using Kruskal-Wallis test (HC 20-35 years n=9, 30-60 years n=4) (MS 20-35 years n=9, 30-60 years n=16).

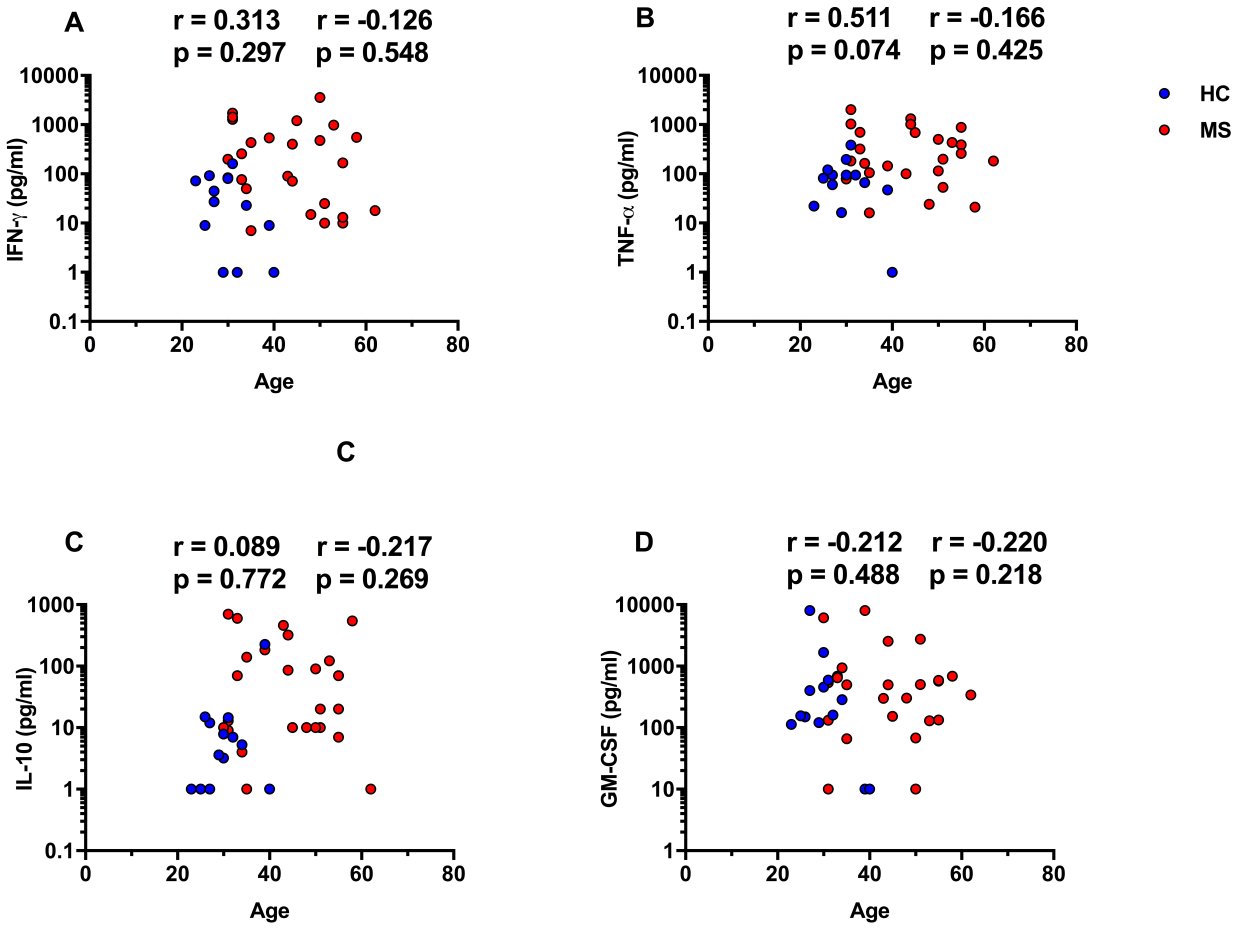


Figure 4.19 No correlation between age and cytokine levels in healthy controls and MS groups. Spearman correlation of cytokine levels (pg/ml) of IFN-γ **(A)** IL-10 **(B)** TNF-α **(C)** and GM-CSF **(D)** compared to the age in culture supernatant of CD4+ CCR6+ memory T cells stimulated with myelin peptide P2 (MOG222-241/MBP85-99/PLP30-49/PLP129-148). (HC n=13) (MS n=25).

4.3.7 Impact of gender on cytokine profile in response to myelin peptides in healthy controls and MS patients

In our study, female healthy controls produced more IFN-γ and IL-10, lower GM-CSF and TNF-α, while there was no difference in IL-17 and IL-4 **(Figure 4.20 A-F, left columns)**. However, female MS groups produced more IFN-γ, TNF-α and IL-4, lower IL-10 and IL-17 and similar levels of GM-CSF as compared to males **(Figure 4.20 A-F, right columns).**

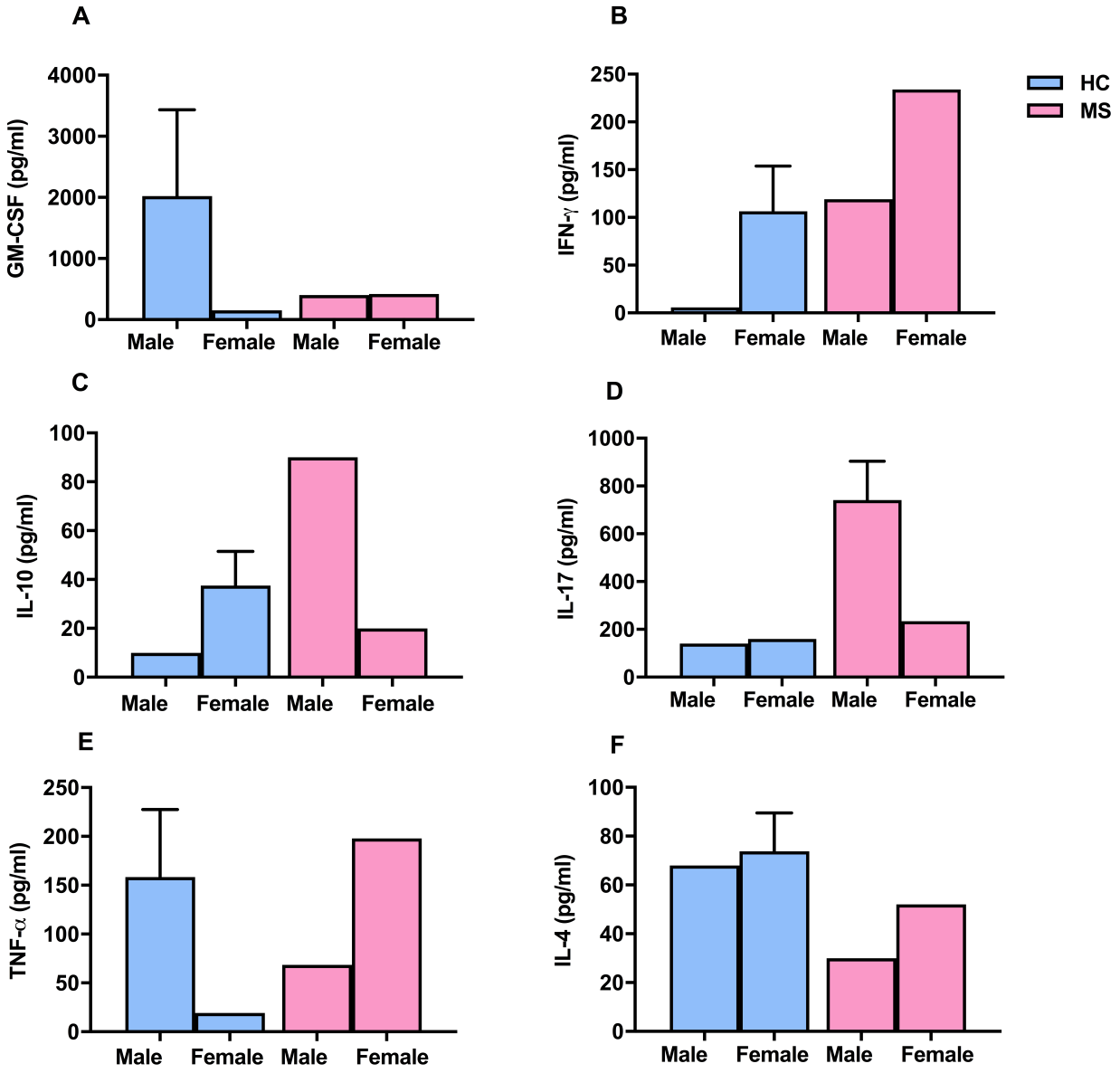


Figure 4.20 Sex difference in cytokine response to myelin peptides in MS. Mean ± SEM bar columns showing production of GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** in culture supernatant of CD4+ CCR6+ memory T cells stimulated with myelin peptide P2 (MOG222-241/MBP85-99/PLP30-49/PLP129-148. Data were not significant using Kruskal- Wallis test (HC male n=7, female n=6, MS male n=10, female n=15).

4.3.8DR15 alleles in MS and its influence in cytokine production

Earlier studies showed that autoimmunity can be related to some HLA genes - HLA-DR4, DR7 and DR15. It has been proven that there was a significant elevation of inflammatory cytokines in MS patients with DR4, DR7 and DR15 than in healthy controls.

In our study, these alleles were higher in MS cohort than healthy controls. We detected a higher frequency of DR15 in MS group than healthy controls **(Figure 4.21 A).**

We couldn't observe any effect of DR15- or DR15+ on production of IL-10 in both healthy controls and MS groups **(Figure 4.21 B)**.

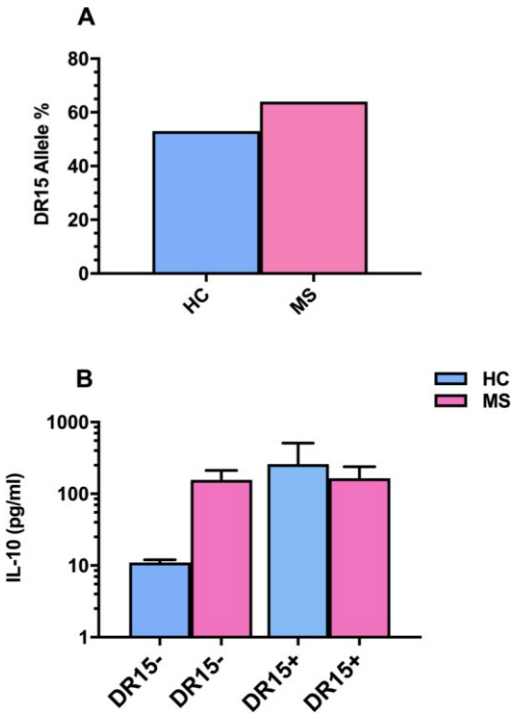


Figure 4.21 Impact of DR15 on IL-10 production in MS group. DR15 alleles % in **(A)** and IL-10 levels in response to P2 myelin peptides in DR15+ and DR15- in healthy controls and MS groups. Data were not significant using Mann-Whitneys and Kruskal-Wallis test (n=13).

4.3.9 Duration of disease and Expanded Disability Status Scale (EDSS) were higher in MS groups treated with IFN-β

It has been considered that duration of disease in MS is varied among patients and depending on many factors such as the stage of disease and whether they are in treatment and the treatment type.

In our study, duration of disease was ranged from few months to 25 years, where most of them have the disease for approximately 5 to 6 years There was a significant difference in the disease duration between untreated and IFN-β treated groups **(Figure 4.22 A).**

One of the tools that has been used to measure the disability of MS is the expanded disability status scale (EDSS) ranges from 0 to 10 (high level off disability) in 0.5 increments, scoring is based on an examination by a neurologist. EDSS steps 1.0 to 4.5 refer to patients who are able to walk without any aid and are based on measures of impairment in eight functional system. EDSS were measured for all patients and was ranged from 0-8 and mostly between 1-4. Although there was no statistical difference in EDSS, there was observational differences between untreated and treated patients **(Figure 4.22 B).**

To explore more about the EDSS score and the disease duration, we examined the correlation between these two factors, we couldn't find any correlation between them **(Figure 4.22 C)**. In addition, we didn't observe any correlation between duration of disease and EDSS on cytokine profile in untreated and treated MS groups **(Figure 4.23 A-F)** and **(Figure 4.24 A-F).** This suggests that the difference in cytokine production between the treated and untreated group was not strongly associated with disease duration or EDSS.

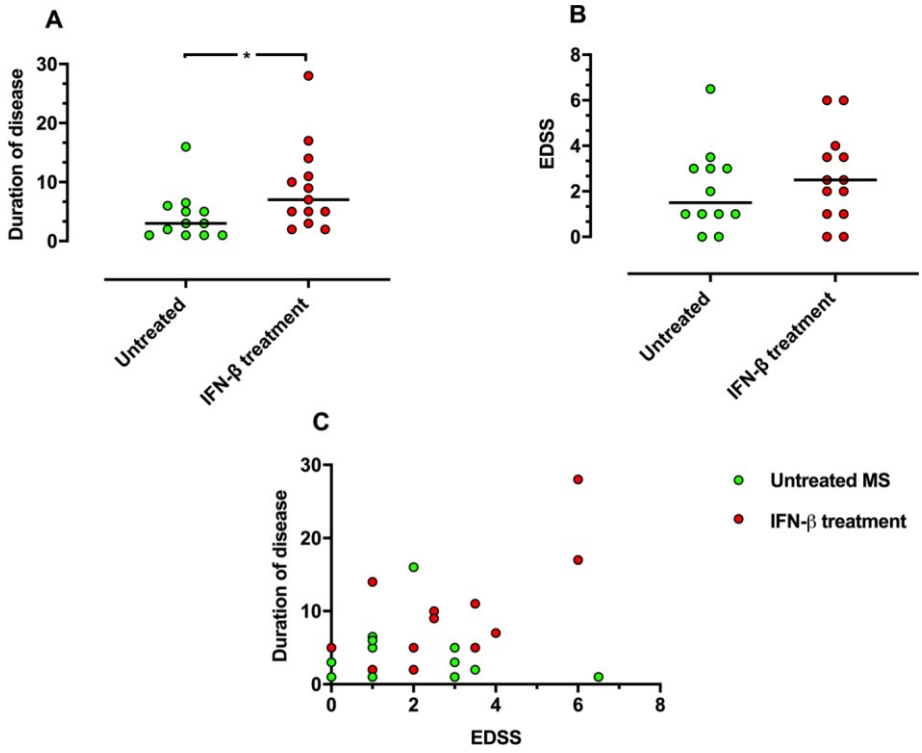


Figure 4.22 No correlation between EDSS and duration of disease between treated and untreated MS. Median values of duration of disease **(A)** score of disease Expanded Disability Status Scale (EDSS) **(B)** and correlation between duration of disease and EDSS **(C)** in untreated in IFN-丿 MS patients. Comparison were significant to * ($P<0.05$) using Mann whitneys test (n=13).

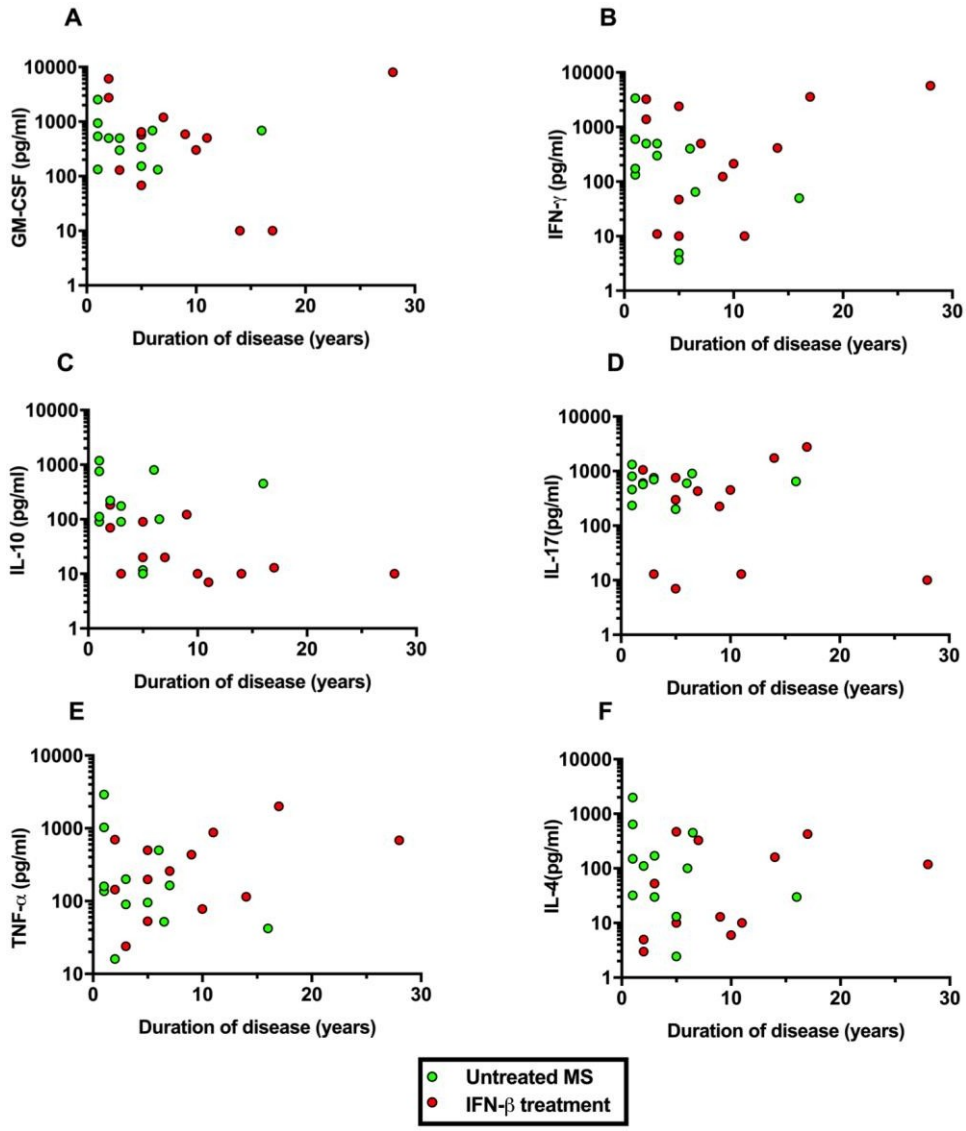


Figure 4.23 Impact of disease duration on inflammatory and regulatory response between untreated and treated MS patients. Plots showing correlation between duration of disease GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-a **(E)** and IL-4 **(F)** in untreated patients and patients under treatment (n=13)

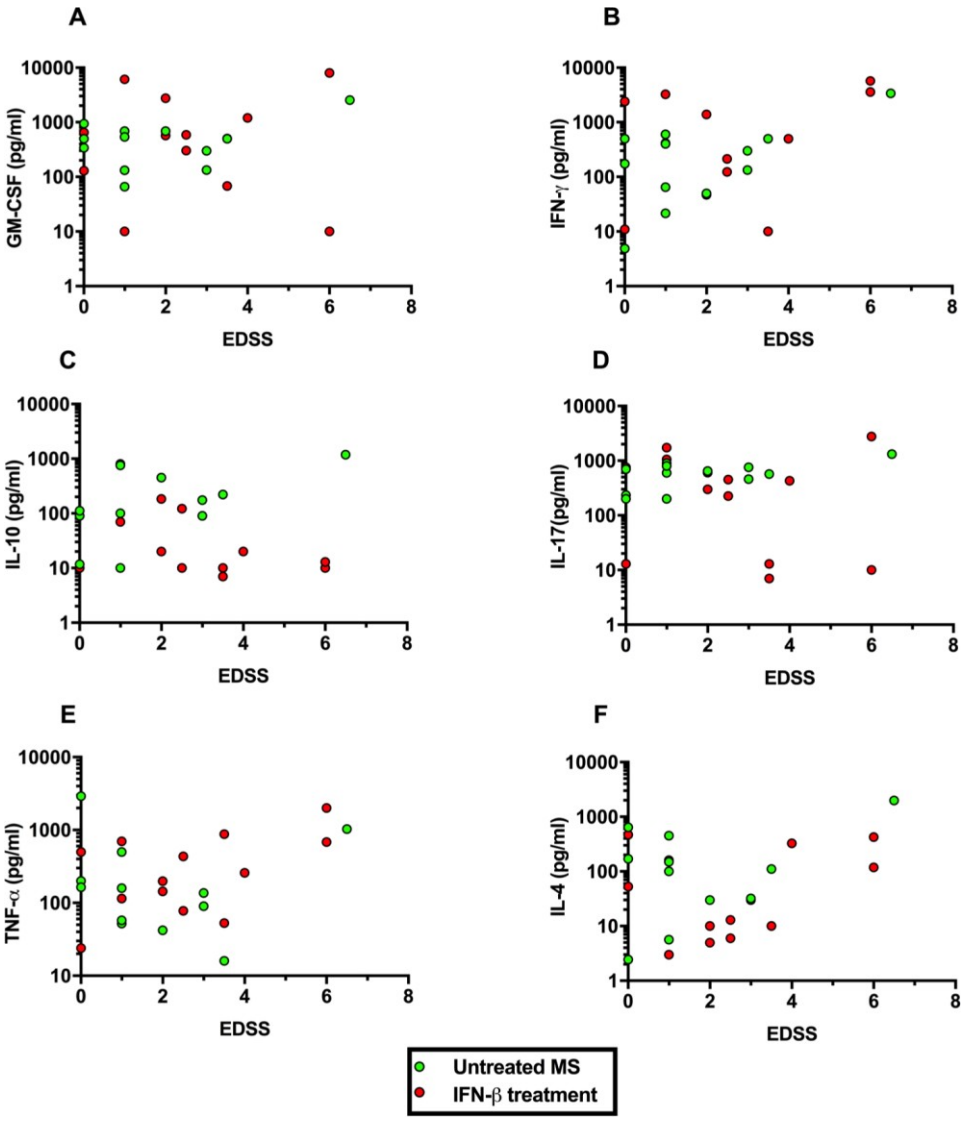


Figure 4.24 Impact of EDSS on inflammatory and regulatory response between untreated and treated MS patients. Plots showing correlation between duration of disease GM-CSF **(A)** IFN-γ **(B)** IL-10 **(C)** IL-17 **(D)** TNF-α **(E)** and IL-4 **(F)** in untreated patients and patients under treatment (n=13)

4.3.10Decreased lymphocytes and CCR6 in MS patients treated with IFN-β

In vitro studies show that it is effectively decreased T-cell migration and also modulates expression of pro-inflammatory cytokines.

We compared the lymphocyte count in untreated patients and with IFN-β treatment, as shown. In our data, total lymphocytes were not different between untreated and treated MS groups **(Figure 4.25 A)**

Additionally, CD4+ memory, non-T-regulatory and CCR6 negative and positive were all statistically decreased after treatment with IFN-β **(Figure 4.25 B-D).**

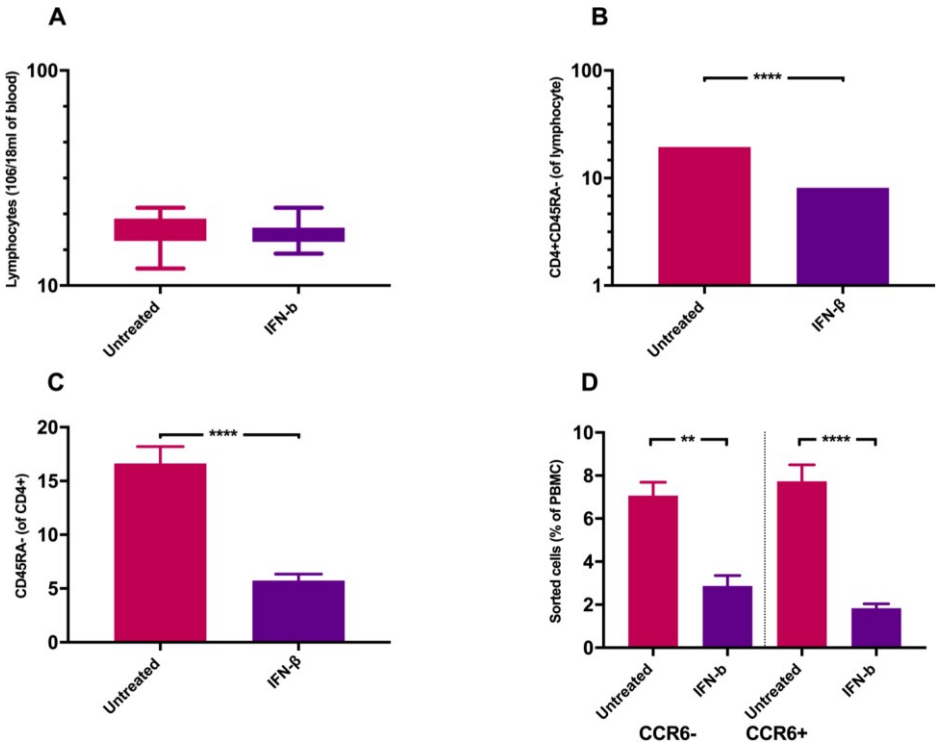


Figure 4.25 Reduced levels of different T cell subsets in blood samples from MS patients after treatment with IFN-β. Graph shows the median percentage of lymphocyte count **(A)** CD4+ memory **(B)** CD45RA- **(C)** and CCR6 negative and positive (D) after cell sorting of total PBMC**.** Comparison were significant to ** (P<0.01) *** (P<0.001) ****(P<0.0001) using Mann whitneys test (n=13).

4.4 Discussion

We demonstrated functional differences in myelin-reactive CCR6 CD4+T cells in MS patients treated with IFN-β as compared to healthy controls, identifying secretion of IL-17, IFN-γ and TNF-α in MS. Further, these autoreactive T cells are CCR6- and CCR6+ memory CD4+ T cells in both patients and controls. CCR6- were enriched with IFN-γ (classical Th1), while CCR6+ were enriched with Th17 (ex Th17).

The characterisation of CCR6+ IFN-γ CD4+ Th cells has been previously reported by a number of different groups, and they are referred to as non-classic Th1, ex-Th17 or non-conventional Th1 cells (Annunziato et al., 2014; Maggi et al., 2010; Maggi et al., 2012, 2015). IFN–γ was detectable in CD4+ CCR6- (classical Th1) and CCR6+ (non-classical Th1) cells which is consistent with published data (Restoric et al., 2017).

It has been known that IFN-γ is a critical mediator of the uptake of myelin antigen by CNS APCs during EAE, while mice deficient with IFN-β can exaggerate the disease (Sosa et al., 2015). Among several mechanism of action proposed for IFN-β, interference with the Th1/Th2 balance including down- modulation of Th1 and induction of Th2 cytokines was reported (Agrawl, 2007). In contrast to published data (Cao et al., 2015), we showed that MS-derived myelin-reactive T cells from both memory CCR6^{-} and CCR6+ populations secrete more pro- inflammatory cytokines (IFN-γ/ IL-17 and TNF-α) in MS patients treated with IFN-β as compared to untreated group. IL-4 was the only cytokine that showed a reduction in the treated groups. Also, regulatory cytokines were reduced in the treatment group.

We observed that there was imbalance in the regulatory-inflammatory cytokine production in untreated MS, which explain the need for the treatment.

IFN-β MS treated groups demonstrated higher disease duration and EDSS score compared to the untreated MS group, also there were some patients with high EDSS that produce more

Inflammatory cytokines. Treated group or patient requires treatment because they have more severe disease (higher EDSS) and disease duration. Also, it could mean that IFN-β treatment is not effective. All of these together could describe the increased inflammatory response in the treated group.

At the beginning of the study, we were aiming to collect blood samples from CIS patients as planned; this was not easy and feasible to do. We decided to classify the early MS, which is diagnosed with MS less than a year. Some of these patients had high levels of inflammatory cytokines, which can explain the need of the treatment. Genetic polymorphisms have been associated with MS, HLA typing of both healthy controls and MS patients was performed to compare the frequency of HLA alleles in both groups. Some HLA polymorphisms increase susceptibility to MS, including DRB1*0401 and DRB1*1501. DR7 carriers of MS have high reactivity of T cell reactivity to myelin peptides suggesting the HLA role in the pathogenesis of MS.

In our data, there were more MS patients with DR7, DR15 and DR4 than HC, consistent with published studies.

In total, the frequent alleles that were found in our MS cohort were DR4, DR15 and DR7 alleles.

The characterisation of CCR6+ IFN–γ CD4+ Th cells has been previously reported by a number of different groups, and they are referred to as non-classic Th1, ex-Th17 or non-conventional Th1 cells (Annunziato et al., 2014; Maggi et al., 2010; Maggi et al., 2012, 2015). In our current study, IFN–γ was detectable from CD4+ CCR6- (classical Th1) and CCR6+ (non-classical Th1, or Th17.1) cells which is consistent with published data on non-antigen specific stimulation of T cells from patients with MS (Restorick et al., 2107).

None of the factors studied (including age, gender, HLA genes, disease duration and EDSS) were significantly correlated with inflammatory cytokine production, which suggests disease activity (inflammation) itself is driving the increased cytokine production in this group of MS patients requiring treatment.

All factors including age, gender, HLA genes, disease duration and EDSS were not significantly correlated which suggest disease activity (inflammation) therefore this group of MS patient require treatment.

With respect to the results of the power analysis, it is arguable that our approach was underpowered to detect clinically significant treatment effects, as the numbers of the samples with other treatment than IFN-β were low.

Patients treated with IFN-β showed a higher response to peptide stimulation compared to the untreated patients and that could be related to the therapy affect.

IFN-β treatment decreased the number of the lymphocytes generally and the CCR6- and CCR6+ memory T cells as they decrease T-cell activation through binding to IFN receptors.

We observed more memory T cells in untreated than treated MS groups, that could be related to the pathogenesis of MS and CNS trafficking, or related to age as we have significant difference in age between the HC and MS groups, as the memory cells increased with age. However, the age has no impact as all the groups were sorted with the same techniques.

4.5 Summary

In Our study, we identified CCR6+CD4+ in the blood from MS treated with IFN-β, where they produced inflammatory and regulatory cytokines in healthy controls in response to different myelin peptides.

Most important is the higher production of inflammatory cytokines (GM-CSF, IFN-γ, IL-17 and TNF-α) in response to myelin peptides were produced in untreated MS patients in myelin specific T cells than healthy controls and that also increased in MS patients receiving IFN−β treatment.

In contrast to published data (Cao et al., 2015), IL-10 production was higher in untreated MS patient compared to healthy controls.

Cytokine response are summarised in table 4.1.

IFN-β is widely and commonly used for MS specifically RRMS, however some of the patients were not responsive to IFN-β which indicates the high demand for an effective therapy for MS.

Cytokine	HC	Untreated MS	IFN−β treatment
GM-CSF			
IFN-γ			
IL-10			
IL-17			
TNF-α			
IL-4			



Table 4.1 Cytokine response to myelin peptides in healthy controls, untreated and IFN-β treated MS patients.

CHAPTER 5

DETAILED IMMUNOPHENOTYPING OF ANTIGEN SPECIFIC CD4+ T CELLS IN MULTIPLE SCLEROSIS PATIENTS

Chapter 5: DETAILED IMMUNOPHENOTYPING OF ANTIGEN SPECIFIC CD4+ T CELLS IN MULTIPLE SCLEROSIS PATIENTS

5.1 Introduction

The use of MHC-II tetramers for detecting human CD4+ T cells specific to EBV has been reported using nine different peptide tetramers (Long et al., 2013).

For central nervous system-specific CD4+ T cells in MS, the detection and frequency of MOG-specific T cells was measured using DRB1*0401/MOG97–109(107E-S) tetramers in MS subjects. Also, the percentage of CD4+ CD25 intermediate DRB1*0401/MOG97–109(107E-S) tetramer–positive T cells was significantly higher in patients with RR-MS compared with healthy control subjects (Raddassi et al., 2011).

The concept of using soluble labeled ligands to detect cell surface receptors is a standard approach for understanding molecular specificity and function. Though, only in the last 15 years, this strategy has been successfully applied to the analysis of antigen-specific T cells using soluble MHC-peptide ligands that engage with the αβTCR. Originally developed for MHC class I recognition in the context of CD8 T cells, over the last decade similar approaches have been successfully used for MHC class II recognition in the context of CD4 T cells for a wide variety of antigens. Hence, interrogating CD4 T cells using soluble tetramers has developed into a robust technology, notably useful in numerous translational and clinical contexts (Altman et al., 1996; Lebowitz et al., 1999; Nepom, 2012).

Variances between $CD4^+$ and $CD8^+$ responses to EBV antigens were found using antigen-specific T-cell clones (Ibisch et al., 2000; Long et al., 2011; Sohn et al., 2015). The specificity of these tetramers was investigated and confirmed for MHC-II–matched alleles by using epitope- specific $CD4^+$ T-cell clones (Long et al., 2013).

In this study, we investigated whether myelin oligodendrocyte glycoprotein (MOG)-PLP and MBP specific T cells can be detected and their frequency measured using DRB1*0401/MOG97–109(107E-S) tetramers in MS patients and healthy controls expressing HLA class II DRB1*0401. In addition, we measured the EBV reactive CD4+ T cells in DR4 and DR7 healthy controls and MS cohorts after staining them with the suitable peptide tetramers.

DRB5*0101 and DRB1*1501, which present the EBV peptide, have been implicated in patients with MS (Hohlfeld et al., 2016; Strautins et al., 2014). It has also been reported that DRB1*1501 present this peptide MVFLQTHIFAEVLKD identified by EBNA-1–specific T-cell clones. Specifically, that was a $CD4^+$ T-cell clone killing EBV-infected T cells positive for DR51 and EBV-infected NK cells transduced with DRB5*0101 (Okamura et al., 2008). Therefore, we used two EBV peptide tetramers, which are DR4 (AEGLRALLARSHVER) and DR7 (PRSPTVFYNIPPMPLPPSQL) restricted. To our knowledge, this is the first time that these responses have been measured using tetramers.

5.5.1 Objectives

1. Quantify EBV (AEG) and myelin reactive CD4+ T cells in (DRB1*0401) healthy controls and MS cohort by applying DR4 peptide tetramers.
2. QEBV (PRS) reactive CD4+ T cells in (DRB1*0701) healthy controls and MS cohorts by applying DR7 peptide tetramers.
3. Define T helper subsets (naive and memory) association with different tetramer staining.

5.2 Methods

Healthy controls and MS cohort (treated and untreated), mostly RRMS, were examined with different MHC-II tetramers. Initially, to confirm the response, we expanded the T cells to verify that they were responding to these peptides.

The cells were stimulated with the specific peptides and re-stimulated on day 7. Responses were confirmed by ELISpot to confirm that the response to these peptides was real by applying controls and different peptides including EBV and myelin peptides.

5.3 Results

5.3.1 Characterisation of CD4+ Tetramer + cells in the blood

Peripheral blood was collected from healthy controls and MS patients with the matched HLA for the compatible tetramers. PBMC from peripheral blood were stained *ex vivo* using series of characteristic markers and activation markers, to identify CD4+ tetramer+ T cells, which were analysed by flow cytometry. For this, an appropriate gating strategy was determined. In the peripheral blood, CD3+CD4+ were gated first with the exclusion of the monocytes (CD14- gate) (**Fig. 5.1A-D,** blue arrows), then gated for the tetramer required; here we stained with the EBV-AEG tetramers for DR4 samples, all other tetramers were stained with the same technique, allowing CCR7 and CD45RA to be used as a marker of activation as naïve and memory T cells **(Fig. 5.1 E-G),** in accordance with the literature, and for cleaner gating to be achieved (James et al., 2009).

In the peripheral blood, EBV tetramers were detected at the average (median) of 0.07 % for mostly all the samples, which is consistent with the previous studies (Long et al., 2013). However, there was no enrichment in the memory fraction for tetramer+ cells; naive (CCR7+ CD45RA+) were shown as the higher percentage (69.7%), followed by central memory (CCR7+CD45RA-) (18.18%), then effector memory (CCR7-CD45RA-), and effector memory RA (CCR7-CD45RA-) (3.03%). These were very similar to the cells not labeling with the tetramer.

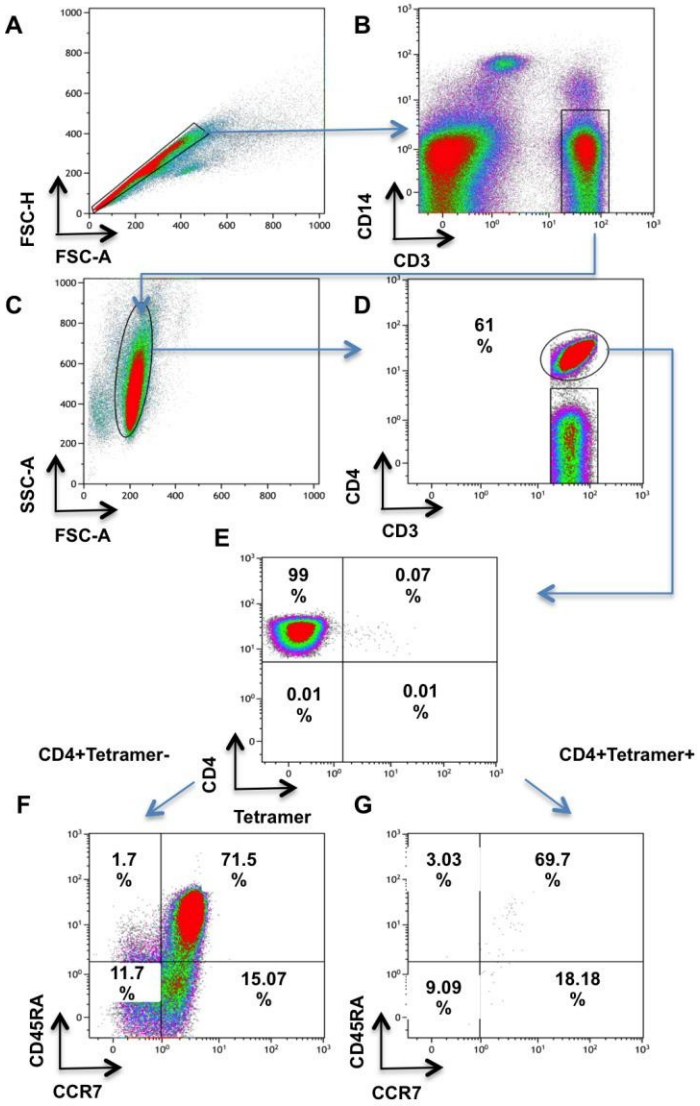


Figure 5.1 Gating strategy to identify EBV^{+} MHC-II tetramer CD4^{+} memory T cells in (DR4*04:01) in the peripheral blood samples. Total PBMC were isolated and stained with EBV (AEG) tetramers followed by surface markers and analysed by flow cytometry in all healthy controls and MS samples. Single cells were selected by FSC-A vs FSC-H **(A)** gated on CD3 $^{+}$ CD14+ **(B)** lymphocyte (**C**) CD3 $^{+}$ CD4 $^{+}$ **(D)** CD4+ tetramer **(E)** based on different populations as Naïve (CCR7 $^{+}$ CD45RA $^{+}$) central memory CM (CCR7^{+} CD45RA^{-}) effector memory EM (CCR7^{-} CD45RA^{-}) effector memory RA (CCR7^{-} CD45RA^{+}) EMRA for CD4+ tetramer – **(F)** and tetramer + **(G)**
Values represent percentage of cells in the gate. Blue arrow representing gating used throughout.

5.3.2 Detection of EBV tetramers in MS DR4 samples, but not in healthy controls

The first tetramers we used to compare our EBV or myelin tetramers were the CLIP. class II-associated invariant chain, known as CLIP (class II-associated invariant peptide) (100-101). CLIP was used in our experiments as a negative control. MHC-II tetramers were used to detect antigen specificity in CD4+ memory T cells for both EBV and CNS antigens (Raddassi et al., 2011; Long et al., 2013). EBV (AEGLRALLARSHVER) and MOG97-109 reactive CD4+ T cells, were isolated with DRB1*0401/ EBV – MOG97-109 tetramers.

We determined by flow cytometry the percentage of CD4+ tetramer+ populations in DR4 MS blood sample stained with CLIP (0.03%), EBV-AEG (0.1%), MOG (97-109) (0.2%) and (PLP 180-199) (0.4%) tetramers **(Fig. 5.2 A)**. There was no significant difference of tetramer+ CD4+ T cells in response to all of the tetramers in comparison to CLIP tetramer in healthy controls **(Fig. 5.2 B).** However, EBV (AEG) and MOG (97-109) tetramers were significantly increased in MS patients but not with PLP (180-199) tetramer **(Fig. 5.2 C)**.

There were no significant EBV (AEG), MOG (97-109) and PLP (180-199) CD4+ T cells between healthy controls and MS patients (**Fig. 5.2 D**). Although there is a slight increase of EBV response in MS patients, but it is not statistically significant. More samples would be required to confirm the difference between healthy controls and MS patients. These data indicate that autoantigen-specific T cells can be detected and enumerated from the blood of subjects using class II tetramers, and the percentage of EBV and MOG 97-109 reactive T cells is greater in patients with MS patients than healthy controls.

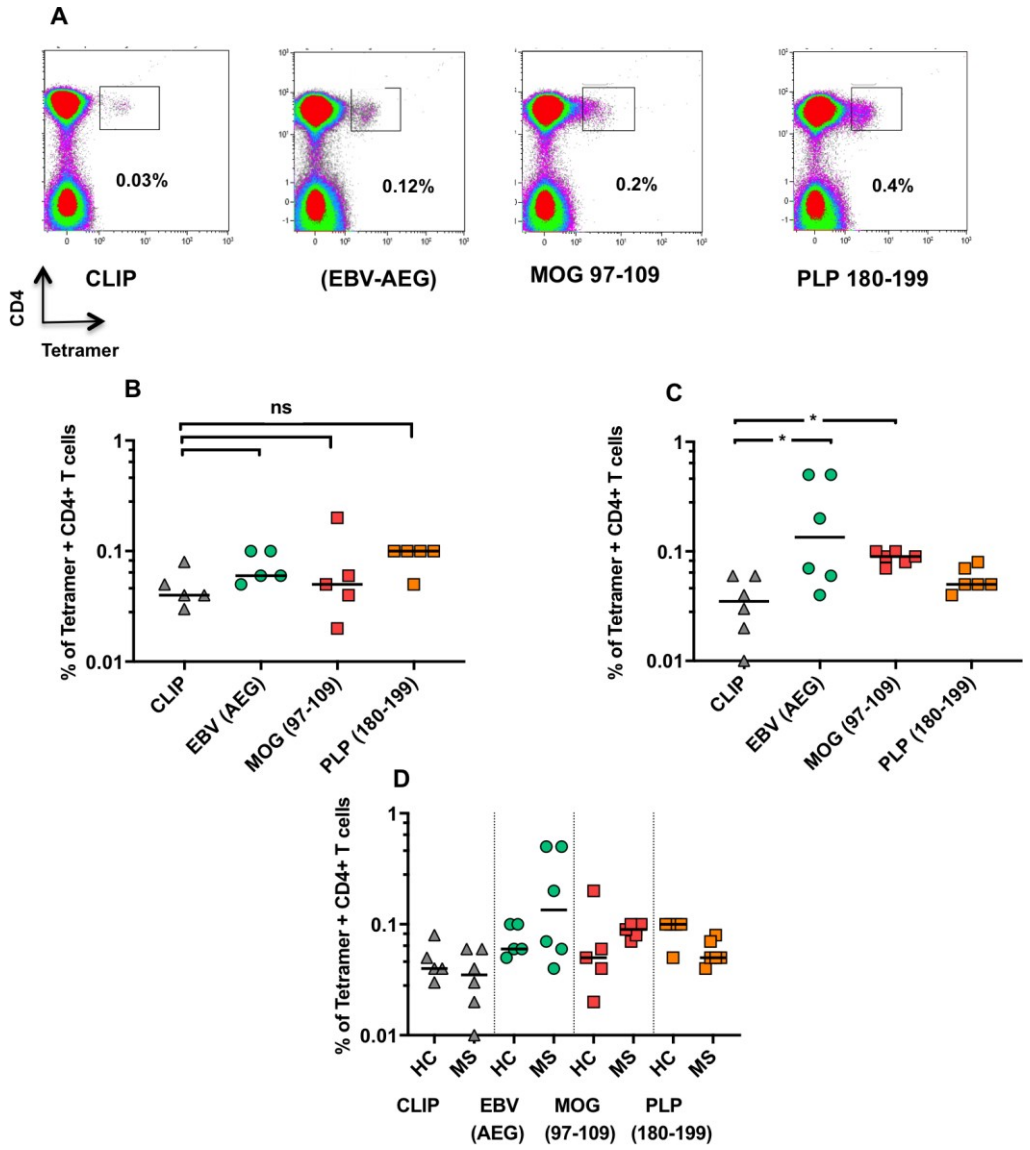


Figure 5.2 Significant increase expression of DR4 EBV and myelin peptide tetramers MHC-II tetramer$^+$ specific CD4$^+$ T cells in (DR4*04:01) blood samples in MS patients. Representative flow charts of (DR4*04:01) MS patients showing percentage of CLIP, EBV, MOG (97-109), PLP (180-199) tetramers by CD4+ T cells. PBMC were isolated and stained with CLIP, EBV, MOG (97-109) and PLP (180-199) tetramers followed by surface markers and analysed by flow cytometry **(A)** Percentage of CLIP, EBV, MOG (97-109), PLP (180-199) tetramers by CD4+ tetramer T cells in DRB1*04:01 from HC **(B)** MS patients **(C)** and HC compared with MS patients **(D)**
Data are significant to *($P<0.05$),) using dunns multiple comparison test HC (n=5) MS (n=6). The data are represented as scatter graphs with each scatter representing a single individual.

CLIP= Class II associated invariant chain
EBV-AEG= AEGLRALLARSHVER

5.3.3 No enrichment of tetramer CD4+ with memory populations in healthy controls and MS blood samples stained with EBV MHC class II tetramers

Cytokine responses to virus lysate, virus proteins, or epitope peptides as the readout, suggest that EBV-specific CD4+ T cells in the blood of healthy carriers lay in the CD45RA$^-$ CCR7$^-$ effector memory (EM) and CD45RA$^-$ CCR7+ central memory (CM) compartments (Long et al., 2019). Here, we analysed the phenotype of MHC II tetramer–positive cells using a panel of antibodies against cell surface differentiation markers in multi-color flow cytometry. Overall, in the EBV(AEGLRALLARSHVER) tetramer-negative and positive populations analysed in 5 healthy controls, the epitope-specific CD4+ T cells were split mainly in naïve subset CCR7$^+$CD45RA$^+$ (50-60%), followed by central memory (CM) CCR7$^+$CD45RA$^-$ (20-25%), then effector memory (EM) CCR7$^-$CD45RA$^-$(5-10%) and the lowest percentages within CCR7$^-$CD45RA+ (EMRA), with no significant differences between the subsets **(Fig. 5.3 A, B).** Also, similar observation were detected in six MS blood samples; where the EBV tetramer-negative and positive highest percentages of the epitope-specific CD4$^+$ T was from the naïve subset CCR7+CD45RA+ (50-60%), followed by central memory (CM) CCR7+CD45RA$^-$(20-25%), then effector memory (EM) CCR7$^-$CD45RA$^-$(5-10%) and the lowest percentages within CCR7$^-$CD45RA+ (EMRA), which show significant difference between the naive and the memory subsets **(Fig. 5.3 C, D).**

Overall, we couldn't detect any enrichment in the memory subset in the blood samples of healthy controls and MS patients.

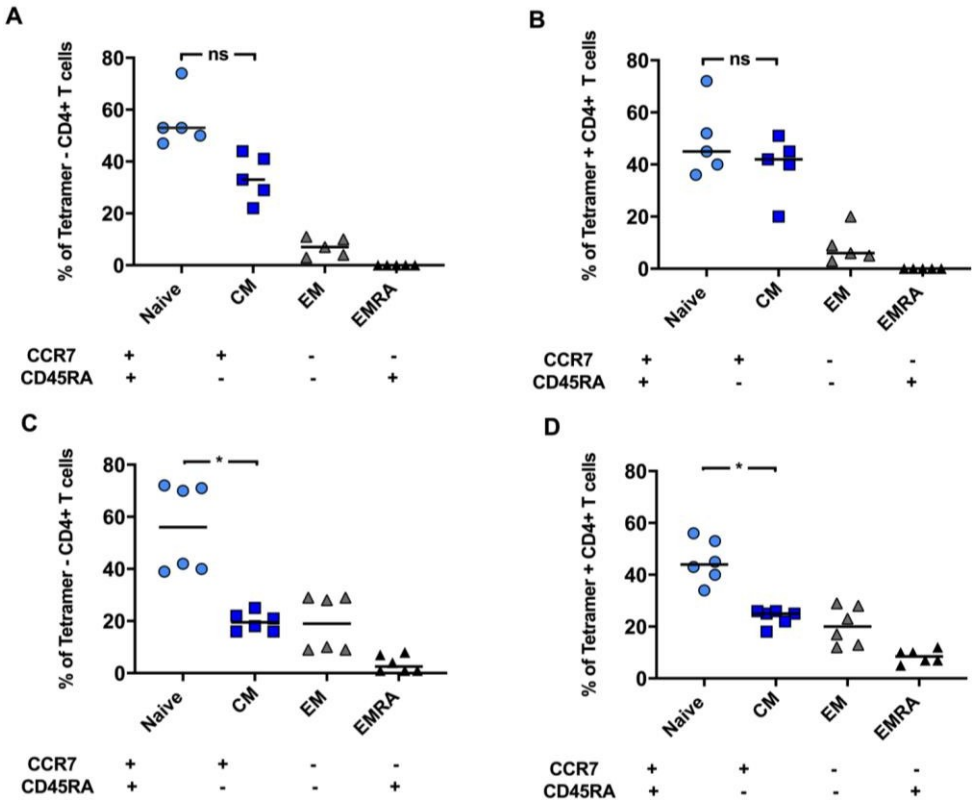


Figure 5.3 Phenotype of EBV (AEG) MHC II tetramer negative/positive-specific CD4 memory populations. Analysis of EBV (AEG) epitope-specific CD4+T cells from DRB1*04:01 HC tetramer- CD4+ **(A)** and tetramer +CD4+ **(B)** and from MS DRB1*04:01 tetramer- CD4+ (C) and tetramer +CD4+ (D)
showing percentage CCR7 and CD45RA expression on MHC II tetramer staining populations of all relevant specificities.

Data are significant to *($P<0.05$) using Dunns multiple comparison test HC (n=5) and MS (n=6). The data are represented as scatter graphs with each scatter representing a single individual.

CM=Central Memory
EM= Effector Memory
EMRA=Effector Memory RA

5.3.4 EBV MHC II tetramer-specific CD4$^+$ T cells were not expanded in blood samples

Next, we explored if the EBV (AEGLRALLARSHVER) MHC II tetramers CD4+ memory T cells were expanded in healthy controls blood samples with DR4 alleles. We didn't observe any significant difference before and after expansion with EBV (AEG) peptide in all blood samples **(Fig. 5.4 A).** EBV specific CD4+ T cell response tetramers were determined by flow cytometry which showed no difference before and after expansion **(Fig. 5.4 A).**

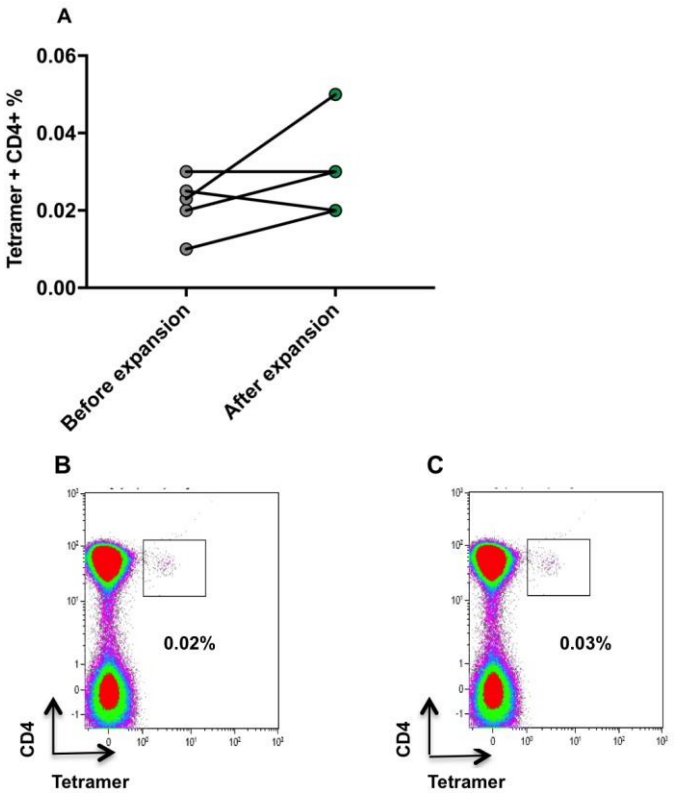


Figure 5.4. Percentage of EBV MHC II tetramer positive-specific CD4 memory populations before and after expansion with EBV peptides. Analysis of EBV epitope-specific CD4+T cells from 5 HC donors stained with DR4 EBV tetramer before and after expansion **(A)** PBMC were isolated and stained with EBV (AEG) tetramers followed by surface markers and analysed by flow cytometry before **(B)** and after expansion **(C).**
Data were not significant by Wilcoxon-paired test (n=5).

5.3.5 Significant production of EBV tetramers in DR7 healthy controls and MS samples

We then applied other epitope tetramers, which are known to induce EBV specific CD4+ T cell response in healthy individuals. EBV (PRSPTVFYNIPPMPLPPSQL) peptide is known as a CD4+ T cell epitope. It was recognized by 40% of the donors tested, and this was largely responsible for the overall frequency of EBNA2-reactive donors reaching 65% (Brook et al., 2016). EBV (PRS) reactive CD4+ T cells were detected in healthy controls at significant levels when compared to blood samples without tetramer staining **(Fig. 5.5 A).**

We also explored if there is an EBV specific CD4+ response in DR7 MS patients. To our knowledge, this is the first attempt for detecting (PRSPTVFYNIPPMPLPPSQL) in DR7 MS patients. We detected a significant production of EBV specific CD4+ tetramer response in DR7 MS patients **(Fig. 5.5 B).** We determined the percentage of CD4+ tetramer+ populations in DR7 healthy controls samples stained with EBV-PRS tetramer **(Fig. 5.5 C)** and MS **(Fig 5.5 D),** respectively.

These data indicate that EBV (PRSPTVFYNIPPMPLPPSQL) -specific CD4+ T cells can be detected from the blood of subjects using class II tetramers, however, similar percentage of EBV (PRSPTVFYNIPPMPLPPSQL) reactive CD4+ T cells was observed between healthy controls and MS patients **(Fig 5.5E).**

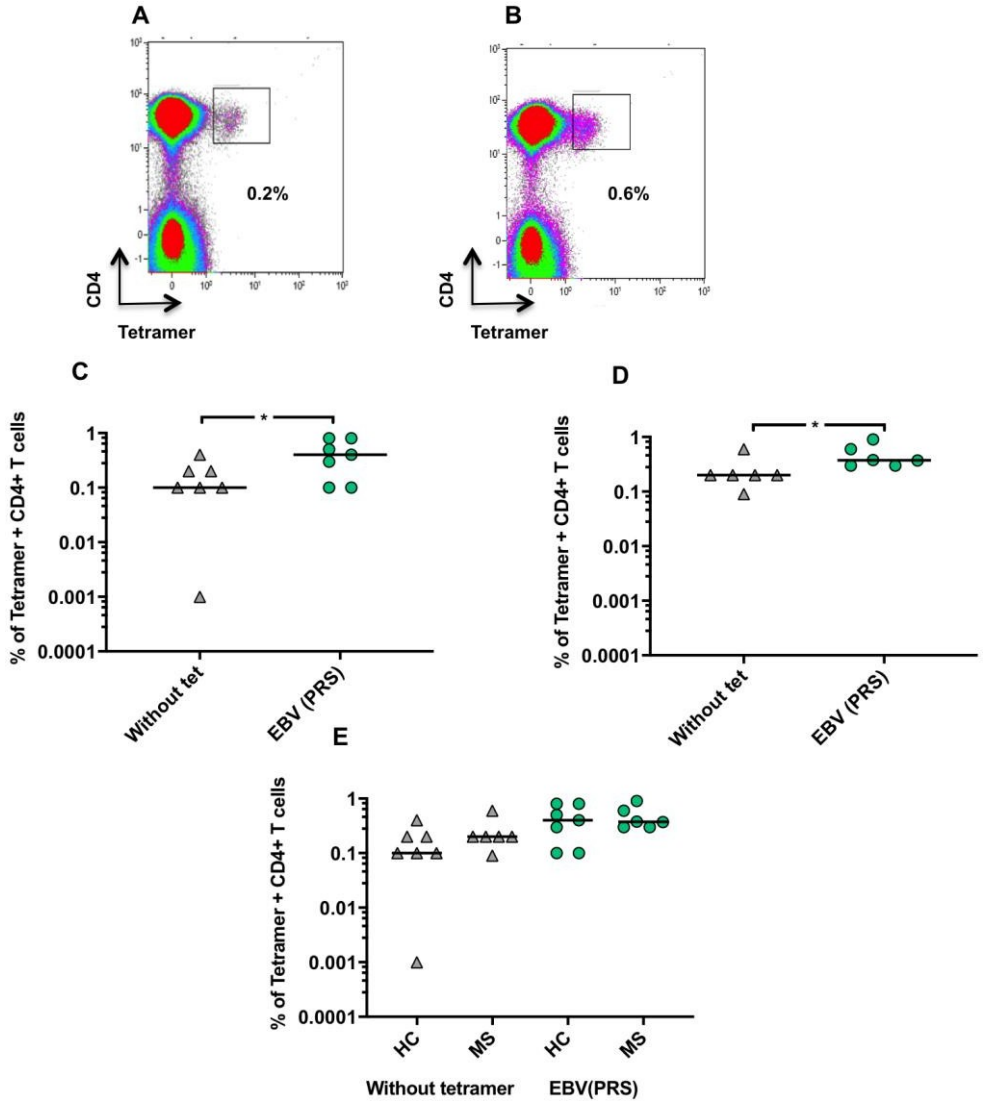


Figure 5.5 High expression of DR7 EBV peptide tetramer MHC-II tetramer^{+} specific CD4^{+} T cells in (DRB1*07:01) blood samples in HC and MS patients. Representative flow charts of (DRB1*07:01) HC **(A)** and MS patients **(B)** showing percentage of EBV tetramers by CD4+ T cells. PBMC were isolated and stained with EBV followed by surface markers and analysed by flow cytometry. Percentage of EBV tetramers by CD4+ tetramer T cells in DRB1*07:01 from healthy controls **(C)** MS patients **(D)** and HC compared with MS patient **(E).**
Data are significant to *($P<0.05$),) using Wilcoxon paired comparison test HC (n=7) MS (n=6). The data are represented as scatter graphs with each scatter representing a single individual.

EBV-PRS= PRSPTVFYNIPPMPLPPSQL

5.3.6. Enrichment of tetramer CD4+ with memory populations in healthy controls and MS blood samples stained with EBV MHC class II tetramers

Long et al., 2013, have revealed that blood from DR7 healthy donors stained with EBV (PRS) tetramers, was enriched with the CD45RA$^-$ CCR7$^+$ central memory (CM) and CD45RA$^-$ CCR7$^-$ effector memory (EM) compartments. As in the previous healthy controls and MS cohort, we analysed the phenotype of MHC II tetramer–positive cells using a panel of antibodies against cell surface differentiation markers in multi-color flow cytometry. Overall, in the EBV (PRSPTVFYNIPPMPLPPSQL) tetramer-negative and positive populations analysed in healthy controls, the epitope-specific CD4$^+$ T cells were significantly enriched with the central memory populations CCR7+ CD45RA- (50%) **(Fig. 5.6 A, B).**

A similar trend was observed in the DR7 MS cohort **(Fig. 5.6 C, D),** with CD4+ tetramer + T cells preferentially exhibiting a CM (70%), naive (25%) and to a lesser extent EM (4%) and EMRA phenotype (1%).

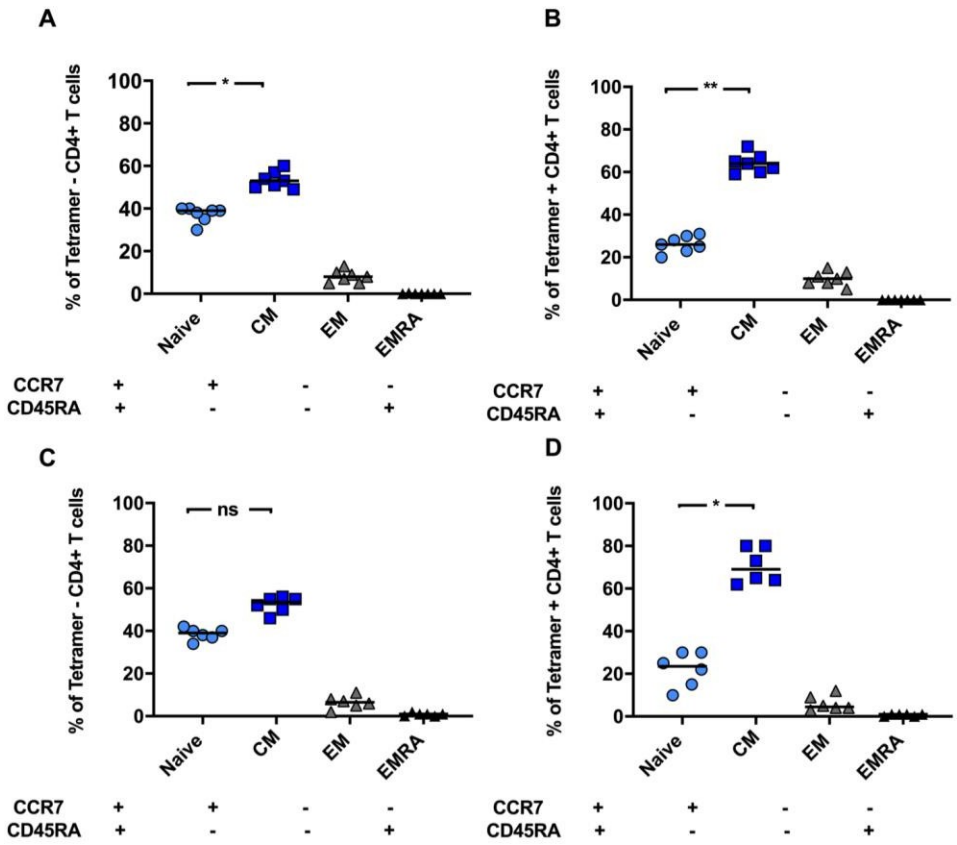


Figure 5.6 Phenotype of EBV (PRS) MHC II tetramer negative/positive-specific CD4 memory populations. Analysis of EBV (PRS) epitope-specific CD4+T cells from DRB1*07:01 HC tetramer- CD4+ **(A)** and tetramer +CD4+ **(B)** and from MS DRB1*07:01 tetramer- CD4+ **(C)** and tetramer +CD4+ **(D)**
Showing percentage CCR7 and CD45RA expression on MHC II tetramer staining populations of all relevant specificities.

Data are significant to *($P<0.05$), **($P<0.01$) using Dunns multiple comparison test HC (n=7) and MS (n=6). The data are represented as scatter graphs with each scatter representing a single individual.

CM=Central Memory
EM= Effector Memory
EMRA=Effector Memory RA

5.3.7 Expansion of EBV (PRS) MHC II tetramer-specific CD4+ T cells in HC blood samples

Long et al., 2013 has shown an expansion in the percentage of EBV (PRS) MHC II tetramers CD4+ T cells in the EBV+ healthy controls blood samples. We next applied EBV (PRS) MHC II tetramers to examine if CD4+ T cells from DR7 healthy control blood samples can be expanded. However, there was no statistically significant difference in the percentage of tetramer+CD4+ T cells before and after expansion with EBV (PRSPTVFYNIPPMPLPPSQL) tetramer, there was an observational difference before and after expansion (**Fig. 5.7 A).** EBV specific CD4+ T cell response to tetramers was determined by flow cytometry, which showed difference before (0.01%) and after (0.3%) expansion **(Fig. 5.4 B, C).**

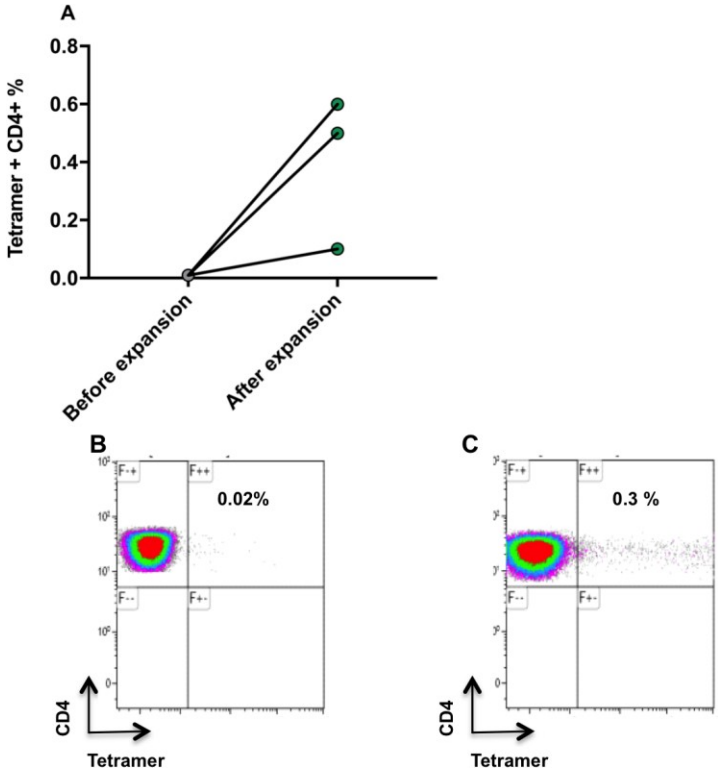


Figure 5.7 Percentage of EBV MHC II tetramer positive-specific CD4 memory populations before and after expansion with EBV peptides. Analysis of EBV epitope-specific CD4+T cells from 3 HC donors stained with DR4 EBV tetramer before and after expansion **(A)** PBMC were isolated and stained with EBV (PRS) tetramers followed by surface markers and analysed by flow cytometry before **(B)** and after expansion **(C)**
Data were not significant by Wilcoxon-paired test (n=3).

5.3.8 Detection of EBV (PRS) but not EBV (EAG) nor CNS antigens specific CD4+ T cells secreting IFN-γ in blood from healthy donors

The final step was to confirm the response to EBV or myelin peptides in blood samples, by measuring the IFN-γ secretion in CD4+ T cells by ELISpot. The enzyme-linked immune absorbent spot (ELISpot) is a type of assay that allows the quantitative measuring of the frequency of cytokine secretion for a single cell.

Previous work using certain EBV peptide including (EBV- PRSPTVFYNIPPMPLPPSQL) panels have enumerated circulating CD4+ memory T cells in *ex vivo* ELISpot analyses of cytokine production (Münz et al., 2000; Amyes et al., 2003; Long et al., 2005; Long et al., 2011b). We used DMSO as a negative control, PHA as a positive control, DR4 control which is an EBNA-2 epitope (GQT) that is known to induce a response in EBV healthy carriers. DR4/DR7 healthy donor that is EBV+ was incubated with different peptides including EBV and myelin peptides. There was a high number of cells secreting IFN-γwith PHA control (>500 cells), DR4 control (200 cells), DR7 EBV (PRS) epitope (40 cells) and no secretion of IFN-γ cells with DR4 EBV (AEG), CNS-1 (MOG97-109/PLP180-199) or CNS-2 (MOG222-241/MBP85-99/PLP30-49/PLP129-148) epitopes **(Fig 5.8 A, B).**

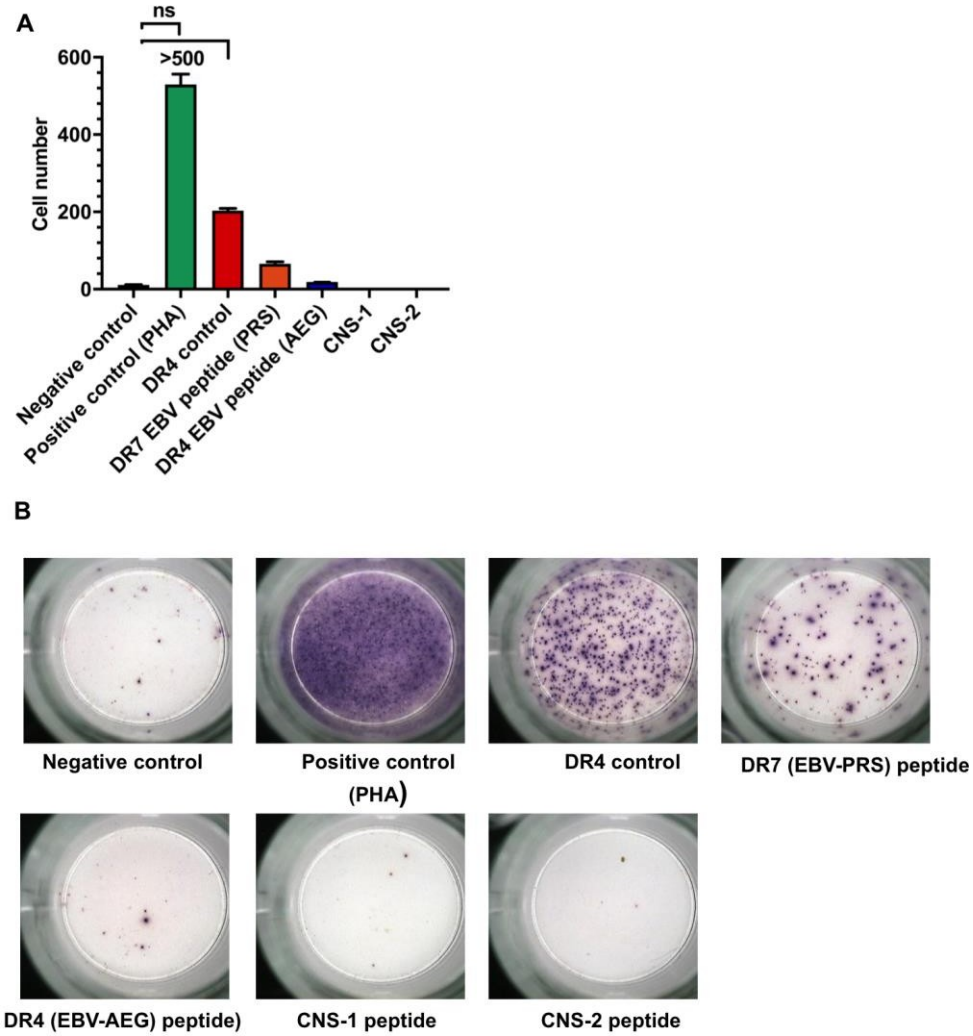


Figure 5.8 ELISpot of IFN-γ cytokine secretion by DRB1*04:01/DRB1*07:01 purified PBMC in healthy donor cells. (50,000 cells/well) were incubated for 20h in the absence or presence of different EBV (1:1000) and the number of cells secreting IFN-γ was analysed by ELISpot. Bar columns expressing IFN-γ cytokine secretion after stimulating with different peptides **(B)** Well images are shown in purple and white.
Data were not significant using Dunn's multiple comparison test. This experiment is representative of one donor done in 3 replicates.

PHA=Phytohemagglutinin
DR4 control=GQTYHLIVDTDSLGNPSLSV (EBNA-2)
EBV-PRS= PRSPTVFYNIPPMPLPPSQL
EBV-AEG= AEGLRALLARSHVER
CNS-1= MOG97-109/PLP180-199
CNS-2=MOG222-241/MBP85-99/PLP30-49/PLP129-148

5.4 Discussion

We first wanted to determine if CNS antigen specific T cells could be detected using myelin MHC class-II tetramers. MOG 97-109 and PLP 180-199 tetramers were detected in all MS cohort **(Fig 5.2 C).** In addition, myelin tetramers were observed in healthy controls but without a statistical difference when compared to CLIP **(Fig 5.2 B).** Although myelin class-II percentage was higher in MS than healthy controls, this was not statistically significant (**Fig 5.2 D).**

When we applied ELISpot; for the DR4 myelin peptides that we used previously in the culture stimulation for the cytokine production (chapter 3), there was no response as compared to PHA or the DR4-EBVcontrol (few spots). This suggests that in the future it will be important to test T-cell clones with peptides before testing the tetramer for staining. These findings suggest that the frequency of cells is low or undetectable in response to the peptide in healthy controls which indicates that there is no antigen specific (dominant) response to this peptide.

There was a CNS peptide-specific response in healthy controls and that was detected previously in the culture supernatant when we measured different cytokines including IFN-γ (chapter 3) after the stimulation with different myelin peptides, which could be a result from molecular mimicry as discussed before. Raddassi et al., 2011, suggested that CNS peptide tetramers such as DRB1*0401/MOG97–109(107E-S) tetramers were higher in MS that HC with an increased percentage of $CD4^+$ CD25 intermediate DRB1*0401/MOG97–109(107E-S) tetramer–positive T cells in MS patients. T cell clones from single cell cloning of DRB1*0401/MOG97–109(107E-S) tetramer+ cells confirmed that these T cell clones were responsive to both the native and the substituted MOG peptide.

It has been difficult to demonstrate differences in the frequency of myelin-specific T cells in the circulation of patients with the disease. Lindert et al, 1999, showed a higher frequency of

anti-MOG^{Igd} in the serum of MS patients, but no difference in the T cell proliferative response between healthy subjects and MS patients with MS. A more recent report by Bahbouhi et al., reported an increased frequency of anti-myelin T cells in MS patients using a sensitive detection method. Soluble MHC–peptide complexes allow the detection and isolation of antigen- specific T cells, and these recombinant MHC class II tetramers have been successfully used to detect antigen- specific CD4+ T cells for viral, bacterial, and self-antigens (Novak et al., 1999; Gebe et al., 2003; Nepom, 2005). Raddassi et al., 2011 have successfully applied this approach to demonstrate an increase in the frequency of myelin-specific T cells in MS patients compared with healthy controls using HLA- DRB1*0401 class II tetramers loaded with MOG peptide.

Our second hypothesis, that there was an increased EBV specific CD4+ T cell response in MS compared to healthy controls. Long et al, 2013, have reported the first tetramer-based analysis of human CD4^{+} T cell responses to a viral pathogen that is not only genetically stable but also naturally highly immunogenic to the T cell system. We examined the T cell response to myelin peptides that are known to have a role in MS. As the majority of MS patients are EBV positive, we also examined the T cell response to EBV peptides.

Although the frequencies of MHC II tetramer-positive CD4^{+} T cells were low (0.005–0.085% of the total CD4^{+} T cells), all values were based on the analysis of an average of 10^{6} cells per CD4^{+} T cell–enriched sample and in every case were reproducible upon repeat testing. First, we tested the EBV DR4 epitope (AEGLRALLARSHVER) peptide tetramers in DR4 healthy controls and MS blood samples, this is the first study to examine this specific epitope.

We were able to detect DR4 EBV epitope (AEGLRALLARSHVER) MCH II peptide tetramers specific CD4+ T cells in healthy controls and in MS blood samples with a higher percentage. However, these specific CD4+ T cells were not enriched within the memory subset and were not expanded after stimulation with the EBV peptides. There are many reasons for this result,

these blood donors may not be responsive (positive) to this specific epitope, and this could be achieved by screening the blood donors with this peptide before staining or expansion with the tetramers. Also, we didn't test the blood samples for the EBV (serotype) and therefore the lack of expansion in these samples could be related to EBV sero-negativity.

The EBV tetramer that we used did not produce a clear response in the donors; one of the reasons could be the low frequency of the responded cells. However, after intensive investigation we noticed that this epitope was not reported before in the literature or in the human data when purchased from the company.

We then applied one of the DR7 EBV (PRSPTVFYNIPPMPLPPSQL peptide tetramers that induced a response in EBV healthy control carriers (Long et al., 2013; Meckiff et al., 2019). There was EBV specific CD4+ T cell responses in healthy controls with a higher percentage in MS cohort in response to EBV peptide. In addition, specific CD4+ T cells were enriched with memory subset and were expanded after stimulation with the EBV peptides, which is consistent with published data (Long et al., 2013). With regard to the DR4 EBV (AEG) tetramers, there was no significant staining of antigen CD4+ specific T cells and that was consistent with the result in the ELISpot where no cells were undetectable, while DR7 EBV (PRS) peptide tetramers were able to stain the antigen specific CD4+ T cells and cells were detected in ELISpot.

The last thing that could explain the unresponsiveness to the tetramer, is that the healthy control donor is different from the healthy control donors examined in response to peptide for cytokine production (chapter 3). ELISpot was performed from one donor in 3 replicates in one single experiment, and therefore these data need to be repeated from at least 6 donors to confirm the result. It may help to examine the EBV- DR4+ donor in future and compare those donors with the EBV+ DR4+ donors to detect the difference for a specific peptide response.

Tetramer assays are widely used for single-cell phenotyping and enumeration and offer an

important advantage over other methods, such as ELISPOT and single-cell PCR, by enabling

the recovery and further study of sorted cells based on fluorescent tetramer binding in flow cytometry. As a cytometry- based application, tetramers also provide the prospect of ease of use and short assay time, analogous to Ab-based flow cytometry studies. The major limitation to their use is the requirement to have some knowledge of the specific pMHC components involved in the recognition events being studied, because these are necessary to construct the tetramer reagents. A consequence of this element of MHC restriction is the consideration that humans have very diverse HLA class II molecules, so that random sampling or clinical monitoring studies may require the use of many different tetramers to ensure a broad representation of the population.

5.5 Summary

In this group study, we were able to detect MOG 97-109 class II tetramers at significant levels but not PLP 180-199 myelin MHC II tetramer-specific $CD4^+$ T cells in the blood of MS patients. However, there was no significant difference between healthy controls and MS patients.

DR4 EBV (AEG) tetramers didn't indicate a confirmative result, as antigen specific T cells were not detected in ELISpot. However, DR7 EBV (PRS) MHC II tetramers allowed detection of specific CD4+ T cells both in healthy controls and MS cohort, without a significant difference between healthy controls and MS patients. Analysis of additional samples would be needed to verify the result of the tetramers.

Chapter 6

GENERAL DISCUSSION

Chapter 6: General discussion

Substantial advances have been made in the understanding of MS through studies of EAE models and samples from human blood and brain tissues. However, the exact pathological mechanism of MS remains unclear (Duffy et al., 2014).

Autoreactive T cells, identified in many autoimmune diseases, including MS, show features of an antigen- specific response *(*Koehler et al., 2002; Goverman et al., 2009*)*. These cells can traffic between the CNS and periphery where they may undergo clonal expansion on both sides of the BBB; though, the relative contributions of recently recruited peripheral T cells versus CNS-expanded T cells remains unclear (Wilson et al., 2010).

Benefit of B cell depletion in patients with MS shows that B cells also have a central role MS (Hauser et al., 2008). B cells could contribute to the pathogenesis of autoimmune disease by production of antibodies that recognize components of myelin, axons or neurons and contribute to demyelination or axonal damage, as antigenpresenting cells for T cells that are specific for CNS antigens and as immunoregulatory cells that produce suppressive cytokines and/or influence regulatory T cell activity (Mclaughlin et al., 2008). The deposition of antibodies and complement on the myelin sheath of lesions in tissues from many patients with MS suggests that antibodies have a role in the pathogenesis of this disease.

Rituximab was the first anti-CD20 therapy to be successfully used in MS (Maloney et al., 1994; Hauser et al., 2008; Bar-Or et al., 2008). By targeting CD20, which is expressed by mature B cells from the pre-B cell to plasmablast stage, these therapies deplete B cells via antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated phagocytosis (ADCP), leading to significant reductions in relapse rates and lesion formation (Sorensen and Blinkenberg, 2016; Agahozo et al., 2016; Geland et al., 2017).

Reactivation of these cells (T helper) leads to increased production of inflammatory cytokines.

These cytokines attract other immune cells, such as macrophages, which contribute to inflammation through the release of injurious immune mediators and direct phagocytic attack on the myelin sheath (Ota et al., 1990). Th cells also help B cells to be activated and produce pathogenic Abs (Albert et al., 2002)

Therefore, Th cells are important in the pathogenesis of MS and they are required for almost all adaptive immune responses. Myelin-reactive CCR6 T cells are the hallmark of MS that contribute to the inflammatory response by production of GM-CSF, IL-17 or IFN-γ. The clinical benefits mediated by monoclonal Ab (natalizumab) suggest that T cells predominantly contribute to disease pathology (Tintore et al., 2019).

In my research I attempted to address some of these unanswered questions by investigating multiple aspects of T cells in MS, including their phenotype and cytokine production in the blood. I also carried out investigations into the immunophenotypes of antigen specific with HLA- peptide tetramers in untreated, treated MS groups and healthy controls.

6.1 How could EBV contribute to the pathogenesis of MS?

Infectious agents may play a crucial role in inducing myelin-reactive pathogenic T cells. Potential mechanisms include cross reactivity with CNS myelin antigens, triggering an already expanded autoreactive immune repertoire or a self-limited infection of the brain that releases myelin antigens. A persistent viral infection of the brain or transmissible agent does not cause MS, although infection with EBV seems to be required.

There are 2 hypotheses about MS pathogenesis: cross reactivity (molecular mimicry) between viral and CNS epitopes that has been previously reported in MS (Holmoy et al., 2004, Lunemann et al., 2008b, Lang et al., 2002). These specific T-cell responses are suggested to have a key role in MS development, but the exact contribution and the mechanism of these cells in the pathology of MS remains elusive

The second hypothesis is the direct role for EBV on B cells. EBV may have peptides with direct sequence homologies with myelin components. Higher prevalence of EBV infection of MS was reported compared to controls (Plavina et al., 2014).

A defect in the $CD8^+$ T cell control of EBV predisposes to MS by allowing EBV-infected autoreactive B cells and plasma cells to accumulate in the CNS. Recent study (Pender et al., 2018) showed that T cells expanded with AdE1-LMPpoly peptide might kill EBV-infected B cells in the CNS and thereby benefit patients with MS and thereby prevent disease progression and lead to clinical improvement **(Fig. 6.1)**

Myelin peptide tetramers were detected in healthy controls (cytokine production was detected culture supernatant after stimulation with same myelin peptides (chapter 3) that would be a consequence of cross reactivity with myelin antigens in the brain. In MS group, myelin CD4+ tetramer T cells were detected as published (Cao et al., 2015), but in similar percentage of healthy controls with little difference in some samples which is in contrast to what was expected since increased antigen reactivity has been shown to relate to disease progression, and it would be hypothesized that that there is no increase in antigen specific CD4+ T cell response in MS group. Though, explanations could be that antigen specific T cells were not detectable, MS group we tested were small in number so we need more samples in the future to detect a significance difference and all blood samples would be screened for all the epitopes applied before proceeding to tetramer staining step to ensure the successful binding of the epitope. Furthermore, MS samples were not classified for the tetramer staining if they were treated or not and that could have an impact on the total percentage of autoreactive tetramer CD4+ T cells.

6.2 What are the dynamics of T cells in early MS?

Mazzanti et al have detected autoreactive T-cells specific for myelin antigens in HC (Zou et al., 2008, Danke et al., 2004, Cao et al., 2015). Detection of myelin antigen-reactive T-cells in the blood of healthy controls raises many questions as to why some individuals develop MS and others do not, and several studies have examined the function and phenotype of autoreactive T-cells in MS to try to explain their presence. Cao et al., 2015, found myelin epitope-specific T-cells from MS patients to have different transcriptional profiles compared to healthy controls. T-cells in our study were also found to produce higher levels of IFN-γ, IL-17 and GM-CSF which might indicate a more pathogenic phenotype of these cells in patients, contributing to local inflammation and further immune cell recruitment to the CNS.

The decreased IL-10 expression observed in the CD4+ CCR6 population in HC is interesting, as its functions include regulating a Th1 response, which seems contradictory considering the elevated GM-CSF/IL1-7 and TNF-a secretion by these cells. Despite this, IFN-γ and IL-4 were not elevated **(Fig. 3.14),** and the lack of significantly elevated IL-10 in CCR6+ T cells in MS suggests that IL-10 do not provide regulatory help in the context of functioning as a suppressor cytokine in disease.

This then could suggest an alternative purpose for CCR6+ T cell-derived IL-10. If GM-CSF, IL-17 plays a role in mediating inflammation, IL-10 may be simultaneously upregulated as a regulatory mechanism to counteract the pro-inflammatory features of these cells.

However, autoreactive T cells are found approximately the same frequency in HC and MS patients, but more activated in MS than HC (Racke et al., 1998; Zhang at al., 1994). In my study, I have demonstrated responses to different myelin peptides (MOG-PLP-MBP) in healthy controls suggesting that autoreactive T cells can be detected in normal conditions (Danke et al., 2004) and could be related to molecular mimicry with gut microbes (Zarata-Blades et al., 2016).

It has been suggested an important role for Th1/Th17 cells in the pathogenesis and the cytokines produced by these T cell subset (Cua et al., 2003; Langrish et al., 2005 Sospedra and

Martin, 2005;). Yang et al., 2009, have shown that, regardless of the cytokine expression of the myelin-specific T cells, it is the transcription factor T-bet that determines pathogenicity of both Th1 and Th17 cells in the mouse model of MS.

CCR6 is important in the initiation of MS (Yamazaki., 2008; Reboldi et al., 2009), the literature shown the presence of CCR6 in the blood, CSF and brain lesions and the cytokines produced in context of Th1/ Th17 (Restorick et al., 2017; Langelaar et al., 2018).

According to Cao et al., 2015; $CCR6^{+}$ memory compartment of patients with MS, but not HC, can be stratified into functionally different groups based on cytokine production in response to peptide-specific stimulation and our data were consistent with the published literature.

Furthermore, there are myelin-reactive T cells within this compartment that produce GM-CSF, IL-17, or IFN-γ, and a smaller population that may be protective IL-10 producers in healthy control. However, in our data, elevated levels of IL-10-secreting myelin-reactive T cells were more frequent in MS, suggesting that CCR6 may participate in their migration through the choroid plexus in the brain.

CCR6 is not restricted to Th17 cells, but can include both regulatory cells and pathogenic subsets including IFN-γ and GM- CSF-secreting $CD4^{+}$ T cells. In a previous study by our group they determined that the dominant CCR6+ CD4+ T cell subset in MS is not the Th17 subset but also non-classical Th1 secrete IFN-γ and GM-CSF (Restorick et al., 2017).

Once a few pathogenic T cells have migrated through into the CNS and been re-activated by antigen this would then drive activation of the parenchymal endothelial barrier, reducing barrier integrity, and allowing a larger influx of Th1 cells to enter during a second phase of disease; this suggest that CCR6+ Th1 cells are involved early in the disease process.

Higher levels of inflammatory cytokines (IFN-γ, IL-17, TNF-α) in response to myelin peptides were produced in untreated MS patients in myelin specific T cells than HC cohort and that is consistent with published data (Cao et al., 2015).

However, lack of regulation (IL-10) was the most striking difference in HC when compared to MS patients, suggesting that IL-10 is regulating the inflammation when there is a pathogenesis occurring in the untreated MS group. Though, MS treated with IFN-β demonstrated higher inflammatory response when compared to the untreated MS group. In addition, MS treated with IFN-β showed higher EDSS and disease duration than untreated MS group, suggesting that IFN−β were not capable of decreasing the inflammatory response in this specific group. There is very low IL-10 in healthy controls, consistent with a low inflammatory cytokine profile. In untreated MS there is an increase in IL-10, presumably sufficient to regulate the increased inflammatory cytokine production. If GM-CSF and IL-17 play a role in mediating inflammation, IL- 10 may be simultaneously upregulated as a regulatory mechanism to counteract the proinflammatory features of these cytokines. Indeed, IL-4 is involved in regulating inflammation and promoting tissue repair, and in the CNS can protect against autoimmune demyelination (Cua et al., 2001; Yang et al.,2009; Trandem et al., 2011). For treated MS patients, there appears to be less IL-10, and yet much higher levels of key pathogenic inflammatory cytokines. Therefore, it is perhaps the lack of balance between the regulatory (IL-10) and inflammatory (IL-17, GM-CSF, TNF-α, IFN-γ) cytokines that results in increased pathogenesis and a requirement to treat the patients.

The data in my thesis, identifying CCR6+ cells with specificity for CNS antigen such as (MBP, MOG and PLP) would point towards them playing a role in the destruction of myelin (Scmidt et al., 1999).

The balance between T helper cells Th2- and Th1-related cytokines plays a key role in MS. A shift from a Th1 towards a Th2 cytokine profile could have a beneficial effect on the clinical course of the disease (Guevara et al., 2012). IL-4 in our data did not exhibit any significance

in healthy controls, untreated and treated MS suggesting that IL-4 may not be a putative cytokine.

Clinically isolated syndrome (CIS) is an early indicator of MS, but not all people with CIS will go on to have definite MS. While, radiologically isolated syndrome (RIS) is another early indicator of demyelination but with no symptoms. RIS is usually detected incidentally in healthy people who have an MRI that shows typical MS lesions. We were trying to examine the response in early stages of MS. In this study, it was not feasible to collect enough CIS samples as it is hard to find patients at this stage as it is a single neurological episode. Instead, we classified patients with disease duration with less than a year to be early MS. The untreated group had a short disease duration when compared to the treated patients who had more disease duration. However, we couldn't detect any correlation between all the cytokine and the disease duration.

6.3 How could the treatment affect the inflammatory profile in MS patients?

Considerable studies have been focused on treatment for MS disease.

However, challenges are still remained, as MS is a heterogeneous disease with unknown etiology. Until now, there is no cure for MS and medications may have side effects. Short-term efficacy and safety of newer treatment is being explored however the long term risks of these treatment, particularly when used in combination or succession will remain uncertain. When the etiology of MS is better understood, then more effective, safe and long lasting treatment can be developed.

We did not have longitudinal data available in MS. A much larger study comparing patients without treatment and after treatment and also at the beginning of treatment and after a year

from treatment may help to focus on cytokine responses. It will also be of interest to determine if there is any prognostic relevance to their production within the blood even before any disability occurs.

We had a very few CIS patients as the diagnosis in this stage is not easy. It will be very useful if we can recruit more CIS patients and measure the cytokine response to get the answer whether they will be as the established RRMS (inflammatory) or regulatory as HC **(Fig. 6.2)**. In addition, clinical trials of new drugs require biomarkers that can predict response to therapy over the course of a 1–2 years trial. These biomarkers need to be rapid, relatively inexpensive, and uniformly administered across multiple centers and clinicians. However, serial MRIs and neurofilamentous light chain have emerged as having clinical utility in longitudinal or prospective studies. Small cohort, cross-sectional studies and moderate cohort longitudinal studies have provided a panel of possible biomarkers for MS. The clear next question is to take these potential markers and compare each of them simultaneously in a multi-center study that is sufficiently powered to conclusively determine optimal biomarkers of disease in both screening and replication cohorts. Until those studies are completed, it is unlikely that an MS biomarker will be sufficiently verified to become clinically relevant.

6.4 Strength and limitations of the study

Investigation of antigen specific T cells give this study both strength and limitations. This is highlighted when we compare to animal models EAE. There are variations in human, which are out of our control as genetic and environmental factors as well as age. First, sample size was relatively small, which prevented an analysis of correlation with stage of disease. The detailed and lengthy protocol used in this study is not suitable for the analysis of large cohorts of samples. Second, healthy controls tended to be younger than the patients and the gender subsets were not matched for level of disability. We did not observe any correlations with

disease duration, EDSS or HLA type. It is likely that the heterogeneity of our patient population we examined contributed strongly to our inability to observe certain disease correlations within the groups. Also, the availability of the criteria of the patients was not easy, as selecting CIS patients was difficult, as very few patients will be at the clinic for investigations.

Radiologically isolated syndrome (RIS) is a clinical situation in which a person has white matter lesions suggestive of multiple sclerosis (MS), as shown on an MRI scan, therefore adding RIS samples would be particularly informative.

Beside longitudinal samples were not available at this stage, as mostly they don't come again to the clinic unless there is something severe. However, we got a good number for our healthy controls and MS cohort (without and with treatment), thus leading to analysis of relevant data. Also, the use of Luminex technique for cytokine analysis allowed us to measure different cytokines at once. This is in contrast to Cao et al., 2015, where they used ELISA method with a heat map method to demonstrate their cytokine responses, which is not as sensitive and clear as our method.

Although a number of studies have demonstrated the ability of both Th1 and Th2 cells to support B cell responses (Stevenes et al., 1988; DeKruyff et al., 1989; Noelle et al., 1989; Lin et al., 1993;), Th2 cells are widely believed to be particularly adept in this respect, as the cytokines they characteristically secrete have all been implicated in various stages of B cell proliferation and differentiation (Snapper et al., 1988; Purkerson et al., 1992; Paul et al., 1994). There are pro and anti-inflammatory B cell subsets. In relapsing MS, B cells serve as primary antigen presenting cells that drive pathogenic T cells. In progressive MS, B cells enhance the compartmentalized CNS responses through lymphoid follicles and secreted factors. Therefore, detection of Th2 cytokines as IL-4 in response to myelin antigens in our cohort could add to the current knowledge in the context of pathogenesis of MS. Th2 cells develop in the presence of IL-4, subsequently producing cytokines such as IL-4, IL-5, IL-13, and possibly IL-

10 (Agrawal et al., 2007)

6.5 Potential avenues for future work

The use of single cell techniques may allow a better understanding of the cells by identifying the phenotype of the cells associated with the TCR clonality. To date, there has been a lack of information about the transcriptome of antigen-specific T cells in the context of MS. Single-cell transcriptomic technique examines the gene expression level of individual cells in a given population by simultaneously measuring the messenger RNA (mRNA) concentration of hundreds to thousands of genes. Measuring the trancriptomes from myelin reactive T cells to differentiate between healthy controls and MS patients, identifying transcription factors (e.g. T-bet and ROR-γ) and receptors such as ICOS. This analysis should compare the cells in the blood and CSF, as well as the brain when possible (although this would be mainly post-mortem).

Further research of the antigen specific response in T-cells in MS can help our understanding of the biology of this response in health and disease and help in understanding the MS pathogenesis.

Appendix I Demographics for healthy controls

HC groups	Age	Sex	HLA
1	23	F	DRB1*13:01 DRB1*15:01
2	27	F	DRB1*04:01 DRB1*07:02
3	30	M	DRB1*04:01 DRB1*13:02
4	32	M	DRB1*07:02 DRB1*03:01
5	27	M	DRB1*01:01 DRB1*03:01
6	31	M	DRB1*13:01 DRB1*11:02
7	34	F	DRB1*04:04 DRB1*15:01
8	29	F	DRB1*01:04 DRB1*11:01
9	30	M	DRB1*01:04 DRB1*15:01
10	26	F	DRB1*01:01 DRB1*15:01
11	39	F	DRB1*01:04 DRB1*15:01
12	27	M	DRB1*01:01 DRB1*08:01
13	40	M	DRB1*01:01 DRB1*08:01

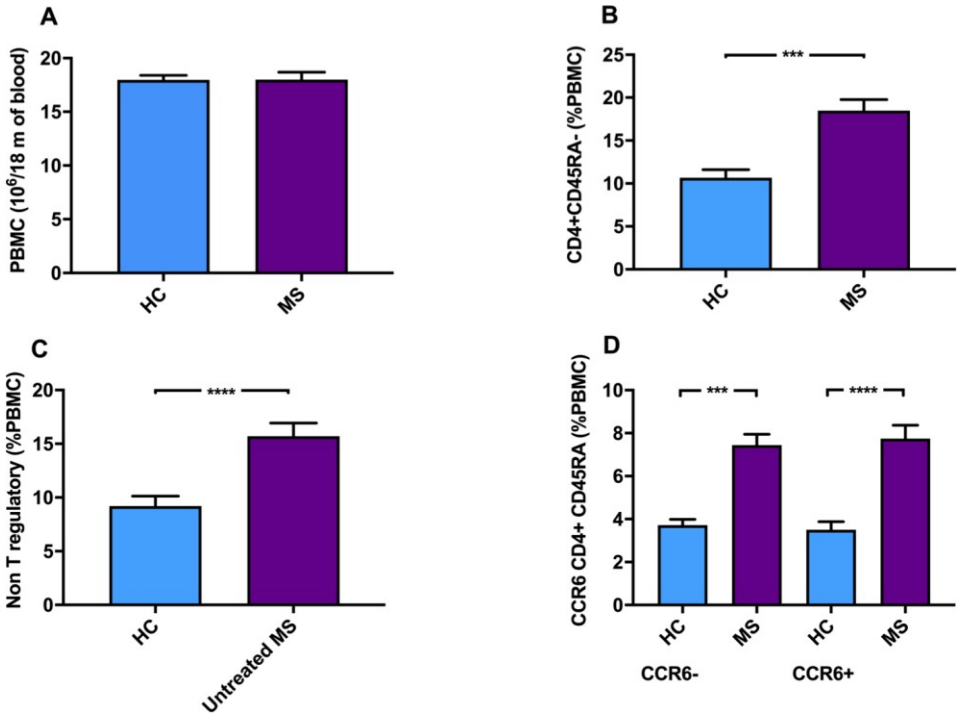
Appendix II Demographics for untreated MS patients

MS groups	Age	Sex	HLA	Duration	Category	EDSS
1	31	F	DRB1*13:01 DRB1*07:01	6.5	CIS	1
2	44	M	DRB1*07:04 DRB1*15:01	5	RRMS	1
3	50	F	DRB1*04:04 DRB1*15:01	2	RRMS	1.5
4	43	F	DRB1*01:01 DRB1*15:01	6	RRMS	1
5	55	M	DRB1*01:04 DRB1*09:01	3	RRMS	2
6	35	F	DRB1*01:01 DRB1*08:01	1	RRMS	1.5
7	31	F	DRB1*03:01 DRB1*04:01	3	RRMS	1
8	33	F	DRB1*07:02 DRB1*03:01	2	RRMS	4
9	44	M	DRB1*18:01 DRB1*09:01	16	RRMS	2
10	34	F	DRB1*14:01 DRB1*15:02	1	SPMS	1.5
11	62	F	DRB1*04:02 DRB1*15:01	5	RRMS	2
12	35	F	DRB1*04:01 DRB1*08:01	1	RRMS	1

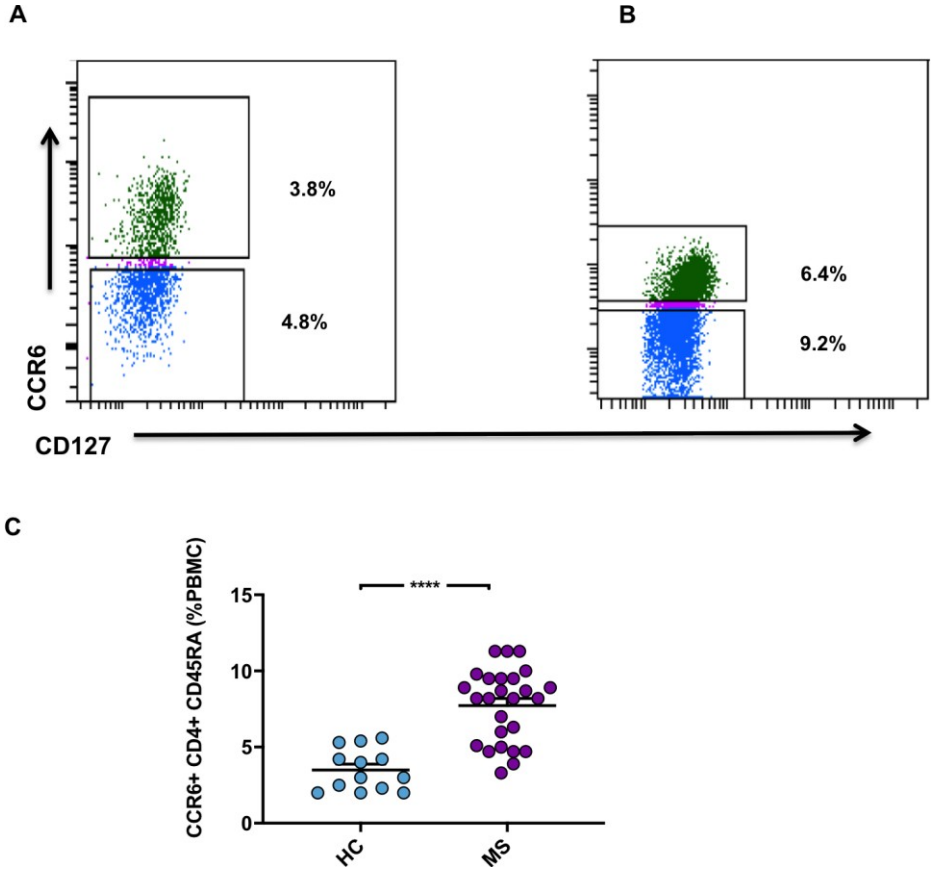
Appendix III Demographics for MS patients treated with IFN-β

MS groups	Age	Sex	HLA	Duration	Category	EDSS
1	45	F	DRB1*04:01 DRB1*15:01	28	RRMS	6
2	39	F	DRB1*04:04 DRB1*13:01	2	SPMS	2
3	51	F	DRB1*03:04 DRB1*07:01	5	RRMS	2
4	55	F	DRB1*03:01 DRB1*15:01	11	RRMS	3.5
5	51	M	DRB1*01:04 DRB1*15:01	5	RRMS	3.5
6	50	M	DRB1*01:01 DRB1*15:01	14	RRMS	1
7	31	F	DRB1*01:01 DRB1*08:01	17	RRMS	6
8	50	F	DRB1*05:01 DRB1*03:01	5	RRMS	0
9	33	M	DRB1*04:01 DRB1*03:01	2	RRMS	1
10	30	M	DRB1*16:01 DRB1*03:02	10	RRMS	2.5
11	48	F	DRB1*01:02 DRB1*05:01	3	RRMS	0
12	53	F	DRB1*01:01 DRB1*04:01	9	RRMS	2.5
13	55	F	DRB1*14:01 DRB1*15:02	7	RRMS	4

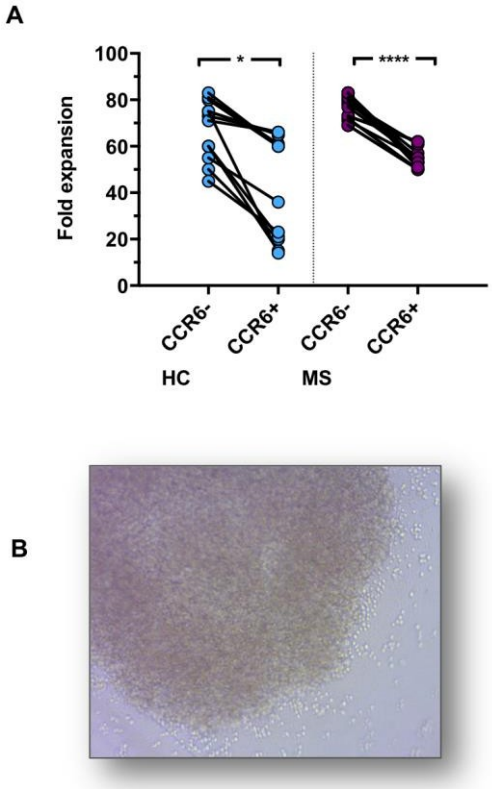
Supplementary



S1. Elevated levels of different T cell subsets in blood samples from MS patients. Lymphocyte count **(A)** percentage of CD4+ memory **(B)** non T-regulatory **(C)** and CCR6 negative and positive (D) after cell sorting of total PBMCs.
Comparison were significant to *** ($P<0.05$) using Mann-Whitneys test (n=13).



S2. Elevated levels CCR6+ CD4+ memory T cells in MS patients.
CCR6+ CD4+ memory T cells were sorted from PBMC of HC **(A)** and untreated MS patient (Representative of one experiment) **(B)**. Scatter blot of CCR6+ CD4+ in HC and MS patients after cell sorting **(C).** Comparison were significant to *** ($P<0.001$) using Mann-Whitneys test (n=13).



S3. Expansion of CCR6 (negative/positive) CD4+ memory T cells in blood samples from healthy controls and MS patients. Fold expansion of CCR6 negative and positive after 2 weeks expansion with irradiated feeder cells IL-2 and PHA-L **(A)** Microscopic photos of CCR6 T-cells culture on day7 (representative of one experiment) **(C)** Comparison were significant to * ($P<0.05$) *** ($P<0.001$) using Mann whitneys test (n=13).

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