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Granulocyte-Macrophage Colony-Stimulating Factor: Expression and Regulation in Human Immune Responses with Relevance to Multiple Sclerosis

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Declaration of Authenticity

I declare that this thesis is my own authentic work, which is not submitted to any other University/Organization for a degree examination. The experimental work which was performed in this thesis is my own work.

I also confirm that I have acknowledged all other sources of information included in this work within the text and at the end of the thesis.

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Publications related to the work in this thesis

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Abstract

Background: Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is a haematopoietic growth factor and a pro-inflammatory cytokine produced by T cells and other immune cells. Recent evidence suggests that GM-CSF plays an important role in multiple sclerosis (MS) pathogenesis. Few recent studies have detected GM-CSF expression by immune cells in MS. In this thesis, the expression of GM-CSF and its receptor by different subtypes of peripheral blood mononuclear cells (PBMCs) in MS was investigated. In addition, GM-CSF regulation was studied in the above-mentioned cells in MS. Finally, GM-CSF neutralization was performed in a phase Ib clinical trial, and some immune-related effects were investigated.

Aims: To evaluate the expression of GM-CSF and its receptor by PBMC subsets in MS; to determine the key factors regulating their expression by PBMC subsets in MS; to detect the differentiation of helper T cells producing GM-CSF (Th-GM) in MS patients, and to detect the frequency of immune cells after GM-CSF neutralization in MS *in vivo*.

Subjects and Methods: Patients were mainly untreated relapsing-remitting MS (RRMS) during remission stage, and some were MS patients during a relapse. Healthy controls were also enrolled. All subjects consented to participation in the study before donating peripheral blood. PBMCs were isolated using Ficoll density gradient centrifugation. Flow cytometry and q-PCR were used to detect the expression of GM-CSF and its receptor. Multiplex bead assay was used to quantify GM-CSF with other pro-inflammatory and anti-inflammatory cytokines.

Results: The frequency of stimulated GM-CSF-expressing cells (helper T (Th), cytotoxic T (Tc), monocytes, NK cells, and B cells) is significantly higher in the mixed PBMC population of untreated RRMS patients when compared to healthy volunteers. The frequency of Th1 cells expressing GM-CSF was higher in MS patients than healthy controls. The expression of GM-CSF by isolated and stimulated NK cells was not different in MS patients and controls. PBMC culture supernatants were shown to contain significantly higher concentrations of IL-2, IL-12, IL-1β, and GM-CSF in MS patients than controls. Blocking IL-2 and IL-12 significantly reduced GM-CSF expression by Tc, NK, and B cells in MS patients, but not in healthy controls. MS patients during relapse had significantly higher frequency of Th-GM (CD3+CD8-IL-17-IFN-γ-IL-3+GM-CSF+) cells than healthy controls. EBV infected B cells expressed GM-CSF receptor in less frequency than non-infected B cells. *In vivo* GM-CSF neutralization in MS patients resulted in significant reduction in the frequency of CD8+ T cells and CD4+CD45RA+CD25++ (naïve) Tregs and an increase in CD4+CD35+foxp3 (total) Tregs.

Conclusions: Th1 (and Th in general), Tc, monocytes, NK and B cells are all high producers of GM-CSF in MS. IL-2 and IL-12 are the main regulators of GM-CSF expression by Tc, NK, and B cells in MS patients. GM-CSF and its receptor may not be major survival or proliferation factors for EBV infected B cells. The newly identified Th-GM cells were detected in higher frequency in MS patients during relapse, which may suggest a new source for GM-CSF production in MS. The recent safety, tolerability, and immune-related results of GM-CSF neutralization in MS are encouraging. Therefore, GM-CSF is a potential therapeutic target in MS.

Abbreviations

a-cytokine	Anti-cytokine (cytokine antibody)
AF	Alexa Fluor
AI	autoimmune
ANOVA	Analysis of variance
APC	Allophycocyanin
APC	Antigen-presenting cell
BBB	Blood-brain barrier
B2M	Beta-2-Microglobulin
BSCB	Blood-spinal cord barrier
BV	Brilliant violet
CCL	Chemokine ligand
CCR1	Chemokine receptor 1
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's Adjuvant
CIS	Clinically isolated syndrome
CNS	Central nervous system
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CrD	Crohn's disease
CSA	Cyclosporin A
CSF	Cerebrospinal fluid
CSF	Colony-stimulating factor
CSF2RA	Colony-stimulating factor 2 receptor-alpha

Ct	Threshold cycle
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CTLs	Cytotoxic lymphocytes
CXCL	Chemokine (C-X-C motif) Ligand
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DMT	Disease-modifying therapy
DNA	Deoxyribonucleic acid
d-NTP	Deoxy-nucleotide triphosphate
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EBNA	Epstein-Barr Nuclear Antigen
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
Fix/perm	Fixation/permeabilization
FMO	Fluorescence minus one
FS	Forward scatter
Foxp3	Forkhead box P3

GA	Glatiramer acetate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMR	Granulocyte-macrophage colony-stimulating factor receptor
GWAS	Genome-wide association study
HC	Healthy control
HERVs	Human endogenous retroviruses
HKG	House-keeping gene
HLA	Human Leukocyte Antigen
HuCAL	Human Combinatorial Library
I	Ionomycin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 receptor
IRA	Innate response activator
iTregs	Inducible regulatory T cells
JAK	Janus kinase
JCV	John Cunningham virus
kDa	Kilo Dalton
LCLs	Lymphoblastoid cell lines
LNCs	Lymph node cells
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6 complex, locus C
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein

MHC	Major Histocompatibility Complex
MOG	Myelin oligodendrocyte glycoprotein
MOR103	Morphosys 103
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NF-κB	Nuclear factor kappa enhancer of activated B cell
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
NTC	No template control
nTregs	Natural regulatory T cells
OCBs	Oligoclonal bands
ODC	Oligodendrocyte
PAP	Pulmonary alveolar proteinosis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PDB	Phorbol 12, 13-Dibutyrate
PE	Phycoerythrin
PeCy (PCy)	Phycoerythrin cyanine
PerCpCy	Peridinin chlorophyll-A protein cyanine
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C

PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear
PNS	Peripheral Nervous system
PPMS	Primary progressive multiple sclerosis
PS	Psoriasis
PT	Pertussis toxin
RA	Rheumatoid arthritis
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
RNS	Reactive Nitrogen species
ROR	Retinoic acid receptor-related orphan receptor
RORC	Retinoic acid receptor-related orphan receptor C
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
RRMS	Relapsing remitting multiple sclerosis
RT	Reverse transcriptase
SAS	Subarachnoidal space
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis
SS	Side scatter
STAT	Signal transducer and activator of transcription
Stim	Stimulated
T-bet	T-box expressed in T cells
Tc	Cytotoxic T cells
TCR	T cell receptor

T1D	Type 1 Diabetes
TGF-β	Transforming Growth Factor-Beta
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
Th	Helper T cells
Th-GM	Helper T cells expressing GM-CSF
TRAP1	TNF receptor-associated protein 1
Treg	Regulatory T cell
q-PCR	Quantitative polymerase chain reaction
UK	United Kingdom
US	Unstimulated
VDR	Vitamin D receptor
VZV	Varicella-zoster virus

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Chapter 1. Review of Literature

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic disease of the Central Nervous System (CNS) characterised by inflammation, demyelination, axonal damage and degeneration, and gliosis (Compston and Coles, 2008). Grey matter, white matter, and spinal cord atrophy can also be demonstrated by magnetic resonance imaging (MRI) (Losseff *et al*., 1996, Fisher *et al*., 2008). MS is considered an inflammatory, autoimmune diseases in which the exact underlying causes are still unknown (Haegert, 2011, Desai *et al*., 2016). Myelin damage (demyelination) and inflammation are the main causes for the loss of axons, dendrites, and neurons (neurodegeneration) that is responsible for all the signs of CNS damage observed in MS (Dutta and Trapp, 2011). Myelin is a complex proteolipid sheath covering some axons in the CNS and the Peripheral Nervous System (PNS). It is made by oligodendrocyte (ODC) cell membrane in concentric layers in the CNS, and Schwann cell membrane in the PNS (Quarles *et al*., 2006). This is because myelin has many important functions like neuronal insulation, nutrition, and support, which are critical for impulse conduction speed (Ravera *et al*., 2009, Ravera *et al*., 2011). Figure 1-1 below is a simplified illustration of the main pathways involved in myelin damage and neurodegeneration.

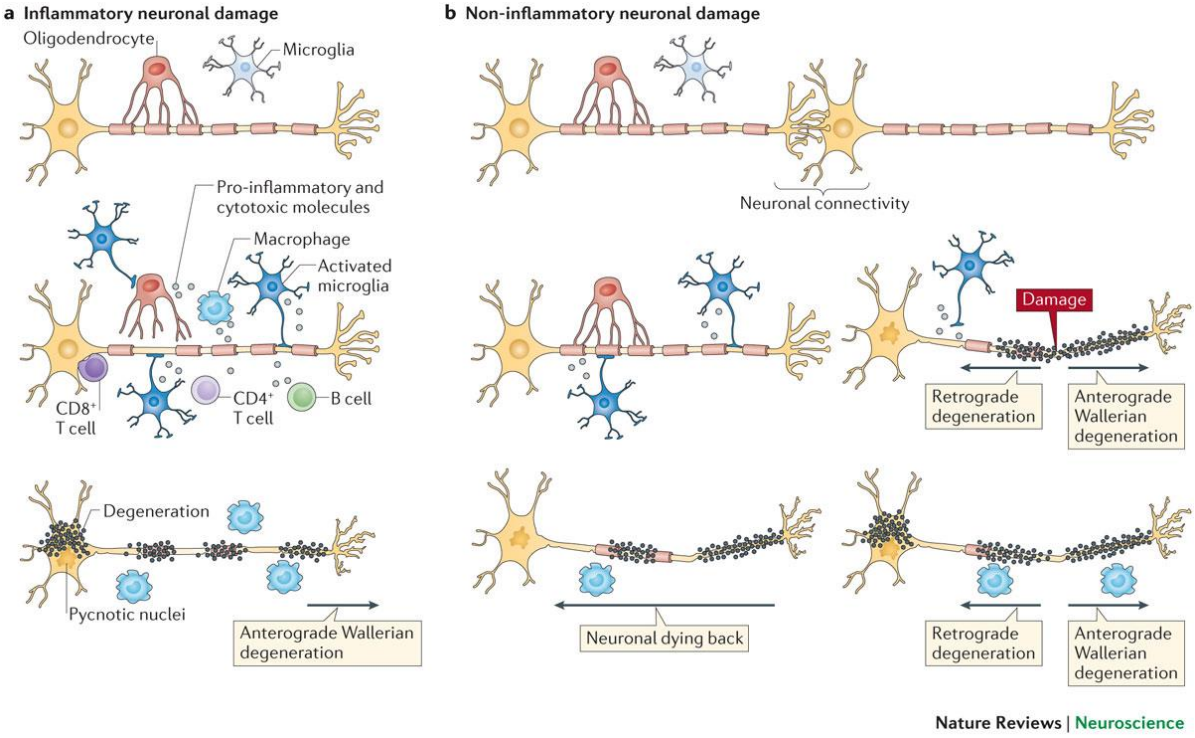


Figure 1-1: Simplified illustration of mechanisms of demyelination and neurodegeneration.

a: neuronal damage via inflammatory pathways. Upper panel: normal undisturbed neuron. Middle panel: activated immune cells (T cells, B cells, macrophages, activated microglia, etc.) contribute to oligodendrocyte damage, demyelination, axonal loss, and neurodegeneration either by direct contact or by the release of pro-inflammatory cytokines and cytotoxic molecules. This may result in downstream neuronal dysfunction (Wallerian degeneration) (lower panel). **b:** neuronal damage via non-inflammatory pathways. Upper panel: normal undisturbed neuronal connectivity. Middle panel: neuronal functions can be impaired by retrograde degeneration, damaging the neuronal network backwards. This will lead to microglial activation and retrograde neuronal degeneration (lower panel). (From Calabrese et al., 2015), reproduced with permission.

1.1.1 Epidemiology of MS

MS is a major CNS disease most often diagnosed in young adults of age ranging between 20-40 years old in developed countries with temperate climate (Koch-Henriksen and Sørensen, 2010). This disease is more common in females than in males, with a ratio of 2-3:1 (Coles, 2009, Ramien *et al*., 2016). In the UK, the prevalence of MS is estimated at about 285/100,000 in women (incidence 11.5/100,000), and 113/100,000 in men (incidence 4.8/100,000) (Mackenzie *et al.*, 2013). At least, 2.5 million people are affected worldwide (Browne *et al*., 2014). Usually it is more common in white Northern people (who are distant from the

equator) (Pugliatti *et al*., 2006) than African (Heydarpour *et al*., 2015), Asian (Kira, 2003), and South American populations (Milo and Kahana, 2010).

1.1.2 Signs and Symptoms of MS

The symptoms of MS can vary depending on the severity and location of CNS damage. They can range from visual problems (e.g. double vision and reduced visual acuity and colour vision) which may lead to blindness, motor disturbances involving fine and coarse movements of hands and legs. In addition, bladder and bowel function problems (e.g. incontinence), sensory disturbances (e.g., loss of touch or neuropathic pain) can also be seen (Hauser and Oksenberg, 2006, Compston and Coles, 2007). Another frequent category of symptoms of MS is the impairment of memory and cognition, which is observed in about half of MS patients. It encompasses low speed of processing and retrieving information from short memory storage (Rao *et al*., 1991, Benedict *et al*., 2006b, Heaton *et al*., 1985). These symptoms have been correlated with brain atrophy, which can be detected using MRI (Benedict *et al*., 2005, Benedict *et al*., 2006a). Moreover, damage to short- or long-term memory may occur permanently and independently, depending on which part of the brain is affected by the autoimmune inflammation (Stepanov *et al*., 2012). Table 1-1 below summarises the main signs and symptoms of MS.

Table 1-1: Signs and Symptoms of MS.

MS can affect any part of the CNS. Main parts affected are the cerebrum, cerebellum, optic nerve, brain stem, and spinal cord. This will result in a range of signs and symptom, some of which are still do not have an effective treatment yet. (From Compston and Coles, 2008), reproduced with permission.

	Symptoms	Signs	Treatment		
			Established efficacy	Equivocal efficacy	Speculative
Cerebrum	Cognitive impairment	Deficits in attention, reasoning, and executive function (early); dementia (late)			Cognitive training
	Hemisensory and motor	Upper motor neuron signs			
	Affective (mainly depression)		Antidepressant drugs		
	Epilepsy (rare)		Anticonvulsant drugs		
	Focal cortical deficits (rare)				
Optic nerve	Unilateral painful loss of vision	Scotoma, reduced visual acuity, colour vision, and relative afferent pupillary defect	Low vision aids		
Cerebellum and cerebellar pathways	Tremor	Postural and action tremor, dysarthria			Wrist weights, carbamazepine, isoniazid, β blockers, clonazepam, thalamotomy, and thalamic stimulation
	Clumsiness and poor balance	Limb incoordination and gait ataxia			
Brainstem	Diplopia, oscillopsia	Nystagmus, internuclear and other complex ophthalmoplegias			Baclofen, gabapentin
	Vertigo			Prochlorperazine, cinnarizine	
	Impaired swallowing	Dysarthria	Anticholinergic drugs		Speech therapy
	Impaired speech and emotional lability	Pseudobulbar palsy	Tricyclic antidepressant drugs		Speech therapy
	Paroxysmal symptoms		Carbamazepine, gabapentin		
Spinal cord	Weakness	Upper motor neuron signs			
	Stiffness and painful spasms	Spasticity	Tizanidine, baclofen, dantrolene, benzodiazepines, intrathecal baclofen	Botulinum toxin, IV corticosteroids	Cannaboids
	Bladder dysfunction		Anticholinergic drugs and/or intermittent self-catheterisation, suprapubic catherisation	Desmopressin, intravesical botulinum toxin	Abdominal vibration, cranberry juice
	Erectile impotence		Sildenafil		
	Constipation		Bulk laxatives, enemas		
Other	Pain		Carbamazepine, gabapentin	Tricyclic antidepressant drugs, TENS	
	Fatigue		Amantadine	Modafanil	Pemoline, fluoxetine
	Temperature sensitivity and exercise intolerance				Cooling suit, 4-aminopyridine

TENS=transcutaneous electrical nerve stimulation.

Table: Symptoms and signs of multiple sclerosis by site

1.1.3 Clinical Subtypes of MS

MS has been recently re-classified clinically into four phases or subtypes, summarized in Figure 1-2 below. The new classification of the clinical course of MS is based on describing two phenotypes, which are relapse and progression. In relapse, the disease activity relapses and/or is presented with new MRI lesions. In progression, there is increased disability (Lublin *et al*., 2014).

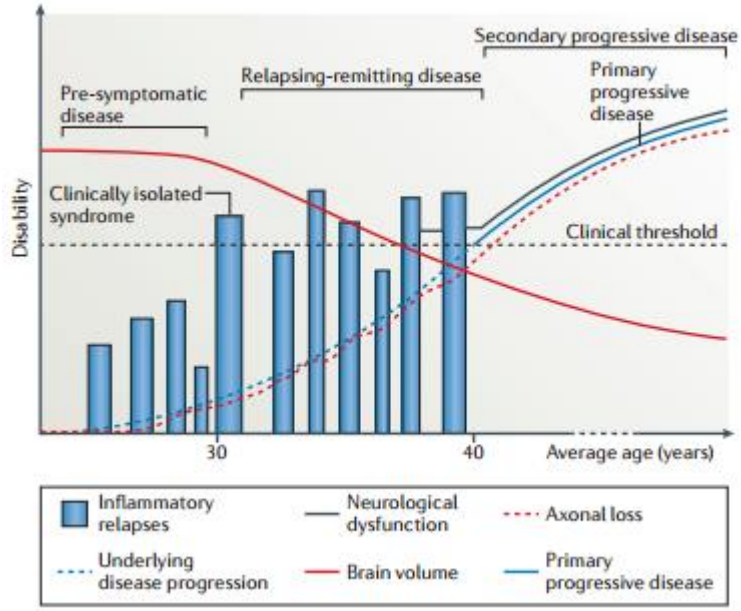


Figure 1-2: Types and Courses of Multiple Sclerosis.

Inflammatory relapses can occur without any symptoms in the early stages of the disease, until the first sign and symptom of disability (clinically isolated syndrome). Later stages of the disease involve the occurrence of inflammatory relapses that either reach clinical threshold or not, as in relapsing-remitting MS. After years of fluctuation between relapse and remission, patients can develop a steady increase in disability without remission (secondary progressive MS). In primary progressive MS, patients will experience increased disability with time after reaching the clinical threshold. Increased disability is accompanied with increase in axonal loss and decrease in brain volume. (From Dendrou *et al*., 2015), reproduced with permission.

The exact molecular mechanisms behind this neurological variability within the disease course is not clear yet. However, it is thought that relapse is an outcome of great influx of immune cells (mainly

autoreactive T cells and B cells) through the disrupted blood-brain barrier (BBB) into the brain, usually leading to new lesions detected by MRI (Disanto *et al*., 2012, Irizar *et al*., 2014). On the other hand, remission is thought to be the result of remyelination of the affected nerves, anti-inflammatory pathways subsiding inflammation, and neuronal plasticity (Irizar *et al*., 2014).

1.1.3.1 Clinically Isolated Syndrome (CIS)

CIS is a term used to describe the first clinical episode as a result of demyelination and inflammation in the CNS. It may lead to relapsing-remitting MS if it is active and fulfilling the 2010 revisions of McDonald's diagnostic criteria of MS (Miller *et al*., 2012, Lublin *et al*., 2014). CIS was not included in the previous classification of MS phenotypes, but studies have shown that CIS combined with brain MRI lesions increases the likelihood of diagnosing MS (O'Riordan *et al*., 1998, Tintore *et al*., 2000). In addition, disease-modifying therapies (DMTs) given to CIS patients in clinical trials have shown reduction in number of patients having another exacerbation (Comi *et al*., 2009, Jacobs *et al*., 2000) and delay clinically definite MS diagnosis (Miller *et al*., 2008).

1.1.3.2 Relapsing-Remitting Multiple Sclerosis (RRMS)

This clinical course of MS is the most common and found in about 85% of all MS patients. It is characterised by repeated attacks followed by remission after each attack. Sometimes patients after relapses may not return to normal baseline remission and may be left with a disability (Hurwitz, 2009). Hence, RRMS can be classified as active (with relapse/new MRI lesions) or inactive (Lublin, 2014).

1.1.3.3 Primary Progressive Multiple Sclerosis (PPMS)

This type comprises about 10-15% of total MS patients. It is characterized by steady devastation in symptoms and neurological degeneration without remission (Sospedra and Martin, 2005).

1.1.3.4 Secondary Progressive Multiple Sclerosis (SPMS)

Approximately 60% of RRMS patients go on to develop SPMS (Coyle *et al*., 2008). It can manifest as at least one attack or relapse, followed by cumulative steadily worsening with time lapse in most cases. SPMS can be developed slowly after CIS or most commonly, after RRMS. With disease progression, the disability is increased and it could be the highest here among all other MS subtypes (Hurwitz, 2009). There is no remission in SPMS, and its diagnosis cannot be asserted unless after at least one year of clinical observation (Coles, 2009).

1.1.4 Risk Factors of MS

Autoimmune inflammation in the CNS is suggested to be one of the pathologic causes for MS, where the cells of the immune system attack the normal CNS tissues. The exact causes that initiate this attack are still not well-known.

1.1.4.1 Genetics and MS

Most of the researchers in the field of MS suggest that there are certain genes responsible for triggering the autoimmune response seen in MS, in conjunction with environmental factors (Willer *et al*., 2003, Dyment *et al*., 2004). A study has strongly argued that MS could have genetic influences by showing that MS risk in monozygotic twins (28.4%) is very much higher than in dizygotic twins (5.6%) (Sadovnick *et al*., 1993). The Major Histocompatibility Complex (MHC) or Human Leukocyte Antigen (HLA) gene complex is found on chromosome 6 and

consists of about 250 recognized genes. The MHC comprises the most polymorphic genes in the genome. Most of HLA genes have immune functions and include the conventional genes DR, DQ, and DP (Figure 1-3) (Hillert, 2010).

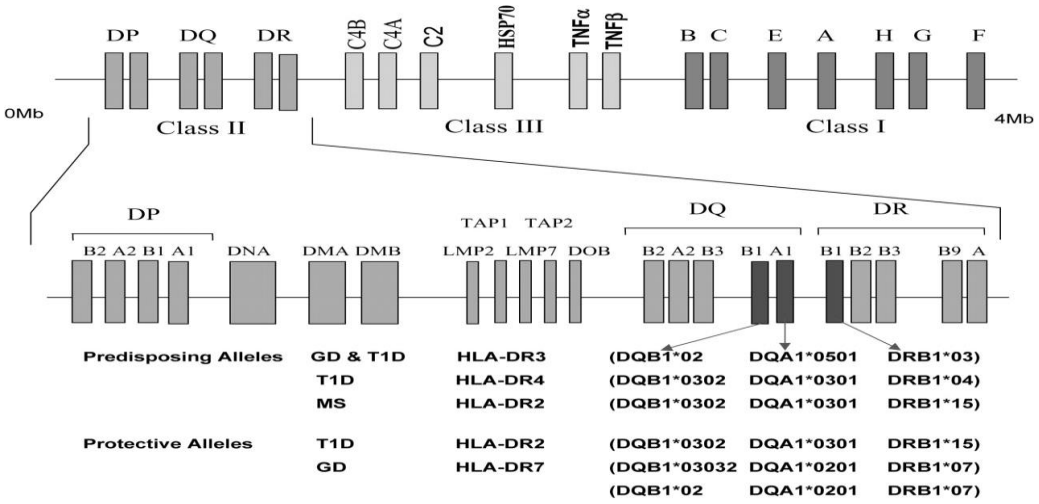


Figure 1-3: A simplified map of the HLA class I and class II gene regions.

HLA class II comprises DP, DQ, and DR regions. These are further classified into different alleles (lower panel of the figure). Predisposing alleles to different autoimmune diseases (GD: Graves' disease, T1D: type 1 diabetes, or MS) were found as sets of haplotypes. C2, C4A, and C4B: genes encoding for the complement system proteins; HSP70: heat shock protein 70; TNFα and TNFβ: tumour necrosis factors alpha and beta. TAP1 and TAP2: transport associated with antigen-processing genes; LMP: large multifunctional protein. (From Simmonds and Gough, 2004), reproduced with permission.

It has been established for over sixty years that there is a strong association between MHC regions and autoimmune diseases (Gough and Simmonds, 2007). MHC serves as an antigen-presenting molecule to T lymphocytes. In order to develop an autoimmune disease, the self-antigen must be presented in a sufficient level by the MHC molecules, and this depends largely on the polymorphism (genotype of the MHC) (Janeway, 2001a).

MHC class II molecules serve to present antigens to CD4+ T cells as a trigger for the immune response. They are expressed normally on

antigen-presenting cells (APCs) that include B cells, and also monocytes/macrophages. During inflammatory conditions, these molecules can be expressed on other cells like astrocytes (Hillert, 2010). Previous studies have found that some MHC-II molecules, especially the HLA-DR2 are the main genes involved in MS pathogenesis (Jersild *et al*., 1973). Later studies have shown a certain haplotype (HLA-DRB1*1501-DRB5*0101-DQA1*0102-DQB1*0602) that was associated with increased MS risk in North Europe (Dyment *et al*., 2004). Many studies have implicated HLA-DRB1*1501 allele in MS susceptibility (Oksenberg *et al*., 2004, Dyment *et al*., 2005, Lincoln *et al*., 2005), and this was confirmed by a large Genome-Wide Association Study (GWAS) (Sawcer *et al*., 2011).

MHC class I molecules can be found in nearly all cell types, they present antigens only to cytotoxic T cells and they are encoded by the classical genes HLA-A, -B, and –C (Hillert, 2010). A study has observed an association between HLA-A*02 and MS (Brynedal *et al*., 2007). Likewise, another study has concluded that HLA-C gene variation has an effect on MS susceptibility (Yeo *et al*., 2007). This is independent of HLA-DRB1 (MHC-II) gene variation. They also observed that HLA-C*05 has a protective effect on MS susceptibility. Another family-based research study has found that the MHC-I and HLA-DRB1*15 haplotypes can influence the susceptibility of MS and control the immune response (Chao *et al*., 2008).

Studies have revealed that MHC proteins were expressed at very low levels in brains that have no inflammation (Redwine *et al.*, 2001). On the other hand, upon stimulation, e.g., during viral infection or inflammation, resident CNS cells such as microglia have shown to express MHC-II proteins (Cash *et al*., 1993, Ulvestad *et al*., 1994, Aloisi *et al*., 1998,

Olson *et al*., 2001, Redwine *et al*., 2001). Oligodendrocytes, neurons, and endothelial cells were shown to express MHC-I proteins (Wong *et al*., 1984, Neumann *et al*., 1995). Astrocytes were also reported to have MHC-II on their surface in MS lesions and after *in vitro* stimulation with IFN-γ (Zeinstra *et al*., 2000, Zeinstra *et al*., 2006). This would likely have bad implications in the course of MS, as antigen (myelin) presentation via MHC proteins is thought to be the driving force of CNS inflammation and demyelination (Williams *et al*., 1993).

Many non-HLA genes were also reported to be involved in MS pathogenesis. Many large-scale GWASs have been conducted to find out more about risk genes associated with MS and have elucidated other genes to be causative factors in MS (Hafler *et al*., 2007, Bahlo *et al*., 2009, Baranzini *et al*., 2009, Jakkula *et al*., 2010, Patsopoulos *et al*., 2011, Sawcer *et al*., 2011, Beecham *et al*., 2013). Figure 1-4 below summarizes most of the up-to-date discovered non-HLA MS risk genes, and reveals the results of a recent GWAS which identified 29 novel non-HLA genes (in bold) associated with MS (Sawcer *et al*., 2011). This study has also suggested no association between MS-related genes and severity or clinical course of MS. Two more recent GWASs have found 48 new (Beecham *et al*., 2013), and seven new (Lill *et al*., 2015) non-HLA alleles that were shown to increase the risk to MS. The non-HLA gene polymorphism that induce MS susceptibility and relevant to this work include IL-2 receptor alpha (IL-2RA), IL-7, IL-7 receptor (IL-7R), IL-12 alpha subunit (IL-12A), IL-12 beta subunit (IL-12B), and IL-12 receptor Beta 1 subunit (IL-12RB1), as seen in Figure 1-4 below.

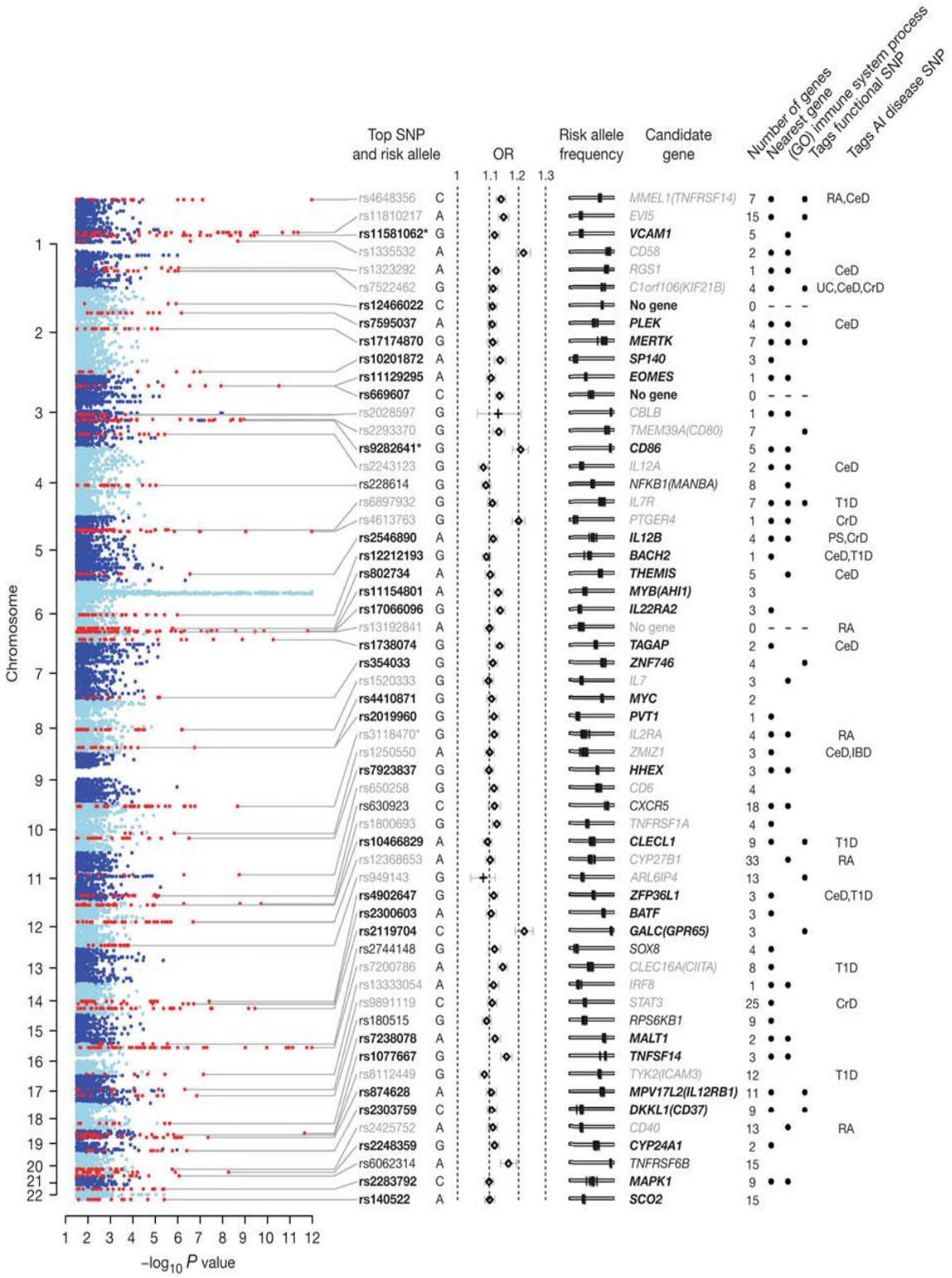


Figure 1-4: Non-HLA Genome Regions Associated with MS.

MS risk alleles are shown in red with their ID (alleles in bold: newly identified, black: with strong evidence, and in grey: reported previously). Odds ratios (OR) from meta-analysis is illustrated with 95% confidence intervals. In each region of MS association, the number of genes is illustrated. SNP: single nucleotide polymorphism; AI: autoimmune; RA: rheumatoid arthritis; CeD: Celiac disease; UC: ulcerative colitis; CrD: Crohn's disease; T1D: Type 1 diabetes; PS: psoriasis; IBD: inflammatory bowel disease. Genes of high relevance for the purpose of this thesis include IL-2RA, IL-7, IL-7R, IL-12A, IL-12B, and IL-12RB1. (From Sawcer *et al.*, 2011), reproduced with permission.

1.1.4.2 Environmental Factors and MS

MS can affect genetically susceptible people who are vulnerable to specific environmental factors. This will stimulate immune cells (especially T cells) to attack and destroy CNS tissues (Compston and Coles, 2008, Correale and Farez, 2011). Recent studies suggest that certain genetic and environmental factors can trigger abnormal DNA methylation that leads to modified expression of certain immune-related genes. DNA methylation is an epigenetic mechanism (occurs before gene expression), in which a methyl group is irreversibly bound to the DNA molecule, which leads to altered or blocked gene expression (Jin *et al*., 2011). This can lead to a series of pathologic autoimmune reactions which are seen in MS, Systemic Lupus Erythematosus (SLE), and type 1diabetes (T1D) (Sun *et al*., 2016).

Viral infection is considered among the important factors that trigger MS. Some studies have demonstrated a doubling effect in MS relapse risk after exposure to a viral infection (mainly upper respiratory and gastro-intestinal infections) (Andersen *et al*., 1993, Buljevac *et al*., 2002). Several other studies have pointed out the role of some viruses in the pathology of MS, such as Epstein-Barr virus (EBV), varicella-zoster virus (VZV), human herpes viruses type 6A and B (HHV-6A and -B), and human endogenous retroviruses (HERVs) (Christensen *et al*., 2007, Lucas *et al*., 2011, Sotelo and Corona, 2011, Nejati *et al*., 2015). EBV infection is considered the most important infectious risk factor for MS, as it is very common. In addition, studies have shown that the titres of anti- Epstein-Barr Nuclear Antigen (anti-EBNA) antibody in serum significantly increases MS risk (Ascherio *et al*., 2001, Levin *et al*., 2005, DeLorenze *et al*., 2006). In addition, MS patients were shown to be nearly 100% seropositive to EBV compared to around 90% in controls

(Wandinger *et al*., 2000, Sundstrom *et al*., 2004, Levin *et al*., 2005, Nejati *et al*., 2016). Besides, some studies have demonstrated the presence of anti-EBV reactivity in oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) of MS patients (Bray *et al*., 1992, Cepok *et al*., 2005). The mechanism(s) behind increased MS risk are not clarified yet; however, molecular mimicry to myelin protein accompanied with genetic risk factors was reported in some studies (Wucherpfennig and Strominger, 1995, Lang *et al*., 2002). In addition, a recent study has found an inverse correlation between EBV viral load and vitamin D concentrations in MS patients, suggesting an interplay between the two factors increasing the risk of MS (Nejati *et al*., 2016). However, others argue that EBV infection is not a risk factor for MS, taking into consideration that it is a very common infection in healthy individuals. The fact that only a minority of people exposed to EBV develop MS can be used as an argument against a causal role of EBV, but can also be explained by the need of both an environmental trigger and a genetic predisposition. It has also been proposed that genetic risk factors for MS can be also risk factors for having an EBV infection, or other infections (Ludwin and Jacobson, 2011, Honarmand *et al.*, 2015). Also, while practically all adults with MS have serological evidence of EBV infection, only 83% of children diagnosed with MS are seropositive for EBV (Alotaibi *et al.*, 2004). Further studies are needed to clarify the exact mechanisms to prove that EBV infection is a causative factor to MS. In conclusion, active infection in general has never been proven as a causative agent of MS. However, infectious triggers are implicated in MS pathogenesis, as their effect on the immune response may lead to MS in genetically susceptible individuals.

With regard to parasitic infection, a study has shown a modulatory role of chronic parasitic infection against active MS with alterations in MS course (Correale and Farez, 2007). This is possibly through activation of regulatory T cells. Therefore, the "hygiene hypothesis" can be applied in MS, where developed (hygienic) countries have low prevalence of parasitic infections and therefore a high autoimmune disease risk.

It has been found that reduced exposure to sunlight or low vitamin D levels could be risk factors for autoimmune diseases including MS (Schwalfenberg, 2012). Conventionally, vitamin D functions as a regulator of calcium and phosphorous homeostasis (Lips, 2006, Cherniack *et al*., 2008). Moreover, it has immuno-regulatory functions with suppression of autoimmune diseases (including MS) through modulating CD4+ T cell differentiation and activity in experimental autoimmune diseases. This leads to reduction of helper T cell (Th1) functions, inhibition of autoimmune T cell production, with enhancing of regulatory T cell (Treg) functions (Cantorna and Mahon, 2004, Muthian *et al*., 2006). The active form of vitamin D was shown to act via binding to vitamin D receptor (VDR), which is a nuclear receptor and a transcription factor. Ligand-activated VDR binds to specific DNA sequences and alter certain gene expressions (Di Rosa *et al.*, 2011). Vitamin D was also shown to inhibit dendritic cell (DC) maturation and differentiation in *in vitro* murine cells (Dong *et al*., 2005). *In vitro* studies have demonstrated that after vitamin D exposure, there was an increase in the number of MHC-II dendritic cells that have high interleukin (IL)-10 and low IL-12 secretions (Griffin *et al*., 2001, Maynard and Weaver, 2008). In addition, vitamin D exposure has reduced the production of pro-inflammatory cytokines (including GM-CSF, IFN-γ, IL-2, and IL-6) (Muller *et al*., 1993, Towers and Freedman,

1998), and increased the production of anti-inflammatory cytokines (including IL-4, transforming growth factor-beta (TGF-β)) (Cantorna *et al.*, 1998). Several studies have shown that MS patients have low vitamin D levels (Kubicka and Pierzchala, 2013, Mazdeh *et al.*, 2013, Shahbeigi *et al.*, 2013). A number of randomised clinical trials were conducted to examine the effects of vitamin D supplementation in MS patients (Shaygannejad *et al.*, 2012, Soilu-Hanninen *et al.*, 2012, Derakhshandi *et al.*, 2013). However, none of them have shown a significant difference in the relapse rate or in the Expanded Disability Status Scale (EDSS). Therefore, the effectiveness of vitamin D as a treatment in MS remains to be elusive. However, high vitamin D dose was shown to be safe when used for a short term (Alharbi, 2015).

Tobacco smoking is also considered one of the risk factors of MS. Many case-control studies have shown a 50% increased risk of MS with cigarette smoking (Antonovsky *et al*., 1965, Simon *et al*., 2010), and many meta-analyses have confirmed this (Hawkes, 2007, Handel *et al*., 2011, Belbasis *et al*., 2015). A recent cohort study in the UK suggested that regular smoking enhances progression of disability and severity of MS, whereas smoking cessation (even after the onset of the disease) slows the disability progression of MS (Manouchehrinia *et al*., 2013). It was postulated that tobacco smoke may lead to changes in the innate and adaptive immune cells (Holt and Keast, 1977). It was observed that cigarette smoke can alter the production of many pro-and anti-inflammatory cytokines. It can also activate macrophages and dendritic cells (DCs) (Arnson *et al*., 2010), induce free radical formation that may lead to autoimmune-related gene activation (Pryor *et al*., 1998), increase auto-reactive B cells (Grimaldi *et al*., 2002), and induce peripheral T cell proliferation (Francus *et al*., 1988).

Obesity is recently considered another genetic-environmental risk factor for MS. Adipose tissue is composed mainly of adipocytes, but also of other cells such as fibroblasts, endothelial cells, and innate and adaptive immune cells (Cildir *et al*., 2013). Adipocytes in obesity produce more pro-inflammatory cytokines (e.g. TNF-α and IL-6) and less anti-inflammatory cytokines (Ouchi *et al*., 2011). This can activate macrophages and increase blood-brain barrier permeability to inflammatory cells that can trigger MS in susceptible patients (Palavra *et al*., 2016).

1.2 Autoimmunity in MS and its Experimental Model

The normal immune system can initiate an immune response against non-self-antigens (and modified-self-antigens), while it should recognise normal (unmodified) self-antigens as self and not be activated by them, in a process of self-tolerance (Aribi, 2017). Autoimmunity occurs as a result of breakdown in self-tolerance. Self-tolerance is a collection of physiological mechanisms that occur centrally and peripherally, which result in inability of the immune cells to react against the body's own antigens (self-antigens or autoantigens) (Ring and Lakkis, 1999). In normal physiology, most of the produced autoimmune cells are deleted in the thymus or bone marrow (central tolerance). However, some of them escape to the bloodstream but are unable to cause organ damage due to regulatory mechanisms (peripheral tolerance) (Miller *et al*., 1997).

The possible physiologic tolerogenic mechanisms to eradicate auto-reactive T and B cells include clonal deletion of auto-reactive cells, and T and B cell ignorance to the target self-antigen. Other mechanisms may be due to inability of these cells either to penetrate through endothelial barriers, or to be fully activated after binding with the self-antigen. In

addition, autoreactive cells may be inactive due to a deficiency in production of co-stimulatory factors. Moreover, these auto-reactive cells may be killed or silenced by the self-antigen or immune-regulatory cells (Miller *et al*., 1997, Goodnow *et al*., 2005).

1.2.1 Autoimmunity in MS

Many autoimmune diseases are tissue/organ-specific, where the autoantigen is present in high levels in specific tissues. These diseases include MS where the autoantigen is expressed in the CNS (neuroantigen: peptides from myelin proteins and other potential molecules).

The nature and identity of the autoantigen in MS is not completely known. The most studied antigens are myelin proteins including myelin basic protein (MBP), myelin-oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and alpha B-crystallin (van Noort *et al.*, 1995, Grau-Lopez *et al.*, 2009). There is increasing interest in the role of other non-myelin antigens, for example axonal or neuronal proteins, such as neurofilament. Moreover, some non-peptide autoantigens presented through molecules that are not part of the MHC have been implicated (Levin *et al.*, 2013).

Evidence implicating the above neuroantigens in MS, in addition to the similarities to the experimental model described below, comes from the demonstration of T cells reactive against peptides from these antigens (and invariant T cells and autoantibodies in the case of non-peptide antigens). These T cells have a higher frequency and/or are in a higher state of activation than potentially self-reactive T cells from healthy individuals (Riedhammer and Weissert, 2015).

One of the causes of autoimmunity in this case may be due to reduced probability of the autoantigen to induce clonal deletion of auto-reactive cells in the thymus. This is because it is locally (and not ubiquitously) frequent. In addition, several defects in central and peripheral tolerance mentioned above have been noticed in MS (Gonsette, 2012). However, autoreactive T cells are also present in healthy individuals, but they are unable to produce autoimmune diseases unless under certain genetic and environmental circumstances (Janeway, 2001a). It is thought that experimental autoimmune encephalomyelitis (EAE) as an animal model of MS is in large part, a good way to try understand pathophysiological mechanisms in MS.

1.2.2 Experimental Autoimmune Encephalomyelitis (EAE) as an MS Model: Similarities and Differences with MS

EAE is an experimental autoimmune inflammatory disease of the CNS in rodents and non-human primates characterised by demyelination of CNS nerves. It shares many similarities in signs and histopathological changes with MS in humans (Kroenke *et al.*, 2010, Constantinescu *et al.*, 2011). Therefore, it is considered the most common animal model used to test the effectiveness of new drugs in MS and to study MS pathophysiology (Zamvil and Steinman, 1990, Baxter, 2007, Miller and Karpus, 2007).

An EAE model was helpful to understand that even healthy humans have autoreactive cells but in an inactive state (Schluesener and Wekerle, 1985). An EAE study states that "...several different models of experimental autoimmune encephalomyelitis were developed, which differ in the immunological reaction, inflammatory process and the neuropathophysiology in the CNS. Each model shares similarities to

multiple sclerosis but also differs in some aspects. Therefore, the proper selection of the most valuable model is essential and strongly depends on the scientific question being addressed" (Kinter *et al*., 2008). There are a number of types of EAE model, including spontaneous and induced models. Spontaneous EAE can develop in transgenic animals expressing a T-cell receptor (TCR) that recognises myelin antigens. These animals are normal at birth but develop spontaneous inflammatory CNS demyelination. They are more useful models in studying the mechanisms of initiating the autoimmune response (Bettelli *et al*., 2003, Lassmann and Bradl, 2017). There are two methods for EAE induction in mice in which the disease onset and severity are almost similar (Zamvil and Steinman, 1990, Miller and Karpus, 2007, Libbey and Fujinami, 2011):

1. Active immunization: cell-mediated immunity will be stimulated when the mouse is directly injected with the encephalitogenic antigen using adjuvants such as Complete Freund's Adjuvant (CFA) and pertussis toxin (PT). Adjuvants are used to activate the innate immune system and induce the immune response. They act via multiple mechanisms, including activation of costimulatory molecules and pro-inflammatory cytokines (Constantinescu and Hilliard, 2005). Mice were then monitored every other day for the appearance of signs and symptoms, which usually takes 12-28 days after the immunization.

2. Adoptive transfer: it is a reproducible method where the cell-mediated immunity is stimulated when the donor mice are immunized with an encephalitogenic antigen in CFA. Then, their lymph node cells (LNCs) or spleen cells are collected after 6-14 days, and re-stimulated with the antigen and specific cytokines of interest. Then, antigen-specific encephalitogenic T cells are isolated and cultured for 3-8 days, then

injected into recipient mice to induce EAE. Mice were monitored for the appearance of signs and symptoms as in active immunization above.

Many factors should be manipulated to obtain a successful EAE resemblance to MS, such as antigen presentation and the expression of adhesion molecules and chemokines which are essential for T cell migration to the CNS (Lassmann and Bradl, 2017). Several studies have shown that EAE can be induced by adoptive transfer of IFN-γ-producing (Th1) or IL-17-producing (Th17) CD4+ cells which are myelin-specific. However, classical EAE (ascending paralysis) was induced in mice with deficient IFN-γ, IL-12 and/or IL-17 signalling pathways (Krakowski and Owens, 1996, Becher *et al*., 2002, Bettelli *et al*., 2004, Kroenke *et al*., 2010). One of the possible mechanisms was suggested to be GM-CSF-related (Kroenke *et al*., 2010). Furthermore, IL-23 signalling pathway has been shown to be crucial in endowing encephalitogenicity to T cells which are myelin-specific (Langrish *et al*., 2005, Cua *et al*., 2003).

The main differences between EAE and MS models are: In EAE, the induction of autoimmune reaction is external and artificial, while in MS it is not (Gran *et al*., 2008). In EAE, brain lesions were mainly composed of CD4+ T cells (Sriram *et al*., 1982, Leuenberger *et al*., 2013), whereas in MS brain lesions mainly contain CD8+ T cells (Rasouli *et al*., 2015). Furthermore, known antigens are capable of inducing autoimmune response in EAE, while in MS it is not clear which are the exact autoantigens. This may be due to differences in how human and mice immune cells are primed and activated (Constantinescu *et al*., 2011). The main advantages of using EAE models are similarities between EAE and MS in the main pathological findings (axonal loss, demyelination, gliosis, and inflammation). In addition, the majority of approved therapies in MS were tested successfully on EAE. Moreover,

EAE models have similar regulatory mechanisms, which include cell-based and neuropathological-based mechanisms (Pachner, 2011). However, the limitations of using any animal model (including EAE model) involve inability to fully reproduce the human disease. Moreover, EAE models cannot detect complications of DMTs such as infections for which humans are the only host (e.g., John Cunningham virus (JCV) after natalizumab therapy). In addition, there are therapies that cannot be tested in mice EAE models for different reasons. These include their toxic effects, molecular differences between human and mouse targets, and absence of the tested target (Constantinescu *et al.*, 2011). Therefore, the next section will discuss the main immune cells and possible pathways involved in the pathogenesis of MS, and will acknowledge important EAE findings.

1.3 Immunological Overview of MS: Main Immune Cells Involved

MS is an autoimmune disease in which many immune cell types are involved. Figure 1-5 below summarizes the most important immune cells involved in MS, their roles are discussed later in this chapter. Some of the main MS treatment options targeting these immune cells or their produced chemicals will be mentioned briefly.

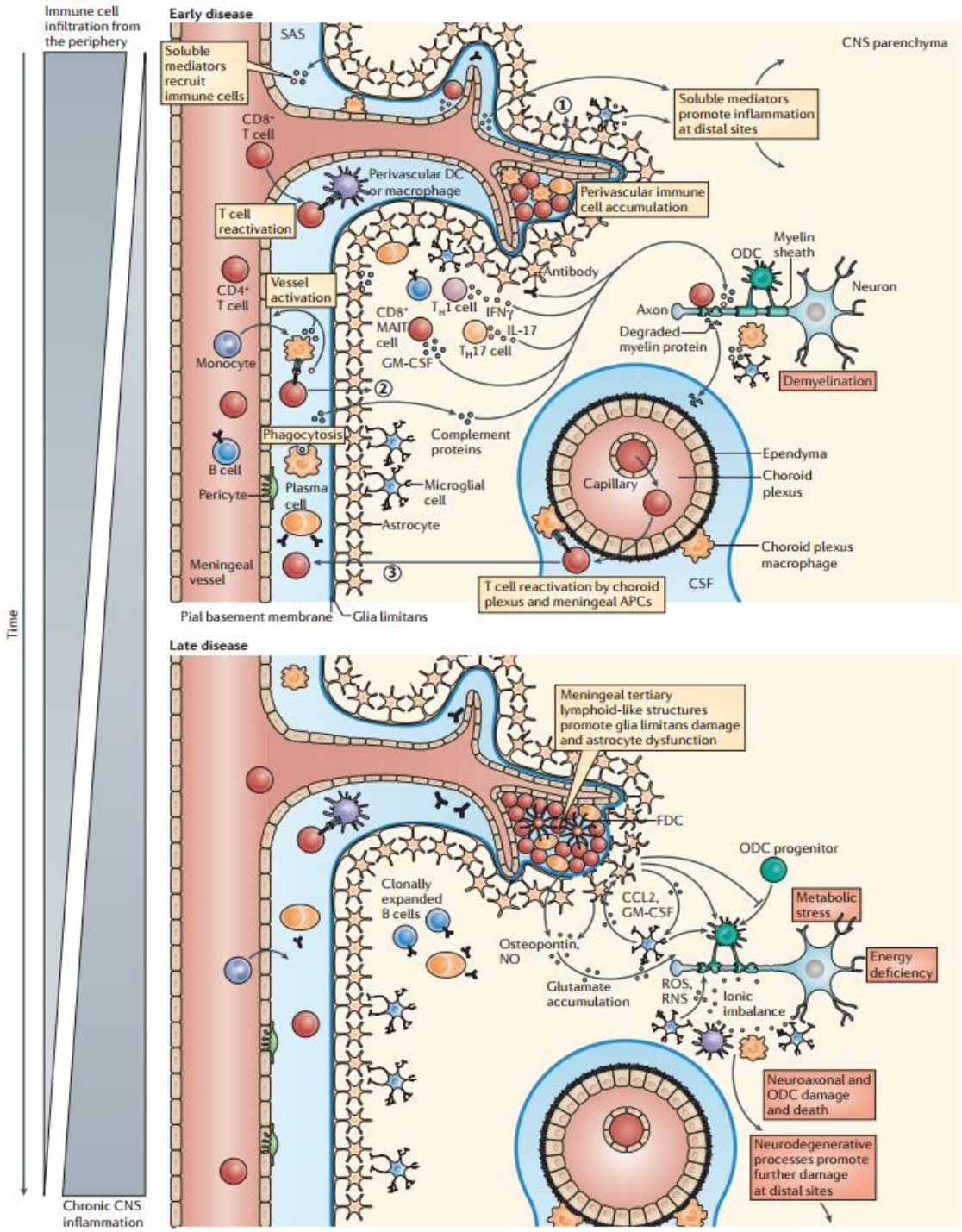


Figure 1-5: A simplified view of the immune-pathogenesis of MS (early and late phases).

In early stages, cells infiltrate the CNS either from BBB (1), subarachnoidal space (SAS) (2), or blood-CSF barrier (3), which contribute to demyelination and inflammation. The main mechanisms of early CNS damage are pro-inflammatory cytokines, direct cell-mediated damage, autoantibodies, and complement proteins. In chronic inflammation, CNS-resident cells mainly contribute to inflammation, with tertiary lymphoid structures formation especially in SPMS (a finding of some pathological studies (Serafini *et al*., 2004) but not confirmed in all studies), which disrupt the BBB. Astrocytes can secrete GM-CSF which can lead to further astrocyte accumulation and inhibition of oligodendrocyte (ODC) maturation. Free radicals and ionic imbalance contribute to CNS damage. FDC, follicular dendritic cell; CCL2, chemokine ligand 2; RNS, reactive nitrogen species; ROS, reactive oxygen species; NO, nitric oxide. (From Dendrou *et al*., 2015), reproduced with permission.

1.3.1 Helper (CD4+) T Cells

Helper T cells (Th) are considered the most important cells in adaptive immunity, because they are crucial for most adaptive immune reactions. Their functions include helping B cell activation to differentiate into plasma cells which secrete antibodies, helping macrophages to eradicate ingested microorganisms, and helping cytotoxic T cells to destroy infected cells (Alberts, 2002). In order for the Th cells to be functional, they must be activated into effector Th cells, via antigen presentation process to the T cell receptor (TCR), accompanied by co-stimulatory signals provided by the interaction between co-stimulatory molecules on T cells and APC (e.g. CD28 binding with CD80 or CD86) (Janeway, 2001b). APCs (innate immune cells) present processed antigens to Th cells; this activation can lead to different and functionally distinct Th subsets which secrete different cytokines. The generated Th subset depends largely on the available cytokines surrounding the activation process (Mosmann and Coffman, 1989, Abbas *et al*., 1996). The most common Th subsets are Th1, Th2, Th17, and most recently Th-GM was identified. In the following sections, these cells and their relevance with MS and EAE will be discussed.

IL-2 is one of the essential pro-inflammatory cytokines that is expressed by human activated CD4+ T cells and naïve CD8+ T cells (Sallusto *et al*., 1999). It is crucial for cell proliferation and growth (Smith, 1988). IL-2 achieves its action by binding to its receptor, which is expressed by different cells, and is composed of three subunits: IL-2R-α (CD25), -β, and -γ (Hatakeyama *et al*., 1989). IL-2R-α subunit is important for initiating high affinity binding of IL-2 to its receptor (Robb, 1986). Blocking IL-2R-α (CD25) subunit with the monoclonal antibody

daclizumab has been shown to be effective in reducing brain inflammation in MS (Martin *et al*., 2010).

1.3.1.1 Th1 Cells

Th1 cells differentiate from naïve Th after the latter being stimulated mainly by IL-12 (Hoeve *et al*., 2006). IL-12 is produced by macrophages and DCs during innate immune response (Langrish *et al*., 2004). Th1 cells represent one of the mediators of CNS autoimmune inflammation like MS and EAE (Goverman, 2009). The main cytokines produced by Th1 are IFN-γ, GM-CSF, and Tumour Necrosis Factor (TNF). Interferon-Gamma (IFN-γ) is the major cytokine produced by Th1 cells (Todd *et al.*, 2015). It is important in monocyte activation and differentiation, targeting intracellular pathogens, and tumour suppression (Perussia *et al*., 1983, Schroder *et al*., 2004). Animal studies have shown that over-expression of IFN-γ in the CNS of transgenic mice has led to enhanced oligodendrocyte death and demyelination. Moreover, it was demonstrated early that MS was exacerbated in patients after IFN-γ treatment (Panitch *et al*., 1987). However, a few years later, it was observed that EAE was exacerbated in IFN-γ receptor knock-out mice, concluding that this cytokine is not crucial for EAE, but may have a protective role (Willenborg *et al*., 1996). This is one of the different findings between MS and EAE. Th1 cells also secrete TNF, which augments Th17 development (discussed below) (Veldhoen *et al*., 2006).

In addition, there are other factors expressed by Th1 and can be involved in aggravating autoimmune inflammation. T-bet is an important transcription factor expressed mainly in Th1 and is involved in direct regulation of IFN-γ with suppression of Th2 cytokines (Lovett-Racke *et al*., 2004, Szabo *et al*., 2000). Many studies have shown that T-bet has an essential role in the encephalitogenicity (ability to induce CNS

inflammation) of CD4+ T-cells, it was also shown to be a therapeutic target in EAE (Lovett-Racke *et al*., 2004, Bettelli *et al*., 2004, Nath *et al*., 2006, Yang *et al*., 2009). Studies have also found that T-bet-deficient mice were unable to develop EAE. Regarding MS, T-bet was shown as a biomarker for disease activity and treatment efficacy (Frisullo *et al*., 2006, Iorio *et al*., 2009).

1.3.1.2 Th2 Cells

Th2 cells are considered crucial in stimulation of eosinophils and IgE production in allergy and asthma. Moreover, they are involved in eliminating extracellular organisms such as helminths and parasites (Murphy and Reiner, 2002). These cells are considered as excellent B cell helpers (Mosmann *et al*., 1997). Their role is essential after exposure to certain chemicals, which also leads to activation of CD4+ T cells and B cells (Druet *et al*., 1995). Th2 cells are differentiated from naive CD4+ T cells after their T cell receptor (TCR) activation in the presence of IL-4. This will lead to activation of Th2-specific cytokine expression (IL-4, IL-5, and IL-13), with simultaneous inhibition of Th1-specific cytokine production (Zheng and Flavell, 1997). There is evidence suggesting that Th1 but not Th2 cells have a pathogenic role in experimental autoimmune diseases (Druet *et al*., 1995). It has been shown that Th2 cells have no role in the majority of organ-specific autoimmune diseases (Jager and Kuchroo, 2010), and they failed to produce EAE after adoptive transfer of myelin proteins-specific Th2 cells (Das *et al*., 1997, Jager *et al*., 2009). As a result of previous findings mentioned above, which have shown Th1 cells being encephalitogenic and Th2 being protective, there are more recent therapeutic trends towards shifting naive T cell differentiation from Th1 to Th2 in MS using some strategies. Among these trends are

administration of IL-4 in ongoing autoimmune inflammation (Jager and Kuchroo, 2010), and administration of glatiramer acetate (GA) which shifts naïve CD4 differentiation from Th1 to Th2 cells (Duda *et al*., 2000, Dhib-Jalbut, 2002). This may have beneficial therapeutic outcomes in MS and may be one of the mechanisms of action of GA in reducing MS relapses. This may be a contributing mechanism; however, it is not proved to be the only mechanism of GA effectiveness in MS.

1.3.1.3 Th17 Cells

They are called Th17 because they produce higher amounts of the cytokine IL-17 than the other T cell subtypes. The initial differentiation of these cells from naive CD4+ T cells requires TCR activation, and cytokines like TGF-β combined with IL-6, IL-9, or IL-21 (Bettelli *et al*., 2006, Mangan *et al*., 2006, Korn *et al*., 2007, Elyaman *et al*., 2009) with the aid of TNF and IL-1β (Veldhoen *et al*., 2006). IL-23 is required for the final differentiation of Th17 cells into mature effector cells (McGeachy *et al*., 2009). The most important inflammatory mediators secreted by Th17 cells are IL-17A, IL-17F, GM-CSF, TNF, IL-22, IL-21, and IL-9 (Ouyang *et al*., 2008, Korn *et al*., 2009, Beriou *et al*., 2010, McGeachy, 2011). Previous studies have pointed out the importance of Th1 in triggering autoimmune response in MS as mentioned above. However, other studies have shown that Th17 (not Th1) cells are the encephalitogenic cells in autoimmune inflammation (Park *et al*., 2005). On the other hand, others have suggested the involvement of both Th1 and Th17 in EAE encephalitogenicity (Fletcher *et al*., 2010, El-Behi *et al*., 2011).

IL-17 is a pro-inflammatory cytokine produced by human activated CD4+ CD45RO+ T cells (Yao *et al*., 1995, Shin *et al*., 1999). It induces phagocytes, chondrocytes, fibroblasts, endothelial and epithelial cells to

express and secrete many catabolic enzymes and inflammatory mediators, like IL-1β, IL-6, IL-8, TNF-α, GM-CSF, PGE2, NO, NO synthase, COX-2, intracellular adhesion molecule-1, and membrane cofactor protein-1 (Aggarwal and Gurney, 2002). It was shown that IL-17 can be produced in human T cells after exposure to IL-2, IL-15, IL-18, IL-21, and IL-23. On the other hand, IL-17 production can be inhibited after exposure of T cells to IL-12, which inhibits IL-23 pathway (IL-23 is secreted by macrophages and stimulates T cells to produce IL-17). IL-12 also stimulates IFN-γ production that co-stimulates macrophages to secrete IL-12 which inhibits IL-17 production. IL-23 can indirectly inhibit IL-17 production through activation of IL-12-IFN-γ pathway (Hoeve *et al*., 2006). IL-17 receptors are distributed and found in many cell types, such as endothelial and myeloid cells (Aggarwal and Gurney, 2002).

IL-17 was observed to play a role in autoimmune diseases like MS (Matusevicius *et al*., 1999), psoriasis (Teunissen *et al*., 1998), and Rheumatoid arthritis (RA) (Ziolkowska *et al*., 2000). Studies involving EAE models have concluded that treatment with IL-17 antibodies provides partial EAE protection (Langrish *et al*., 2004), and IL-17-knockout mice were resistant to EAE (Komiyama *et al*., 2006). These findings suggest the critical inflammatory role of IL-17 in the pathogenesis of autoimmune diseases in humans (Langrish *et al*., 2004).

RORC (Retinoic acid receptor-related orphan receptor C) (or called ROR-γt in animals) is one of the most important transcription factors in Th17 development and IL-17 production (Yang *et al*., 2009). It was also shown to be involved in Th17 pathogenicity in EAE, and ROR-γt

knockout mice had attenuated EAE with no infiltrating Th17 cells (Ivanov *et al*., 2006).

1.3.1.4 Th-GM Cells

Recent studies have demonstrated that upon stimulation of naïve CD4 T cells with either IL-2 or IL-7, a phenotypically distinct population was produced that is neither IL17- nor IFN- γ-producing (they lack RORγt and T-bet transcription factors). Instead, they were high GM-CSF and IL-3 producers. These cells were called Th-GM (Noster *et al*., 2014, Sheng *et al*., 2014). IL-3 is considered mainly as a hematopoietic growth factor (Heike and Nakahata, 2002, Nitsche *et al*., 2003). However, few later studies have shown IL-3 as a pro-inflammatory cytokine that is involved in autoimmune diseases such as arthritis and spinal cord motor neuron autoimmunity (Chavany *et al*., 1998, Bruhl *et al*., 2009). Th-GM cells were shown to be more severe than Th1 or Th17 in inducing EAE (Noster *et al*., 2014, Sheng *et al*., 2014). Further research is needed to identify them and examine their effects in MS and autoimmune diseases.

1.3.2 Cytotoxic (CD8+) T Cells (Tc)

These are CD8+ cells which have two subsets (Tc1 –Major subset, and Tc2). Both subsets have cytotoxic functions mediated by perforin, Fas, and stimulation of delayed type hypersensitivity (DTH) reactions that involve oedema and granulocyte infiltration. However, each cell subtype secretes specific cytokines (Tc1 secrete IFN-γ, and Tc2 secrete IL-4 and IL-5) during DTH reaction (Mosmann *et al*., 1997). Later studies have shown a third Tc subset capable of producing IL-17 (named as Tc17) (Huber *et al*., 2009), and another subset expressing both IFN-γ and IL-17 (Tajima *et al*., 2011). It has been shown that Tc cells produce lower amounts of cytokines than Th cells, possibly because they kill their antigen-presenting cells before their full activation. In addition, there are

cytokines which may interfere with Tc1 CD8+ T cell differentiation, such as IL-4, IL-10 (Mosmann *et al*., 1997), and others which increase their proliferation, such as IL-12 (Vukmanovic-Stejic *et al*., 2000). Tc cells were also shown to produce GM-CSF (Min *et al*., 2010).

Figure 1-6 below summarizes the theoretical role of Tc cells in MS. Many studies have found evidence supporting this theory and suggesting the involvement of Tc cells in MS pathogenesis (Babbe *et al*., 2000, Neumann *et al*., 2002, Frischer *et al*., 2009). In susceptible individuals, APCs become activated and present self-peptides to CD8 T cells (through MHC-I). This leads to the differentiation of CD8 T cells into regulatory and effector cytotoxic lymphocyte (CTL) subsets. The latter cells migrate to the CNS, become reactivated, expanded and attack oligodendrocytes and neurons. On the other hand, regulatory CD8+ T cells may have anti-inflammatory actions by directly killing the effector autoreactive CD4 T cells. This is a pathway that was seen to be induced after treatment with glatiramer acetate (GA), an immunomodulatory drug (Saxena *et al*., 2011).

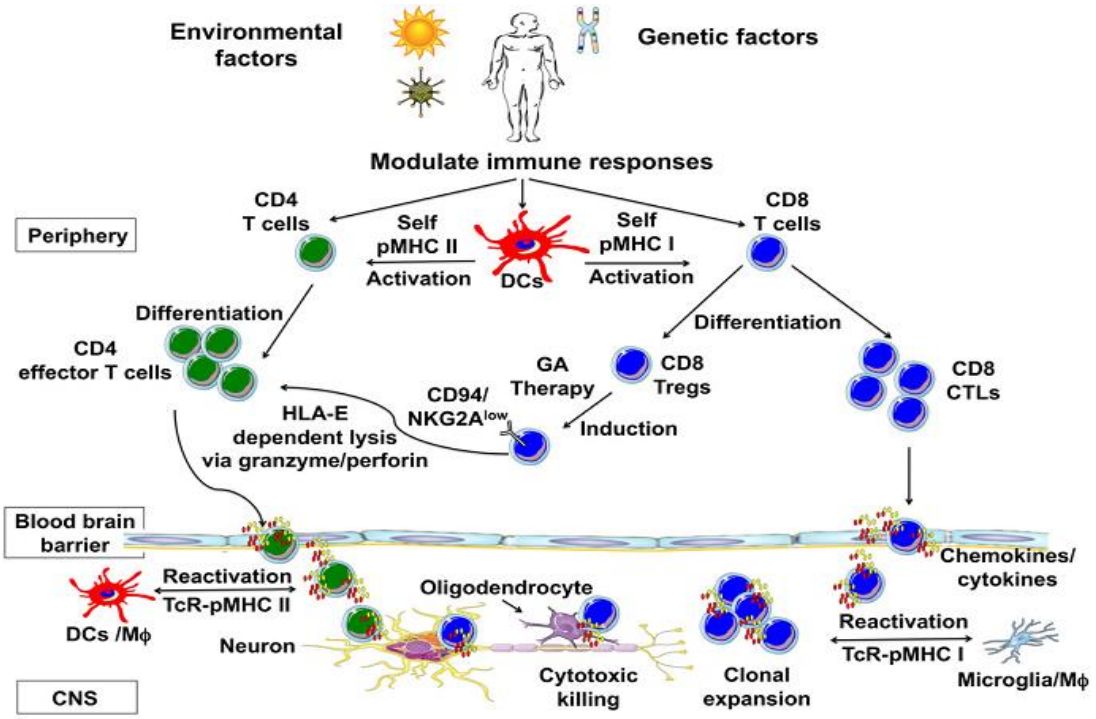


Figure 1-6: Theoretical scheme of the contribution of CD8 T cells in MS pathogenesis.

In genetically susceptible individuals exposed to environmental factors (e.g., low vitamin D, viral infection), self-peptides will be presented to CD4 and CD8 T cells via MHC-I and II, respectively. This will induce T cell expansion into effector CD4 and CD8 cells and migration into the CNS, where they will be reactivated by antigen-presenting cells (DCs, microglia, or macrophages). This will induce clonal expansion of these cells and damaging neurons. CD8 Tregs can also be differentiated in the periphery, this pathway is induced by glatiramer acetate (GA). pMHC I and II: peptide-bound major histocompatibility complex class I or II, respectively. CTLs: cytotoxic T lymphocytes. (From Saxena *et al.*, 2011), reproduced with permission.

1.3.3 Regulatory (CD4+) T Cells (Tregs)

Tregs belong to a heterogeneous group of cells whose primary function is to prevent or contain abnormal or exaggerated immune responses. T cells with regulatory functions include Tr1 cells, which produce IL-10 and are responsive to vitamin D and steroids; Th3 cells which express produce TGF-β and act during tolerance induction, and Treg cells (Workman *et al.*, 2009). The latter are typically CD4+ cells that suppress inflammatory and tissue-destructive pathways mediated by Th cells, largely through antigen-dependent pathways. The main functions of

Tregs are to prevent autoimmune diseases, suppress allergic inflammation that occurs in asthma and pathogen-induced responses, and maintain self, oral, and foeto-maternal tolerance (Corthay, 2009, Joller *et al*., 2012). There are several types of identified Tregs, natural (nTregs) that are produced in the thymus, and induced (iTregs) produced in the periphery. Both types are differentiated from naive CD4+ T cells (Rudensky, 2011, Josefowicz *et al*., 2012). In addition, there is another type of Tregs which are CD8+, and have similar anti-autoimmune functions to CD4+ Tregs (Correale and Villa, 2010).

Foxp3 is the most reliable Treg cell marker and has crucial functions as the main transcription factor in Tregs (Fontenot *et al*., 2005b, Joller *et al*., 2012). *In vitro* studies have shown that production of Foxp3+ Tregs is mediated by TGF-β, and the differentiation is facilitated by IL-2 and retinoic acid (Chen *et al*., 2003, Fontenot *et al*., 2005a, Mucida *et al*., 2007). Moreover, cytokines produced by tolerogenic DCs, and antigen presentation by immature DCs, both can induce Foxp3+ Treg formation from Foxp3- T cells (Kretschmer *et al*., 2005). Some studies (Brunkow *et al*., 2001, Wildin *et al*., 2001) have observed that mutations in Foxp3 gene are associated with multi-organ immune disease in mice and humans, indicating the importance of Foxp3 expression in normal functioning of Tregs.

Tregs secrete the anti-inflammatory cytokine TGF-β (Todd and Spickett, 2010), which is also required for the development and differentiation of Th17 cells; but has inhibitory effects on GM-CSF expression (El-Behi *et al*., 2011). Furthermore, Tregs also secrete other anti-inflammatory cytokines, such as IL-10 and IL-35 that suppress APCs and effector T cells (Asseman *et al*., 1999, Collison *et al*., 2007). Tregs also inhibit inflammatory responses by direct cell-to-cell contact. Activated Tregs

can suppress any effector T cells regardless of their antigen specificity (Takahashi *et al*., 1998). Some studies (Corthay, 2009, Rosenblum *et al*., 2011) suggest that the suppressive activity of Tregs to Th cells may be more effective and physiologically relevant when both cell subtypes share the same antigen specificity.

The protective role of Tregs in MS and its experimental models seems to be crucial. It has been reported that targeted Treg deletion has resulted in the development of EAE spontaneously in transgenic mice with T cell receptor specific for enecephalitogenic MOG peptides, which develop spontaneous EAE at high rates. Supporting Treg function by either injecting Tregs or enhancing their differentiation with different therapies has led to prevention or amelioration of EAE (Zozulya and Wiendl, 2008). MS can be a result of altered Treg cell homeostasis (Venken *et al*., 2010, Nyirenda *et al*., 2015). Moreover, studies have demonstrated that Tregs have impaired immunosuppressive function or migration ability to the CNS in RRMS patients (Viglietta *et al*., 2004, Schneider-Hohendorf *et al*., 2010). Tregs in MS were shown to have antigen specificity against myelin peptides, which means that activation of Tregs may restore the immunological tolerance in MS (Rodi *et al*., 2016).

It has been shown that most of the therapies used to treat MS are enhancing Treg activities by many pathways, such as IFN-β, GA, and fingolimod (Hong *et al*., 2005, de Andres *et al*., 2007, Haas *et al*., 2009, Haas *et al*., 2015). In addition, it was reported that Treg expression was lower in peripheral blood of stable MS patients than healthy controls. However, it returns to normal during acute clinical attacks, as a compensatory mechanism to restore homeostasis during the attacks (Dalla Libera *et al*., 2011). On the other hand, other studies have found contradicting results to Dalla Libera and colleagues, showing no

difference in Treg frequencies among healthy controls and MS patients with different clinical subtypes, but with impaired functioning in MS patients than healthy controls (Putheti *et al*., 2004, Viglietta *et al*., 2004, Haas *et al*., 2005, Feger *et al*., 2007). Recent studies have revealed genetic variants in MS that impair Treg activity (Piedavent-Salomon *et al*., 2015, Severin *et al*., 2016). It was also shown that reduced gut microbiome (chiefly bacterial species) may contribute to modulating EAE by elevating Treg frequency (Mielcarz and Kasper, 2015). In MS, there are recent attempts to induce Treg response by parasite/helminth therapy (Correale, 2014, Maizels, 2016). Moreover, it was found that during acute MS exacerbation, the induction of Tregs from memory T cells is inhibited (Mohiuddin *et al.*, 2016).

1.3.4 B Cells

B cells are considered as part of adaptive immune system. They develop in the bone marrow and mature in the spleen and lymph nodes and express CD20 on their surface (LeBien and Tedder, 2008, Pieper *et al*., 2013). There is strong evidence that B cells are involved in the pathogenesis of MS, through the observation of oligoclonal bands (OCB) (mainly clonal IgG produced intrathecally) on CSF analysis in more than 95% of MS patients (Reiber *et al*., 1998, Freedman *et al*., 2005, Villar *et al*., 2005). The role of OCB in MS is not well understood yet, although they are very useful for diagnosis; but antibodies, produced by plasma cells, the last stage in the development of B cells, have a role in complement-mediated demyelination and IgG deposition (Breij *et al*., 2008, Weber *et al*., 2011). In addition, plasma cells that produce these antibodies have a role in disease progression. However, there are many studies suggesting that B cells have other roles in MS, including acting as APC to activate T cells (Lanzavecchia, 1985, Constant *et al*., 1995,

Weber and Hemmer, 2010) and secretion of cytokines. B cells were reported to express GM-CSF in some studies (Harris *et al*., 2000, Weber *et al*., 2014), but not consistently in others (Zupo *et al*., 1992). They can be subdivided into effector and regulatory subtypes according to their cytokine profile. Effector B cells produce IL-2, IL-4, IFN-γ, TNF-α, IL-4, IL-6, IL-12 and IL-17; while regulatory B cells produce TGF-β and IL-10 (Pistoia, 1997, Lund *et al*., 2005, Mizoguchi and Bhan, 2006, Bermejo *et al*., 2013, Schlegel *et al*., 2013). Furthermore, monoclonal antibody treatment targeting B cells (anti-CD20, e.g., rituximab) was found to be effective in MS (Disanto *et al*., 2012). This is by rapid reduction in the number of new CNS lesions detected with MRI in RRMS patients, and lower number of relapses (Hauser *et al.*, 2008, Hawker *et al.*, 2009, Kappos *et al.*, 2011).

1.3.5 Natural Killer (NK) Cells

NK cells are important components of innate and adaptive immunity. They have a role in disease suppression (regulatory function) or progression (effector function) through cytolysis and production of growth factors, as well as pro- and anti-inflammatory cytokines (Cooper and Yokoyama, 2010, Mayo *et al*., 2012). They are involved in cancer prevention and therapy through NK cytotoxicity receptors that recognise abnormal cancer cells. They were also used in HIV treatment (Maghazachi and Al-Aoukaty, 1998, Moretta *et al*., 2001). These cells can be activated at the inflammation site. Among different classification types, NK cells can be classified into NK1 (IFN-γ-producing), and NK2 (IL-5- and IL-13-producing) cells (Peritt *et al*., 1998). However, NK cells may secrete other cytokines, like IL-17, IL-21, and IL-22, and GM-CSF (Cooper *et al.*, 2001c, Todd *et al.*, 2015).

NK cells play a role in autoimmune diseases like systemic lupus erythematosus (SLE) and RA (Dalbeth and Callan, 2002, Schleinitz *et al*., 2010). Furthermore, there is evidence about the involvement of NK cells in MS (Morandi *et al*., 2008, Maghazachi, 2012). However, so far, there is a controversy regarding the exact role of NK cells in pathogenesis of MS and EAE. Some studies have suggested a protective role of NK cells in MS and EAE, probably through cytolysis of cells that cause exacerbation of the disease (mostly Th17 cells), alteration in DC and microglial cell functioning, and production of neurotrophic factors (Huang *et al*., 2006, Hao *et al*., 2010). Regarding MS, the frequency of NK cells has been increased during the remission stage of the disease (Takahashi *et al*., 2001, Takahashi *et al*., 2004). On the other hand, other studies have demonstrated a stimulating and aggravating role of NK cells in RRMS, mainly by secretion of pro-inflammatory cytokines, such as IFN-γ which activate Th1 cells (Kastrukoff *et al*., 1998, Shi *et al*., 2000). Some *in vitro* studies have shown that NK cells directly lyse primary oligodendrocytes and neurons, with a partial lysis of DCs, T cells, astrocytes, and microglial cells (Lunemann *et al*., 2008, Zhang *et al*., 2011). Therefore, more research is needed to elucidate the role of NK cells more clearly in MS, especially after the recent discovery of NK17/NK1 cells that require more investigation in using these cells as therapy in MS treatment (Maghazachi, 2013).

1.3.6 Monocytes

Monocytes are myeloid cells that belong to the innate immune system. They represent a link between innate and adaptive immune responses against microorganisms. They have an important role in homeostasis and inflammation, mainly by their scavenging role to pathogens. They also act as precursors for macrophages and DCs that are essential APCs

(Auffray *et al*., 2009). They express CD14 abundantly (Simmons *et al*., 1989), which functions as a co-receptor with Toll-like receptors (TLRs) for binding of lipopolysaccharides (LPS) or other pathogen-associated molecules. This is the first step in presentation of these molecules to T cells (Kitchens, 2000, Tapping and Tobias, 2000, Ranoa *et al*., 2013). Another study has shown that CD14 plays a role in IL-2-associated monocyte activation (Bosco *et al*., 1997). This is due to that CD14 was found to be involved in IL-2 binding to its receptor on the monocytes.

Monocytes also express CD16, hence they are classified according to their co-expression of CD14 and CD16 into classical monocytes (CD14++CD16-), intermediate monocytes (CD14++CD16+), and non-classical monocytes (CD14+CD16++) (Ziegler-Heitbrock and Hofer, 2013). Classical and intermediate monocytes were suggested to be involved in MS. They were found to me more activated and produced more pro-inflammatory factors (Chuluundorj *et al.*, 2014). Intermediate monocytes were found to be at lower frequencies in peripheral blood of MS patients, located in higher frequencies in the CSF and in the perivascular regions of active MS lesions (Waschbisch *et al.*, 2016).

Monocytes produce many inflammatory cytokines, such as IL-1β, IL-12, IL-18, IL-23, and GM-CSF (see below). It was noticed that before EAE relapses, GM-CSF plays a crucial role in mobilization of CD11b+ CD62L+ $Ly6C^{hi}$ monocytes from bone marrow to the blood. Then these cells pass through the blood-brain barrier to the CNS where they induce production of pro-inflammatory mediators, and differentiate into macrophages and DCs which are considered crucial for autoimmune demyelination in the CNS (King *et al*., 2009).

IL-1β is produced by monocytes and it enhances Th17 development, RORα expression, as well as GM-CSF and IL-17A production (Veldhoen *et al*., 2006). It also enhances Th1 production of GM-CSF (El-Behi *et al*., 2011) and recruitment of macrophages to the site of inflammation (Rider *et al*., 2011)

IL-12 is produced by monocytes, macrophages, and DCs. It plays an important role in the differentiation of IFN-γ-producing-Th1 cells from naive T cells. It also plays a role in regulation of Th1 cell response (O'Garra and Arai, 2000, Trinchieri, 2003, Schaller *et al.*, 2015). It is involved in the antimicrobial response against intracellular pathogens. Various cytokines can control IL-12 production, such as IL-4, IL-18, IFN-γ, GM-CSF, and even IL-12 itself, probably through upregulation of IL-12R by these cytokines (Langrish *et al*., 2004). There is a controversy regarding the role of IL-12 in EAE and MS. An EAE study has suggested that IL-12 acts as an inhibitor for GM-CSF and RORγt expression in Th cells (Codarri *et al*., 2011). However, the opposite was shown (i.e., IL-12 induced GM-CSF expression by CD4 Th cells) in more recent EAE and MS studies (Noster *et al*., 2014, Grifka-Walk *et al*., 2015). IL-12 has an identical protein subunit to IL-23 (p40), and p35 subunit unique to IL-12. Other EAE studies have shown that IL-12 or IL-12p35 knockout mice developed EAE after induction, concluding that IL-12 is not essential for EAE (Becher *et al.*, 2002, Gran *et al.*, 2002, Chu *et al.*, 2000, Cua *et al.*, 2003, Gutcher and Becher, 2007). However, other studies have demonstrated the opposite and suggested a pro-inflammatory role of IL-12. Exposure of macrophages and DCs to IL-12 *in vivo* was shown to increase the antigen-presenting activity and stimulate DTH reactions (Bianchi *et al*., 1996, Grohmann *et al*., 1997). Moreover, other studies (Segal *et al*., 1998, Kroenke *et al*., 2010) have

concluded that IL-12 has a potent enhancer of encephalitogenicity in CD4+ Th1 cells which is independent on IFN-γ or IL-17A/F. With regard to MS, some studies have shown no change or reduction of IL-12 levels in the PBMCs of MS patients (Rohowsky-Kochan *et al.*, 1999). The administration of anti-IL-12/23p40 monoclonal antibody has failed to produce any effects in MS (Segal *et al*., 2008). On the other hand, others have demonstrated an increase in IL-12 or its receptor's expression in blood and MS plaques of MS patients (Windhagen *et al*., 1995, Balashov *et al*., 1997, Comabella *et al*., 1998, Ozenci *et al*., 2001).

IL-23 is a heterodimeric cytokine that belongs to IL-12 family and is secreted by activated APCs in humans. It binds to a receptor that has two subunits, one is IL-23R and the other is IL-12Rβ. However, it was noticed that IL-23 acts mainly on memory T cells, while IL-12 acts mainly on naive T cells (Oppmann *et al*., 2000). A study has suggested that IL-23 is responsible for the final differentiation of Th17 cells (that specifically secrete IL-17) from Th cells or primed CD4+ T cells in mice (Aggarwal *et al*., 2003). On the other hand, in the presence of IL-12, naïve T cells or primed CD4+ T cells will differentiate into IFN-γ-producing Th1 cells (O'Garra and Arai, 2000, Trinchieri, 2003). Figure 1-7 below summarises the role of IL-23 in the innate immunity.

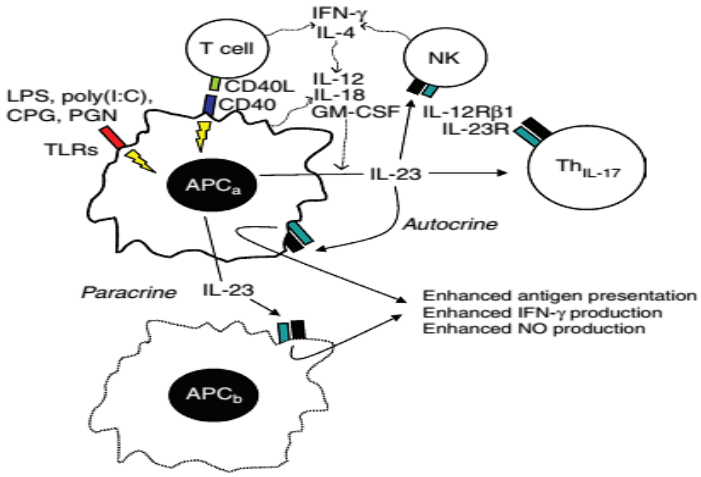


Figure 1-7: Role of IL-23 in innate immunity.

After APC activation (by binding with activated T cells or TLR-mediated activation), they produce IL-23 and other pro-inflammatory cytokines (IL-12, IL-18, and GM-CSF) which also induce IL-23 production. IL-23 can act on NK and Th17 cells via binding to its receptor (paracrine effect). It also has an autocrine effect inducing its own production, antigen presentation, and pro-inflammatory factor production. (From Langrish *et al*., 2004), reproduced with permission.

In EAE, it was shown that IL-23 has induced the frequency of GM-CSF-expressing Th17 cells (Codarri *et al*., 2011, El-Behi *et al*., 2011). Many other studies (Gyulveszi *et al*., 2009, McGeachy *et al*., 2009, Codarri *et al*., 2011) have found that Th cells unresponsive to IL-23 were not encephalitogenic, unable to produce GM-CSF, and had an impaired ability to enter the CNS. Therefore, IL-23 can convert non-pathogenic T cells into pathogenic ones. It has been reported that the expression of IL-23 receptor on T cells is very important for IL-17-secreting Th cells to invade the CNS *in vivo* (Gyulveszi *et al*., 2009, McGeachy *et al*., 2009). Based on the above-mentioned studies, monocytes are crucial for MS pathogenesis. Therefore, blocking their differentiation, migration to the CNS (for which GM-CSF is crucial, as discussed in the sections below), their activation or production of key inflammatory cytokines, may significantly attenuate the pathogenic course of MS.

1.4 Functions of GM-CSF

GM-CSF is considered as a haematopoietic growth factor that stimulates the formation of granulocytes and macrophages (mature myeloid cells) form precursor cells in the bone marrow (Stanley *et al*., 1994). It has been shown that GM-CSF is also a pro-inflammatory cytokine that can act on mature myeloid cells *in vitro* to induce their activation (Handman and Burgess, 1979, Hamilton *et al.*, 1980, Whetton and Dexter, 1989, Hamilton and Achuthan, 2013). GM-CSF induces DC maturation, survival and activation of macrophages, neutrophils, and eosinophils, differentiation of alveolar macrophages, and mobilization of myeloid cell population from bone marrow into blood (Figure 1-8) (Hamilton, 2008). GM-CSF is involved in the pathogenesis of many diseases, including cancer, chronic obstructive pulmonary disease (COPD), obesity, nephritis, arthritis, and MS (Hamilton, 2008).

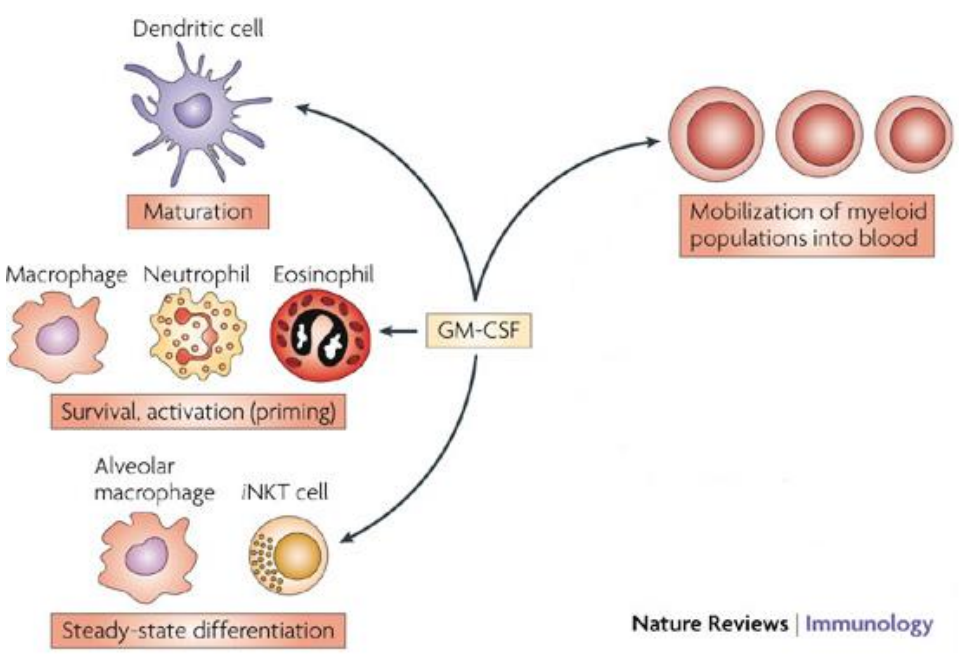


Figure 1-8: The major functions of GM-CSF.

GM-CSF is involved in DC maturation, survival and activation of macrophages, neutrophils, and eosinophils, and steady-state differentiation of alveolar macrophages and invariant NKT cells. GM-CSF also functions in mobilization of myeloid populations into blood. These processes are relevant to MS pathophysiology (see also fig. 1-9). (Adapted from Hamilton, 2008), reproduced with permission.

1.4.1 The Role of GM-CSF in Autoimmune Diseases (EAE and MS)

GM-CSF is a glycosylated protein of a 23 kDa molecular weight (Whetton and Dexter, 1989). It is expressed by many cell types (Table 1-2). GM-CSF is produced by various T cell subsets, including CD4+ and CD8+ T cells. However, its secretion is regulated differently in each cell type, as naive CD4+ cells do not secrete GM-CSF, while naive CD8+ cells do (Min *et al*., 2010). It has been shown that other cell types can express GM-CSF, including NKT cells and epidermal T cells, with undetectable amounts produced by Tregs and (Min *et al*., 2010). However, it was observed that activated γδ T cells that migrate into the CNS produce GM-CSF in mice with EAE (Lukens *et al*., 2012). It has been found that B cells (both normal and malignant types) can also produce GM-CSF, which is crucial for their survival (Harris *et al*., 2000). GM-CSF was shown to be able to cross the BBB, blood-spinal cord barrier (BSCB), and blood-testis barrier in mice (McLay *et al*., 1997a, McLay *et al*., 1997b). Regarding healthy CNS, GM-CSF was detected in humans and mice and is produced mainly by astrocytes. It may have a role in local microglial functions regulation (Malipiero *et al*., 1990), and stimulation of antigen-presentation in inactive microglia (Re *et al*., 2002).

Th1 and Th17 are the main GM-CSF-producing cells that have important pathogenic role in autoimmune inflammation in mice. In mice, myelin-reactive Th1 and Th17 could induce EAE. However, Th cells lacking GM-CSF were unable to induce EAE, and GM-CSF-deficient mice were also unable to induce or develop EAE. This has led to suggesting that it is the most important cytokine for development of EAE and Th cells pathogenicity (Ponomarev *et al*., 2007, Kroenke *et al*., 2010, Codarri *et al*., 2011, El-Behi *et al*., 2011).

Table 1-2: Expression of GM-CSF and GM-CSF receptor (GM-CSF-R) subunits.

Cell Type	GM-CSF expression (ref)	GM-CSF-R expression (ref)
Activated Th1, Th2, Th17	+ (Infante-Duarte *et al*., 2000, Mosmann *et al*., 1986, Codarri *et al*., 2011)	
Monocytes/ macrophages	+ (Hamilton, 1994)	+ (Baldwin *et al*., 1989)
Smooth muscle cells	+ (Filonzi *et al*., 1993)	
Chondrocytes	+ (Campbell *et al*., 1991)	
Parenchymal cells in kidney	+ (Timoshanko *et al*., 2005)	
Endothelial cells	+ (Bagby *et al*., 1986)	+ (Soldi *et al*., 1997)
Epithelial cells	+ (Hamilton, 2008)	
Fibroblasts	+ (Zucali *et al.*, 1986, Fitzgerald *et al.*, 2003)	+ (Dedhar *et al.*, 1988, Montagnani *et al.*, 2001)
Tumour cells		+ (Baldwin *et al.*, 1989, Uemura *et al.*, 2007)
Keratinocytes	+ (Mascia *et al.*, 2010)	+ (Hancock *et al.*, 1988, Braunstein *et al.*, 1994)
CD8+ cells	+ (Min *et al*., 2010)	
B cells	+ (Harris *et al*., 2000)	+ (Harris *et al.*, 2000)
NK Cells	+ (Cooper *et al.*, 2001c)	+ (Kubista *et al*., 2003)
NKT cells	+ (Min *et al*., 2010)	+ (Hamilton, 2008)
Epidermal T cells	+ (Min *et al*., 2010)	
γδ T cells	+ (EAE mice)(Lukens *et al*., 2012)	
	- (Min *et al*., 2010)	
Astrocytes	+ (Malipiero *et al*., 1990)	+ (Sawada *et al*., 1993)
Tregs	- (Min *et al*., 2010)	
Dendritic cells	+ (Sonderegger *et al*., 2008)	+ (Sonderegger *et al*., 2008)
Microglial cells	+ (Lee *et al*., 1994)	+ (Ponomarev *et al*., 2007)
Neutrophils	+ (Kita *et al*., 1991)	+ (Arnaout *et al.*, 1986, Lee *et al.*, 2008)
Eosinophils	+ (Kita *et al*., 1991)	+ (Hamilton, 2008)
Neurons		+ (Sawada *et al*., 1993)
Oligodendrocytes	+ (Kim *et al.*, 2014)	+ (Sawada *et al.*, 1993, Kim *et al.*, 2014)

In humans, on the other hand, CD4+ Th cells were the main GM-CSF sources that are associated with MS (Noster *et al*., 2014). However, it is not yet identified which Th subset express GM-CSF. A murine study has reported that GM-CSF produced by CD4+ T cells rather than by DCs was essential for development of autoimmune myocarditis. This is because it will stimulate DCs to produce IL-23 which is crucial for Th17 differentiation. They have also noticed that CD4+ T cells isolated from EAE mice expressed high amounts of IL-17A and GM-CSF, which may further suggest the importance of GM-CSF in pathogenesis of EAE (Sonderegger *et al*., 2008). Another study has shown that GM-CSF-deficient mice were resistant to EAE induced by immunisation with MOG (McQualter *et al*., 2001). This was due to reduced proliferation of T cells and reduced secretion of IL-6 and IFN-γ, which suggests decreased Th1 response. However, this effect is not direct on T cells since they lack receptors for GM-CSF (Kelly *et al*., 2001). Rather, this effect is thought to be due to generation and activation of inflammatory DCs by GM-CSF, which, in turn, activate CD4+ T cells by presenting the antigen to antigen-specific T cells (Kelly *et al*., 2001). The activated T cells then can activate antigen-specific B cells to differentiate and produce specific antibodies (Smith *et al*., 2000).

Endogenous GM-CSF is thought to increase tumour growth by suppressing immune response needed to kill cancer cells. This occurs through elevating the presence of myeloid suppressor cells (Young *et al*., 1996). On the other hand, exogenous GM-CSF is used clinically after chemotherapy to elevate neutrophil numbers and induce bone marrow recovery (Grant and Heel, 1992). Moreover, it is used as a substitute for bone marrow cell transfer because it mobilizes myeloid cells from bone marrow and restore the immune system in the blood (Antman *et al*.,

1988). In addition, GM-CSF administration can increase blood supply in mice brains after brain injury (Schneider *et al*., 2007). Moreover, endogenous GM-CSF may have a role in wound healing (Kaplan *et al*., 1992) and in foetal and placental development (Robertson, 2007).

In summary, it has been observed that GM-CSF neutralization can be beneficial in some inflammatory diseases such as asthma (in mice) and RA (in mice and humans), due to its pro-inflammatory effects (Cook *et al*., 2001, Yamashita *et al*., 2002, Behrens *et al*., 2015). However, GM-CSF administration in other cases of inflammatory diseases has resulted in suppression of inflammation through elevating host defences and lowering microorganism load which was useful in Crohn's disease (Stanley *et al*., 1997, Sweet and Hume, 2003, Korzenik *et al*., 2005). Moreover, GM-CSF-deficient mice have been shown to have higher susceptibility to microorganism infection. This supports the possible role of GM-CSF as an activator of phagocytes and inducer of inflammatory response necessary for fighting the infection (Zhan *et al*., 1998). One of the factors that influence colony-stimulating factors' effects in general may be the route of administration. Local use causes activation, division, and differentiation of myeloid cells, while systemic administration results (in addition to the above) in myeloid cell mobilization from bone marrow into bloodstream (Takahashi *et al*., 1999, Shiba *et al*., 2007).

Up to now, there is no direct evidence for the exact pathogenic action of GM-CSF in MS (El-behi *et al*., 2013). However, high GM-CSF amounts were found in the CSF of active-phase MS patients, suggesting that GM-CSF might have a role in MS pathogenesis (Carrieri *et al*., 1998). Figure 1-9 below illustrates GM-CSF-producing immune cells, possible mechanisms of GM-CSF in MS, and simplified pathophysiology of neuronal and myelin damage in MS.

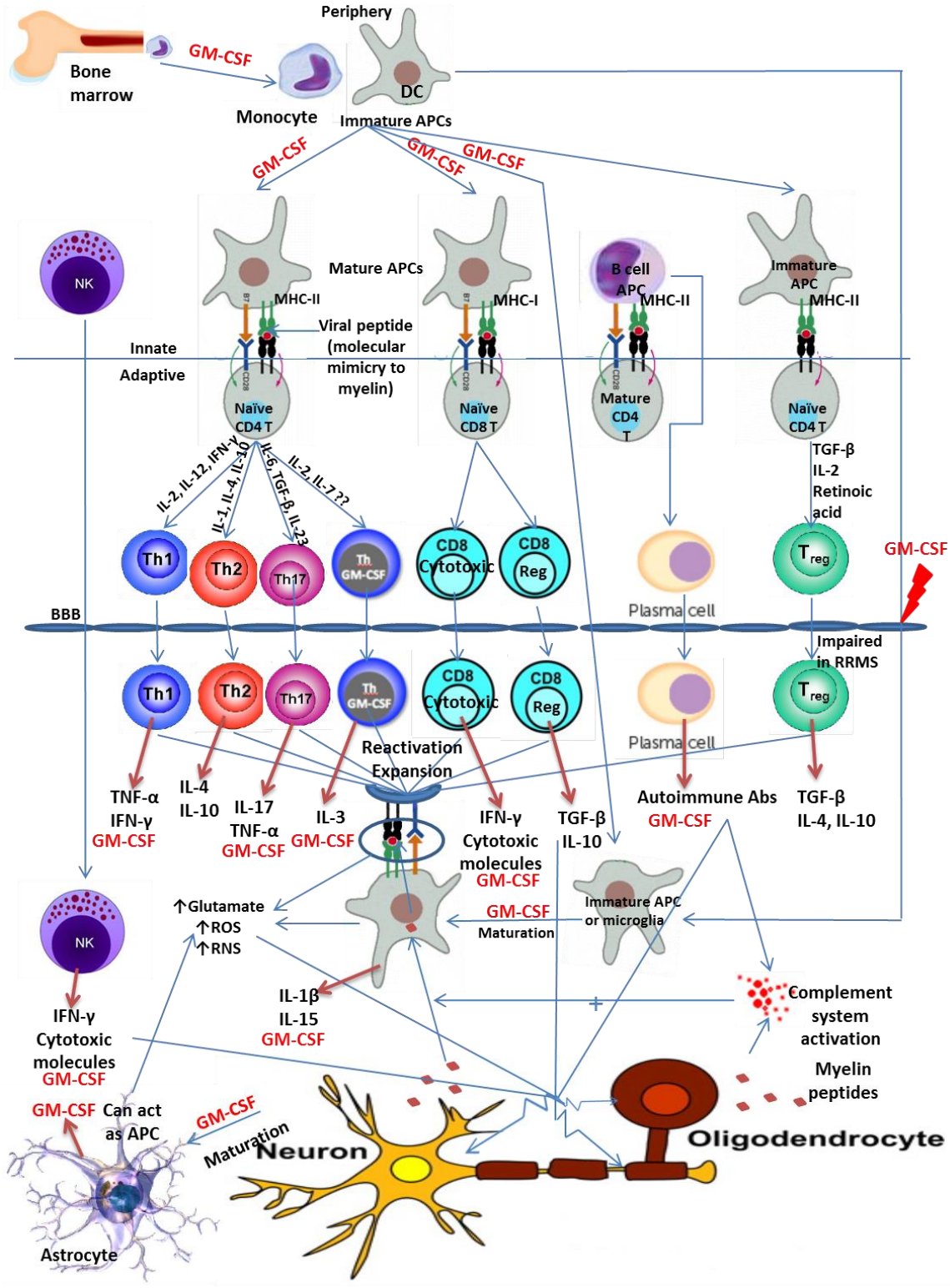


Figure 1-9: Simplified sheme of MS pathophysiology highlighting the role of GM-CSF.

In the periphery, GM-CSF induces mobilization and maturation of myeloid cells, which function as APCs to present autoantigenic peptides or mimics of myelin peptides. This leads to expansion of a wide range of immune cells and their migration to CNS. In the CNS these cells are re-activated, and recruit and activate other inflammatory cells, and result in damage to the myelin, axons and neuroglial elements, . In addition, other pathways were also simply illustrated, involving autoimmune antibodies (Abs), reactive oxygen species (ROS), direct cytotoxicity, and complement-mediated damage.

1.4.2 The Structure-Activity Relationship of GM-CSF Receptor (GMR) and Signalling Pathways

The GM-CSF receptor (GMR) is expressed by many cell types, including monocytes, macrophages, APCs, neurons, astrocytes, and oligodendrocytes. This suggests that GM-CSF is involved in physiological regulation of these cells (Sawada *et al*., 1993). GMR consists of a low affinity, but high ligand-specificity α chain (GM-CSF-α), and a high affinity chain that is shared among GM-CSF, IL-3, and IL-5 (β common or β_c chain) (Nicola *et al*., 1997, Hercus *et al*., 2009). The α chains of the latter three ligands bind to their cognate ligand with low affinity. Then the complexes produced compete with each other for binding to the β_c chain. This can be found in humans as well as in mice, except that in mice, there is an additional specific β chain for IL-3 (β_{IL-3}). This similarity in overall receptor structure of GM-CSF, IL-3, and IL-5 has resulted in similarities in some biological actions of these ligands.

The α chains of GMR (as well as IL-3 and IL-5 receptors) contain 200 amino acids in the extracellular (haemopoietin) domain, and a very short intracellular domain. These chains are not capable to transmit signals after the ligand is bound to them (Nicola *et al*., 1997), except the signalling for the induction of glucose transport in some cell types (Golde *et al*., 1994). The β chains, however, consist of a duplicated haemopoietin domain extracellularly, and a much longer domain than α chains intracellularly (Miyajima *et al*., 1993). β chains are responsible for most GM-CSF receptor activities (Figure 1-10).

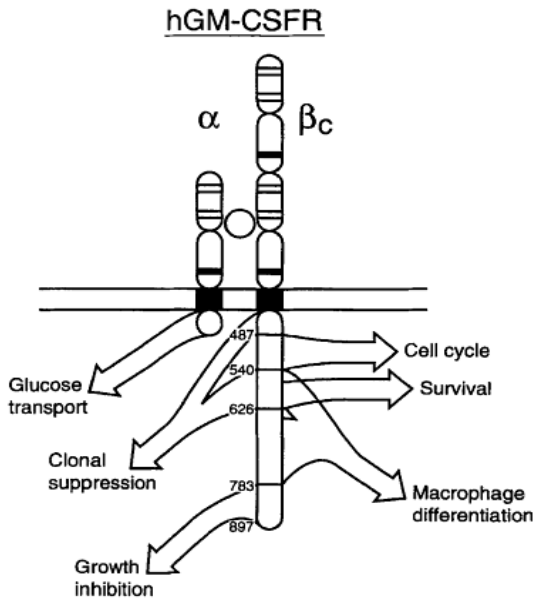


Figure 1-10: Human GM-CSF Receptor (hGM-CSFR) and its Main Functions

hGM-CSFR is composed of two subunits. The α subunit has a shorter extracellular and intracellular domains than the β common subunit (βc). Bc is a common subunit among GM-CSF, IL-3, and IL-5 receptors. In hGM-CSFR, βc is involved in most of the functions of GM-CSF. The closest part to the cytoplasmic membrane is called box-1 and is responsible for cell proliferation. The next region is called box-2, which is responsible for cell survival, clonal suppression, differentiation, or growth inhibition. (From Nicola *et al.*, 1997, pp25), reproduced with permission.

When GM-CSF binds to its receptor, it is known to activate at least three intracellular pathways: the Janus Kinase-Signal Transducer and Activator of Transcription (JAK2-STAT5) pathway, phosphoinositide 3-kinase (PI3K) pathway, and mitogen-activated protein kinase (MAPK) (Fleetwood *et al.*, 2005). These are important pathways for cell survival and proliferation (Figure 1-11). It was stated that the main function of α chains is to dimerize the intracellular domain of β chains (Nicola *et al.*, 1997, pp20). The receptor region of β chain, which is close to the cytoplasmic membrane is responsible for cell proliferation (Sato *et al.*, 1993). This region is called Box-1. After receptor dimerization, JAK kinase is activated leading to phosphorylation (activation) of STAT (transcription factors), that will be transferred to the nucleus to stimulate

the expression of specific genes (Mui *et al*., 1995). However, there are other pathways (other than JAK-STAT) that are suggested to have a role in cell proliferation after GM-CSF binding to its receptor (Miyajima *et al*., 1993). The next adjacent region to Box-1 is called Box-2 and is responsible for cell proliferation and other functions (Nicola *et al*., 1997, pp23 and 24). The intracellular region of β chain falling between the amino acid sequence of 624 and 778 is thought to activate Sos-Raf-Ras-MAPK pathway which leads to tyrosine kinase activation (Sato *et al*., 1993).

Other studies have shown that in order for GMR to be activated, it must form a dodecamer structure. This is formed by the head-to-head assembling of two ligand-receptor hexamers. This will allow β_c-associated Jak2 to transphosphorylate and perform its intracellular signalling. In addition, the combination of two GMR-α chains in the dodecamer structure is also important in the initiation of intracellular signalling (Muto *et al*., 1995, Lilly *et al*., 2001). Furthermore, GM-CSF concentration controls which signalling pathways are activated. Low GM-CSF concentrations lead to selective survival-only signalling pathway. On the other hand, higher GM-CSF concentrations result in the dodecamer assembly and lead to activation of survival and proliferation signalling pathways, as shown in Figure 1-11 below. Therefore GM-CSF receptor is considered as a therapeutic target in diseases including autoimmune diseases (Greven *et al.*, 2015, Wicks and Roberts, 2016).

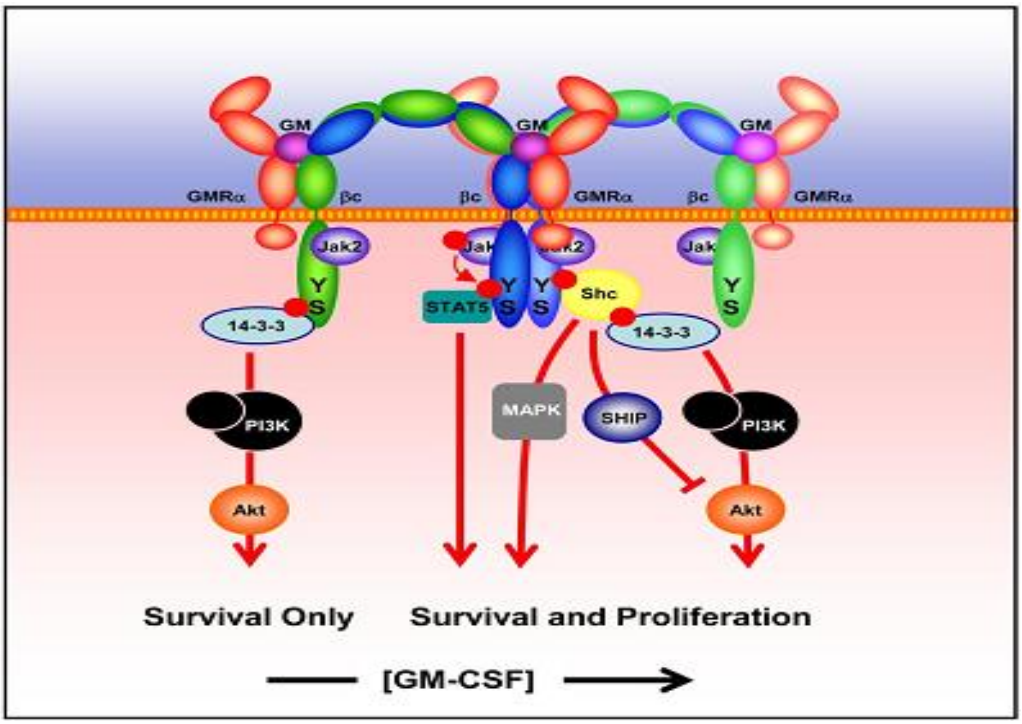


Figure 1-11: Concentration-dependent activation of GM-CSF receptor and its signalling pathways

This figure shows the dodecamer structure of GM-CSF receptor required for full action. In order to have this structure, four receptor molecules (or two hexamers formed by two receptor molecules) should bind to each other via the extracellular domains of βc chains. This structure is formed when the concentration of GM-CSF is sufficient to bind to the receptor molecules. Low GM-CSF concentration will signal survival pathways only. When the dodecamer structure is formed, this will activate survival and proliferation signalling pathways. GMRα: alpha subunit of GM-CSF-receptor, JAK: Janus kinase, STAT: signal transducer and activator of transcription, PI3K: phosphoinositide 3-kinase, Akt: protein kinase B, Shc: SH2-containing collagen-related proteins, MAPK: mitogen-activated protein kinase, SHIP: SH2-containing inositol 5-phosphatase. (From Hercus *et al*., 2009), reproduced with permission.

1.4.3 Mechanism of Action of GM-CSF in MS and EAE

There are many possible mechanisms by which GM-CSF can induce and maintain chronic autoimmune inflammation. It can stimulate APCs which are either local and/or infiltrating into the CNS to increase the expression of MHC-II molecules (Alvaro-Gracia *et al*., 1989, Wei and Jonakait, 1999). It also can induce IL-6 secretion by DCs, which increase the lifespan of auto-aggressive T cells (Sonderegger *et al*., 2008). Some studies (Shinohara *et al*., 2000) have elucidated that GM-CSF has a role in regulation of the synthesis of certain chemokines in

mouse polymorphonuclear (PMN) cells and macrophages. These chemokines have a positive role in accumulation and mobilization of immune cells to the site of inflammation in EAE (Kennedy *et al*., 1998, Gerard and Rollins, 2001). In addition, GM-CSF selectively induces M1 macrophage development (inflammatory macrophage lineage) (Fleetwood *et al*., 2007). GM-CSF acts mainly on macrophages and neutrophils to enhance their effector, phagocytic functions, and cytokine production (Cornish *et al*., 2009). Furthermore, GM-CSF secreted by auto-aggressive Th cells invading the CNS plays an important role in initiation and propagation of EAE. This is through inducing microglial activation, increasing infiltration of systemic myeloid cells into the CNS, and mediating their differentiation into DCs (Ponomarev *et al*., 2007). GM-CSF acts also through stimulating the maturation, differentiation, and activation of macrophages (Kroenke *et al*., 2010, Codarri *et al*., 2011). It also acts via activation of resident microglial cells, stimulating their phagocytic and antigen-presenting action (Morrissey *et al*., 1987, von Zahn *et al*., 1997, Hofstetter *et al*., 2005, Ponomarev *et al*., 2007). Moreover, GM-CSF stimulates the expression of MHC-II on monocytes and macrophages (Alvaro-Gracia *et al*., 1989); it also increases the adherence of monocytes and granulocytes to blood vessel endothelia (Arnaout *et al*., 1986, Gamble *et al*., 1989). Neutralization of systemic GM-CSF has resulted in decreased circulating Ly6+ myeloid cells infiltration into the CNS (King *et al*., 2009). GM-CSF can be produced at the BBB and uni-directionally transported to the CNS through the BBB and BSCB. This occurs via a selective and saturable transport system in addition to simple diffusion and leakage of GM-CSF. This causes higher levels of GM-CSF in the CNS; here it plays a role in microglial activation and APC migration and recruitment during CNS

inflammation (McLay *et al*., 1997b, Parajuli *et al*., 2012, Vogel *et al*., 2015).

1.4.4 Regulation of GM-CSF Production

GM-CSF secretion is dependent on IL-12/IL-23 receptor complex activity and on RORγt in Th17 cells (Codarri *et al*., 2011). Both IFN-γ and IL-12 function as negative regulators for GM-CSF production in Th17 cells and inhibit RORγt expression in mice (Codarri *et al*., 2011). However, recent studies have shown that GM-CSF production is correlated with Th1 regulation (which is mediated by IL-12 and IFN-γ) (Noster *et al*., 2014, Grifka-Walk *et al*., 2015). In addition, a recent study has also shown that IL-2 and IL-12 stimulation of naïve helper T cells has led to significantly higher GM-CSF production (Noster *et al*., 2014). Results of other studies have supported the stimulatory effects of IL-12 on GM-CSF production by T and NK cells (Aste-Amezaga *et al*., 1994, Duhen and Campbell, 2014). IL-2 was seen to counteract the inhibitory effects of glucocorticoids on GM-CSF production in human mononuclear cells (Larsson *et al*., 1999). A new study has reported that IL-2R polymorphism in MS has resulted in higher expression of GM-CSF by Th cells induced by IL-2 (Hartmann *et al*., 2014).

GM-CSF produced by T cells can be regulated by many cytokines. Based on murine studies, TGF-β, IFN-γ (from Th1), and IL-4 (from Th2) inhibit GM-CSF production. Th1-derived GM-CSF expression can be inhibited by IL-27. On the other hand, IL-23 is a strong enhancer of Th17-derived GM-CSF (Codarri *et al*., 2011, El-Behi *et al*., 2011, Young *et al*., 2012). IL-1β strongly increases GM-CSF production by CD4+ T cells in mice (El-Behi *et al*., 2011, Lukens *et al*., 2012) and in humans (Duhen and Campbell, 2014). There is a relation between TNF

and IL-1, inducing the production and activity of colony-stimulating factors (CSFs) in general (Hamilton, 2008). Table 1-3 below summarises the effects of the above cytokines on GM-CSF expression.

Table 1-3: Major Cytokines that regulate GM-CSF production.

Cytokine	Effect on GM-CSF expression	Reference(s)
IL-2	Stimulatory	(Noster *et al.*, 2014)
IL-12	Stimulatory in Th1 cells Inhibitory in Th17 cells	(Noster *et al.*, 2014, Grifka-Walk *et al.*, 2015) (Codarri *et al.*, 2011)
IL-23	Stimulatory in Th17 cells	(Codarri *et al.*, 2011, El-Behi *et al.*, 2011)
IL-4	Inhibitory	(Jansen *et al.*, 1989, Codarri *et al.*, 2011)
IL-10	Inhibitory	(Hashimoto *et al.*, 2001, Sagawa *et al.*, 1996)
TGF-β	Inhibitory	(Jacobsen *et al.*, 1991)
IFN-γ	No effect in Th1 cells Inhibitory in Th17 cells	(Noster *et al.*, 2014, Grifka-Walk *et al.*, 2015) (Codarri *et al.*, 2011)
IL-27	Inhibitory in Th1 cells	(Young *et al.*, 2012)
IL-1β	Stimulatory	(Lukens *et al.*, 2012, Duhen and Campbell, 2014)
TNF	Stimulatory	(Hamilton, 2008)
IL-7	Stimulatory	(Sheng *et al.*, 2014)

1.4.5 GM-CSF Neutralisation Testing as Suggested Therapy for MS

As mentioned above, GM-CSF may have a pathogenic role in many diseases, including some types of cancer, COPD, autoimmune diseases such as RA and MS. In some studies, cancer cells were suppressed after using anti-GM-CSF monoclonal antibodies (mAbs) *in vitro* (Rigo *et al*., 2010, Padron *et al*., 2012). On the other hand, GM-CSF immunotherapy was shown to be useful in some types of cancer (Waller, 2007). Using GM-CSF neutralising antibodies in animal models of COPD revealed suppression in pulmonary inflammation and oxidative stress (Vlahos *et*

al., 2006, Lötvall *et al*., 2012). Some studies have found beneficial effects upon targeting GM-CSF using neutralising antibodies in experimental models of RA (Plater-Zyberk *et al*., 2007, Cook *et al*., 2012). The pharmaceutical companies have designed different monoclonal antibodies against GM-CSF, among which are KB002 and KB003 (humanised mAbs), and Morphosys 103 (MOR103). MOR103 is a fully human mAb from the Human Combinatorial Library (HuCAL) against GM-CSF with a high affinity (Cornish *et al*., 2009). It was tested for safety and efficacy in RA patients via phase 1b/2a clinical trial. The results were promising because of the significant improvement with placebo-range side effects, which encourage further clinical development of MOR103 (Behrens *et al*., 2012). This is likely because GM-CSF neutralization will block maturation of APCs, which is a crucial step in autoimmune inflammation. In addition, it will inhibit GM-CSF-mediated myeloid cell migration to the CNS.

We also conducted a first, novel phase I clinical trial supported by Morphosys to evaluate the safety and efficacy of MOR103 in MS patients. A small immunology sub-study is presented in 0 in this thesis.

1.4.6 Possible Side Effects of CSF Neutralization

Suppressed immunity against microorganisms was observed in mice and humans with neutralizing anti-GM-CSF antibodies (Hamilton and Anderson, 2004, Seymour, 2006). Therefore, patients may become more susceptible to infection after GM-CSF neutralization. Therefore, clinical trials testing GM-CSF-neutralizing antibodies were focusing on detection of any mild or serious infections.

Lack of GM-CSF action can result in a rare syndrome called pulmonary alveolar proteinosis (PAP), in which high amounts of the surfactant

protein are accumulated in the alveoli with dysfunctional myeloid cells (Trapnell *et al*., 2003).

Using GM-CSF-neutralizing agents locally (which is difficult to do in MS) may be beneficial in localizing their action to the desired site with minimal systemic undesired effects (Hamilton, 2008).

1.5 Hypothesis, Aims and Objectives of the Study

In MS, there is an imbalance between the pro-inflammatory and anti-inflammatory pathways, favouring the former. As GM-CSF is a pro-inflammatory cytokine; therefore, in this study we hypothesize that the frequency of GM-CSF-producing immune cells is higher in MS patients when compared to healthy controls, possibly due to impaired regulatory mechanisms leading to dysregulation of GM-CSF across immune cell lineages. Based on data implicating GM-CSF as an autocrine growth factor for B cells (Harris *et al.*, 2000), we also hypothesize that GMR expression is changed (up- or down-regulated) in NK and B cells, due to GM-CSF expression changes in MS.

One of our objectives was to measure the cytokines that, according to the available data, regulate GM-CSF expression. Then we planned to block the high concentration cytokines *in vitro* in MS to detect their effects on GM-CSF and GMR expression.

We also hypothesize that the T cell subset called Th-GM, which have been identified in the mouse immune system and implicated in the MS model (Sheng *et al.*, 2014), are also present in the human immune system. In addition, our hypothesis is that these cells are expressed at higher frequency in MS patients.

We also propose that EBV infection (one of MS environmental risk factors) will induce GMR expression by EBV infected B cells and autocrine production of GM-CSF, as a mechanism of GM-CSF-supported B cell survival.

Finally, we hypothesize that anti-GM-CSF treatment of MS will reduce the frequency of T cell activation, accompanied by increased Treg frequency in MS patients. This is due to an indirect effect of GM-CSF neutralization on T cells, mediated via inhibiting APC maturation and differentiation, and therefore inhibiting APC-mediated T cell activation.

The main objectives of this thesis project are to:

1. Evaluate the expression of GM-CSF in different types of cells of the immune system in MS *in vitro*, with focus on stable untreated MS.
2. Determine expression of GM-CSF during relapse and remission stages of the disease for the same patients, or after IFN-β treatment, all *in vitro*.
3. Examine what cytokines that regulate GM-CSF production in different types of cells of the immune system in the healthy immune system and in MS *in vitro*.
4. Examine Th-GM differentiation from naïve CD4 T cells in healthy volunteers and MS patients *in vitro*.
5. Determine the effect of GM-CSF neutralization *in vivo* on the balance between Th1/Th17 and Treg cells, and other immune effects of GM-CSF neutralization as part of a phase I clinical trial.

Chapter 2. Methods

All the below mentioned sample collection and experimental work was approved by Nottingham Research Ethics Committee, as shown in Appendix II.

2.1 PBMC Isolation and Counting Using Trypan Blue

PBMC isolation was performed using density gradient centrifugation. Venous blood (up to 50 ml) was drawn into green top 10 ml-heparinised vacutainer tubes (BD Biosciences, UK). Histopaque (Sigma Aldrich, UK) placed into 50 ml-sterile universal tubes and blood was added slowly onto it. The volume of histopaque added was similar to the volume of the blood tested. The tubes were then centrifuged at 800 x g for 20 minutes, with the brake off. The buffy layer in the middle (PBMCs) was collected using a 1ml-micropipette into a 50-ml Falcon tube. Phosphate buffered saline (PBS) was added up to 50 ml (first wash), and the samples were centrifuged (500-600 x g, 5-10 minutes). The supernatant was removed and the cells were re-suspended in 10 ml PBS for counting. 10 µl of the cell suspension was added into 90 µl 20% trypan blue dye (Sigma Aldrich, UK) in an Eppendorf tube. Then 10 µl of the final mixture was loaded into the Neubauer WBC counting chamber (Superior Marienfeld, Germany). Using a light microscope (X40 lens), live cells (colourless and bright) were counted in the four large margin squares (X), then total PBMCs were calculated as follows:

Total PBMCs ($x\ 10^6$) = X/4 x 10,000 x Y x Z

Y= total parts after dilution with trypan blue

Z= volume of cell suspension with PBS

After cell counting, cells were re-suspended in PBS (up to 50 ml) and centrifuged (500-600 x g, 5-10 minutes) (second wash). The supernatant was removed, and the cells were re-suspended in 10% FCS (Sigma Aldrich, UK) in RPMI medium (which also contained 100 units per ml of penicillin, 0.1 mg/ml of streptomycin, and 2 mM of glutamine) (all from Sigma Aldrich, UK). Cells then were divided into labelled sterile Fluorescence Activated Cell Sorting (FACS) tubes for PBMC stimulation and staining, so that each tube contained about 1 x 10^6 cells.

2.2 PBMC Freezing and Thawing

Cells were re-suspended in RPMI medium (up to 30 ml) and centrifuged (300 x g, 10 minutes) (third wash). The supernatant was discarded and freezing medium (9 parts FCS and 1 part DMSO-both from Sigma) was added. The volume was calculated to get a final concentration of 10-15 x 10^6 cells per 1 ml freezing medium. The cell suspension was divided into 1-ml labelled cryovials which were transferred into a cool cell™ in a -80°C freezer. After the cells were frozen, the cryovials were transferred into a liquid nitrogen canister for later use.

For PBMC thawing, cryovials were taken from liquid nitrogen and placed on ice, warmed in 37°C water bath for less than 1 minute. Few drops of warm medium (10% FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin to prevent bacterial contamination of the cultures, and 2 mM glutamine, an essential amino acid that enables nitrogen transfer and storage and serves as an energy source for proliferating cells, in RPMI medium-all from Sigma) were then added to the cryovial. The cell suspension was withdrawn to be added slowly on 10 ml warm medium, mixed together, and centrifuged (300 x g, 5 min). The supernatant was discarded, 10 ml of fresh warm medium was added and left overnight in

37°C incubator with 5% CO_2. Next day, cells were counted, centrifuged and re-suspended and distributed on 24-well plates to have 1 x 10^6 cells/ml/well-plate.

2.3 PDB/Ionomycin Stimulation

Fresh PBMCs were divided into FACS tubes in a concentration of 1 x 10^6 cells/ml/tube. They were either left unstimulated or stimulated with 20 ng/ml phobol dibutyrate (PDB) (Sigma) and 0.5 μg/ml ionomycin (Sigma) in the presence of 10μg/ml brefeldin A (Sigma) and left for 16 hrs in a 37°C incubator with 5% CO_2. After overnight incubation, cells were processed for staining as in section 2.5 below.

2.4 Anti-CD3+anti-CD28 Stimulation and Cytokine Antibodies

Fresh or thawed PBMCs were cultured in 24-well plate with RPMI medium containing 10% FCS, 100 units per ml of penicillin, 0.1 mg/ml of streptomycin, and 2 mM of glutamine in a concentration of 1 x 10^6 cells/ml/well-plate. Cells were either left unstimulated, or stimulated with soluble anti-CD3 and anti-CD28 (1 μg/ml each) (BD Biosciences), and incubated for 5 days in 37°C incubator with 5% CO_2. This was adapted from a recently published work that we collaborated on (Hartmann *et al.*, 2014). They found high GM-CSF expression using this protocol, and we confirmed this in the work for this thesis.

For cytokine blocking, specified cultured and stimulated thawed cells were treated with one of the following human cytokine antibodies (all from R&D Systems) to reach a final concentration of 10 μg/ml each: anti-IL-2 and anti-IL-2R-alpha, anti-IL-12p70, all the previously mentioned, anti-IL-12/23p40, anti-IL-1β and recombinant human IL-1RA, and mouse IgG1 isotype control.

Before re-stimulation and adding brefeldin A in the last 5 hrs of cultured PBMCs, 0.5 ml supernatant was taken from each well to perform Multiplex Bead Assay (Section 2.8). In addition, 0.25 ml cell culture (1-1.5 x 10^6 cells) were taken from each well to perform RNA extraction and proceed with q-PCR assay (Section 2.9). Then 0.75 ml fresh warm medium was added to each well plate and cells were re-stimulated with Phorbol myristate acetate (PMA) (50 ng/ml), Ionomycin (I) (0.5 μg/ml), in the presence of brefeldin A (10 μg/ml) (All from Sigma).

2.5 Flow cytometry Staining

Cultured cell suspensions were placed in FACS tubes, including the negative control, single colour controls, isotype controls (PDB/I protocol), and fluorescence minus one (FMO, see below) (anti-CD3+anti-CD28 protocol) controls. They were centrifuged (all centrifuges were at a speed of 300 x g for 5 min), washed once with PBS (1ml), then re-suspended in 1 ml PBS. They were then stained with dead cell stain (near IR 1:1000) (Invitrogen) (in PDB/I protocol, dead cell stain was not used). Cells were left at room temperature in the dark for 20 minutes, then washed once in FACS buffer (PBS + 2% FCS). Then, cells were fixed by adding 0.25 ml of Fix/Perm solution (BD Biosciences) and keeping them at 4°C for 20 minutes. After fixation, cells were washed twice with perm/wash solution 1X (BD Biosciences), and then FACS tubes were blotted to remove excess fluids. If more than two antibodies with brilliant violet fluorochrome were planned to be used in the same FACS tube, these fluorochromes may interact with each other resulting in staining artefacts and misinterpretation of data. This effect was minimised by re-suspending the cells in 50 μl of brilliant staining buffer (BD Biosciences) to improve the appearance of these

dyes. Surface and intracellular staining was performed afterwards, and cells were incubated for 30 minutes at 4°C. Cells then were washed once with perm/wash 1X (1ml) and once with FACS buffer (1ml), and then re-suspended in 0.3ml fixation buffer (BD Biosciences). Table 2-1 below illustrates the antibodies and isotype controls used.

2.6 Experimental Controls and Machine Setup in Flow Cytometry

Unstained control, single colour controls, and isotype controls were used during flow cytometry staining after PDB/Ionomycin stimulation. For anti-CD3+anti-CD28 stimulation, isotype controls were replaced with fluorescence minus one (FMO) controls. These controls involve using all the fluorescent dyes planned to be used in one tube except one of these dyes. This is to detect any spillage of signal from other channels to the one of the missing dye. FMO is also used to set the quadrants of the flow cytometry plots. Single colour controls were used for each fluorescent dye bound to the antibody of interest. This was done to detect the signal in the specific channel in which it should be detected. Single colour controls are also used to perform the compensation of the detected signals in the flow cytometer. Compensation is a mathematical process used when multiple colours are used simultaneously to remove any spillage of fluorescence to channels other than the channel of interest (Maecker and Trotter, 2006). In the experimental work performed in this thesis, compensation was done while acquiring data either by Dr David Onion or Nicola Croxall. Flow cytometer setup was kindly performed either by Dr David Onion, the manager of the flow cytometry facility in the university, or Nicola Croxall, the technician in this facility. Table 2-2 below illustrates the list of antibody panels used in this work, their design was done by me and revised by Dr David Onion.

Table 2-1: Antibodies and Isotype controls used in flow cytometry.

Antibody/ Isotype Control	Fluoro-chrome	Composition/ Isotype	Clone	Manufacturer	Amount 10^6 Cells
Isotype control	PE	Mouse IgG1, κ	P3.6.2.8.1	eBioscience	5 µl
Isotype control	FITC	Mouse IgG1, κ	MOPC-21	Biolegend	5 µl
Isotype control	APC	Mouse IgG1, κ	MOPC-21	Biolegend	5 µl
Isotype control	PeCy7	Mouse IgG2b, κ	MPC-11	Biolegend	5 µl
Isotype control	AF488	Mouse IgG1, κ	MOPC-21	Biolegend	5 µl
GM-CSF	Efluor® 660	Mouse IgG1, κ	GM2F3	eBioscience	5 µl
GM-CSF	BV421	Rat IgG2a	BVD2-21C11	BD Biosciences	5 µl
CD3	ECD	Mouse IgG1, κ	UCHT1	Beckman Coulter	5 µl
CD4	PE-CF594	Mouse IgG1, κ	RPA-T4	BD Biosciences	5 µl
CD8	PeCy7	Mouse IgG1	SFCI21Thy2D3	Beckman Coulter	5 µl
CD8	BV785	Mouse IgG1, κ	RPA-T8	Biolegend	5 µl
IL-17	PE	Mouse IgG1, κ	EBio64CAP17	eBioscience	5 µl
INF-γ	FITC	Mouse IgG1	45.15	Beckman Coulter	5 µl
CD56	PE	Mouse IgG1, κ	HCD56	Biolegend	10 µl
CD56	BV605	Mouse IgG2b, κ	NCAM16.2	BD Biosciences	5 µl
CD14	FITC	Mouse IgG1, κ	HCD14	Biolegend	5 µl
CD14	PerCpCy5.5	Mouse IgG1, κ	61D3	eBiosciences	5 µl
CD16	PeCy5	Mouse IgG1, κ	3G8	BD Biosciences	10 µl
CD19	PE	Mouse IgG1, κ	HIB19	Biolegend	5 µl
CD20	PeCy7	Mouse IgG2b, κ	2H7	Biolegend	5 µl
CD25	PE	Mouse IgG2a	B1.49.9	Beckman Coulter	10 µl
CD116 (GMR)	APC	Recom-human IgG1	REA211	Miltenyi Biotech	10 µl
CD45RA	FITC	Mouse IgG2b, κ	HI100	Biolegend	5 µl
CD45RA	PeCy7	Mouse IgG2b, κ	HI100	Biolegend	5 µl
CD154	PeCy5	Mouse IgG1, κ	24-31	Biolegend	5 µl
Foxp3	AF488	Mouse IgG1, κ	259D	Biolegend	5 µl
Annexin V	FITC	Protein	None	BD Biosciences	5 µl
PI	PI	Dye	None	BD Biosciences	5 µl
IL-3	APC	Rat IgG1, κ	BVD3-1F9	Miltenyi Biotech	10 µl

Table 2-2: Full panels used in all experiments performed in this work.

Experiment	Full panel
GM-CSF expression: monocytes and B cells (PDB/I stimulation)	CD3-ECD, CD14-FITC, CD20-PeCy7, GM-CSF-eFluor 660 (emission similar to APC)
Cytokine expression: Tc and NK cells (PDB/I stimulation)	CD3-ECD, CD8-PeCy7, CD56-PE, GM-CSF-eFluor 660, IFN-γ-FITC
Cytokine expression: Th1 and Th17 cells (PDB/I stimulation)	CD3-ECD, CD8-PeCy7, IL-17-PE, IFN-γ-FITC, GM-CSF-eFluor 660
Cytokine and GMR expression: Th, Tc, NK, and B cells (anti-CD3+anti-CD28 stimulation)	CD3-ECD, CD8-BV785, CD56-BV605, CD20-PeCy7, CD116-APC, IL-17-PE, IFN-γ-FITC, GM-CSF-BV421, dead stain-near IR
NK cell purity test	CD3-ECD, CD56-PE, CD16-PeCy5
Cytokine expression in isolated NK cells	CD3-ECD, CD56-PE, IFN-γ-FITC, GM-CSF-BV421, dead stain-near IR
Cytokine and GMR expression in isolated B cells	CD20-PeCy7, CD116-APC, IL-17-PE, IFN-γ-FITC, dead stain-near IR
Naïve CD4 T cell purity	CD4-PE-CF594 (emission similar to ECD dye), CD45RA-FITC
Cytokine expression in naïve CD4 T cells	CD3-ECD, CD8-PeCy7, IL-17-PE, IFN-γ-FITC, GM-CSF-BV421, IL-3-APC, dead stain-near IR
T cell frequency and cytokine expression-Morphosys	CD3-ECD, CD8-PeCy7, CD154-PeCy5, IL-17-PE, IFN-γ-FITC, GM-CSF-eFluor660
Treg frequency- Morphosys	CD4-PE-CF594, CD25-PE, CD45RA-PeCy7, Foxp3-AF488

2.7 Data Acquisition, Statistics and Analysis

Cells were acquired on Beckman Coulter Mo-Flo XDP (the map of which is illustrated in Table 2-3 below), and analysed using Kaluza 1.5 software. Data were combined and statistical analysis was performed using GraphPad Prism 6. The type of statistical test applied depends on whether data were normally distributed or not (measured with

D'Agostino & Pearson normality test), and the number of groups measured, which is specified in each result section.

Table 2-3: Beckman-Coulter MoFlo XDP flow cytometer map.

Colours reflect the laser colour. This machine has 14 channels (FL1-FL14) with different excitation/emission spectra (wave lengths are shown in nanometres). The last column illustrates some examples of fluorophores that can be detected in each channel, including the ones that were used in this work.

Channel	Laser		Emission Filter	Emission Bandwidth (nm)	Fluorochromes	
FL1	488		529/28	515-543	FITC, CFSE, AlexaFluor (AF) 488, GFP, DyLight488, Bodipy FL	
FL2	488		580/23	568.5-591.5	Phycoerythrin (PE / R-PE)	
FL3	488		625/26	612-638	PE-Texas Red (ECD), Propidium Iodide	
FL4	488		670/30	655-685	PE-Cy5, PE-Cy5.5, PE-AF680, DRAQ5	
FL5	488		720/13	703.5-716.5	PE-AF700	
FL6	488		785/29	770.5-799.5	PE-Cy7	
FL7	405	561	450/65	417.5-482.5	Pacific Blue, BV421, AmCyan	N/A
FL8	405	561	542/50	517-567	QDot 530, Pacific Orange	N/A
FL9	405	561	575/25	562.5-587.5	QDot 585, Brilliant violet 570	DsRed, mOrange, tdTomato
FL10	405	561	740LP	>740	QDot 800, BV785	PE-Cy7
FL11	405	561	615/20	605-625	QDot 625, BV605	mCherry, Texas Red
FL12	640		670/30	655-685	APC, AF647, TO-Pro-3, Cy5	
FL13	640		720/13	713.5-726.5	APC-AF700	
FL14	640		785/62	754-816	APC-Cy7, APC-H7, near IR	

2.8 Multiplex Bead Assay

After fresh PBMC isolation and stimulation for 5 days with anti-CD3+anti-CD28, 0.5 ml culture supernatants were taken from

unstimulated and stimulated well plates, stored in -20°C until they were thawed and used in multiplex bead assay. In this assay, the concentrations of multiple cytokines can be measured simultaneously in the same well using magnetic beads. Each magnetic bead is internally color-coded with a unique spectral signature so it can be detected in a specific region. Multiple beads with different detection regions can be detected in the same well plate, and each bead is coated with a different protein (cytokine) antibody. Biotinylated detection antibodies are added to bind with and quantify the protein. This is via the fluorescence emitted from biotin-bound streptavidin phycoerythrin (PE) conjugates (Figure 2-1). Laser is used in Multiplex (Luminex) machines to allow fluorescence emission to quantify the beads and bound analyte concentrations.

Three kits were used in this experiment. The first one was Milliplex® MAP kit (EMD Millipore) which was used to detect eight cytokines: GM-CSF, IFN-γ, IL-2, IL-7, IL-10, IL-12p70, IL-17, and IL-23. The second kit, ProcartaPlex® (Affymetrix eBioscience), was used to detect four cytokines: IL-1β, IL-6, IL-15, and IL-18. The third one was an ELISA kit to detect free active TGF-β1 (Biolegend). Samples were processed according to manufacturer's protocols. Multiplex samples were run on Bio-Rad (Bio-Plex® 200 System) machine and analysed using Bio-Plex Manager Software. The machine setup and plate running was done by Dr Ian Spendlove. ELISA samples were run on Benchmark plus microplate reader (Bio-Rad). Data were transferred to an excel sheet and then to GraphPad Prism to draw collective data graphs. The standard curves for all the analytes detected are illustrated in Figure 2-2 and Figure 2-3 below. The type of statistical test used depends on how

many groups selected, and whether or not they are normally distributed, which was measured with D'Agostino & Pearson normality test.

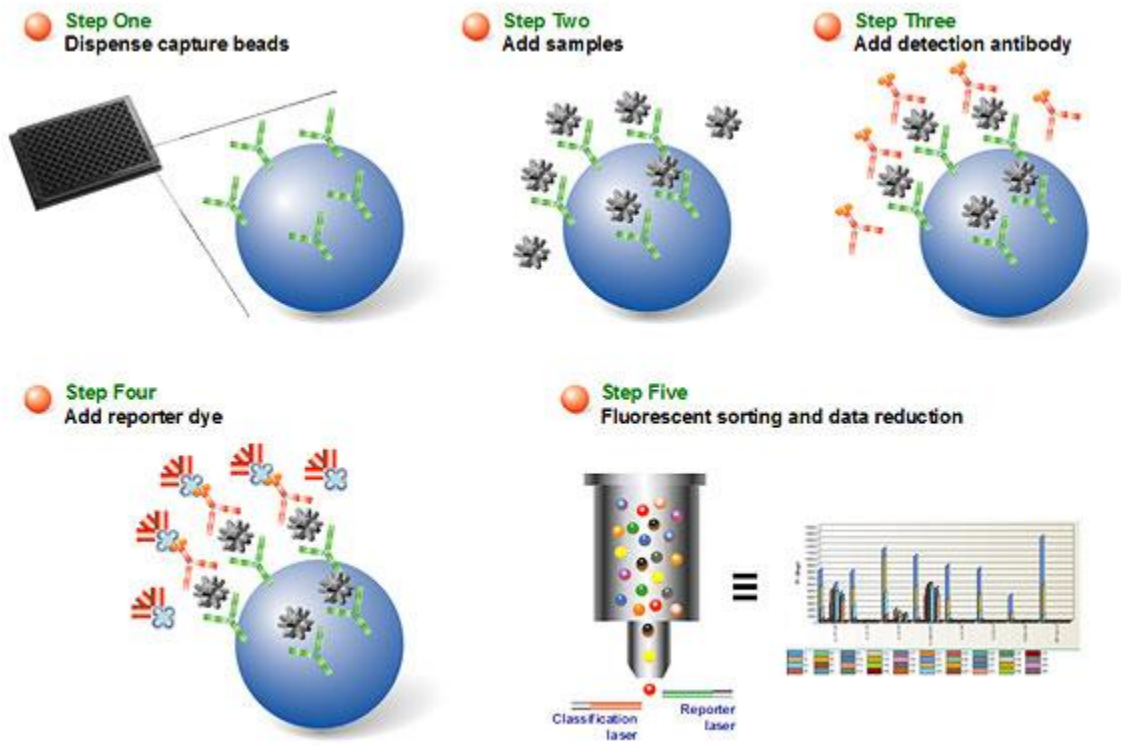


Figure 2-1: Multiplex Bead Assay principle.

Each bead is covered with the specific antibody to be tested. It is also internally colour-coded in a unique way to be distinguished from the other beads that are covered with different antibodies when used all in one well-plate. After adding different beads to the well-plate, the sample is added. Then, biotinylated detection antibodies are added to bind to the protein-antibody complexes. The next step is adding the reporter dye, then running the plate in a special machine, where two lasers are used. One is the classification laser to classify the unique beads, and the other is the reporter laser, which measures the intensity of light emitted from the reporter dye. The emission signals then are quantified in the machine to determine the concentration of each analyte per one millilitre. From (http://www.bio-rad.com/en-ug/applications-technologies/bio-plex-multiplex-immunoassays).

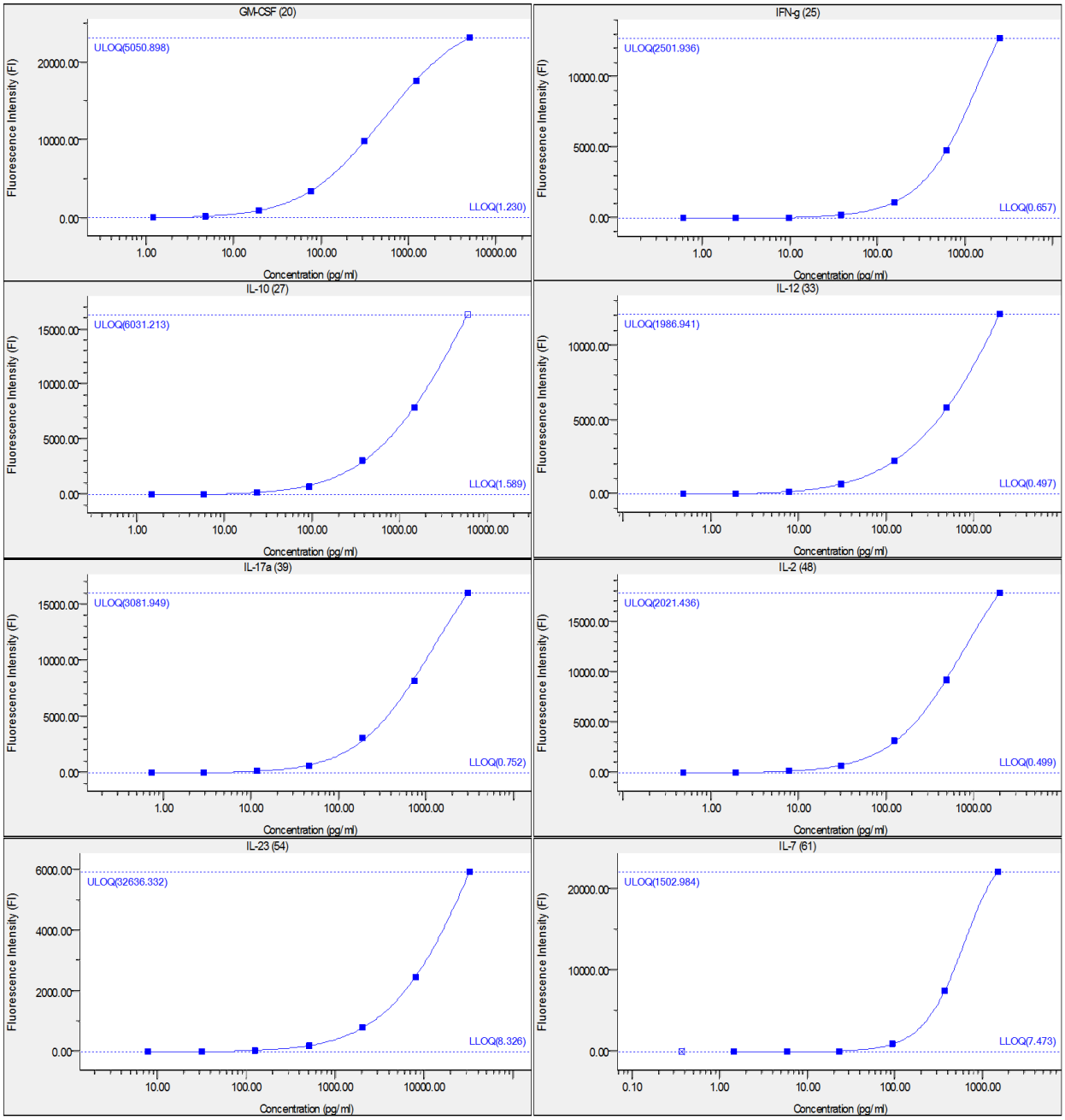


Figure 2-2: Standard curves of analytes tested with Merck Millipore® Multiplex kit.

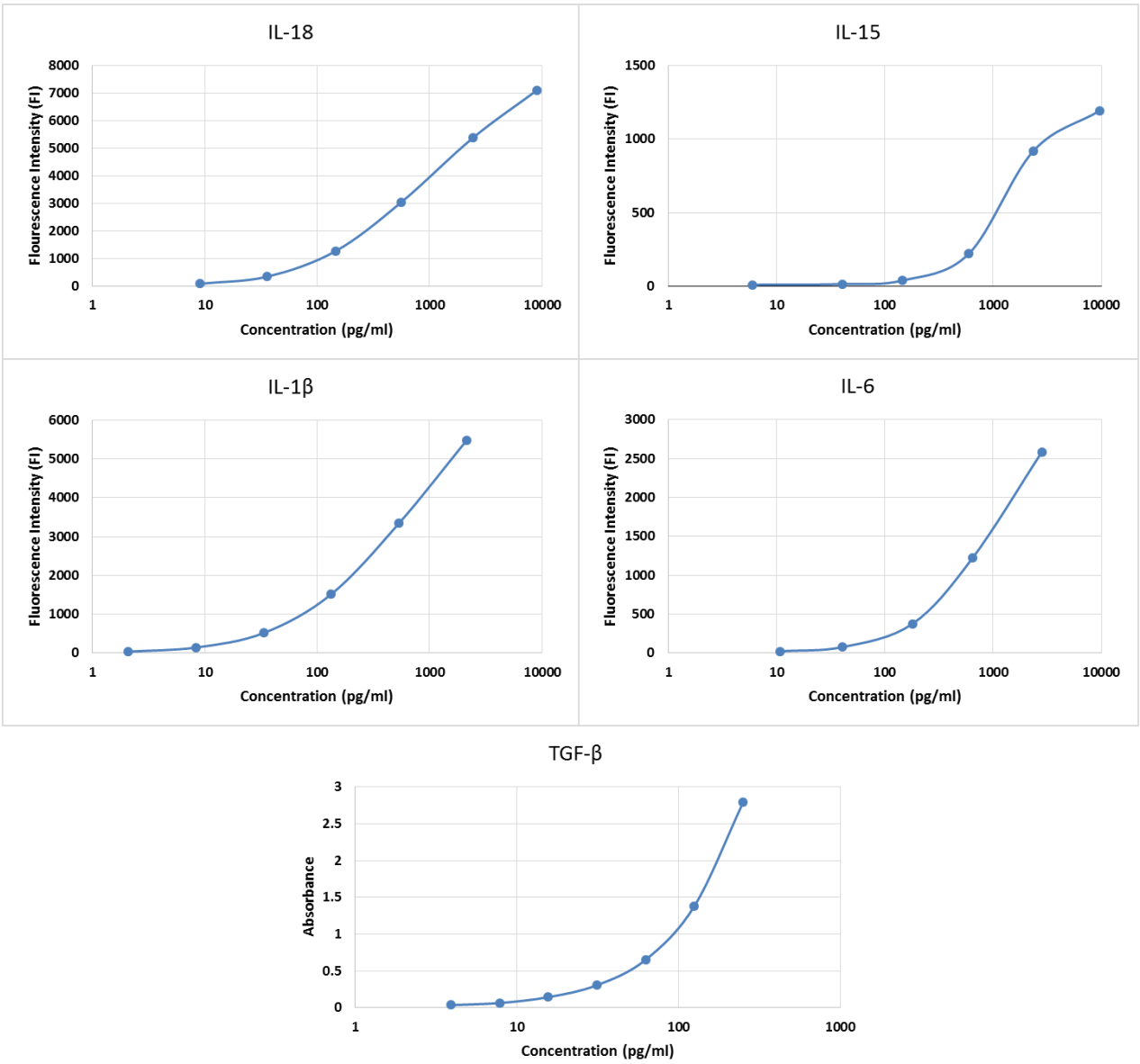


Figure 2-3: Standard curves for analytes tested with eBioscience and Biolegend kits.

Concentrations of IL-18, IL-15, IL-1β, and IL-6 were measured via eBioscience multiplex kit. Concentration of TGF-β was measured via Biolegend ELISA kit.

2.9 Quantitative Polymerase Chain Reaction q-PCR-TaqMan® Assay

2.9.1 Total RNA Extraction of Cultured PBMCs and Whole Blood

Fresh blood (20-30 ml) was taken from healthy volunteers and untreated RRMS patients, PBMCs were isolated (Section 2.1) and stimulated (Section 2.4). 0.25 ml of cell suspension was taken on the fifth day

before PMA/I re-stimulation and processed for RNA extraction using RNA extraction kit (Roche) following manufacturer's instructions. Whole blood was collected in Paxgene® tubes and left for at least 2 hours at room temperature before freezing to ensure complete blood lysis. These tubes were thawed by leaving them at room temperature for further 2 hours before starting RNA extraction. Total RNA extraction was performed using PAXgene® Blood RNA Kit (PreAnalytiX®) according to manufacturer's instructions. Quantification of total extracted RNA was measured using NanoDrop® ND1000 (Thermo Scientific). Purity was checked by 260/280 nm ratio, including samples that have ratio range of 1.8-2.3. RNA composition, quality, and quantity were also measured for part of the samples using Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with intact RNA composition with RNA integrity number (RIN) of ≥ 7 were selected.

2.9.2 Reverse Transcription and Complementary DNA (cDNA) Synthesis

All the process was done on ice. Reagents and total RNA were left to thaw on ice too. 2 µl of random hexamers 1:10 (Promega) were added to new Eppendorf tubes for each sample, then 1 µl of dNTPs mixture 1:10 (Promega) was added to each sample. Then, a certain volume (maximum 10 µl) of total RNA (or water for –RNA controls) solution was taken from each sample to contain total of 0.5 µg and added to the above mixture. The final volume was completed with Diethypyrocarbonate (DEPC) water to reach 13 µl. The tubes were given a quick spin (800 x g, 10-15 sec), then were set in the Master-Cycler (Eppendorf®) and run for 5 min at 65°C, and then the tubes were placed on ice. While running the samples in the Master-Cycler, the master mix was prepared so that it contained 4 µl of the enzyme buffer, 1 µl of Dithiothreitol DTT, and 1 µl

of RNAse inhibitor for each sample (all from Promega). While samples were on ice, 6 µl of the master mix, then 1 µl of SuperScript® III Reverse Transcriptase (RT) (Promega) were placed on the sides of the Eppendorf tube (in case of –RT control, water was added instead of RT). Final volume was 20 µl, samples were put for a quick spin (800 x g, 10-15 sec), then were set in the Master-Cycler (Eppendorf®). They were run in the following programme: 5 min at 25°C+ 1 hr at 50°C+ 15 min at 70°C. Samples were taken out of the thermal cycler and given a quick spin, then placed in -80°C freezer until used in q-PCR assay.

2.9.3 q-PCR TaqMan® Assay for GM-CSF and GM-CSF Receptor (GMR) Gene Expression

Quantitative (real time) polymerase chain reaction (q-PCR) is an assay used for amplification of a desired and specific gene sequence using its specific cDNA sequence and quantitatively measure its expression or amplification. TaqMan® q-PCR assay is performed by the presence of specific primers and a probe that binds in proximity to the primer and is cleaved by the Taq Polymerase during the second strand synthesis (Figure 2-4). Through the cleavage of the probe, fluorescence is released and detected. In the next cycle, another probe will be bound to the target sequence, and cleaved after DNA polymerase synthesizes the new strand, releasing more fluorescence. This will add more specificity to the assay. Fluorescence will increase in an exponential manner after each cycle when the desired cDNA sequence is amplified after a number of repeated cycles. This process continues until the threshold cycle (Ct) is reached for the desired gene sequence, which is the cycle in which the desired fluorescence exceeds the background or threshold fluorescence. Ct is used in data analysis to detect relative gene expression. It reflects if

the gene is expressed more (lower Ct) in certain samples than others (higher Ct).

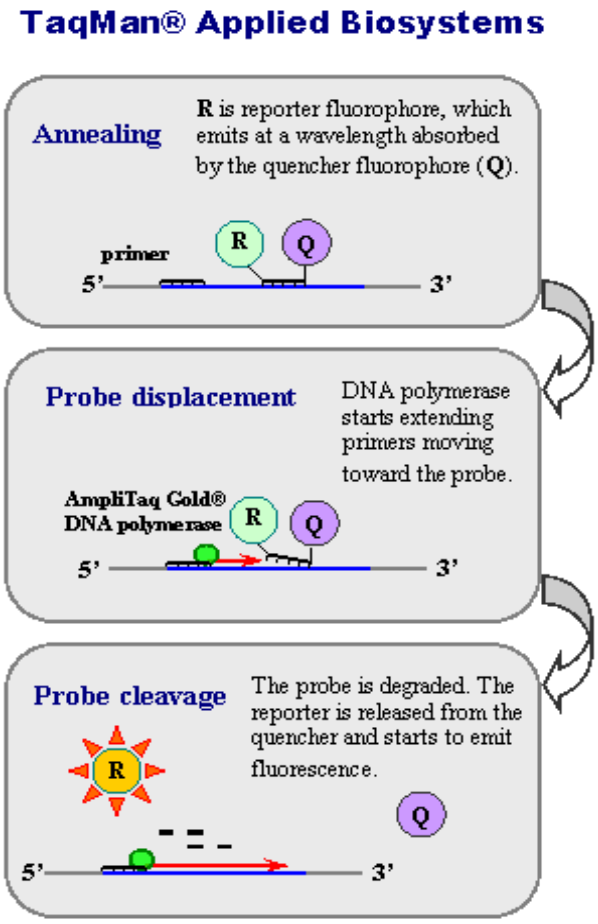


Figure 2-4: q-PCR TaqMan® Assay Principle.

In TaqMan assay, the primers are bound to (annealed) their specific region on the cDNA, with the probe annealed in a specific location at some distance from the primer. The probe is bound to a reporter and quencher fluorophores. The reporter fluorophore emits light absorbed by the quencher fluorophore, so no signal is observed. In the extension step, Taq DNA polymerase starts extending the primers. When it reaches to the region where the probe is bound, it displaces the probe which will be degraded and the reporter fluorophore separates from the quencher fluorophore, resulting in fluorescence emission that is detected by the q-PCR machine. In the meantime, the extension of the desired sequence by Taq DNA polymerase continues. The process is repeated and the fluorescence emission will increase exponentially. From (http://www.ncbi.nlm.nih.gov/probe/docs/techqpcr/).

There were a number of controls used in the TaqMan® q-PCR assay:

1. No RT control: where no RT was added during cDNA synthesis. q-PCR signal from this sample indicates DNA contamination in the RNA-cDNA synthesis level.
2. No RNA control: where no RNA was added during cDNA synthesis. q-PCR signal in this sample indicates DNA contamination in the cDNA synthesis level.
3. No template control (NTC): where no cDNA was added to the q-PCR well plate. q-PCR signal in this plate indicates DNA contamination in the q-PCR level.
4. Internal sample control: where the same sample is measured twice in 2 plates for the same gene amplification, more than 1 cycle difference in the Ct indicates technical error.
5. PCR duplicates: where the same is added in 2 well plates for each sample and for each gene, more than 1 Ct difference indicates technical error.
6. House-keeping genes (HKGs): these are constitutive genes that are expressed similarly under different conditions (e.g., stimulation, disease, infection, etc.). They are used to normalize the test gene expression with, and to calculate relative folds change of expression of the test gene in comparison to the HKG.

Depending on the sample from which RNA was extracted (cultured PBMCs or whole blood), different HKGs were tested to find out the best one for each. HKGs were selected based on published works that have shown no change in their expression under similar conditions to this work. For cultured PBMCs, Beta-2-Microglobulin (B2M) (Vilisaar *et al*., 2015), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)

(Christophi *et al*., 2008, Vordenbaumen *et al*., 2011), and TNF receptor-associated protein 1 (TRAP1) were tested on 18 different samples. TRAP1 appeared to be the best HKG, with the lowest standard deviation SD (0.42) and the lowest maxCt-minCt value (1.3).

For RNAs extracted from whole blood samples, B2M, TRAP1, and Hydroxymethyl-bilane synthase (HMBS) (Stamova *et al*., 2009) HKGs were tested on 26 samples. The best one appeared to be TRAP1, with the lowest SD (0.56) and the lowest maxCt-minCt value (1.14).

TaqMan® Primers-probes for each gene were predesigned from ThermoFisher Scientific® and received as a single tube for each human gene. The reference numbers were: GM-CSF (CSF2): Hs00929873_m1; GMR (CSF2RA): Hs00531296_g1; TRAP1: Hs00212476_m1. According to manufacturer's instructions, no PCR optimization was needed for those primer-probe mixtures.

All reagents (including cDNA) were left on ice to thaw slowly for 1 hour before q-PCR start, and all work was on pre-cooled PCR cooler (Sigma). The q-PCR well contained the following reagents: primer-probe (1.25 µl), FastStart Universal Probe Master Mix (Roche) (12.5 µl), and DEPC water (Sigma) (9.25 µl). cDNA (50 ng or 2 µl) was added in the final step. The plate was sealed with thermal sealer (Alphalabs), and the following thermal profile was used for all samples on 7900HT Fast Real-Time PCR System (Applied Biosystems):

Denaturation	95°C	10 min	
Denaturation	95°C	10 sec	X 40 cycles
Annealing and extension	60°C	30 sec	

2.9.4 Agarose Gel Electrophoresis

Agarose gel (2%) with ethidium bromide (0.5 µg/ml) was used to make sure that specific amplification of the desired gene transcripts was obtained. Samples loaded contained 5 µl loading buffer 10X (ThermoFisher Scientific), and 15 µl q-PCR product. The ladder contained 3 µl 50 base pair (bp) DNA ladder (Invitrogen), 12 µl Tris EDTA (TE) buffer (prepared in lab), and 5 µl loading buffer 10X. Samples were run at 110 V for 1.5 hr. Single band was detected under UV light with each RNA amplicon with the following lengths: GM-CSF 85 bp, GMR 131 bp, and TRAP1 66 bp (Figure 2-5).

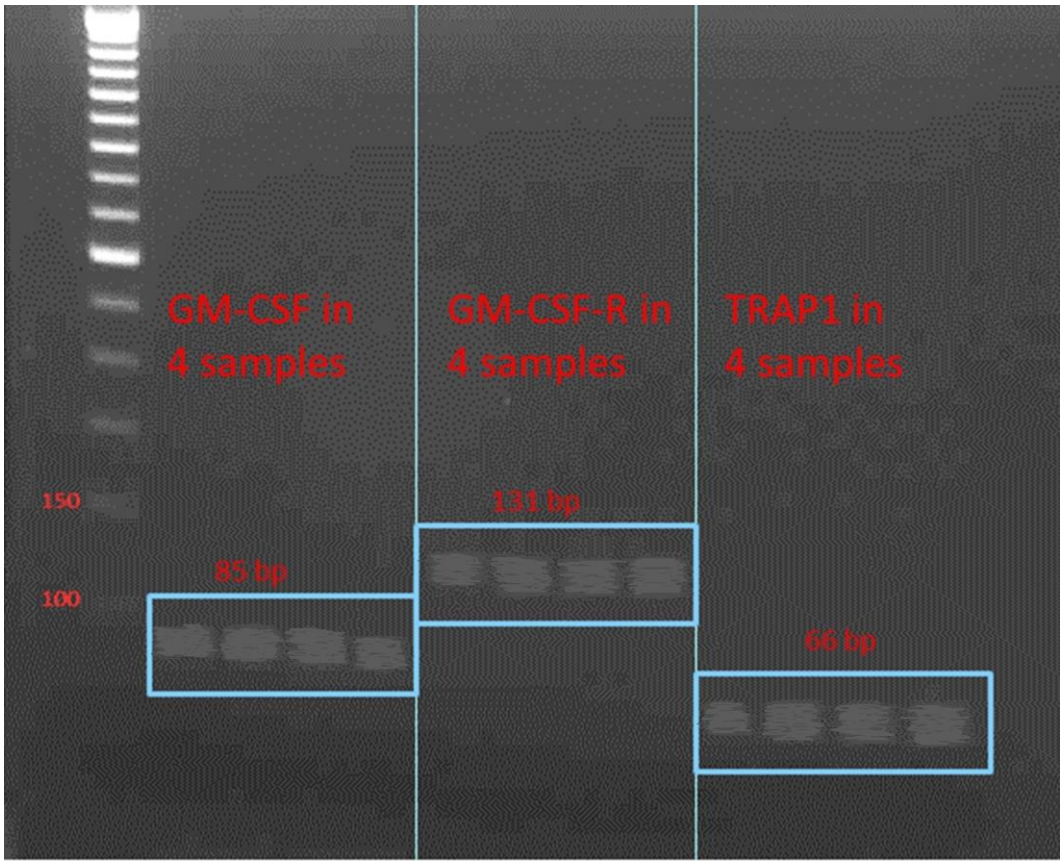


Figure 2-5: Assessing amplicon specificity with gel electrophoresis.

Desired amplicon bands appear on gel electrophoresis after q-PCR TaqMan® Assay for cultured PBMCs in 4 samples for each gene detected, 2 unstimulated and 2 stimulated, respectively. GM-CSF amplicon length = 85 base pairs (bp); GM-CSF-R amplicon length = 131 bp; TRAP1 amplicon length = 66 bp. Single bands were detected at the desired amplicon length of each product, which reflects the specificity of the product and absence of contamination. A shade of grey was added to the bands to improve their visibility.

2.9.5 Data Analysis

Ct values were exported to excel sheets, and then delta Ct (Δ Ct) was calculated as follows:

$\Delta Ct = Ct_{test\ gene} - Ct_{HKG}$

Then Δ Δ Ct was calculated as follows:

$\Delta \Delta Ct = \Delta Ct_{test} - \Delta Ct_{control}$

Fold change of test gene = $2^{-\Delta \Delta Ct}$

Fold change values were transferred to GraphPad Prism and graphs were drawn. Statistical test used depending on whether or not data were normally distributed, which was measured with D'Agostino & Pearson normality test.

With regard to sample size in each of our experiments, we did not calculate it or use pre-determined numbers. Instead, exploratory numbers were estimated, largely based on other published related studies (Hartmann *et al*., 2014, Noster *et al*., 2014, Rasouli *et al*., 2015). In addition, statistics were usually performed for exploratory purposes, to get ideas of differences and trends between groups; therefore, p value was not corrected for multiple comparisons. In addition, it is likely that many of the results are collinear (i.e., likely to vary together and thus correlated to each other) and interdependent, whereas correction for multiple comparisons requires independence of the variables measured.

Chapter 3. GM-CSF and GM-CSF receptor (GMR): Expression and Regulation by T cells and Monocytes, and relevance to MS

3.1 Introduction

GM-CSF exerts its effects through binding to its receptor, which is expressed by many cell types. This binding initiates a series of intracellular signalling that lead to activation/priming, differentiation, maturation, and mobilization of myeloid populations from bone marrow into the circulation (Hamilton, 2008). There is growing evidence in the literature for a crucial role of GM-CSF in inflammatory autoimmune conditions, including EAE and MS. In this chapter, we have studied the expression of GM-CSF and its receptor by the main PBMC subtypes, including Th cells (mainly Th1 and Th17 cells) and Tc cells in healthy controls and untreated RRMS patients in the remission stage of the disease. Our working hypothesis was that there is a higher proportion of GM-CSF expressing cells in MS patients than in healthy controls.

We also aimed to identify the pro-inflammatory cytokines that are more likely to up-regulate GM-CSF expression, and to determine whether they are increased in MS compared with controls. In addition, we planned to determine the effect of the antibody blockade of these cytokines on the expression of GM-CSF.

It is unclear whether GM-CSF is expressed in Th1 or Th17 in humans. It is also unclear if GM-CSF expression in Th cells is preferential in Th1 or Th17 cells. Only recently, a study has shown that Th1 pathway produces GM-CSF (Noster *et al.*, 2014).Therefore, GM-CSF expression was examined in Th1 (expressing IFN-G) and Th17 (expressing IL-17) in the same PBMC culture. In this thesis work, T cells were phenotyped

based on their surface markers. CD4+, and CD3+CD8+CD56- T cells are referred to as Th and Tc cells, respectively. CD3-CD14+ cells are referred to as monocytes.

Finally, GMR expression was investigated in T cells in untreated RRMS patients compared to healthy controls. However, it was shown that resting T cells do not express the β chain of GM-CSF receptor, but it was suggested that α chains are triggered in these cells by higher than normal levels of GM-CSF (Shi *et al*., 2006). Therefore, it was important to investigate α chain (CD116) expression on activated T cells in the presence of higher levels of GM-CSF in MS. We also investigated whether blocking the most elevated cytokines in cultured cells would affect the receptor's expression.

3.2 Subjects and Methods

3.2.1 Subjects (Inclusion/Exclusion Criteria)

Patients who contributed samples for PDB/I stimulation, anti-CD3+anti-CD28 stimulation (fresh and frozen/thawed samples), multiplex bead assay, and q-PCR for cultured PBMC experiments presented in this chapter had untreated RRMS. Whole blood was obtained from MS patients during relapse and remission stages of the disease to examine GM-CSF and GMR expression via q-PCR. Another set of whole blood samples were collected from patients before and after treatment with IFN-β to examine GM-CSF and GMR expression. The following criteria were applied for PDB/I and anti-CD3+anti-CD28 stimulation protocols. Patients had to be ambulatory, ≥ 18 years old, with Expanded Disability Status Scale (EDSS) range of ≥ 2 to ≤ 6.5, they should not be having exacerbation of disease within 1 month of recruitment. Exclusion criteria were: being pregnant or breast-feeding, treated with immuno-modulatory

therapies within 1-12 months (depending on the type of therapy) of recruitment or having a disease that needed to be treated with such medications, having serious infections or conditions (hepatic, renal, psychiatric, addiction, pulmonary, cardiac, or malignancy), having vaccination within 6 months of blood collection, being treated with B or T cell depleting medications, stem cells, cytotoxic agents, and having undergone total lymphoid irradiation or bone marrow transplantation.

For MS patients who participated in the q-PCR assay using whole blood during relapse/remission, the inclusion criteria were being ≥ 18 years old, and having MS with relapse; the exclusion criteria were advanced pregnancy ≥ 24 weeks, and having severe concurrent medical condition. All the important details of healthy controls and MS patients and the experiment(s) each subject has participated in are listed in appendix I.

3.2.2 Methods

All methods used in this chapter are detailed in chapter 2. All the below mentioned sample collection and experimental work was approved by Nottingham Research Ethics Committee, as shown in Appendix II. PBMCs were isolated, counted, frozen and thawed as in section 2.1 and 2.2; either PDB/I or anti-CD3+anti-CD28 stimulation were performed as in sections 2.3 and 2.4, respectively. Flow cytometry staining and data acquisition/analysis was done according to sections 2.5 and 2.7. In this thesis work, T cells were phenotyped based on their surface markers. CD4+, and CD3+CD8+CD56- T cells are referred to as Th and Tc cells, respectively. CD3-CD14+ cells are referred to as monocytes. Multiplex bead assay was performed as in section 2.8. q-PCR assay was performed as in section 2.9.

3.3 Results

3.3.1 Demographic and Clinical Characteristics of the Participants

The tables below demonstrate the demographics and clinical characteristics of healthy controls and MS patients recruited for each experiment.

Table 3-1: Demographic and clinical characteristics of subjects who contributed samples to the studies of PDB/I stimulation protocol.

Demographics	**Healthy Controls**	**Untreated RRMS**
Total no. of participants	18	17
Age mean (y) (SD)	38.61 (10.67)	46.23 (11.53)
Female no. (%)	10 (55.5)	11 (64.7)
Ethnicity:		
White (%)	9 (50)	11 (64.7)
Indian (%)	2 (11.1)	2 (11.7)
Chinese (%)	1 (5.6)	-
Other (%)	6 (33.3)	4 (23.5)
Time since MS diagnosis (y) (SD)	-	10.65 (4.2)
EDSS mean (SD)	-	3.8 (1.52)
Smoking status:		
Smoker (%)	2 (11.1)	3 (17.6)
Ex-smoker (%)	0	0

Table 3-2: Demographic and clinical characteristics of subjects who contributed samples to the studies of fresh PBMCs stimulated according to anti-CD3+anti-CD28 protocol

Demographics	**Healthy Controls**	**Untreated RRMS**
Total no. of participants	10	9
Age mean (y) (SD)	40 (8.71)	43.89 (7.62)
Female no. (%)	7 (70)	6 (66.6)
Ethnicity:		
White (%)	7 (70)	8 (88.8)
Chinese (%)	1 (10)	-
Other (%)	2 (20)	1 (11.1)
Time since MS diagnosis (y) (SD)	-	9.77 (3.1)
EDSS mean (SD)	-	3.2 (1.8)
Smoking status:		
Smoker (%)	0	1 (11.1)
Ex-smoker (%)	1 (10)	0

Table 3-3: Demographic and clinical characteristics of subjects who contributed samples to the studies of frozen PBMCs stimulated according to anti-CD3+anti-CD28 protocol

Demographics	**Healthy Controls**	**Untreated RRMS**
Total no. of participants	11	10
Age mean (y) (SD)	36 (11.4)	35.58 (8.78)
Female no. (%)	9 (81.8)	8 (80)
Ethnicity:		
White (%)	8 (72.7)	8 (80)
Other (%)	3 (27.2)	2 (20)
Time since MS diagnosis (y) (SD)	-	5.37 (2.47)
EDSS mean (SD)	-	2.75 (0.65)
Smoking status:		
Smoker (%)	0	0
Ex-smoker (%)	0	0

Table 3-4: Demographic and clinical characteristics of subjects who contributed samples to the studies of Multiplex Bead Assay experiment.

Demographics	**Healthy Controls**	**Untreated RRMS**
Total no. of participants	10	14
Age mean (y) (SD)	40 (10.3)	44.86 (10.73)
Female no. (%)	3 (60)	5 (71.4)
Ethnicity:		
White (%)	8 (80)	12 (85.7)
Chinese (%)	1 (10)	-
Other (%)	1 (10)	2 (14.2)
Time since MS diagnosis (y) (SD)	-	8.46 (3.52)
EDSS mean (SD)	-	3.31 (1.6)
Smoking status:		
Smoker (%)	0	1 (7.1)
Ex-smoker (%)	1 (20)	1 (7.1)

Table 3-5: Demographic and clinical characteristics of subjects who contributed samples to the studies of q-PCR/cultured PBMCs experiment

Demographics	Healthy Controls	Untreated RRMS
Total no. of participants	9	8
Age mean (y) (SD)	40.56 (8.83)	39 (10.66)
Female no. (%)	6 (66.6)	4 (50)
Ethnicity:		
White (%)	7 (77.7)	7 (87.5)
Other (%)	2 (22.2)	1 (12.5)
Time since MS diagnosis (y) (SD)	-	9.27 (2.9)
EDSS mean (SD)	-	3.3 (1.51)
Smoking status:		
Smoker (%)	0	1 (12.5)
Ex-smoker (%)	1 (11.1)	0

Table 3-6: Demographic and clinical characteristics of subjects who contributed samples to the studies of GM-CSF/GMR expression during MS relapse and remission experiment

Demographics	Healthy Controls	MS patients during relapse
Total no. of participants	15	11
Age mean (y) (SD)	41.07 (9.15)	39.91 (10.18)
Female no. (%)	10 (66.6)	7 (63.6)
Ethnicity:		
White (%)	13 (86.6)	11 (100)
Other (%)	2 (13.3)	-
Time since MS diagnosis (y) (SD)	-	6.77 (3.7)
EDSS mean (SD)	-	4 (1.22)
Smoking status:		
Smoker (%)	1 (6.6)	3 (27.2)
Ex-smoker (%)	1 (6.6)	0

Table 3-7: Demographic and clinical characteristics of subjects who contributed samples to the studies of GM-CSF/GMR expression before/after IFN-β therapy experiment.

Demographics	Healthy Controls	MS patients during relapse
Total no. of participants	15	7
Age mean (y) (SD)	41.07 (9.15)	42.43 (9.82)
Female no. (%)	10 (66.6)	5 (71.4)
Ethnicity:		
White (%)	13 (86.6)	7 (100)
Other (%)	2 (13.3)	-
Time since MS diagnosis (y) (SD)	-	4 (2.9)
EDSS mean (SD)	-	2 (0.67)
Smoking status:		
Smoker (%)	1 (6.6)	0
Ex-smoker (%)	1 (6.6)	1 (14.2)

3.3.2 Th1 and Th17 Cells Express GM-CSF, with higher frequency of GM-CSF-Expressing Th1 in MS patients

PBMCs were thawed and stimulated with anti-CD3+anti-CD28 for 5 days then re-stimulated with PMA/I in the last 5 hours. They were stained and gated for live CD4 lymphocytes. To understand T cell frequencies and their cytokine expression in MS, a number of parameters were set for comparison between healthy controls and MS patients. Th1 and Th17 proportions, as well as co-expression of IFN-γ/GM-CSF and IL-17/GM-CSF were assessed in healthy controls and MS patients. Figure 3-1 illustrates how cell were gated and how quadrants were drawn in flow cytometry plots based on using FMO controls. Consistent with the literature (Rasouli *et al.*, 2015), Th1 cells were in significantly higher proportion than Th17 cells among all CD4 lymphocytes (Figure 3-2 A). However, there was no difference in Th1 or Th17 cell frequency between healthy controls (n=11) and MS patients (n=10). As a consequence, the percentage of GM-CSF+IFN-γ+ (GM-CSF-producing Th1 cells) was significantly higher than GM-CSF+IL-17+ (GM-CSF-producing Th17) in stimulated live CD4+ T cells in both

healthy controls (n=11, p=0.001 Wilcoxon test) and MS patients (n=10, p=0.002, Wilcoxon test) (Figure 3-2 B).

After gating for Th1 and Th17 cells only (not among CD4+ PBMCs), their GM-CSF expression was also examined (Figure 3-3). Generally, we found that GM-CSF was expressed by Th1 and Th17 cells in healthy controls and MS patients. In addition, the frequency of GM-CSF-expressing cells differed with the type and duration of stimulation, the lowest was with 16 hours PDB/I stimulation (median around 15%) (Figure 3-3 A). Higher frequency of thawed cells expressed GM-CSF after stimulation following anti-CD3+anti-CD28 protocol (around 30%) (Figure 3-3 B). The frequency of Th1 cells expressing GM-CSF generally appeared to be higher in MS patients than in healthy controls, which has reached statistical significance (healthy n=10, MS n=9, p=0.024 unpaired t-test) in fresh PBMCs stimulated with anti-CD3+anti-CD28 protocol (Figure 3-3 C). In these cells, GM-CSF expression by Th17 was not done, while Th17 responded with a similar frequency of GM-CSF expression outcome in healthy controls and MS patients. Cell frequencies were also checked based on cell marker expression (CD3-CD8+, CD3+CD8+) and were not different before/after anti-CD3+anti-CD28 stimulation.

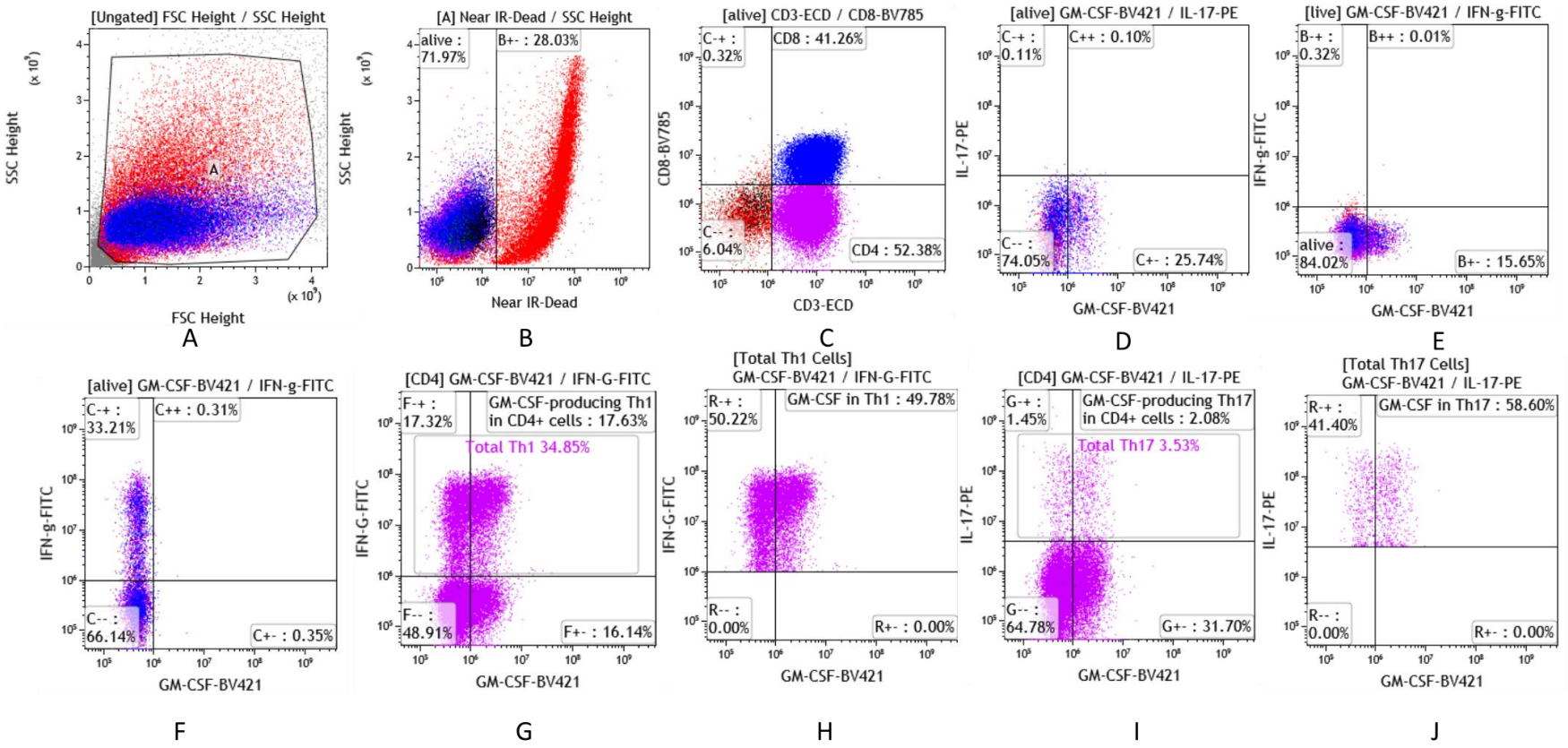


Figure 3-1: Flow cytometry plots of GM-CSF expression in Th1 and Th17 cells.

Cells were gated according to the following pattern after anti-CD3+anti-CD28 stimulation protocol. **A:** forward scatter (FSC) and side scatter (SSC) plot of cultured PBMCs **B:** only live cells were included in the plots after excluding the dead cells (red coloured cells) **C:** identification of CD4 (CD3+CD8-) T cells (purple coloured cells) **D, E, F:** FMO for IL-17, IFN-γ, and GM-CSF, respectively, all in live cells. **G, I:** GM-CSF versus IFN-γ or IL-17 plots in CD4 cells only (total Th1 and Th17 frequencies are calculated from these plots). **H, J:** GM-CSF expression in Th1 and Th17 populations, respectively.

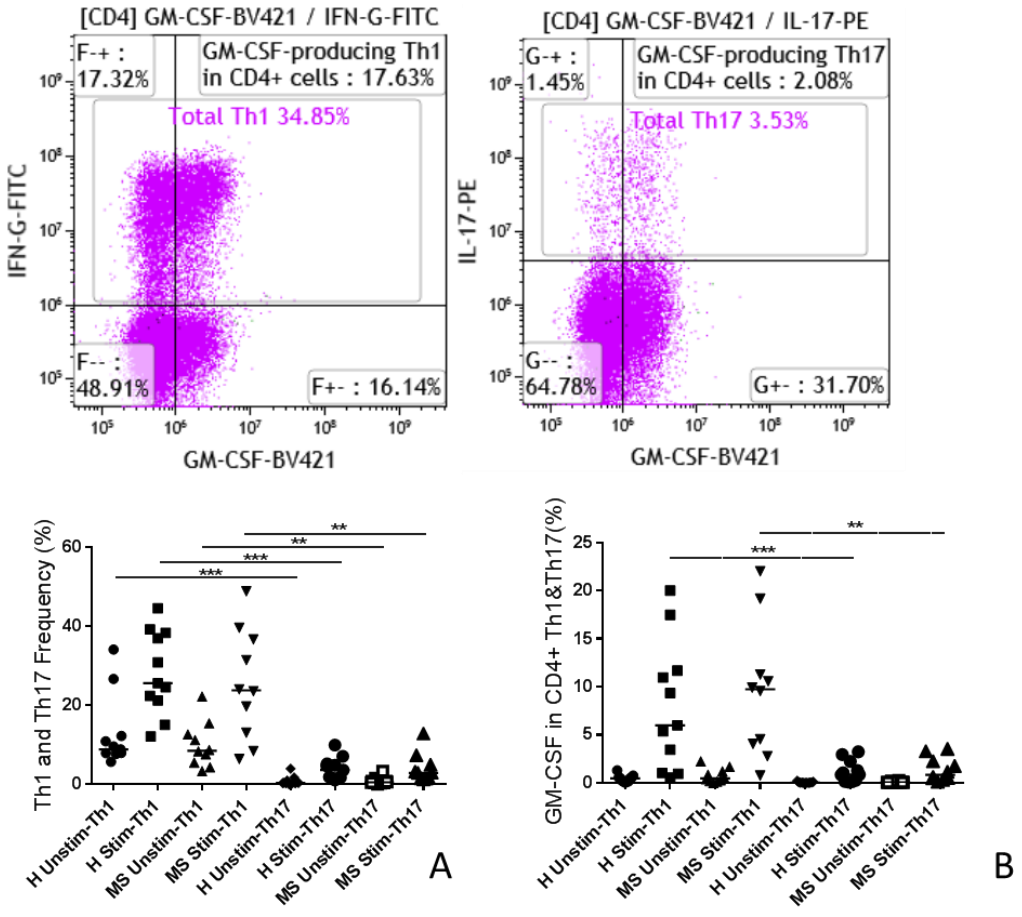


Figure 3-2: Th1 and Th17 cell frequencies and their GM-CSF expression among CD4+ cells in healthy controls and MS patients.

After culturing PBMCs following PDB/I protocol, only live CD3+CD8- cells (representing CD4+ cells) were selected in the flow cytometry plots above, gating is shown in Figure 3-1. **A:** frequency of Th1 (CD4+IFN-γ+) and Th17 (CD4+IL-17+) cells among CD4 T cells. **B:** frequency of GM-CSF-producing Th1 and Th17 among CD4 cells. Only double positive populations are selected in this plot (from the representative flow cytometry plots in the upper panel) of GM-CSF with either IFN-γ or IL-17, respectively. Unstim: unstimulated; Stim: stimulated; H: healthy controls; MS: multiple sclerosis patients. ** $p \leq 0.01$; *** $p \leq 0.001$. Horizontal lines are medians.

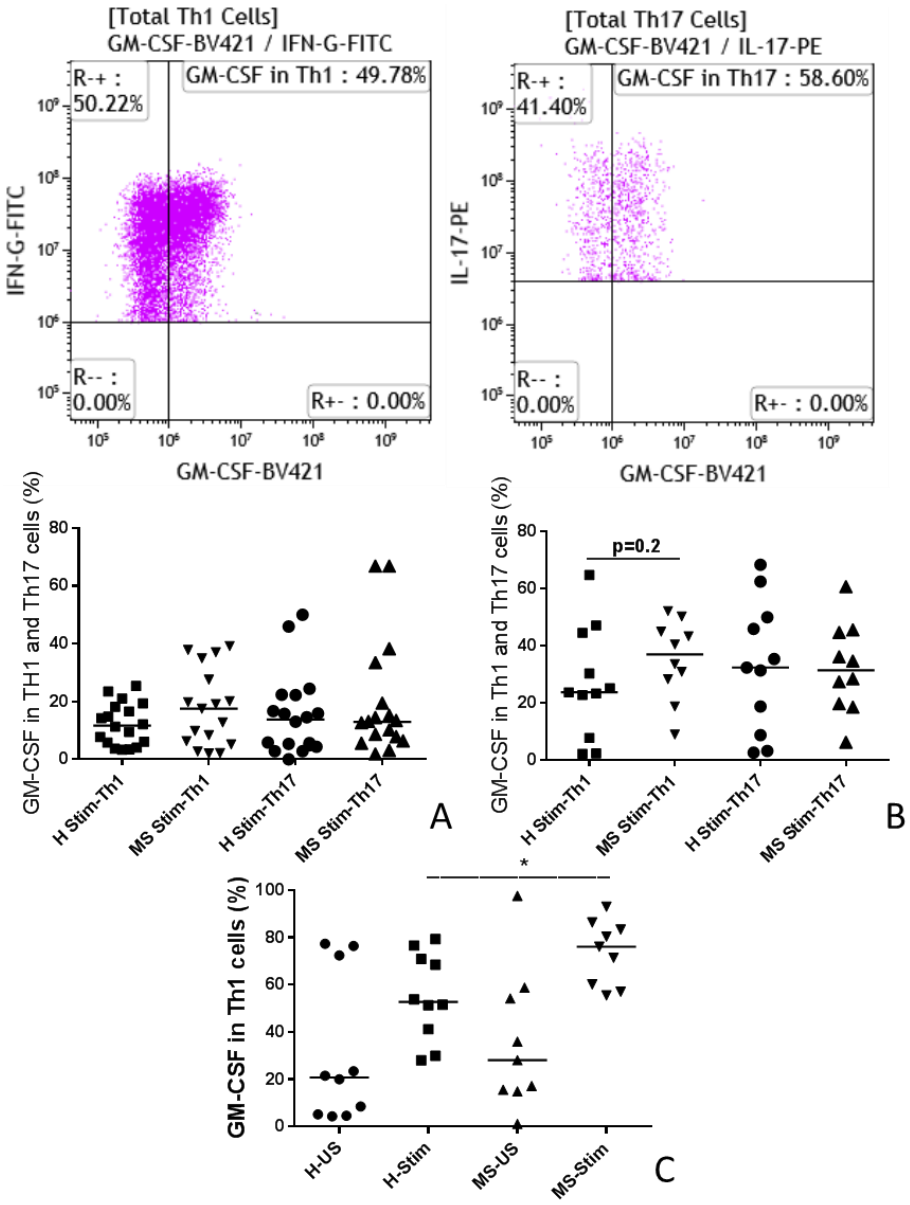


Figure 3-3: Frequency of GM-CSF-expressing Th1 and Th17 cells in healthy controls and MS patients.

After culturing PBMCs following different protocols (specified below), cells were gated as in Figure 3-1, selecting only live CD4 cells. Then, only Th1 (CD4+IFN-γ) and Th17 (CD4+IL-17+) were drawn in the flow cytometry plots (upper panel). Lower plots represent the double positive populations from the upper panels in this figure. This was measured in after culturing PBMCs in different conditions: A: following PDB/I stimulation protocol; B: frozen and thawed cells following anti-CD3+anti-CD28 protocol; C: fresh cells following anti-CD3+anti-CD28 protocol. US: unstimulated; Stim: stimulated; H: healthy controls; MS: multiple sclerosis patients. * $p \leq 0.05$. Horizontal lines are medians.

3.3.3 GM-CSF Expression by Th and Tc is Significantly higher in MS Patients

Fresh and frozen/thawed PBMCs were stimulated with anti-CD3/anti-CD28 for 5 days and re-stimulated with PMA/I for the last 5 hours. The percentage of gated CD4+ lymphocytes (Th) expressing GM-CSF was significantly higher in MS patients than in healthy controls using unpaired t-test (fresh cells: healthy n=10, MS n=9, $p<0.0001$; frozen/thawed: healthy n=11, MS n=10, $p=0.02$) (Figure 3-4). In addition, the frequency of gated CD3+CD8+CD56- lymphocytes (Tc) expressing GM-CSF was also significantly higher in MS patients than in healthy controls using unpaired t-test (fresh cells: healthy n=10, MS n=9, $p=0.0002$; frozen/thawed: healthy n=11, MS n=10, $p=0.03$). GM-CSF gene expression in the same stimulated PBMCs showed statistically significant increase in MS when compared to unstimulated samples (paired t-test $p=0.02$, n=8), but no difference when compared to stimulated healthy control samples (unpaired t-test $p=0.18$, healthy n=9, MS n=8) (Figure 3-4).

There was a difference between the frequency of GM-CSF-expressing cells in fresh and frozen conditions, which reached significance in MS patients but not in healthy controls. In comparing fresh and frozen GM-CSF-expressing CD3+CD8- cells, unpaired t-test was used (fresh MS n=9, frozen MS n=10, $p < 0.0001$) (fresh healthy n=10, frozen healthy n=11, $p=0.08$). Unpaired t-test was also used in comparing fresh and frozen GM-CSF-expressing CD3+CD8+CD56- cells (fresh MS n=9, frozen MS n=10, $p < 0.0001$) (fresh healthy n=10, frozen healthy n=11, $p=0.09$).

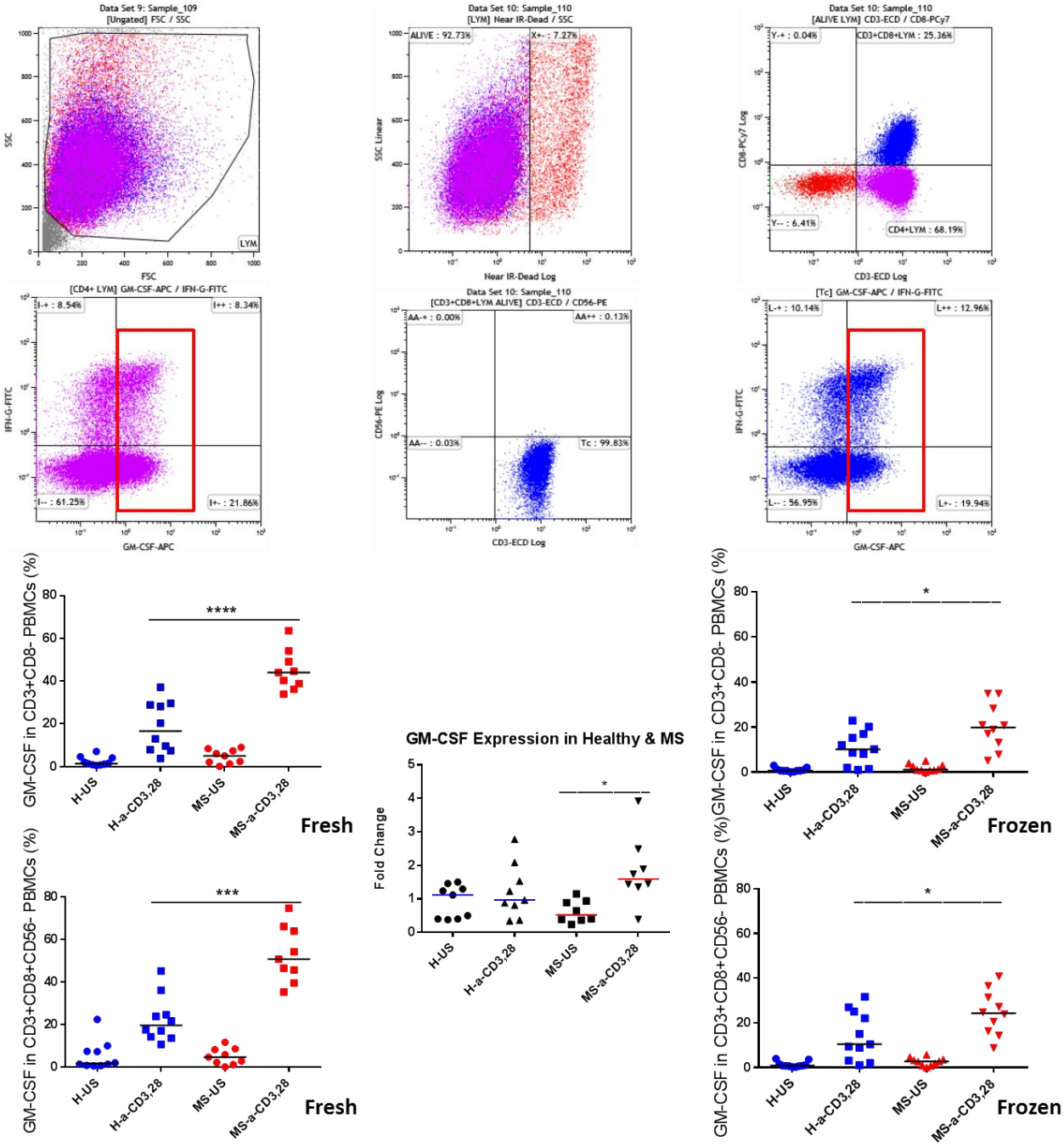


Figure 3-4: GM-CSF Expression in Th and Tc lymphocytes.

Fresh and frozen/thawed PBMCs were cultured under same stimulation conditions (anti-CD3+anti-CD28 protocol). Upper part shows representative flow cytometry plots. Gating cells and quadrants were drawn as in Figure 3-1. Th cells were identified as CD3+CD8- (pink coloured cells), Tc cells were identified as CD3+CD8+CD56- (purple coloured cells). Percentages calculated are the ones outlined in red in flow cytometry plots above. Middle graph: q-PCR results showing fold change in GM-CSF gene expression following same stimulation protocol. H-US, MS-US: unstimulated samples from healthy controls and MS patients, respectively. H-a-CD3,28, MS-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from healthy controls and MS patients, respectively.*p<0.05; ***p<0.001; ****p<0.0001. Horizontal lines are medians.

3.3.4 Anti-CD3+anti-CD28 Stimulation Yielded More Expression of GM-CSF, IFN-γ, and IL-17 than PDB/I Alone in PBMCs

Fresh PBMCs were stimulated with either PDB/I for 16 hours or anti-CD3+anti-CD28 for 5 days then PMA/I for the last 5 hours. Our results show significant differences, with higher frequencies of CD4+ and CD3+CD8+CD56- cells expressing the pro-inflammatory cytokines GM-CSF, IFN-γ, and IL-17 when using the latter protocol (Figure 3-4, Figure 3-5, Figure 3-6, and Figure 3-7). To confirm this finding, three of the healthy controls have participated in both experiments, and the latter protocol has shown more induction of the pro-inflammatory cytokines GM-CSF, IFN-γ, and IL-17 (Figure 3-8).

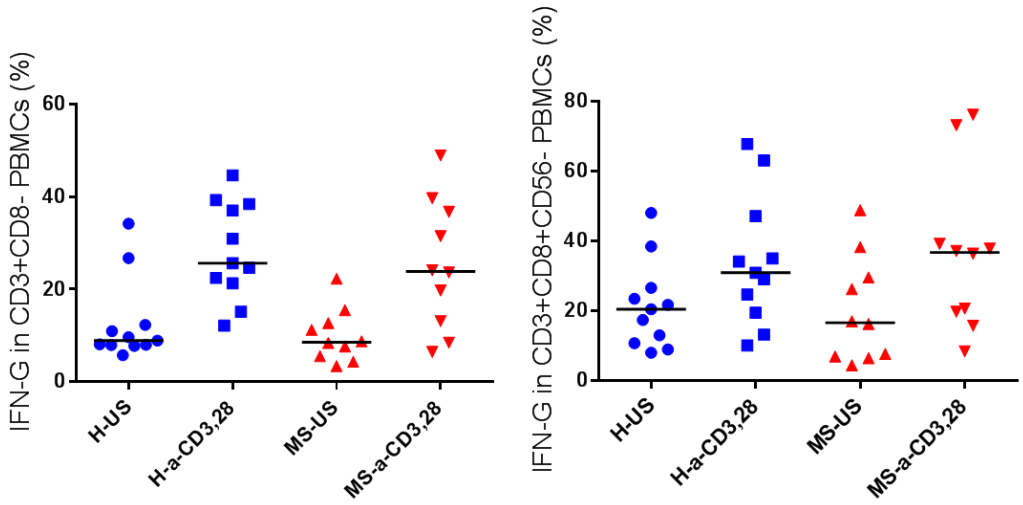


Figure 3-5: IFN-γ Expression in Th and Tc lymphocytes.

Frozen/thawed PBMCs were cultured and stimulated following anti-CD3/anti-CD28 protocol. Gating and drawing quadrants was done as in Figure 3-1 and 3-4 above. Th cells are identified as CD3+CD8- cells, Tc cells are identified as CD3+CD8+CD56- cells. H-US, MS-US: unstimulated samples from healthy controls and MS patients, respectively. H-a-CD3,28, MS-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from healthy controls and MS patients, respectively. Horizontal lines are medians.

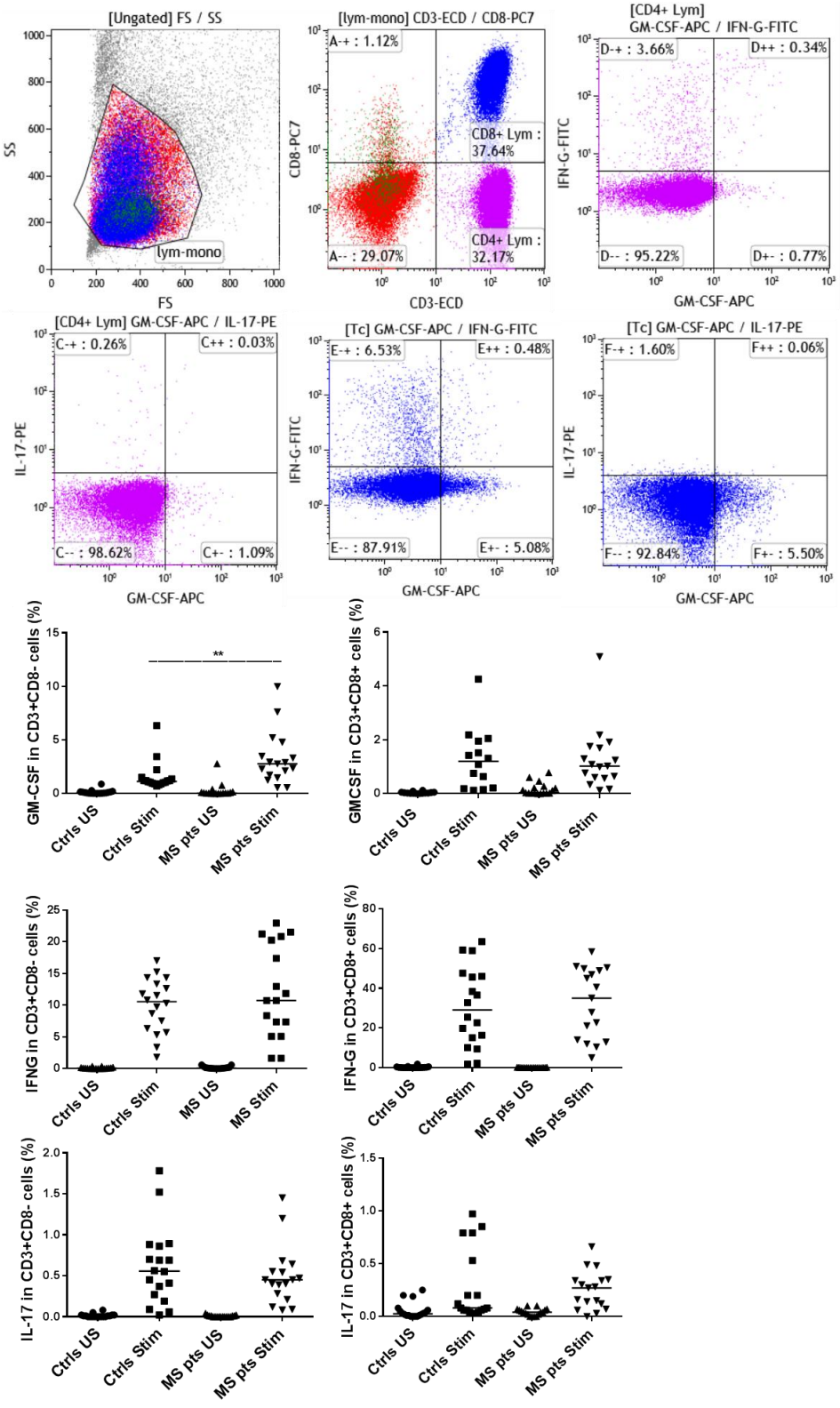


Figure 3-6: Expression of GM-CSF, IFN-G, and IL-17 in Th and Tc lymphocytes.

Fresh PBMCs were left either unstimulated or stimulated with PDB/I for 16 hrs. Healthy controls (Ctrls) (n=18) and MS patients (MS pts) (n=17). Gating and drawing quadrants was done as in Figure 3-1 and 3-4 above, except that dead cell stain was not used in this experiments. Th (CD3+CD8- cells), Tc (CD3+CD8+ cells). US: unstimulated; Stim: stimulated. ** $p<0.01$. Horizontal lines are medians.

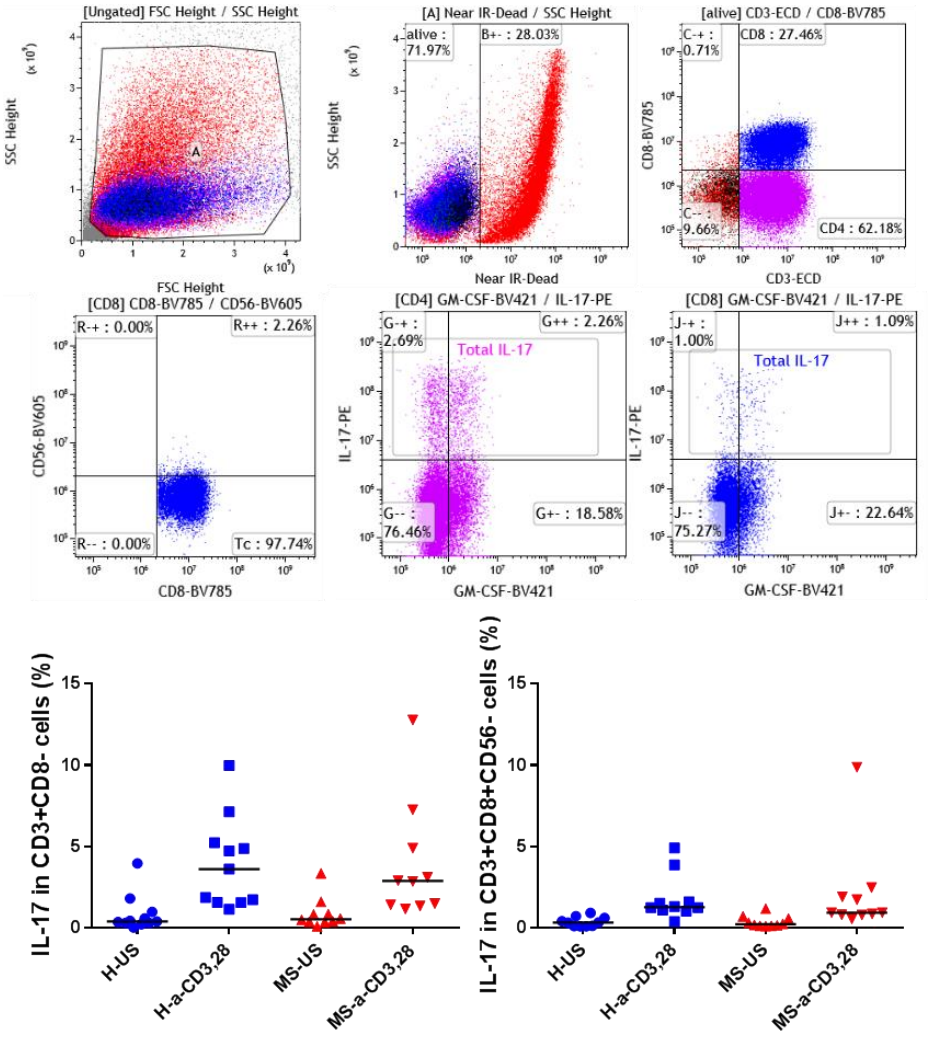


Figure 3-7: Expression of IL-17 in Th and Tc lymphocytes.

Frozen PBMCs were cultured with/without stimulation (anti-CD3+anti-CD28 protocol) in healthy controls (n=11) and untreated RRMS (n=10). Gating and drawing quadrants was done as in Figure 3-1 and 3-4 above. Percentages calculated are the ones outlined within the last two flow cytometry plots and named (total IL-17). Th cells are identified as CD3+CD8- cells, Tc cells are identified as CD3+CD8+CD56- cells. H-US, MS-US: unstimulated samples from healthy controls and MS patients, respectively. H-a-CD3,28, MS-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from healthy controls and MS patients, respectively. Horizontal lines are medians.

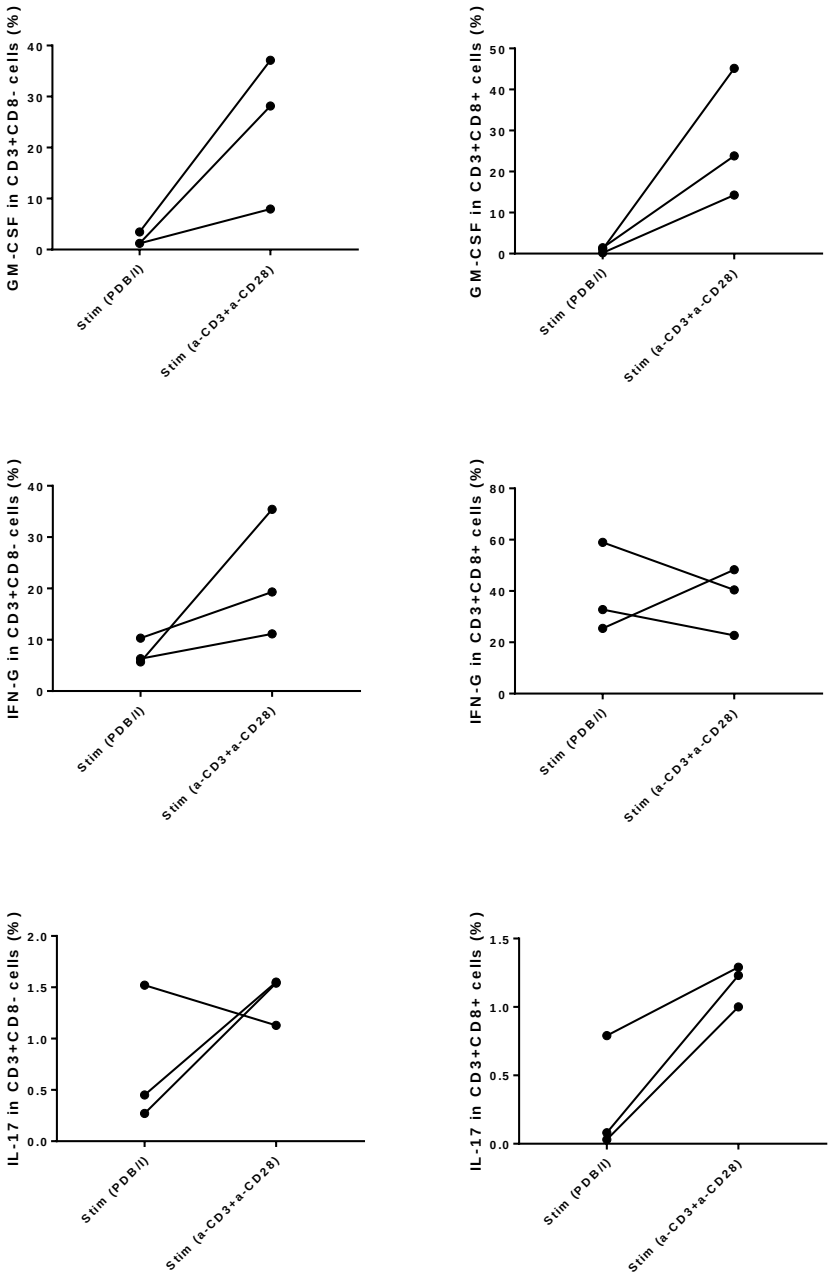


Figure 3-8: Frequency of GM-CSF, IFN-γ, and IL-17 in same healthy volunteers using different stimulation protocol.

Left panel (from top to bottom): frequency of GM-CSF, IFN-γ, and IL-17 in CD3+CD8- cells. Right panel (from top to bottom): frequency of the above-mentioned cytokines in CD3+CD8+ cells. PBMCs were isolated from three healthy volunteers and stimulated with either following PDB/I (Stim (PDB/I)) or anti-CD3+anti-CD28 (Stim (a-CD3+a-CD28)) protocols.

3.3.5 Frequencies of Th and Tc Cells Expressing GM-CSF+IFN-γ+ and GM-CSF+IL-17+ in MS Patients and Healthy Controls

Fresh and frozen/thawed PBMC stimulation with anti-CD3+anti-CD28 for 5 days, then re-stimulated with PMA/I for the last 5 hours have led to elevated expression of Th and Tc cells expressing GM-CSF+IFN-γ+ in MS patients (i.e., increased frequency of Th1 and Tc1 cells expressing GM-CSF among Th and Tc cells, respectively). However, statistical significance was achieved in fresh but not in thawed cells using unpaired t-test (healthy n=10, MS n=9, p=0.005 in Th cells, p=0.001 in Tc cells) (Figure 3-9). On the other hand, frozen PBMC stimulation (anti-CD3+anti-CD28 protocol) revealed no changes in the frequency of Th and Tc cells expressing GM-CSF+IL-17+ (Th HC n=11, MS n=10, p=0.76 unpaired t-test) (Tc HC n=11, MS n=10, p=0.69 Mann-Whitney test).

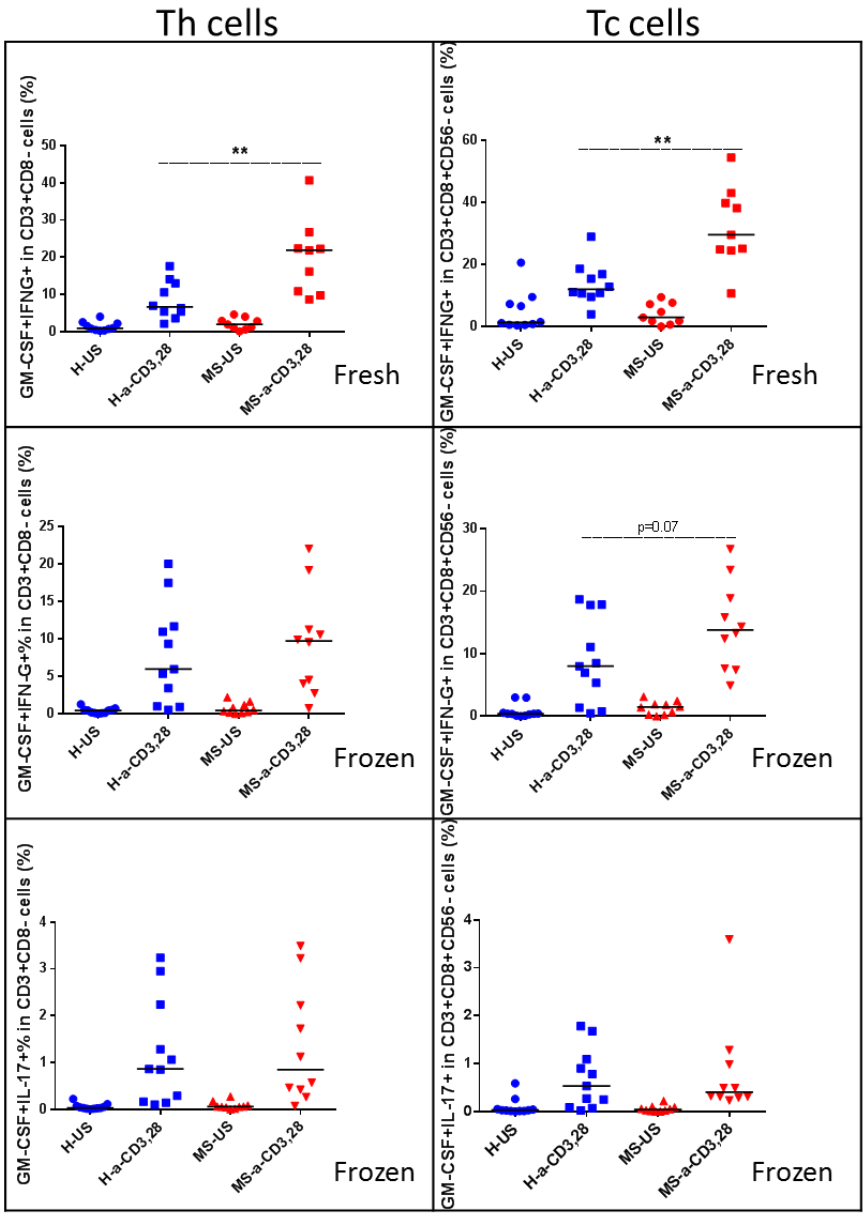


Figure 3-9: Co-expression of GM-CSF/IFN-G and GM-CSF/IL-17 in Th and Tc Lymphocytes.

Upper panel: fresh PBMCs. Middle and lower panel: frozen/thawed PBMCs. All cells were stimulated following anti-CD3+anti-CD28 protocol in healthy controls and untreated RRMS patients. Th cells were identified as CD3+CD8- cells, and Tc cells were identified as CD3+CD8+CD56- cells. Gating and drawing quadrants was done as in Figure 3-1 and 3-7 above. H-US, MS-US: unstimulated samples from healthy controls and MS patients, respectively. H-a-CD3,28, MS-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from healthy controls and MS patients, respectively. ** $p<0.01$. Horizontal lines are medians.

3.3.6 CD3-CD14+ Cells Exhibit Higher GM-CSF Expression in MS

Fresh PBMCs were either left unstimulated or stimulated with PDB/I for 16 hours and stained for GM-CSF in monocytes (CD3-CD14+) in healthy controls (n=18) and MS patients (n=17). Isotype control antibody was used in this experiment. Results show significantly higher frequency of GM-CSF-expressing gated stimulated monocytes in MS patients than controls (Mann-Whitney test, p=0.0449) (Figure 3-10). Removing the two outliers yielded only a non-significant trend (p=0.18); however, looking at the demographic and clinical characteristics of the two outliers showed no special features that would provide a rationale for removing them.

3.3.7 Supernatants of PBMC from MS Patients Contain Higher levels of IL-2, IL-12, IL-1β, GM-CSF, and lower levels of TGF-β

After PBMC isolation and stimulation (anti-CD3+anti-CD28) for 5 days, supernatants were tested to detect the following cytokine concentrations (GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-7, IL-10, IL-12p70, IL-15, IL-17, IL-18, IL-23, and TGF-β1). The concentrations of GM-CSF, IL-2, IL-12, and IL-1β were significantly increased in MS. On the other hand, there was a statistically significant reduction in TGF-β1 levels in culture supernatants of MS patients, with no differences among the rest of the cytokines tested (Figure 3-11). No detectable IL-15 or IL-18 levels were seen. After obtaining these results, the hypothesis was that blocking one or more of these cytokines in PBMC culture can reduce GM-CSF expression by PBMC subtypes tested.

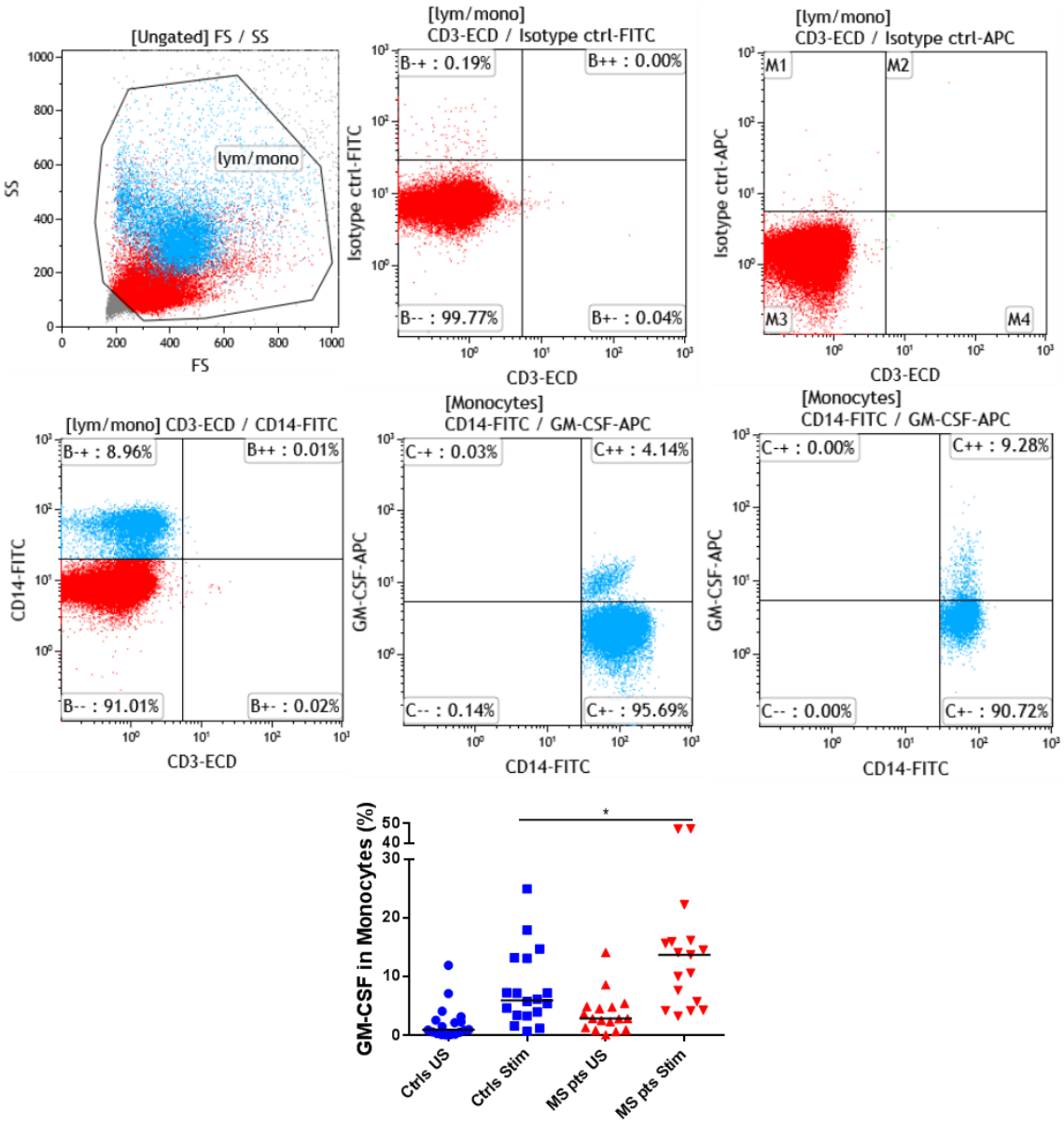


Figure 3-10: GM-CSF expression by monocytes in cultured PBMCs.

Gated monocytes (CD3-CD14+ Cells), unstimulated (lower middle plot) or stimulated (lower right plot) with PDB/I for 16 hours in healthy controls (Ctrls) (n=18) and MS patients (MS pts) (n=17). Quadrants were set according to isotype controls in this experiment. US: unstimulated; Stim: stimulated * $p<0.05$. Horizontal lines are medians.

3.3.8 Blocking IL-1β Reduced Frequency of Th cells expressing IL-17 in Healthy Controls but not in MS Patients

Frozen stimulated PBMCs (anti-CD3+anti-CD28 protocol) were treated with antibodies for IL-2, IL-12, IL-12/23, and IL-1β. We found that the percentage of IL-17-expressing CD4+ cells was reduced by blocking IL-1β in healthy controls but not in MS patients (Figure 3-12 E) (MS n=7, Wilcoxon test p=0.21; healthy n=7, Wilcoxon test p=0.03).

3.3.9 Blocking IL-12 Reduced GM-CSF-Expressing Tc Cells in MS

Thawed PBMCs were stimulated (anti-CD3+anti-CD28 protocol) in the presence of cytokine blocking, followed by flow cytometry staining. We show that blocking IL-12 significantly reduced the frequency of Tc lymphocytes expressing GM-CSF in MS patients but not in healthy control samples (Figure 3-13 A and B). Wilcoxon test was used for comparison, (MS n=7, p=0.046; HC n=7, p=0.57).

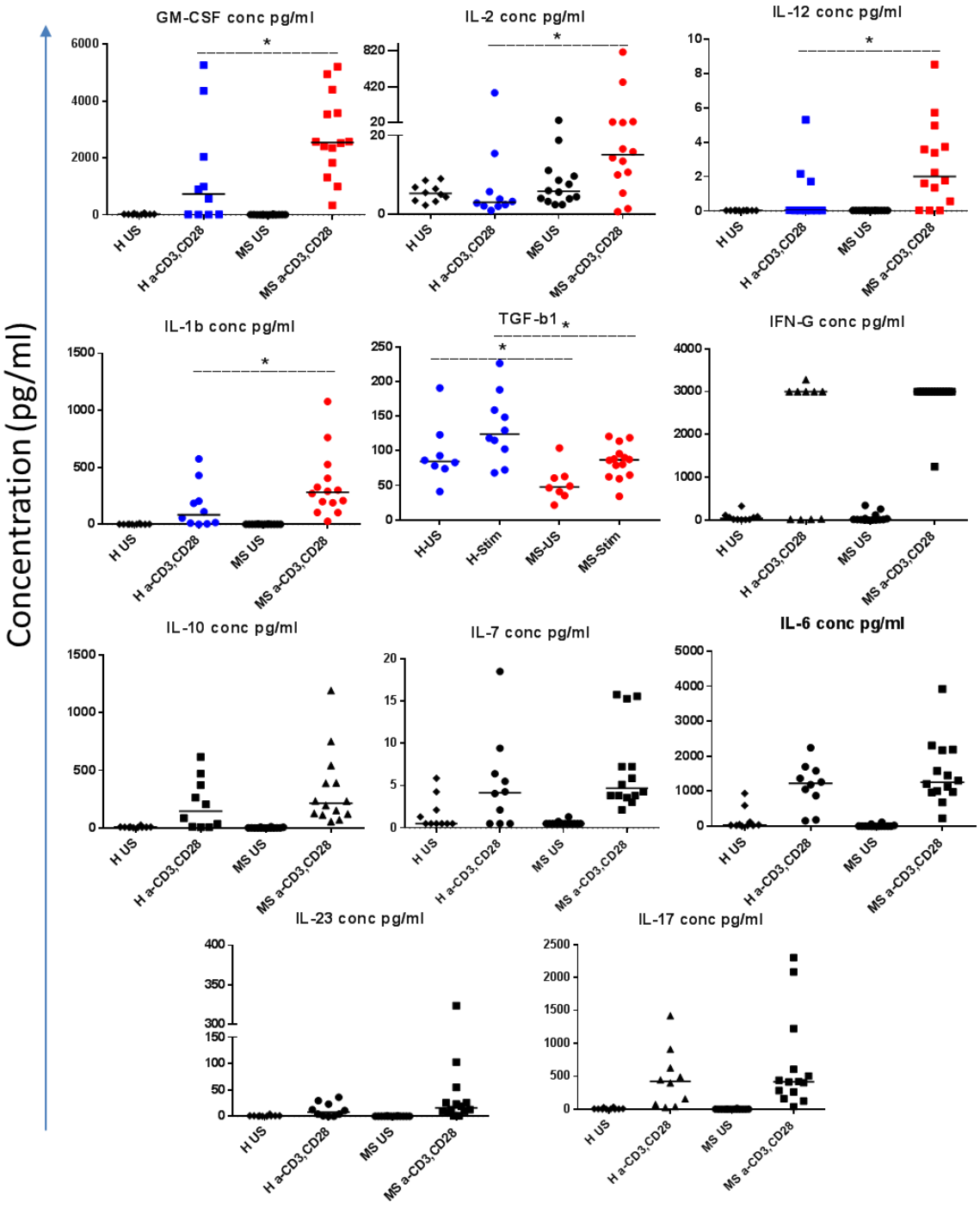


Figure 3-11: Cytokine concentrations (pg/ml) in PBMC culture supernatants.

Fresh PBMCs were cultured following anti-CD3+anti-CD28 protocol. Culture supernatants were taken on day 5 from healthy controls (n=10) and MS patients (n=14). H-US, MS-US: unstimulated samples from healthy controls and MS patients, respectively. H-a-CD3,28, MS-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from healthy controls and MS patients, respectively. * $p<0.05$. Horizontal lines are medians.

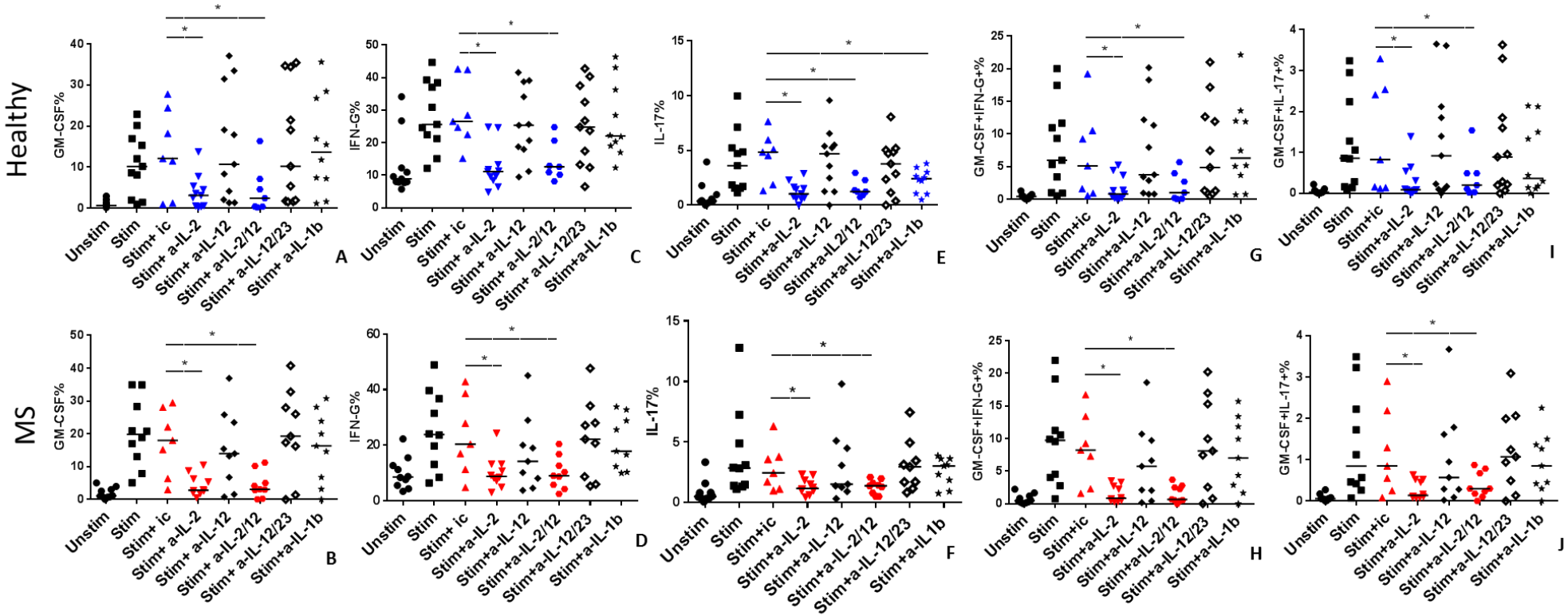


Figure 3-12: Effects of anti-cytokine antibodies on the frequency of CD4 lymphocytes expressing GM-CSF, IFN-γ, IL-17, GM-CSF/IFN-γ, and GM-CSF/IL-17.

Frozen/thawed cultured PBMCs in healthy controls (H) (n=11) and MS patients (n=10). PBMCs were cultured either unstimulated (Unstim) or stimulated (Stim) with anti-CD3+anti-CD28 for 5 days. This was done with or without adding one of the antibodies to block the following cytokine (a-cytokine: cytokine antibody): IL-2, IL-12, IL-2+IL-12 (IL-2/IL-12), IL-12+IL-23 (IL-12/IL-23), and IL-1β. In addition, cells were cultured with antibody isotype control (ic) as controls. Only live CD3+CD8- cells were selected and analysed for the above-listed parameters. Upper row represents healthy control results, lower row represents MS patient results * $p<0.05$. Horizontal lines represent medians.

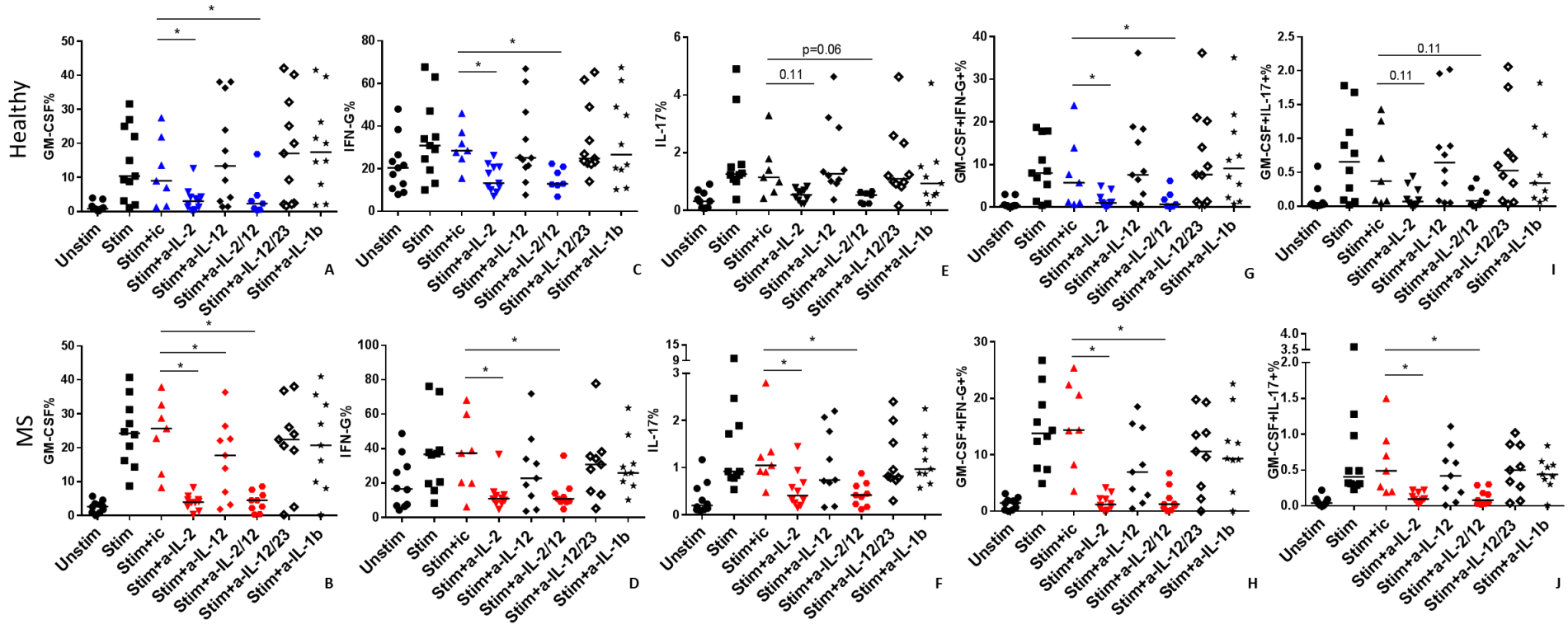


Figure 3-13: Effects of anti-cytokine antibodies on the frequency of Tc lymphocytes expressing GM-CSF, IFN-γ, IL-17, GM-CSF/IFN-γ, and GM-CSF/IL-17.

Frozen/thawed cultured PBMCs in healthy controls (H) (n=11) and MS patients (n=10). PBMCs were cultured either unstimulated (Unstim) or stimulated (Stim) with anti-CD3+anti-CD28 for 5 days. This was done with or without adding one of the antibodies to block the following cytokine (a-cytokine: cytokine antibody): IL-2, IL-12, IL-2+IL-12 (IL-2/IL-12), IL-12+IL-23 (IL-12/IL-23), and IL-1β. In addition, cells were cultured with antibody isotype control (ic) as controls. Only live CD3+CD8+CD56- cells were selected and analysed for the above-listed parameters. Upper row represents healthy control results, lower row represents MS patient results * $p<0.05$. Horizontal lines represent medians.

3.3.10 No Difference in GM-CSF or GMR Gene Expression in MS Patients during Relapse or Remission or After IFN-β Therapy

Total RNA isolation, cDNA synthesis, and TaqMan® q-PCR assay were performed. Our data analysis show no difference in GM-CSF or GMR gene expression among healthy (n=15) and the same MS patients during relapse and remission stages of the disease (n=11) (Figure 3-14 A and B) (Table 3-8). Similarly, no difference was noticed among healthy controls (n=15) and the same MS patients before, 6 months, and 1 year after IFN-β therapy (n=7) in GM-CSF and GMR gene expression (Kruskall-Wallis test p=0.98 and 0.06, respectively) (Figure 3-14 C and D) (Table 3-9).

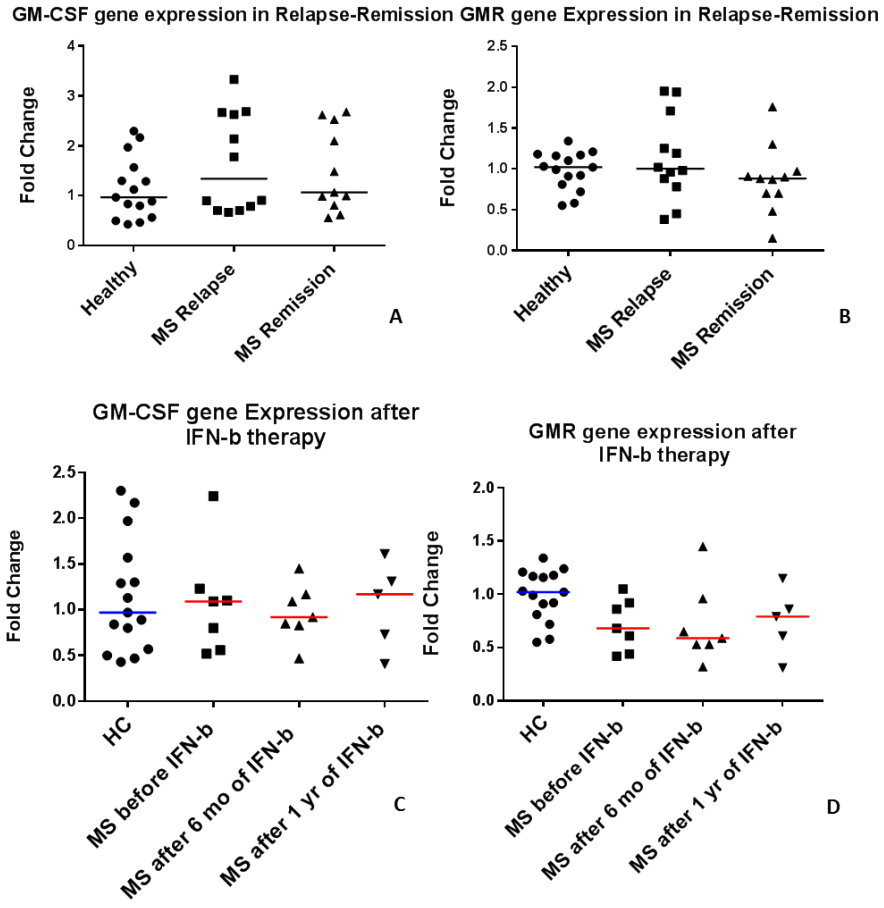


Figure 3-14: GM-CSF and GMR gene expression under different *in vivo* conditions.

Whole blood was used, total RNA was isolated, cDNA was synthesized, and q-PCR was performed on these samples. A and B: Healthy controls (n=15) and MS patients (n=11) during relapse and remission, one of whom had two relapses then remission; C and D: healthy controls (HC) and RRMS patients before and after IFN-β therapy. Horizontal lines are medians.

Table 3-8: GM-CSF and GMR fold change in gene expression in healthy controls and MS patients during relapse and remission stages.

Numbers represent medians. HC: healthy controls (n=15); MS patients (n=11) were checked during relapse and remission (same population of MS patients).

Fold change	HC	MS relapse	MS remission
GM-CSF	0.97	1.3	1.1
GMR	0.98	0.99	0.88

Table 3-9: GM-CSF and GMR fold change in gene expression in healthy controls and MS patients before/after IFN-β therapy.

Numbers represent medians. HC: healthy controls (n=15); MS patients (n=7), all of them where checked before and 6 months (6 Mo) after IFN-β therapy, while five of them were checked after 1 year (1 yr) of IFN-β therapy.

Fold change	HC	MS before IFN-β therapy	MS 6 Mo after IFN-β therapy	MS 1 yr after IFN-β therapy
GM-CSF	0.97	1.1	0.92	1.2
GMR	1.02	0.68	0.59	0.79

Table 3-10 below illustrates a summary of the significant findings in this chapter that will be discussed in the next section.

Table 3-10: Main findings of work illustrated in chapter three.

Result (compared to healthy controls)	Significance test	P value
Higher frequency of GM-CSF-expressing cells Th1 in MS	Unpaired t-test	0.024
Higher frequency of GM-CSF-expressing Th cells in MS	Unpaired t-test	<0.0001
Higher frequency of GM-CSF-expressing Tc cells in MS	Unpaired t-test	0.0002
Higher frequency of GM-CSF+IFN-γ+-expressing Th cells in MS	Unpaired t-test	0.005
Higher frequency of GM-CSF+IFN-γ+-expressing Tc cells in MS	Unpaired t-test	0.001
Higher frequency of GM-CSF-expressing monocytes in MS	Mann-Whitney	0.044
Higher levels of GM-CSF in culture supernatants in MS	Unpaired t-test	0.02

Higher levels of IL-2, IL-12, and IL-1β in culture supernatants in MS (p values are shown respectively)	Mann-Whitney	0.04 0.02 0.04
Lower levels of TGF-β in culture supernatants in MS	Unpaired t-test	0.014
Lower frequency of IL-17-expressing Th cells after blocking IL-1β in healthy controls but not in MS patients	Wilcoxon test	0.03
Lower frequency of GM-CSF-expressing Tc cells after blocking IL-12 in MS	Wilcoxon test	0.046

3.4 Discussion

Our results show that both Th1 and Th17 cells express GM-CSF in stimulated human PBMCs. Th1 and Th17 phenotyping was based upon their surface staining (CD3+CD8-), and cytokine expression (IFN-γ and IL-17, respectively). The anti-CD3+anti-CD28 stimulation protocol was followed by several research groups (Hartmann *et al*., 2014, Rasouli *et al*., 2015). It is still unclear whether Th1 or Th17 are more encephalitogenic due to their GM-CSF production, although the data from EAE suggests so (El-Behi *et al.*, 2011). Therefore, the exact role of GM-CSF derived by either Th1 or Th17 in encephalitogenicity is not yet completely clarified.

Some EAE studies have shown that Th17-derived GM-CSF is essential for their encephalitogenicity (Codarri *et al*., 2011, El-Behi *et al*., 2011). We, on the other hand, showed that the frequency of GM-CSF–expressing Th1 cells is higher in MS patients than in healthy controls. This is consistent with the results of another recent study on humans, which has found that Th1-derived GM-CSF pathway (IL-12-T-bet) is predominant over Th17-derived GM-CSF pathway (IL-23-RORγt) (Noster *et al*., 2014). This is also supporting the results of older EAE

studies which have demonstrated that T-bet-deficient mice were resistant to EAE despite a strong Th17 response (Bettelli *et al*., 2004, Lovett-Racke *et al*., 2004, Harrington *et al*., 2005). A variation in the frequency of Th1 or Th17 cells expressing GM-CSF was noticed among the participants (healthy controls and MS patients) in our results. The exact reasons for this are not identified. However, this was maybe because the population selected for analysis was smaller (live Th1 or Th17 cells) than live Th cells in general. It may also be a part of normal distribution of samples in the population.

It was shown that both PDB and PMA have similar low nanomolar potency on activating protein kinase C (PKC) (Dunn and Blumberg, 1983); however, our results have shown that using only phorbol esters with ionomycin are not enough to stimulate GM-CSF, IFN-γ, and IL-17 expression by the mixed PBMC population. On the other hand, another group has detected high frequency of freshly isolated CD4+ T cells expressing GM-CSF in healthy controls and MS patients using PMA/I only (Noster *et al*., 2014). However, they did not state the concentrations they used and used a smaller number of participants (n=7), in which the MS patients were recently diagnosed. Time since MS diagnosis may influence T cell autoreactivity, but such effects are not studied yet. The PDB/I stimulation assay was done first and yielded low GM-CSF expression in general. Therefore, the anti-CD3+anti-CD28 stimulation assay was performed on another set of subjects and was much better in terms of increasing cell capability to produce GM-CSF. In addition, we wanted to maintain consistency with the stimulation protocols used by our collaborators (Hartmann *et al.*, 2014).

Anti-CD3+anti-CD28 stimulation is important for autoreactive T cell stimulation (Reynolds *et al*., 2000, Groh *et al*., 2003). It may serve as a

re-stimulation of these cells mimicking what happens during antigen presentation after their first stimulation *in vivo* by myelin peptides or myelin peptide decoys (neuroantigens in general). Therefore, cytokine expression may be increased, and this is what is shown in fresh stimulated PBMCs gated for Th1 cells in terms of GM-CSF expression. Frozen and thawed Th and Tc cells expressed less GM-CSF than fresh cells in MS patients. Freezing impacts PBMC proliferation, cytokine production, and mRNA expression of proteins. This is achieved via different mechanisms that lead to cell damage, such as oxidative stress, disturbed ion homeostasis, osmotic injury, changes in physical properties of cellular structures, and mechanical injury as a result from ice crystal formation (Mallone *et al.*, 2011). The effects of freezing PBMCs on GM-CSF expression in particular have not been investigated yet; therefore, more work is needed to confirm this.

Our results reveal that stimulated Th and Tc cells express GM-CSF in higher frequency in MS patients than in healthy controls. We showed this at the protein level, and a trend of increase ($p=0.18$) in the stimulated mixture of PBMC population at the gene expression level. This supports the growing evidence of GM-CSF being involved in MS pathogenesis. This is consistent with others' findings (Hartmann *et al*., 2014, Rasouli *et al*., 2015). However, these results contradict the finding of another recent study which demonstrated no difference in frequency of GM-CSF-expressing CD4+ T cells *in vitro* or after 5 day stimulation with anti-CD3 then PMA/I for the last 4-5 hours (Peelen *et al*., 2015). Although the latter protocol looks similar to ours, Peelen and colleagues had not used anti-CD28, which is a strong T cell co-stimulus that increases T cell activation and acts in synergy with anti-CD3 to increase GM-CSF expression (Thompson *et al*., 1989).

Our study also showed that the frequency of Th and Tc cells expressing GM-CSF+IFN-γ+ (but not GM-CSF+IL-17+) is higher in MS patients than healthy controls in fresh and frozen/thawed PBMCs. However, in fresh PBMCs, the percentages were higher and reached statistical significance. This is consistent with Rasouli's group findings (Rasouli *et al*., 2015), and is supported by Noster's group findings of Th1-GM-CSF pathway being stronger than Th-17-GM-CSF pathway (Noster *et al*., 2014). In addition, the last study has shown higher frequency of CD4+ T cells expressing GM-CSF+IFN-γ+ (and not GM-CSF+IL-17+) in the CSF of recently diagnosed untreated RRMS patients compared to healthy controls. Our results indicate that more Th1 cells in MS patients produce GM-CSF than in healthy controls. This means that Th1 in MS may be one of the main CD4+ T cell subsets that are responsible for higher pathogenic GM-CSF expression. This may suggest new targets for GM-CSF suppression in MS via Th1 axis. Our results also show no difference in the frequency of Th or Tc cells expressing IL-17 in the PBMCs between healthy controls and MS patients.

Studies have shown that IL-17 expressing cells were present in higher numbers in active brain MS lesions compared to inactive lesions (Tzartos *et al.*, 2008, Kebir *et al.*, 2009). However, the results regarding IL-17 levels in blood are variable in the literature, and are affected by age, time of MS diagnosis, and disease activity (Matusevicius *et al.*, 1999, Kallaur *et al.*, 2013, Guerrero-Garcia Jde *et al.*, 2016). These effects on IL-17 were not studied in this work.

Our results also show a significant increase in the frequency of monocytes (CD3-CD14+ cells) expressing GM-CSF in MS patients when compared to healthy controls after PDB/I stimulation of PBMCs. It was shown that blood-borne monocytes/macrophages are seen in

abundance in active MS lesions (Bruck *et al*., 1995, Hauser *et al*., 1986), and GM-CSF was shown to have a role in increasing migration ability of monocytes through the BBB (Vogel *et al*., 2015). It is also possible that GM-CSF might have an autocrine/paracrine role stimulating the same monocyte or those which are nearby (Hamilton, 2008). PDB and ionomycin stimulation *in vitro* leads to T cell activation through PKC activation which leads to cell induction of IL-2 expression that leads to cells activation and proliferation (Larsen, 1990). Ionomycin is a Calcium ionophore that leads to Calcium mobilization and T cell activation (Chatila *et al*., 1989). T cell activation will lead to GM-CSF production, which leads to monocyte activation and stimulates them to express GM-CSF (Hamilton, 2008). In addition, PDB/I was shown to induce monocyte activation (Kelley *et al*., 1987, Bjork *et al*., 1997). Our results have shown that more monocytes are capable of producing GM-CSF in the remission stage of MS than healthy controls, which was not shown before. This suggests that monocytes in MS may contribute to the higher GM-CSF production, and the latter may play an autocrine role in monocyte differentiation and activation.

High GM-CSF levels in MS culture supernatants are possibly a result of the significant increase in frequency of the main PBMC populations (Th and Tc) expressing GM-CSF in MS patients as shown above. IL-2 is produced by activated CD4+ T cells (Sallusto *et al*., 1999) which are one of the major PBMC components after PBMC activation using anti-CD3+anti-CD28 protocol. We show significantly higher IL-2 secretion in MS patients during remission stage, which may reflect higher CD4+ T cell activity in that stage.

IL-12 is a pro-inflammatory cytokine produced by monocytes, macrophages, and DCs (O'Garra and Arai, 2000, Trinchieri, 2003). No

monocytes were detected in our cultured cells after 5 days of anti-CD3+anti-CD28 stimulation. However, IL-12 can be secreted by monocytes during the first two days of stimulation. IL-12 is also produced by B cells (Sartori *et al*., 1997, Trinchieri, 2003). IL-12 is crucial for Th1 responses, and this finding may be involved in Th1 stimulation in untreated RRMS patients in remission stage.

IL-1β is a pro-inflammatory cytokine produced mainly by APCs; however, NK and B cells can also produce IL-1β (Dinarello, 2009). In addition, another study has suggested a distinct CD4+ T cell population that secrete IL-1β in response to IL-2 (Numerof *et al*., 1990).

TGF-β is an anti-inflammatory cytokine that inhibits the differentiation, proliferation and functioning of Th1 and Th2 cells. However, it is involved in Th17 differentiation in the presence of IL-6. It also involved in the differentiation of Tregs in the presence of IL-2. TGF-β also supresses NK and B cell functioning and maturation (Yoshimura and Muto, 2011, Zheng, 2013). Our finding of lower TGF-β levels in culture supernatants of MS patients is relevant and consistent in correlation with induced GM-CSF (pro-inflammatory functioning) of Th1, NK and B cells (as shown in chapter 4 and 5, respectively). Moreover, low TGF-β may result in low frequency of Tregs in MS patients, which inhibit GM-CSF expression by immune cells. This may explain the dysregulation of GM-CSF in MS. Other studies have shown contradicting findings in terms of serum levels of TGF-β in MS, being elevated in some (Nicoletti *et al.*, 1998), and reduced in others (Rollnik *et al.*, 1997). However, our study is different in testing cultured supernatants and not serum levels.

IL-23 is a pro-inflammatory cytokine mainly produced by APCs (Oppmann *et al*., 2000). There is no evidence of other PBMCs producing

IL-23. We have detected IL-23 produced by cultured and anti-CD3+anti-CD28-stimulated PBMCs in healthy controls and MS patients. We found that IL-23 levels were similar in healthy controls and MS patients, which may explain similar Th17 responses discussed above.

IFN-γ is secreted by many PBMC subtypes, including Th1, Tc1 (Mosmann *et al*., 1997), NK1 cells (Peritt *et al*., 1998), B cells (Ballesteros-Tato *et al*., 2014, Bao *et al*., 2014), and stimulated monocytes (Kraaij *et al*., 2014). It was detected in high amounts in our stimulated culture supernatants, many of which were above the maximum standard value (3000 pg/ml). Hence, the comparison was difficult to be done. IL-10 is an anti-inflammatory cytokine produced by monocytes/macrophages, DCs, various CD4+ and CD8+ T cell subsets (mainly Tregs and Th2) (Moore *et al*., 2001). Controversial results were demonstrated about its levels in MS cultured and stimulated PBMCs, where some researchers have found its increase in active and stable MS (Crucian *et al*., 1995, Ferrante *et al*., 1998). On the other hand, some have found the reverse (Hedegaard *et al*., 2008), and others have found no change (Inoges *et al*., 1999) consistent with our findings. IL-10 levels may reflect the stage of the disease in RRMS, where a decrease in IL-10 occurs preceding MS attacks (Segal, 2014). This may explain the above-mentioned variation in IL-10 response.

IL-7 is a hematopoietic growth factor and a pro-inflammatory cytokine produced by non-hematopoietic cells such as stromal cells (Onder *et al*., 2012). It can also be secreted by DCs (Guimond *et al*., 2009). To date, there is no evidence of IL-7 being produced by other PBMCs, and detection of little amounts of IL-7 indicates that there may be some IL-7-producing cells (may be activated monocytes/DCs) in cultured PBMCs. Polymorphism in IL-7 and its receptors are among the genetic risk

factors involved in MS pathogenesis. IL-7 induces T cell proliferation and survival (Zuvich *et al*., 2010, Sawcer *et al*., 2011).

IL-6 is a pro-inflammatory cytokine produced by brain cells (endothelial cells, microglia, neurons, and astrocytes) (Erta *et al*., 2012), T cells (Li and He, 2006), mast cells (Conti *et al*., 2002), and mainly B cells (Yehudai *et al*., 2012). B cell-derived IL-6 plays a role in EAE and MS, being crucial for T cell activation in EAE and being elevated in MS (Barr *et al*., 2012). On the other hand, IL-6 may be protective for oligodendrocytes (Schonrock *et al*., 2000). Our results showed no differences in IL-6 levels between healthy controls and MS patients in the population studied. This may indicate that it may not directly explain the differences in GM-CSF expression and regulation that we observed.

We also show that IL-17 levels were similar in PBMC culture supernatants in healthy controls and MS patients. IL-17 is a pro-inflammatory cytokine produced by human activated memory CD4+ T cells (Yao *et al*., 1995, Shin *et al*., 1999), mainly by a distinct subset (Th17). It is regulated mainly by IL-1β, IL-6, IL-23, and TGF-β (Park *et al*., 1986, Harrington *et al*., 2005, Wei *et al*., 2007, Mills, 2008). IL-6 and IL-23 were in similar levels in MS patients and healthy controls in our results. However, IL-1β was elevated in PBMC supernatants of MS patients. The importance of IL-1 in Th17 development and IL-17 production was confirmed by several studies (Mills, 2008, Korn *et al*., 2009). However, it may be redundant in the presence of IL-6, IL-21, IL-23, and TGF-β (Korn *et al*., 2009). Lower TGF-β1 levels in MS patient culture supernatants may account for the overall null effects on IL-17 levels in PBMC cultures of MS patients. Our results also show that IL-12 blocking significantly reduced the percentage of Tc cells expressing

GM-CSF in MS patients (p=0.04) but not in healthy controls (p=0.57), n=7 for both groups. IL-12 is involved in Tc cell generation. It works in synergy with IL-2 to increase GM-CSF expression (Trinchieri, 1995). The reason behind this difference in healthy controls and MS patients was not investigated in this study. However, it was shown that IL-12A (p35 subunit) and IL-12B (p40 subunit) genetic polymorphism is one of the genetic risk factors for MS (Sawcer *et al*., 2011), which may lead to increased IL-12-induced GM-CSF expression. However, the exact role of IL-12 polymorphism on GM-CSF expression by CD8+ T cells in MS is not yet investigated.

With regard to GM-CSF and GMR expression, we were also interested in finding if there are any differences in their expression during relapse and remission stages in the same patients. This is because previous studies have shown higher levels of GM-CSF during relapse in CSF samples (Carrieri *et al*., 1998). To achieve this aim, we analysed whole blood mRNA gene expression in Paxgene® tubes, and analyse their mRNA gene expression. We found no changes in GM-CSF and GMR gene expression among patients in relapse-remission stages and healthy controls. The reason behind this finding may be the use of unstimulated whole blood cells, and we have already shown that unstimulated PBMCs do not produce much GM-CSF. There is a study in which GM-CSF gene expression in whole blood was not analysed as pre-planned due to its low levels (Romme Christensen *et al*., 2012). Another study has not detected GM-CSF gene expression in unstimulated PBMCs via northern blot (Cairo *et al*., 1991). With regard to GMR gene expression, there are studies which detected GMR gene expression in whole blood of healthy controls, although in low levels (Lee *et al*., 2008, Goldstein *et al*., 2011).

We hypothesized that GM-CSF and GMR gene expression will have different regulation during relapse that could be detected in whole blood.

Finally, we were interested to look at GM-CSF and GMR expression differences before, 6 months, and 1 year after IFN-β therapy of RRMS patients during remission stage. This is because Rasouli and colleagues have recently found that *in vitro* treatment of PBMCs with IFN-β significantly reduced the frequency of GM-CSF only-expressing CD4+ and CD8+ T cells at the protein level (Rasouli *et al*., 2015). We could not detect any differences in GM-CSF or GMR expression at the RNA level in the whole blood among healthy controls or in patients before and after treatment. On the other hand, another recent study analysed GM-CSF (among other markers) mRNA gene expression in PBMCs after IFN-β therapy for 37 RRMS patients during remission stage (Milosevic *et al*., 2015). They have found that GM-CSF gene expression reduces significantly only after the first 6 months of treatment, and keeps increasing to become similar to before treatment levels after 1 year of treatment. Then GM-CSF expression keeps going up until it becomes significantly higher after 3 years of treatment. Our results are consistent with them at the one year time point, and may indicate no long term effects of IFN-β on GM-CSF expression by PBMCs.

In conclusion, we identified GM-CSF-expressing Th1 and Th17 in humans, and that GM-CSF-expressing Th1 cells are in higher frequency in MS patients. This may be due to lower TGF-β levels in MS. In addition, we found more Th cells, Tc cells, and monocytes expressing GM-CSF in MS patients. Finally, IL-12 may have a role behind higher frequency of GM-CSF-expressing Tc cells in MS. In the next chapter, we presented work done to look at GM-CSF and GMR expression and regulation in another PBMC subset (NK cells) with relevance to MS.

Chapter 4. GM-CSF and GM-CSF receptor (GMR): Expression and Regulation in NK cells and Relevance to MS

4.1 Introduction

In the previous chapter, we have investigated GM-CSF and GMR expression and regulation in 3 PBMC subsets (Th, Tc, and monocytes) with relevance to MS. In this chapter, we investigated GM-CSF and GMR expression and regulation in another PBMC subset, the NK cells, in MS. NK cells are important immune cells involved in innate and adaptive immune responses. They play an important role in killing cancer cells, viral and bacterial infection. (Sun *et al*., 2009, Hall *et al*., 2010, Paust *et al*., 2010). Based on cytokine secretion profile, NK cells can be classified to NK1 (secrete IL-10 and IFN-γ), NK2 (secrete IL-5 and IL-13), and NK17/NK1 (secrete IFN-γ and IL-17) (Peritt *et al*., 1998, Pandya *et al*., 2011). NK1 and NK2 can produce GM-CSF (Peritt *et al*., 1998). NK cells can be also classified according to their CD56 and CD16 co-expression into 5 subtypes: $CD56^{bright}CD16$-, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16$-, $CD56^{dim}CD16^{bright}$, and CD56-$CD16^{bright}$ (Poli *et al.*, 2009). The majority of NK cells in the peripheral blood (more than 90%) are $CD56^{dim}CD16^{bright}$, while $CD56^{bright}$ cells represent around 10% of the circulating NK cells (Cooper *et al.*, 2001a, Caligiuri, 2008).

With regard to autoimmune diseases including MS, the reports are controversial, suggesting either a protective or disease-aggravating roles. Some studies have suggested a protective role of NK cells in MS and EAE, probably through cytolysis of cells that worsen the disease (mostly Th17 cells), alteration in DC and microglial cell phenotypes, and production of neurotrophic factors (Huang *et al*., 2006, Hao *et al*., 2010).

Regarding MS, NK cells have been increased during the remission stage of the disease, which were NK2 subtype with anti-inflammatory actions including suppressing Th1 responses (Takahashi *et al.*, 2001, 2004).

On the other hand, other studies have demonstrated a stimulating and aggravating role of NK cells in RRMS. This is achieved mainly by secretion of pro-inflammatory cytokines, such as IFN-γ, which activates Th1 cells (Kastrukoff *et al*., 1998, Shi *et al*., 2000). Some *in vitro* studies have shown that NK cells directly lyse primary oligodendrocytes and neurons, with a partial lysis of DCs, T cells, astrocytes, and microglial cells (Lunemann *et al*., 2008, Zhang *et al*., 2011).

NKT cells have also been shown to produce GM-CSF (Jeong *et al*., 2014). They usually play a protective role in autoimmune diseases including MS and EAE (Van Kaer *et al*., 2015). The expression and regulation of GM-CSF in NKT cells was not studied in this work. This is because NKT cells constitute a very small population (0.9-1.2%) of the mixed PBMC population (Dellabona *et al*., 1994). Our experiments resulted in less percentage of NKT cells than the above-mentioned (around or less than 0.5%), as they were measured after 5 days of PBMC stimulation with anti-CD3+and-CD28, which mainly stimulate T cell proliferation. Attempts were carried out to detect GM-CSF expression in NKT cells. However, it was hard to detect GM-CSF or other cytokines with flow cytometry in such a small population as part of the mixed PBMC population. However, investigating NKT cells producing GM-CSF in MS is important for future studies.

Our aim was to investigate GM-CSF expression and regulation in PBMC subsets, including NK cells in MS. This may depict one of the pro-inflammatory pathways exerted by NK cells in MS. It was previously

shown that NK cells secrete GM-CSF (Levitt *et al*., 1991). However, the expression of GM-CSF by NK cells has not been assessed in MS. Therefore, in this study we examined GM-CSF expression by NK (CD3-CD56+) cells in stimulated mixed PBMC population in untreated RRMS patients compared to healthy volunteers. In addition, we wanted to investigate if GM-CSF-expressing NK cells produce other pro-inflammatory cytokines, mainly IFN-γ and IL-17. Therefore, expression of IFN-γ and IL-17 was studied in NK cells. It was previously shown that NK cells express IFN-γ and IL-17 (Cooper *et al.*, 2001b, Pandya *et al.*, 2011). However, their expression and correlation to GM-CSF expression by NK cells was not investigated in MS. We have also examined GM-CSF and IFN-γ expression in isolated and stimulated NK cells (then only CD3-CD56+ cells were selected) in untreated RRMS patients compared to healthy volunteers. After that, Multiplex bead assay was performed to determine changes in a number of pro-and anti-inflammatory cytokines in cultured PBMCs of MS patients compared to healthy controls. We examined the effects of blocking these cytokines on GM-CSF, IFN-γ, and IL-17 expression in CD3-CD56+ gated cells in mixed PBMC population in MS patients and healthy controls. In addition, fresh and frozen/thawed PBMCs were also examined in terms of their cytokine expression under similar stimulation conditions.

On the other hand, NK cells were shown to express GMR (Kubista *et al*., 2003). However, there was controversy in the literature on the effects of GM-CSF on NK cell functions, which needs further clarification. GMR expression in NK cells in MS is not studied yet, so we determined GMR expression in CD3-CD56+ in the mixed PBMC population (but not in isolated NK cells) in MS patients and healthy controls. Finally, we examined the effects of blocking cytokines which were significantly

increased in PBMC culture supernatants in MS patients on GM-CSF and GMR expression in healthy controls and MS patients.

4.2 Subjects and Methods

4.2.1 Subjects

All patients participated in the experiments presented in this chapter were untreated RRMS. The same inclusion/exclusion criteria mentioned in chapter 3 section 3.2.1 were applied to subjects enrolled in the experiments included in this chapter. All the important details of healthy controls and MS patients and the experiment(s) each subject has participated in are listed in appendix I.

4.2.2 Methods

All the below mentioned sample collection and experimental work was approved by Nottingham Research Ethics Committee, as shown in Appendix II. PBMCs were isolated, counted, frozen and thawed as in sections 2.1 and 2.2; anti-CD3+anti-CD28 stimulation was performed as in section 2.4. Flow cytometry staining and data acquisition/analysis was done according to sections 2.5 and 2.7. Multiplex bead assay was performed as in section 2.8.

4.2.2.1 NK Isolation, Purity Determination, and Stimulation

PBMCs were isolated and counted as in section 2.1. NK cells were magnetically isolated using NK isolation kit (Miltenyi Biotech) via negative selection. Collected flow-through contained NK cells which were counted and checked for purity (≥ 90%) (Figure 4-1). Cells were re-suspended in RPMI medium with 15% FCS, 100 units per ml of penicillin, 0.1 mg/ml of streptomycin, and 2 mM of glutamine (concentrations were chosen according to standard protocols in the

literature) and distributed in 24-well plates at a concentration of $1x10^5$ cells/ml/well-plate. NK cells were either left unstimulated or stimulated with one of the following: IL-15 (100 ng/ml) (R&D Systems) + IL-1β (10 ng/ml) (Peprotech), IL-15 (100 ng/ml) + IL-18 (100 ng/ml) (R&D Systems), and IL-2 (10 ng/ml) + IL-12 (10 ng/ml) (Peprotech). Cells were incubated for 3 days at 37°C with 5% CO_2, then re-stimulated with PMA/I in the presence of brefeldin A for the last 5 hrs (concentrations are as section 2.4 above). Cells were stained following section 2.5 above; except there was no dead cell staining were applied in this procedure.

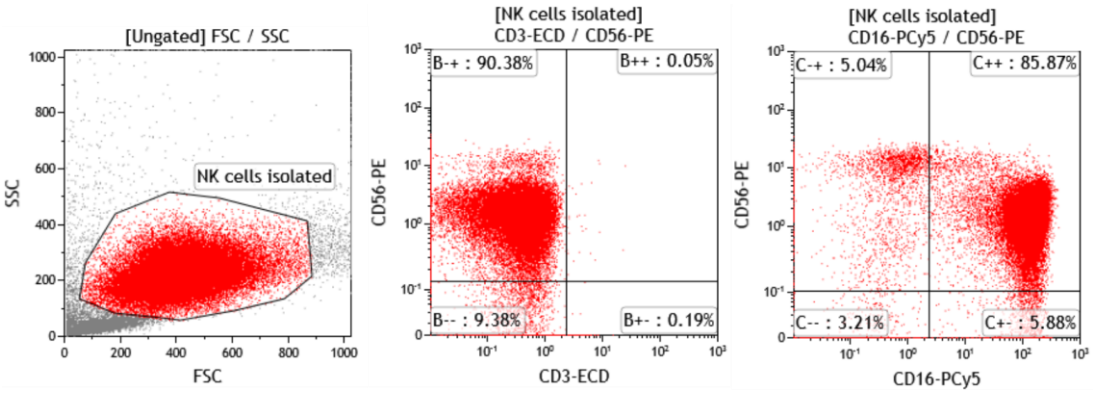


Figure 4-1: Determination of NK cell purity after magnetic isolation.

After plotting the forward scatter (FSC) versus side scatter (SSC) plot and excluding the debris, NK cells were displayed in flow cytometry plots. ≥ 90% of cells were CD3-CD56+ cells, of which the majority were CD16+.

4.2.2.2 Flow Cytometry Staining and Data Analysis

Data acquisition and analysis was done as in sections 2.5 and 2.7. We used CD3-CD56+ lymphocytes as a marker of NK cells.

4.3 Results

4.3.1 Demographic and Clinical Characteristics of the Participants

Same subjects were used in the following experiments as in chapter 3 section 3.3.1: anti-CD+anti-CD28 stimulation (fresh: Table 3-2, and frozen: Table 3-3), and multiplex bead assay (Table 3-4). For NK

isolation and stimulation, Table 4-1 below demonstrates the demographics and clinical characteristics of subjects recruited.

Table 4-1: Demographic and clinical characteristics of subjects who contributed samples to the studies of NK isolation and stimulation experiment

Demographics	**Healthy Controls**	**Untreated RRMS**
Total no. of participants	5	7
Age mean (y) (SD)	40 (10.3)	44.86 (10.73)
Female no. (%)	3 (60)	5 (71.4)
Ethnicity:		
White (%)	4 (80)	7 (100)
Other (%)	1 (20)	-
Time since MS diagnosis (y) (SD)	-	6.33 (2.08)
EDSS mean (SD)	-	3.71 (1.7)
Smoking status:		
Smoker (%)	0	1 (14.2)
Ex-smoker (%)	1 (20)	0

4.3.2 Higher Frequency of GM-CSF-Expressing NK Cells in MS

Both fresh and frozen/thawed PBMCs from healthy volunteers and MS patients were stimulated (anti-CD3+anti-CD28 protocol) and gated for live CD3-CD56+ lymphocytes. Frequency of NK cells expressing GM-CSF was significantly higher in MS patients than healthy controls using unpaired t-test ($p=0.0008$ in fresh cells, and $p=0.0197$ in frozen cells) (Figure 4-2). With regard to frequency of NK cells expressing IFN-γ (Figure 4-2), it increased in a statistically significant manner in MS patients when compared with healthy controls in fresh but not in thawed samples (unpaired t-test, fresh HC n=10, fresh MS n=9, $p=0.0357$) (unpaired t-test, frozen HC n=11, frozen MS n=12, $p=0.69$). IL-17 expression in NK cells was determined in thawed samples only and has shown no statistical difference. However, the percentage of CD3-CD56+ cells expressing IL-17 had a trend to be higher in MS patients than controls (unpaired t-test, HC n=10, MS n=12, $p=0.13$) (Figure 4-2).

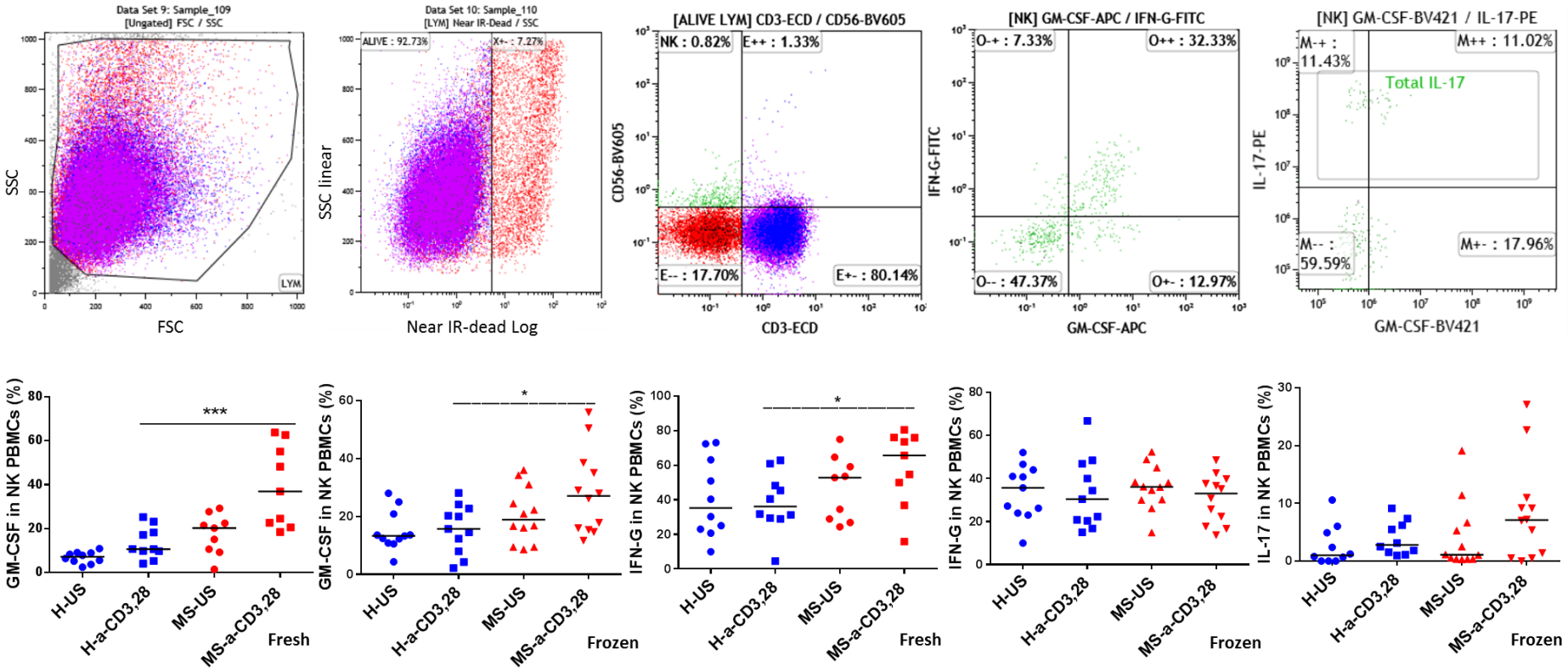


Figure 4-2: Expression of GM-CSF, IFN-γ, and IL-17 in NK cells.

Fresh and frozen/thawed PBMCs were stimulated following anti-CD3/anti-CD28 protocol in healthy controls and MS patients. After selecting only live cells by excluding the debris (first plot) and then selecting the cells that did not stain with the near IR dead stain (second plot), only CD3-CD56+ cells were selected to determine their cytokine expression. Quadrants were set the same way as displayed in Figure 3-1. H-US, MS-US: unstimulated sample from healthy control and MS patient, respectively. H-a-CD3,28, MS-a-CD3,28: anti-CD3+anti-CD28-stimulated sample from healthy control and MS patient, respectively. *p<0.05, ***p<0.001. Horizontal lines are medians.

4.3.3 Frequencies of NK Cells Expressing GM-CSF+IFN-γ+ and GM-CSF+IL-17+ in MS patients

The stimulation of fresh and frozen/thawed PBMCs with anti-CD3+anti-CD28 protocol led to elevated percentage of NK cells expressing GM-CSF+IFN-γ+ in MS patients. However, statistical significance was achieved in fresh but not with frozen/thawed cells (only a trend) using unpaired t-test (fresh HC n=10, MS n=9, p=0.022; thawed HC n=11, MS n=12, p=0.12) (Figure 4-3). In addition, there was a non-significant numerical increase in the percentage of NK cells expressing GM-CSF+IL-17+ in thawed samples of MS patients (HC n=11, MS n=12, Mann-Whitney p=0.13).

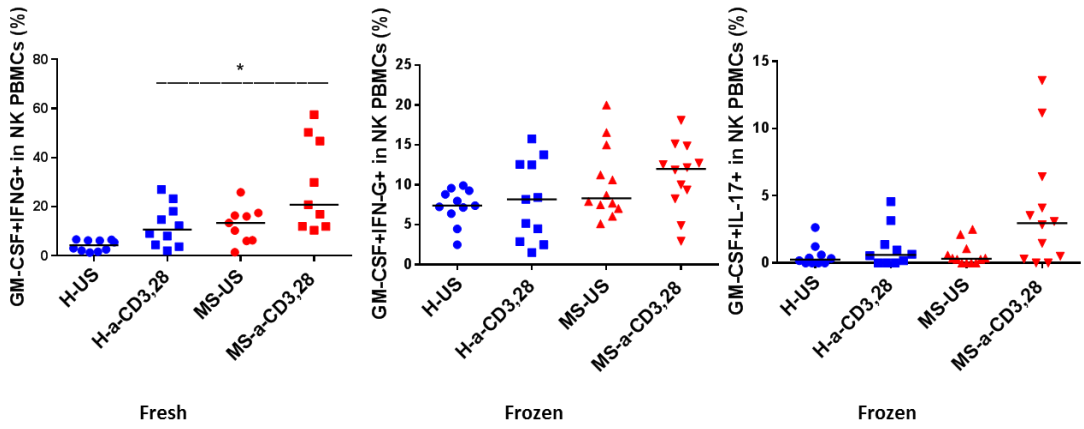


Figure 4-3: Co-expression of GM-CSF/IFN-G and GM-CSF/IL-17 by NK cells.

Fresh and frozen/thawed PBMCs were stimulated following anti-CD3/anti-CD28 protocol in healthy controls and MS patients. Double positive populations were selected from plots as displayed in Figure 4-2. H-US, MS-US: unstimulated sample from healthy control and MS patient, respectively. H-a-CD3,28, MS-a-CD3,28: anti-CD3+anti-CD28-stimulated sample from healthy control and MS patient, respectively. *p<0.05. Horizontal lines are medians.

4.3.4 GM-CSF and IFN-γ Profile in NK Cells before and after Isolation in Healthy Controls and MS Patients

As shown in section 4.3.2 of our results, the percentage of NK cells expressing GM-CSF was higher in MS patients when compared to healthy controls. Therefore, GM-CSF expression by isolated NK cells was investigated to determine whether other cells or factors in the mixed PBMC population might have played a role in GM-CSF expression by NK cells in MS. We have looked in the literature for the stimuli that were shown to be strong in terms of GM-CSF expression by NK cells. Therefore, IL-15+ IL-18, IL-15+IL-1β, and IL-2+IL-12 were selected. NK cells were freshly isolated, stimulated for 3 days with IL-15+ IL-18 and IL-15+IL-1β and re-stimulated with PMA/I. These cells were stained for flow cytometry, their gating is illustrated in Figure 4-4. These cells did not exhibit any change in the frequency of CD3-CD56+ NK cells expressing GM-CSF or IFN-γ in MS patients when compared to healthy controls (Figure 4-5 A, B, and C). However, CD56+ population was a minor population among all isolated and gated NK cells, knowing that the purity of CD3-CD56+ NK cells was ≥ 90% before culturing and stimulating them (Figure 4-1). This indicates a strong down-regulation of CD56 upon NK cell culture and stimulation (Figure 4-4). However, these stimuli were strong GM-CSF producers in CD56+ cultured NK cells (Figure 4-5 A, B, and C). When CD3- cells were gated (regardless of CD56 expression), similar results were obtained (Figure 4-5 D, E, and F). However, the frequency of GM-CSF-expressing cells were significantly less when considering all NK cells regardless their CD56 expression than when considering CD56+ NK cells in healthy controls and MS patients.

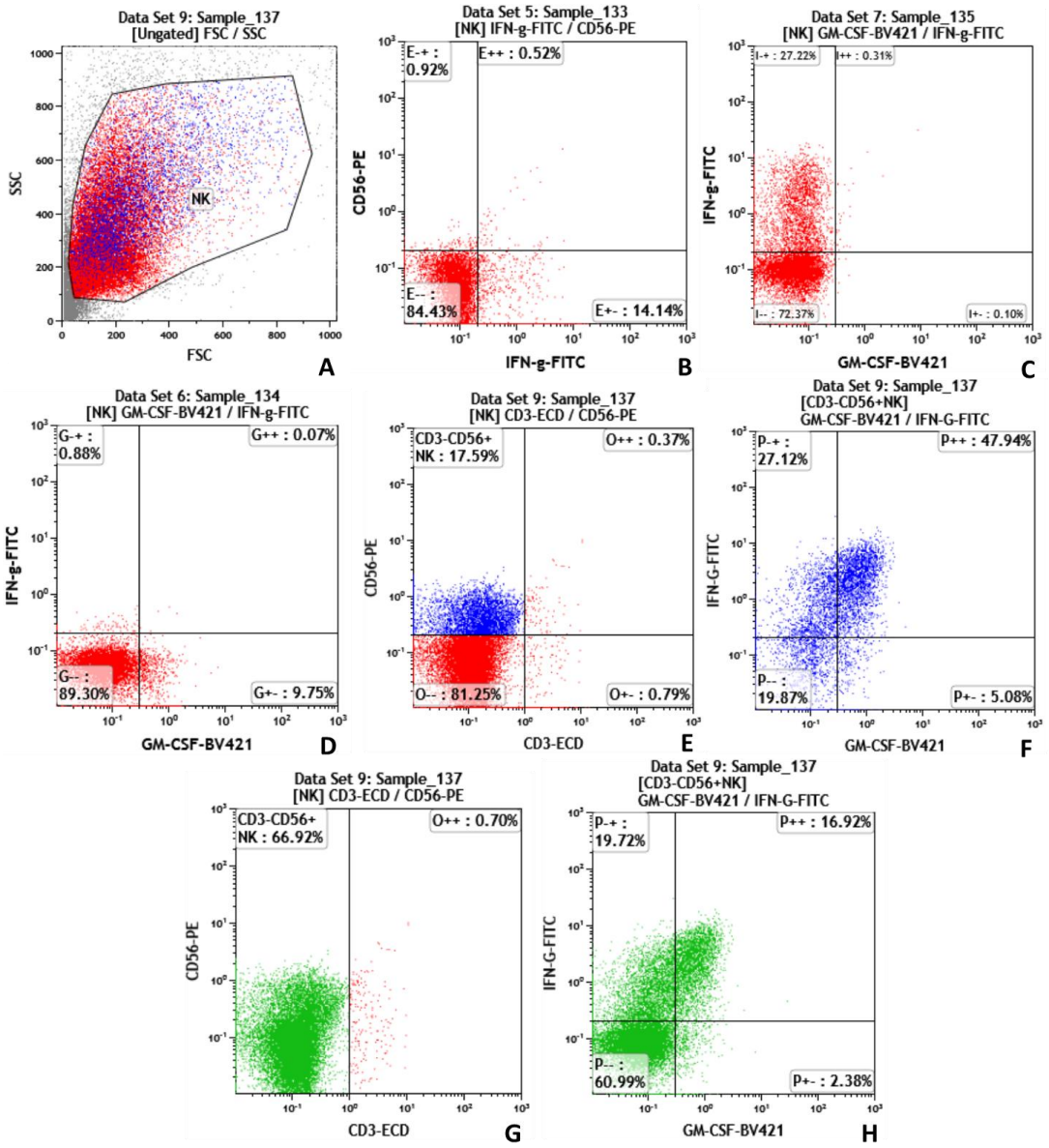


Figure 4-4: Gating of isolated and stimulated NK cells.

After magnetic isolation of NK cells, they were either unstimulated or stimulated with different stimuli for 3 days, then re-stimulated for the last 5 hrs with PMA/I in presence of brefeldin A. cells were stained and run in the flow cytometer. A: the forward scatter (FSC) and side scatter (SSC) plot to gate the possible NK cells. B: FMO control of CD56-PE, C: FMO control of GM-CSF-BV421, D: FMO control of IFN-γ-FITC. E: gating cells to select CD3-CD56+ NK cells (blue-coloured population). F: CD3-CD56+ NK cells plotted to observe their GM-CSF and IFN-γ expression. G: gating cells to select CD3- NK cells (green-coloured population) regardless of their CD56 expression. H: GM-CSF and IFN-γ expression by total NK cells.

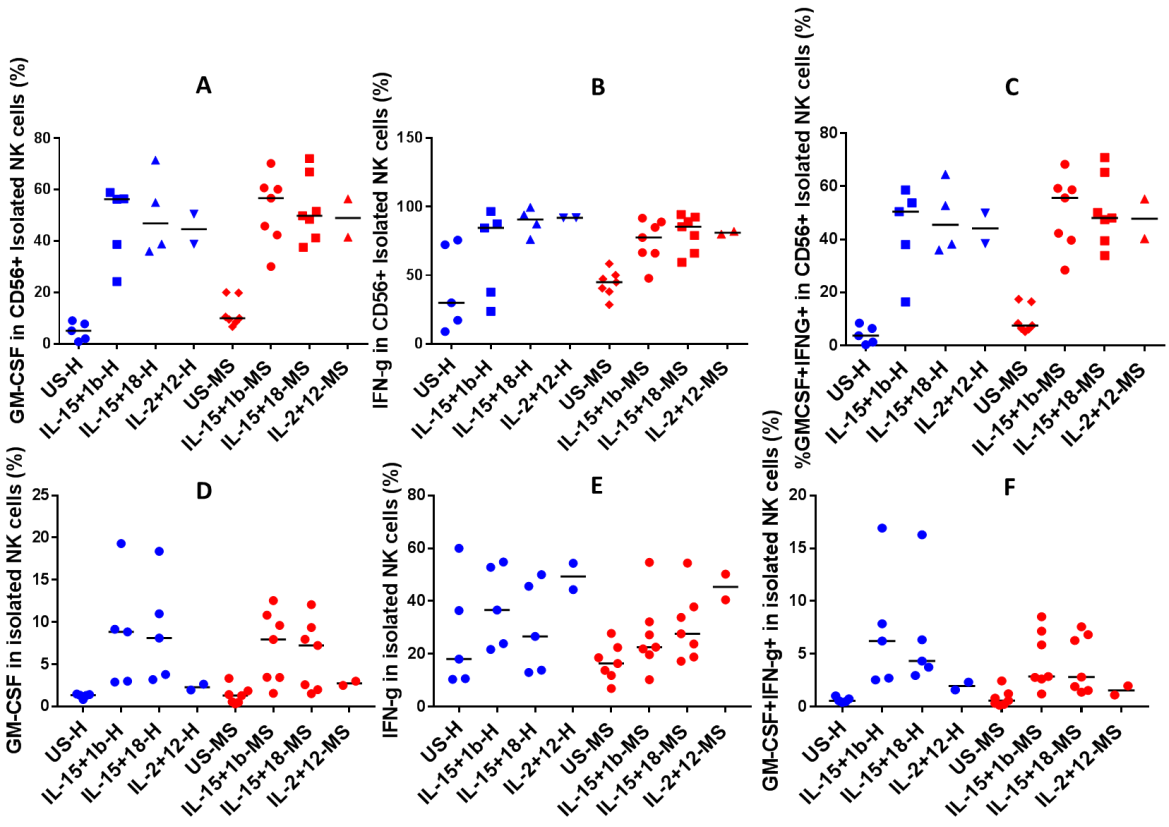


Figure 4-5: The expression of GM-CSF, IFN-γ, and their co-expression in isolated NK cells.

After gating the cells as in Figure 4-4, the expression GM-CSF, IFN-γ, and the combination of both, was examined in healthy controls (n=5) and MS patients (n=7). A, B, C: cytokine profile in isolated NK cells gated for CD3-CD56+ (blue-coloured population in Figure 4-4). D, E, and F: cytokine profile in isolated NK cells gated for CD3- (regardless of their CD56 expression after culture and stimulation) (green-coloured population in Figure 4-4). US-H: unstimulated cells from healthy controls; IL-15+1b-H: cells stimulated with IL-15 and IL-1β from healthy controls; IL-15+18-H: cells stimulated with IL-15 and IL-18 from healthy controls; IL-2+12-H: cells stimulated with IL-2 and IL-12 from healthy controls (n=2); US-MS: unstimulated cells from MS patients; IL-15+1b-MS: cells stimulated with IL-15 and IL-1β from MS patients; IL-15+18-MS: cells stimulated with IL-15+IL-18 from MS patients; IL-2+12-MS: cells stimulated with IL-2 and IL-12 from MS patients (n=2). Horizontal lines are medians.

These results indicate that there are stimuli that may be more abundant in the mixed PBMC population than the isolated NK cells medium in MS patients but not in healthy control samples. These stimuli may be responsible for the increase in GM-CSF expression. Therefore, we performed Multiplex Assay to evaluate the concentration of a number of pro- and anti-inflammatory cytokines in culture supernatants of mixed PBMC populations taken after 5 days of stimulation just before addition of PMA/I and brefeldin A (results are found in chapter 3 section 3.3.7).

We included the cytokines that were more likely, based on the existing knowledge, to represent stimuli/inhibitors for GM-CSF production in T cells and NK cells.

4.3.5 Blocking IL-2 and IL-12 Reduced Frequency of CD3-CD56+ Cells Expressing GM-CSF in MS Patients

Frozen and thawed PBMCs from healthy controls and MS patients were cultured and stimulated with anti-CD3+anti-CD28 along with adding neutralizing antibodies to IL-2, IL-12, IL-2+IL-12, IL-12/23, and IL-1β for 5 days. The percentage of GM-CSF-expressing CD3-CD56+ cells was significantly reduced in MS patients but not in healthy controls after IL-2+IL-12 blocking (Figure 4-6 A and B) (MS n=10, Wilcoxon test, p=0.01; HC n=7, Wilcoxon test, p=0.53).

4.3.6 Blocking IL-2 and IL-12 reduced Frequency of NK Cells Expressing GM-CSF+IFN-γ+ and GM-CSF+IL-17+ in MS Patients

The same procedure mentioned above led to significant reduction in the percentage of NK cells expressing GM-CSF+IFN-γ+ in MS patients when compared to healthy volunteers. This was achieved after blocking IL-2 (MS n=12 paired t-test p=0.01; HC n=11 paired t test p=0.12), IL-12 (MS n=10 paired t-test p=0.03; HC n=11 paired t-test p=0.19), and both together (MS n=10 paired t-test p=0.04; HC n=7 Wilcoxon test p=0.21) (Figure 4-6 G and H). With regard to GM-CSF/IL-17 co-expression by NK cells, blocking IL-2, IL-12, and IL-2+IL-12 show significant reduction in this population in MS patients but not in healthy controls (Figure 4-6 I and J). In blocking IL-2 (MS n=12 Wilcoxon test p=0.01; HC n=10 Wilcoxon test p=0.64); in blocking IL-12 (MS n=9 paired t-test p=0.03; HC n=10 Wilcoxon test p=0.22); and in blocking IL-2+12 (MS n=10 paired t-test p=0.04; HC n=7 Wilcoxon test p=0.84).

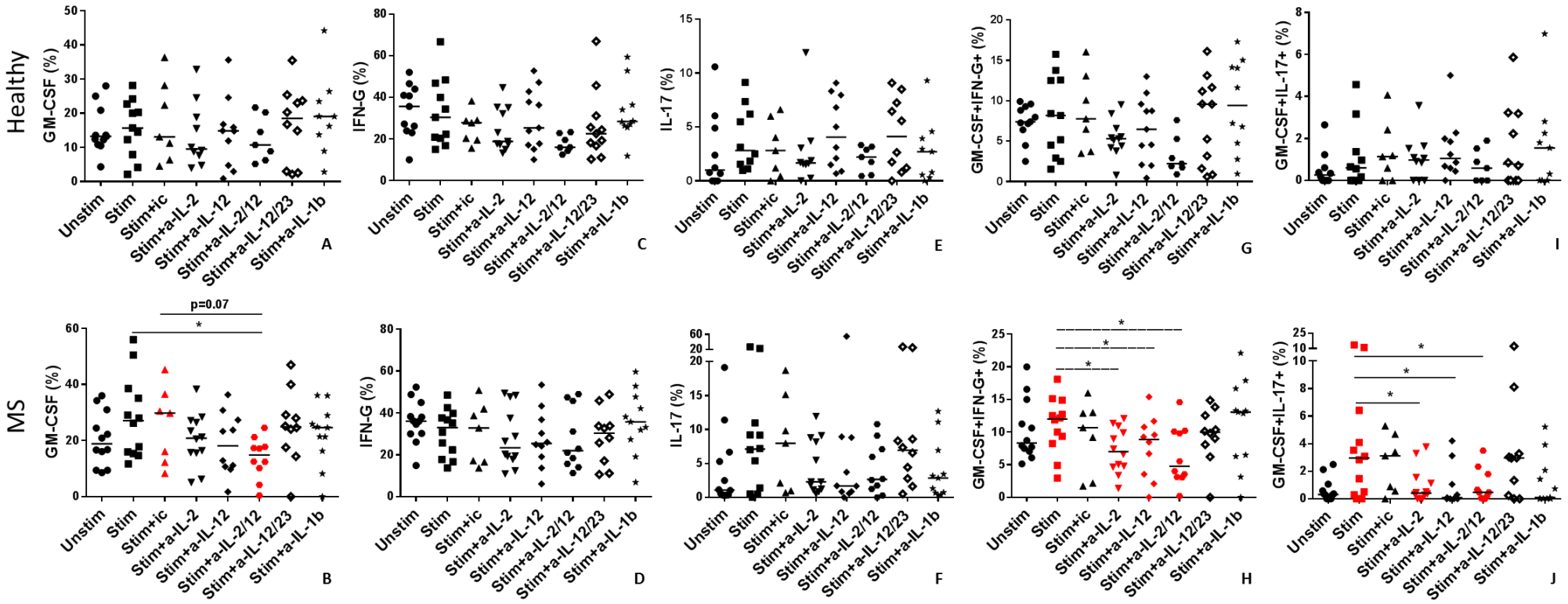


Figure 4-6: Effects of anti-cytokine antibodies on the frequency of NK cells expressing GM-CSF, IFN-γ, IL-17, GM-CSF/IFN-γ, and GM-CSF/IL-17.

Frozen/thawed cultured PBMCs in healthy controls (H) (n=11) and MS patients (n=12). PBMCs were cultured either unstimulated (Unstim) or stimulated (Stim) with anti-CD3+anti-CD28 for 5 days. This was done with or without adding one of the antibodies to block the following cytokine (a-cytokine: cytokine antibody): IL-2, IL-12, IL-2+IL-12 (IL-2/IL-12), IL-12+IL-23 (IL-12/IL-23), and IL-1β. In addition, cells were cultured with antibody isotype control (ic) as controls. Only live CD3-CD56+ cells were selected and analysed for the above-listed parameters. Upper row represents healthy control results, lower row represents MS patient results * $p<0.05$. Horizontal lines represent medians.

4.3.7 Higher Frequency of NK Cells Expressing GMR upon IL-2+IL-12 Blocking in MS Patients

Higher percentage of GMR-expressing NK cells was noticed in unstimulated conditions (median around 7%) in healthy controls and MS patients. This percentage decreased upon anti-CD3+anti-CD28 stimulation (around 1%) in healthy controls and MS patients. This decrease reached statistical significance in healthy controls (n=7, Wilcoxon test p=0.015) but not in MS patients (n=10, Wilcoxon test p=0.1) (Figure 4-7). Upon IL-2+IL-12 blocking, the decrease in the frequency of NK cells expressing GM-CSF in MS patients was accompanied by an increase in the frequency of NK cells expressing GMR in MS patients but not in healthy subjects. Wilcoxon test was used for comparison (MS n=8, p=0.007; HC n=4, p=0.12) (Figure 4-7).

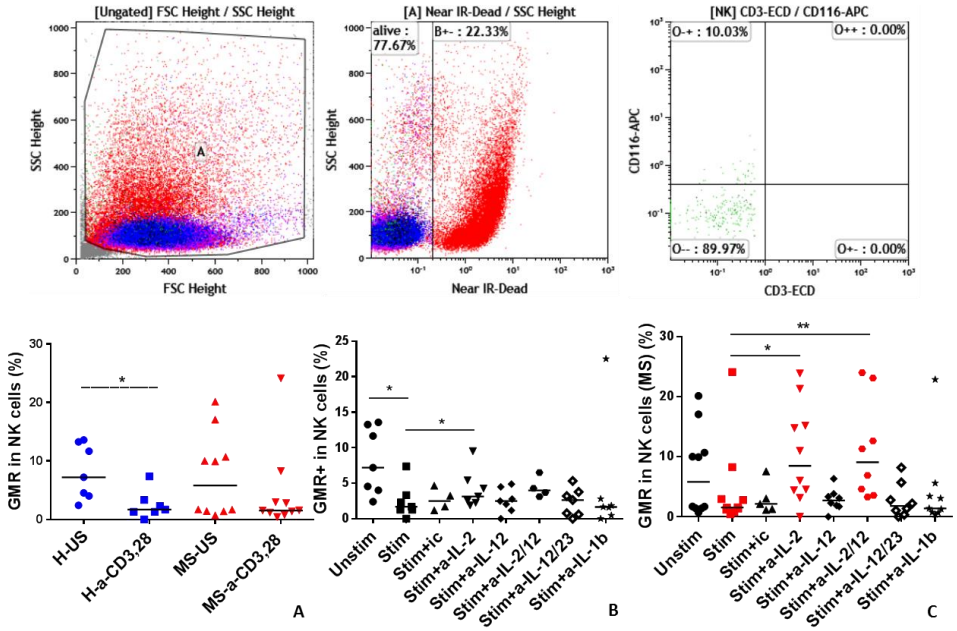


Figure 4-7: GMR expression in NK cells under different stimulation/blocking conditions.

A: GMR expression in NK cells of healthy controls and MS patients. B: GMR expression by NK cells in healthy controls. C: GMR expression by NK cells in MS patients. PBMCS were cultured either unstimulated (Unstim) or stimulated (Stim) with anti-CD3+anti-CD28 for 5 days. This was done with or without adding antibody isotype control (ic) or one of the antibodies to block the following cytokine (a-cytokine: cytokine antibody): IL-2, IL-12, IL-2+IL-12 (IL-2/IL-12), IL-12+IL-23 (IL-12/IL-23), and IL-1β. Only live CD3-CD56+ cells were selected as in Figure 4-2 and their GMR (CD116) expression is examined (green-coloured population). H-US: unstimulated samples from healthy controls; H-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from healthy controls; MS-US: unstimulated samples from MS patients; MS-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from MS patients. * p<0.05; **p<0.01. Horizontal lines are medians.

Table 4-2 below illustrates a summary of the significant findings in this chapter that will be discussed in the next section.

Table 4-2: Main findings of work illustrated in chapter four

Result (compared to healthy controls)	Significance test	P value
Higher frequency of GM-CSF-expressing NK cells in stimulated mixed PBMC population in MS	Unpaired t-test	0.0008
Similar frequency of GM-CSF-expressing isolated and stimulated NK cells in healthy controls and MS patients	Mann-Whitney test	0.34
Lower frequency of GM-CSF-expressing NK cells in stimulated mixed PBMC population after blocking IL-2+IL-12 in MS	Wilcoxon test	0.01
Higher frequency of GMR-expressing NK cells in stimulated mixed PBMC population after blocking IL-2 in MS	Wilcoxon test	0.015

4.4 Discussion

NK cells in the mixed PBMC population were phenotyped as CD3-CD56+, according to previous studies (Lanier *et al*., 1986, Fauriat *et al*., 2010). There are several studies that assessed the role of NK cells in MS. However, the results seem to be controversial as mentioned in the introduction. In addition, there are no published data regarding GM-CSF production by NK cells in MS to date.

Our results have shown a statistically significant increase in the frequency of NK cells expressing GM-CSF in MS patients when compared to healthy controls. This was detected in fresh and frozen/thawed mixtures of PBMC populations following anti-CD3+anti-CD28 stimulation protocol. It was not intended to examine GM-CSF expression and compare it between fresh and frozen/thawed cells. The work was first conducted using fresh cells in which the expression of GM-CSF and IFN-γ was examined. However, it was then planned to examine IL-17 and adding more conditions to block the cytokines which

were elevated in multiplex bead assay. To perform this experiment, it was not feasible to collect fresh blood from MS patients. However, we had ready to use frozen PBMCs from MS patients. Thawed PBMCs were allowed to rest overnight before stimulation (see methods section 2.2). Despite that, our results show reduced frequency of GM-CSF-expressing NK cells in frozen/thawed PBMCs than fresh PBMCs. Possible causes for this difference are due to freezing effects on cells which are discussed in Chapter Three section 3.4. Higher GM-CSF expression may be one of the mechanisms of NK cells exerting their pro-inflammatory role in MS. This may be simply by providing more GM-CSF in the inflammatory milieu. In addition, GM-CSF was shown to induce NK cell cytotoxicity (Kubista *et al.*, 2003), suggesting another pro-inflammatory pathway that is GM-CSF-NK-related.

Isotype controls were used to exclude any non-specific binding of the cytokine blocking antibodies. They have similar structure of the blocking antibody (constant chains) except for the antigen-binding sites (variable chains) to detect the presence of non-specific binding or effects of the antibody that is not related to the specific antigen-binding regions. In experiments where different anti-cytokine conditions were used in addition to isotype control conditions, a different number of samples was included in each condition. This is because some conditions were not included due to lack of sufficient cell numbers in each sample to include all the conditions. However, isotype control condition was favoured as it is the control to which the rest will be compared. Moreover, in few times during the staining process, one of the antibodies runs out so the conditions with incomplete staining were excluded from the experiment.

In general, cytokine frequencies in stimulated cells with isotype control were very close to those without adding isotype controls, including this set of results. This indicates very few non-specific binding during the cytokine blocking experiments. In this set of results, comparing frequency of GM-CSF-expressing NK cells between two conditions (IL-2+IL-12 antibodies and isotype control) did not reach statistical significance in MS patients (MS n=7, Wilcoxon test, p=0.07) (Figure 4-6 A and B). On the other hand, statistical significance was achieved in MS patients when comparing frequency of GM-CSF-expressing NK cells between two conditions (IL-2+IL-12 antibodies and stimulated cells without isotype control) (MS n=10, Wilcoxon test, p=0.01). This may be due to higher number of MS samples compared without adding the isotype control (n=10) than when we added the isotype control condition (n=7).

Based on our results, it is difficult to categorize our CD3-CD56+ cells as NK1 or NK2, as we have not detected the full cytokine profile of each subset. Our selected cells were producing the pro-inflammatory cytokines GM-CSF, IFN-γ, and some IL-17. This may indicate that these cells still maintain the pro-inflammatory profile when stimulated more than healthy controls, even during the remission stage of the disease. To date, the role of NK-derived GM-CSF in MS is not investigated; therefore, more experiments are needed to examine its role.

NK cytotoxicity in MS is an important aspect that should be studied. However, the main aims of this work were to evaluate their pro-inflammatory cytokine expression in MS, and mainly GM-CSF. Future experiments are needed to investigate NK cell cytotoxicity in MS. In addition, further investigation of NK cell subsets based on their CD16 expression was not performed in this work (CD16 was used only for NK

cell purity detection). This is because the main aim of this work was to investigate GM-CSF expression in traditional NK cells (CD3-CD56+) without subdividing them. It may be an important future work plan. We also tried to investigate NKT cells and their GM-CSF production in MS, but they were a very small population (less than NK cells) in these experiments (less than or around 0.5%), therefore it was difficult to look at their GM-CSF expression.

There is controversy with regard to the role of NK cells producing IFN-γ in EAE and MS. EAE studies have shown that IFN-γ-producing NK cells are essential in induction of the disease and stimulating M1 macrophages (Shi *et al*., 2000, Dungan *et al*., 2014). Others have demonstrated a protective role of IFN-γ produced by NK cells via different mechanisms. These included supressing effector Th17, induction of nitric oxide (NO) production in neutrophils, macrophages, and DCs, which directly suppress CD4+ T cell proliferation (Huang *et al*., 2006, Hao *et al*., 2010, Arellano *et al*., 2015). Therefore, it is suggested that the role of NK-derived IFN-γ is stage-specific (as NK cells are part of the innate immune system), being essential for the initiation (via pro-inflammatory pathways) but not the progression of EAE (via anti-inflammatory pathways) (Arellano *et al*., 2015). Based on the above, It is important to investigate the role of NK-derived IFN-γ during the course of MS (early-late, and relapse-remission).

With regard to IL-17 expression in NK cells, the percentage of CD3-CD56+ PBMCs expressing IL-17 showed a trend towards being higher in MS patients when compared to healthy controls (HC n=10, MS n=12, unpaired t-test p=0.13). NK cells are a source of IL-17 (Cua and Tato, 2010). IL-17-producing NK cells were reported to be enriched in the CSF of MS patients (Fletcher and Mills, 2012). The causes of this trend

in IL-17 increase were not investigated in this work; however, possible mechanisms may be implicated. Its production may be induced by IL-23 which acts via IL-23 receptor expressed on activated T cells and NK cells (Parham *et al*., 2002). IL-23 receptor signalling is via STAT3, which is reported previously as one of the risk factors in MS pathogenesis (Sawcer *et al*., 2011).

Our results also show an increase in the percentage of NK cells expressing GM-CSF+IFN-γ+ and GM-CSF+IL-17+ in MS patients when compared to healthy controls. This may be due to the higher expression of NK cells expressing GM-CSF, IFN-γ, and IL-17 in MS patients. The role of these cells and their expression in MS is not investigated yet.

Anti-CD3 stimulation does not work directly on NK cells, as they lack CD3 receptors. However, anti-CD28 may work on some activated CD3-CD56+ NK cells which were shown to express CD28 on their surface. Anti-CD28 was shown to be involved as a stimulus in signalling NK cytotoxicity and IFN-γ production (Hunter *et al*., 1997, Galea-Lauri *et al*., 1999, Chen *et al*., 2006). In addition, NK cell activation can be secondary to anti-CD3+anti-CD28-mediated T cell activation that leads to cytokine secretion, e.g., IL-2, which activates NK cells (Kerdiles *et al.*, 2013).

Based on these results, it was important to examine the expression of GM-CSF and IFN-γ by isolated NK cells from MS patients. After isolation, NK cells were stimulated with cytokine combinations that were previously shown to be the best inducers of GM-CSF and IFN-γ expression: IL-15+IL-1β, and IL-15+IL-18 (Cooper *et al.*, 2001c). We stimulated some NK cells with PMA/I only as in the same study (Cooper

et al., 2001c), or with anti-CD3+anti-CD28 only, but we found low GM-CSF or IFN-γ expression in healthy controls and MS patients after using the latter two stimulants (not shown in the results). Few preliminary samples were stimulated with IL-2 with either IL-15 (concentrations are in methods section in this chapter) or TNF-α (50 ng/ml) (Peprotech) (or the three cytokines combined). However, little cytokine expression was detected (results are not shown). Few samples were also stimulated with a combination of IL-2+IL-12, as previous reports have illustrated that this cytokine combination is involved in inducing NK cytotoxicity and cytokine (GM-CSF and IFN-γ) production (Lehmann *et al*., 2001, Parihar *et al*., 2002, Leong *et al*., 2014).

Culturing freshly isolated NK cells (of verified purity ≥ 90%) and stimulating them for three days has resulted in a significant down-regulation in CD56 expression. There are possible reasons for this finding. $CD56^{bright}$ NK cells are considered precursors for the $CD56^{dim}$ NK cells, and upon maturation, $CD56^{bright}$ cells lose their CD56 (Poli *et al.*, 2009). Furthermore, PMA/ionomycin stimulation was shown to down-regulate CD56 expression on NK cells (Mendes *et al.*, 2000). Cytokine expression was examined in isolated and stimulated NK cells expressing CD56 (CD3-CD56+) and in all NK cells regardless of their CD56 expression (CD3-). Our results show similar percentage of NK cells expressing GM-CSF and IFN-γ after stimulation with IL-15+IL-1β, IL-15+IL-18, and IL-2+IL-12, but no differences between healthy controls and MS patients in both NK cell populations examined. Although the number of samples used in isolated NK cell experiment (Healthy n=5, MS n=7) is relatively less than the mixed PBMC population experiment (healthy n=10, MS n=9), the results of isolated NK cell experiment may indicate that factor(s) which are more abundant

in the mixed PBMC population but not in isolated NK are responsible for higher GM-CSF and IFN-γ expression in MS. Therefore, we have performed a Multiplex bead assay for a number of cytokines that, according to the literature, are involved in GM-CSF regulation. The main outcomes were increased concentrations of GM-CSF, IL-2, IL-12, and IL-1β and decreased concentrations of TGF-β in MS patients when compared to healthy controls. Therefore, we performed another experiment using mixed PBMC population and following anti-CD3+anti-CD28 stimulation protocol, in the presence or absence of cytokine neutralization by antibodies. Antibodies used were to neutralize IL-2, IL-12, IL-12/23, and IL-1β.

The results of this work show a reduction in frequency of CD3-CD56+ PBMCs expressing GM-CSF after blocking both IL-2 and IL-12 in MS patients but not in healthy controls. Unlike what was illustrated in CD4+ cells, it appears that blocking of either IL-2 or IL-12 has a reducing effect on the percentage of GM-CSF-expressing CD3-CD56+ PBMCs that did not reach statistical significance when each antibody was used alone. However, when both IL-2 and IL-12 antibodies were added together, each had an additive effect to the reduction, which resulted in statistical significance in MS patients but not in healthy controls.

We show in the experiment on isolated NK cells that adding IL-2 and IL-12 in the same concentration to samples of healthy controls and MS patients resulted in high but similar frequency of GM-CSF-expressing CD3-CD56+ cells in both groups. In addition, we also show that the percentage of anti-CD3+anti-CD28-stimulated CD3-CD56+ PBMCs expressing GM-CSF is significantly higher in MS patients, where we showed both cytokines to be in significantly higher levels in MS patients than healthy controls. Taken together, this may indicate that higher IL-2

and IL-12 concentration in the MS PBMC culture is responsible for the higher frequency of GM-CSF-expressing NK cells in the PBMCs of MS patients. This is because blocking these cytokines resulted in significant reduction of GM-CSF production. Our results are consistent with previous reports which have shown that both IL-2 and IL-12 induce NK cell cytotoxicity and GM-CSF and IFN-γ production (Lehmann *et al*., 2001, Parihar *et al*., 2002, Leong *et al*., 2014). However, our results did not show any significant effects of blocking IL-2 or IL-12 on IFN-γ expression by CD3-CD56+ PBMCs. This may indicate that IL-2 and IL-12 may be redundant in IFN-γ expression by NK cells. This may suggest that there are other pathways involved in IFN-γ expression in the PBMCs of both groups, such as IL-1 or IL-21 (Cooper *et al.*, 2001b, Roda *et al.*, 2006).

The results of this work show that blocking IL-2 and/or IL-12 significantly reduced the percentage of NK cells co-expressing GM-CSF plus IFN-γ and GM-CSF plus IL-17 in the PBMCs of MS patients but not healthy controls, although there was a trend towards a reduction in the frequency of NK cells co-expressing GM-CSF plus IFN-γ in healthy controls. The exact reasons behind these results are not identified in this work. More experiments are needed to evaluate the role of these cells the pathogenesis of MS and progression of inflammation.

Finally, this work revealed that GMR was expressed by NK cells, which was shown indirectly by another study which has shown the effects of GM-CSF on the cytotoxicity of isolated NK cells in humans (Kubista *et al*., 2003). However, upon anti-CD3+anti-CD28 stimulation for 5 days and PMA/I for the last 5 hours, the frequency of NK cells expressing GMR was reduced in MS patients and healthy controls. However, it reached statistical significance in healthy controls ($p=0.01$) but not in

MS patients (p=0.1). GMR regulation in NK cells has not been studied yet. However, it was shown that PMA (PKC agonist) has led to rapid down-regulation of GMR expression in monocytes and neutrophils. Moreover, GM-CSF itself had a down-regulating effect on its receptor expression (Cannistra *et al*., 1990, Brizzi *et al*., 1991).

We show for the first time that NK cells express GMR with no differences in MS patients and healthy controls. GM-CSF has induced NK cytotoxicity after NK cells being treated with IFN-α, which induced GMR expression by NK cells (Kubista *et al*., 2003). Therefore, GMR regulation via IFN-α may be important in the involvement of NK cells in MS. On the other hand, our results show that blocking IL-2 significantly increased GMR expression by NK cells in healthy controls and MS patients despite the PMA/I stimulation. This may indicate that the previous studies findings of PMA/I down-regulatory effect on GMR (Cannistra *et al*., 1990, Brizzi *et al*., 1991) may be attributed to IL-2. In addition, increased GMR expression may be attributed to the reduction in GM-CSF levels upon IL-2 blocking. However, this may need further experiments to be confirmed. In addition, blocking IL-2 and IL-12 together resulted in significant increase in GMR-expressing NK cells in MS patients but not in healthy controls. This effect appeared to be IL-2-related, because IL-12 alone did not change GMR expression in NK cells in MS patients or healthy controls. Despite higher IL-2 levels in MS patients' culture supernatants, GMR expression was reduced to the same low level as in healthy controls after anti-CD3+anti-CD28 stimulation. This is possibly because the frequency of NK cells expressing GMR is approaching zero% in healthy controls and MS patients. This may indicate that direct role of GM-CSF on NK cells in

MS may be minor. However, it is interesting to find out changes in GMR expression and NK cell activity after DMT for MS patients.

In conclusion, our results show that GM-CSF-expressing NK cells (CD3-CD56+) are in higher frequency in MS patients in the mixed PBMC population. However, isolated NK cells did not show any difference in GM-CSF expression in MS patients and healthy controls. This is possibly due to higher levels of IL-2 and IL-12 in the mixed PBMC population. That was confirmed with blocking IL-2 and IL-12, which reduced the frequency of NK cells expressing GM-CSF in MS patients. Finally, we showed that IL-2 and IL-12 may be involved in down-regulation of GMR expression on NK cells in MS patients. This may indicate that GM-CSF may not have a direct role on NK cell activity in MS. In the next chapter, the presented work was done to investigate GM-CSF and GMR expression and regulation in another PBMC subset (B cells) with relevance to MS.

Chapter 5. GM-CSF and GM-CSF Receptor (GMR): Expression, Regulation, and Effects on B cells and Relevance to MS

5.1 Introduction

In the previous chapters, the expression and regulation of GM-CSF and GMR in T cells, monocytes, and NK cells and the relevance to MS were investigated. In this chapter, the presented work was done to investigate the expression of GM-CSF and GMR in B lineage cells (CD20+ cells from the peripheral blood) in MS. In addition, the effects of blocking cytokines found to be elevated in MS on the expression of GM-CSF and GMR were investigated in the mixed PBMC population in MS patients, with a focus on B cells. The expression of GMR in isolated B cells with or without EBV infection was also examined. Finally, the effects of blocking GM-CSF on the viability of B cells in the absence of presence of EBV transformation were investigated.

The majority of B cells exert their pro-inflammatory role in MS via a number of pathways, including antigen presentation to T cells, production of auto-antibodies, and secretion of pro-inflammatory cytokines (e.g., IL-6 and TNF-α) (Lehmann-Horn *et al*., 2013). In addition, IL-10-secreting B cells exert a regulatory role, and were found in MS patients in lower frequencies compared with healthy controls (Iwata *et al*., 2011). Previous studies have found that B cells express higher IL-6, TNF-α, and less IL-10 in MS patients (Duddy *et al*., 2007, Barr *et al*., 2012). A recent study demonstrated higher GM-CSF expression in isolated B cells in MS patients, which contributes to myeloid cell activation (Li *et al*., 2015). In addition, another study has shown IL-17-producing B cells contributing to RA inflammation

(Schlegel *et al*., 2013). With regard to IFN-γ, its role is controversial in MS. A relatively small percentage of B cells were shown to produce IFN-γ (Harris *et al*., 2005a, Ballesteros-Tato *et al*., 2014, Bao *et al*., 2014), and the role of B cell-derived IFN-γ is not yet determined in MS. All the above-mentioned cytokines were shown to be produced by B cells at the protein level (via using ELISA or flow cytometry). However, to our knowledge, GM-CSF expression has not been investigated in B cells in PBMCs, where B cells can interact with other cells (e.g., T cells) and respond to other cytokines/factors produced by the other cell types. B cell-derived GM-CSF may contribute to the pathogenicity of B cells in MS. We also investigate IFN-γ and IL-17 expression by B cells in MS, which to our knowledge was not done before.

B cells were also shown to express GM-CSF receptor (Harris *et al*., 2000, Weber *et al*., 2014). Autocrine GM-CSF was shown to increase viability of normal and malignant B cells (Harris *et al*., 2000). However, GMR expression on B cells has not been investigated in MS so far. Any significant changes in GMR expression by B cells may indicate changes in the sensitivity of B cells to GM-CSF. We hypothesized that in MS, GM-CSF and/or GMR expression will be increased as a mechanism to induce B cell survival. Hence, we determined GMR expression in B cells in PBMCs and in isolated B cells in MS. After being able to detect the receptor, we proceeded with investigating the effects of GM-CSF blockade on B cell viability and GMR expression in MS patients in comparison to healthy controls.

EBV infection involves B cells mainly. EBV is one of the most important environmental risk factors for MS (Ascherio *et al*., 2001, Levin *et al*., 2005, DeLorenze *et al*., 2006). EBV is involved in MS pathogenesis through several pathways, including immortalization of B

cells, making them a long-term reservoir of the virus, and effective antigen-presenting cells. This is accomplished via the EBV latent antigen EBNA, which also was shown to upregulate the expression of CXCL10 (IFN-γ-inducible protein-10) (Kang and Kieff, 2015). In addition, EBV infection induces the expression of a molecule in transformed B cells called transcription factor 2 (TF2), which upregulates the expression of chemokine receptor 1 (CCR1) (Avasarala *et al*., 2008). Both CXCL10 and CCR1 are involved in inducing T cell and monocyte migration.

Moreover, molecular mimicry to myelin peptides may play a role in MS pathogenesis when accompanied with genetic risk factors. Some EBV peptides may share similarities in structure with neuroantigens in general. In genetically susceptible individuals to MS, EBV infected B cells present EBV peptides to T cells, inducing the activation and proliferation of specific T cell clones against these viral peptides. When these activated T cells migrate to the CNS and encounter structurally similar neuroantigens, they will be re-stimulated and proliferate, producing inflammatory factors resulting in damage to the CNS tissues(Wucherpfennig and Strominger, 1995, Lang *et al*., 2002). EBV viral load was found to be significantly higher in MS patients than healthy controls (Nejati *et al*., 2016). There are reports of GM-CSF being produced in variable levels by some EBV-lymphoblastoid cell lines (LCLs) (Whittingham *et al*., 1993, Rochford *et al*., 1997); however, others failed to detect GM-CSF in LCLs (Wroblewski *et al*., 2002). This indicates variability in GM-CSF expression among different LCLs. Therefore, we have not detected GM-CSF in EBV infected B cells.

As mentioned above, a previous study has shown that autocrine GM-CSF induced survival of healthy and malignant B cells, which indicates

that GMR is expressed by those cells (Harris *et al*., 2000). Therefore, we aimed to investigate if GMR is expressed on EBV infected B cells and whether GM-CSF exerts any role on their viability. In addition, there are no reports of GMR expression by EBV LCLs. Hence, we investigated GMR expression by isolated B cells in the same healthy controls before and after EBV infection. We also investigated whether GM-CSF blockade would have effects on GMR expression and B cell viability.

In summary, the hypotheses tested in this chapter were that the percentage of GM-CSF-expressing B cells is higher in the mixed PBMC population in MS than in healthy controls. In addition, blocking specific GM-CSF-inducing cytokines that are elevated in MS may reduce the frequency of GM-CSF-expressing B cells, with changes in GMR expression. We also tested the hypothesis that GM-CSF enhances B cell survival in MS more than in healthy controls, and that EBV infection affects GMR expression on B cells.

5.2 Subjects and Methods

5.2.1 Subjects (Inclusion/Exclusion Criteria)

For anti-CD3+anti-CD28 stimulation, and multiplex bead assay experiments, the same inclusion/exclusion criteria as in section 3.2.1 were applied. All patients were with untreated RRMS in the above-mentioned experiments. With regard to patients participating in the isolated B cells with/without EBV infection, their inclusion criteria are being ≥ 18 years old having current relapse and EDSS range of ≥ 2 to ≤ 6.5. They were also included even if they were receiving DMT treatment. Their exclusion criteria were as in chapter 3 section 3.2.1. All the important details of healthy controls and MS patients and the experiment(s) each subject has participated in are listed in appendix I.

5.2.2 Methods

All the below mentioned sample collection and experimental work was approved by Nottingham Research Ethics Committee, as shown in Appendix II. PBMCs were isolated, counted, frozen and thawed as in section 2.1 and 2.2; anti-CD3+anti-CD28 stimulation was performed as in section 2.3. Flow cytometry staining and data acquisition/analysis was done as in sections 2.5 and 2.7. Multiplex bead assay was done as in section 2.8.

5.2.2.1 B cell Isolation and Stimulation

PBMCs were isolated and counted from freshly collected venous blood (30-40 ml). B cells were isolated by positive selection of magnetically labelled CD20 cells using magnetic CD20 cell sorting kit (Miltenyi Biotech) and following the manufacturer's instructions. Purity was checked and samples with ≥ 90% were included (Figure 5-1). Cells (with or without EBV infection, see below) were cultured in 96-well round-bottom plates and divided into two wells for each sample ($3x10^5$ cells/200 μl/well). Cells in one well were left unstimulated, and cells in the other cells were stimulated with soluble CD40L (1μg/ml) (eBioscience) + F(ab')2 Anti-Human IgM+IgG (10μg/ml) (eBioscience) for 24 hrs, then stained with fluorescent labelled antibodies and run for flow cytometry. With regard to detection of the proportion of apoptotic cells, B cells (with or without EBV infection) were cultured and either left unstimulated or stimulated as above, with or without adding GM-CSF 10 ng/ml (Peprotech), anti-GM-CSF monoclonal antibody, or Mouse IgG1 isotype control (200 μg/ml) (R&D Systems). Cells were incubated at 37°C with 5% CO_2 for 4 days, then stained and run for flow cytometry.

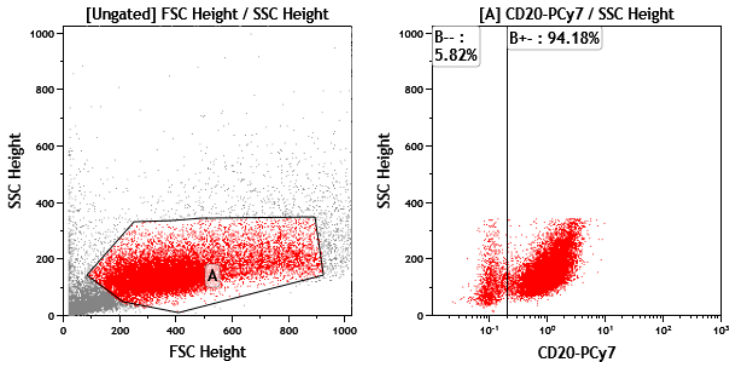


Figure 5-1: Determination of B cell purity after magnetic isolation.

After magnetic isolation of CD20 cells from fresh PBMCs, cells were stained with CD20 and run on the flow cytometer to examine their purity. Samples were included when purity was ≥ 90%.

5.2.2.2 EBV Infection of B cells-Generation of B95.8 Lympho-blastoid Cell Line (LCL)

Frozen B95.8 marmoset cells (kindly provided by Jill Brooks in Birmingham) (~10 x 10^6 cells) were thawed and cultured for 1 week in 10 ml medium (10% FCS, 100 units per ml of penicillin, 0.1 mg/ml of streptomycin, and 2 mM of glutamine in RPMI medium), in a 37°C incubator with 5% CO_2 without changing the medium. After PBMC isolation, 5-10 x 10^6 cells were taken and centrifuged (300 x g, 5 min) to remove the supernatant. 3-4 ml B95.8 LCL culture supernatant was taken and centrifuged (200 x g, 5 min) and added to the PBMCs through 0.45 µm syringe filter (Sartorius Stedim Biotech Ministart®) and 10 ml syringe. Then, a few drops of FCS were added to the cell mixture and incubated overnight in 37°C 5% CO_2 incubator. On the next day, cell/virus mixture were centrifuged (300 x g, 5 min) to discard the supernatant, re-suspended in 2 ml of Cyclosporin A (CSA) medium (usual medium with 1µg/ml Cyclosporin A (all from Sigma Aldrich)) to suppress cytotoxic T cells that may hinder the growth of EBV-infected B cells, and placed in one well of a 24-well plate. Cells were incubated in a

37°C 5% CO_2 incubator and fed after 1 week taking 1 ml supernatant and replacing it with fresh CSA medium. Medium change was performed twice a week, until a confluent layer was formed at the bottom of the well plate, then they were split into 2 wells. The same was repeated, then cells were split into 4 wells. After more proliferation, all cells were transferred into a T25 flask (vent cap, Corning Flask®) using usual medium. When a confluent layer was seen, the cells were transferred to a T75 culture flask (vent cap, Corning Flask®). Passage of the cells was performed twice a week by taking half of the cell suspension (which was frozen or discarded) and replacing it with fresh medium. Once new LCL were established from each donor, these were expanded in culture and used for further experiments. The small variations in the volume of LCL supernatant or PBMC count did not affect the results, because a cell line was created; i.e., if the cells were cultured for one month or for 6 months, same results were obtained. My colleague Dr Elena Morandi has shown me how to infect B cells with EBV only once, after this I have done everything by myself. Infection of the cells with EBV and the handling of EBV-infected cells were performed following the University of Nottingham risk assessment protocols for Biological agents.

5.2.2.3 Determination of Apoptosis, Flow Cytometry and Data Analysis

After B cells were stimulated for 4 days as described above, cells were transferred to FACS tubes, washed twice with cold PBS, and re-suspended in 100 µl 1X binding buffer (eBioscience). Cells were stained with 5 µl of Annexin V-FITC and 5 µl Propidium iodide (PI) (Apoptosis Kit, eBioscience), then incubated for 15 minutes in the dark at room

temperature (25°C). Cells were then re-suspended in 200-300 µl in 1X binding buffer and analysed by flow cytometry within one hour.

5.3 Results

5.3.1 Demographic and Clinical Characteristics of the Participants

The tables below demonstrate the demographics and clinical characteristics of all participants recruited for each experiment.

Table 5-1: Demographic and clinical characteristics of subjects who contributed samples to the studies of B cells isolation and 24 h stimulation experiment

Demographics	Healthy Controls -EBV	MS in relapse -EBV	Healthy Controls +EBV	MS in relapse +EBV
Total no. of participants	6	6	9	3
Age mean (y) (SD)	42.5 (11.5)	46.5 (11.9)	41.11 (12.5)	50 (10.8)
Female no. (%)	5 (83.3)	6 (100)	7 (77.7)	2 (66.6)
Ethnicity:				
White (%)	4 (66.6)	4 (66.6)	6 (66.6)	3 (100)
Other (%)	1 (16.6)	2 (33.3)	1 (11.1)	-
Indian (%)	1 (16.6)	-	2 (22.2)	-
Time since MS diagnosis (y)(SD)	-	9.6 (2.07)	-	15 (1)
EDSS mean (SD)	-	2.4 (1.1)	-	4 (2)
Smoking status:				
Smoker (%)	0	1 (16.6)	0	1 (33.3)
Ex-smoker (%)	0	0	0	0

Table 5-2: Demographic and clinical characteristics of subjects who contributed samples to the studies of B cells isolation and 96 h stimulation-apoptosis experiment

Demographics	Healthy Controls -EBV	MS in relapse -EBV	Healthy Controls +EBV	MS in relapse +EBV
Total no. of participants	3	5	5	0
Age mean (y) (SD)	36.3 (8.3)	43.8 (6.9)	38.2 (15.45)	-
Female no. (%)	3 (100)	5 (100)	5 (100)	-
Ethnicity:				
White (%)	2 (66.6)	4 (80)	4 (80)	-
Other (%)	0	1 (20)	0	-
Indian (%)	1 (33.3)	-	1 (20)	-
Time since MS diagnosis (y)(SD)	-	9.1 (2.1)	-	-
EDSS mean (SD)	-	2.3 (1.2)	-	-
Smoking status:				
Smoker (%)	0	1 (20)	0	-
Ex-smoker (%)	0	0	0	-

5.3.2 Increased Frequency of B cells Expressing GM-CSF and GM-CSF+IFN-γ+ in MS Patients

Stimulation of PBMCs (anti-CD3+anti-CD28 protocol) led to a significant increase in the percentage of CD3-CD20+ lymphocytes (B cells) expressing GM-CSF in MS patients (HC n=9, MS n=11, unpaired t-test p=0.0375) (Figure 5-2 A). In addition, the frequency of B cells co-expressing GM-CSF and IFN-γ was significantly increased in MS patients (HC n=9, MS n=11, unpaired t-test p=0.007) (Figure 5-2 C). Interestingly, this was accompanied by a significant reduction in the frequency of IFN-γ-expressing B cell population in MS patients in comparison with healthy controls (HC n=9, MS n=11, Mann-Whitney test p=0.003) (Figure 5-2 B). The total population of B cells expressing IL-17, and the subpopulation co-expressing GM-CSF and IL-17 showed no differences between healthy controls and MS patients (Figure 5-2 D and E).

5.3.3 Blocking IL-2, IL-12, IL-2/12, and IL-12/23 Reduces the Frequency of B Cells Expressing GM-CSF in MS Patients

Frozen isolated PBMCs from healthy controls and MS patients were stimulated (anti-CD3+anti-CD28 protocol). Antibodies of IL-2, IL-12, IL-12/23, and IL-1β were also added to the cell culture for 5 days. PBMC staining showed significant reduction in the frequency of B cells expressing GM-CSF upon blocking of IL-2, IL-12, IL-2 plus IL-12, and IL-12/23 in MS patients but not in healthy controls. Wilcoxon test was used for comparison (Table 5-3) (Figure 5-3 A and B).

Table 5-3: List of cytokine antibodies that reduced the frequency of GM-CSF-expressing B cells in MS.

PBMCs were isolated and stimulated with anti-CD3+anti-CD28 for 5 days with the cytokine antibodies listed. The details listed are the number of samples (n) and the p value in each group.

Cytokine blocked	MS group details	HC group details
IL-2	n=11, p=0.042	n=9, p=0.18
IL-12	n=10, p=0.027	n=9, p=0.97
IL-2+IL-12	n=10, p=0.048	n=6, p=0.43
IL-12+IL-23	n=10, p=0.037	n=9, p=0.19

5.3.4 Other Effects of IL-2, IL-12, IL-12/23, and IL-1β Antibodies on CD20+ PBMCs

Stimulation of PBMCs from healthy controls and MS patients with anti-CD3+anti-CD28 led to the following effects illustrated in Table 5-4 below.

Table 5-4: Different effects of cytokine antibodies on the frequency of B cells expressing IFN-γ, IL-17, and their co-expression with GM-CSF in healthy controls and MS patients.

Frozen/thawed PBMCs were stimulated following anti-CD3+anti-CD28 protocol. Live CD3-CD20+ cells were selected. IL-2+IL-12 antibodies significantly reduced IL-17 expression in MS patients. IL-12 and IL-12/23 antibodies reduced GM-CSF+IFN-γ+ expression in MS patients. IL-2, IL-2+IL-12, and IL-1β antibodies reduced GM-CSF+IL-17+ expression in MS patients. On the other hand, IL-2, IL-12, and IL-2+IL-12 antibodies reduced IFN-γ expression in healthy controls. Significant p values were in red font and ≤ 0.05.

Cytokine blocked	Effect	Healthy (n)	P value	MS (n)	P value
IL-2+IL-12	Reduced IL-17	6	0.21	7	0.03
IL-12	Reduced GM-CSF+IFN-γ+	9	0.75	10	0.02
IL-12/IL-23		9	0.64	10	0.04
IL-2	Reduced GM-CSF+IL-17+	9	0.99	11	0.01
IL-2+IL-12		6	0.99	10	0.02
IL-1β		9	0.43	10	0.03
IL-2	Reduced IFN-γ	9	0.01	11	0.32
IL-12		9	0.03	10	0.84
IL-2+IL-12		6	0.03	10	0.37

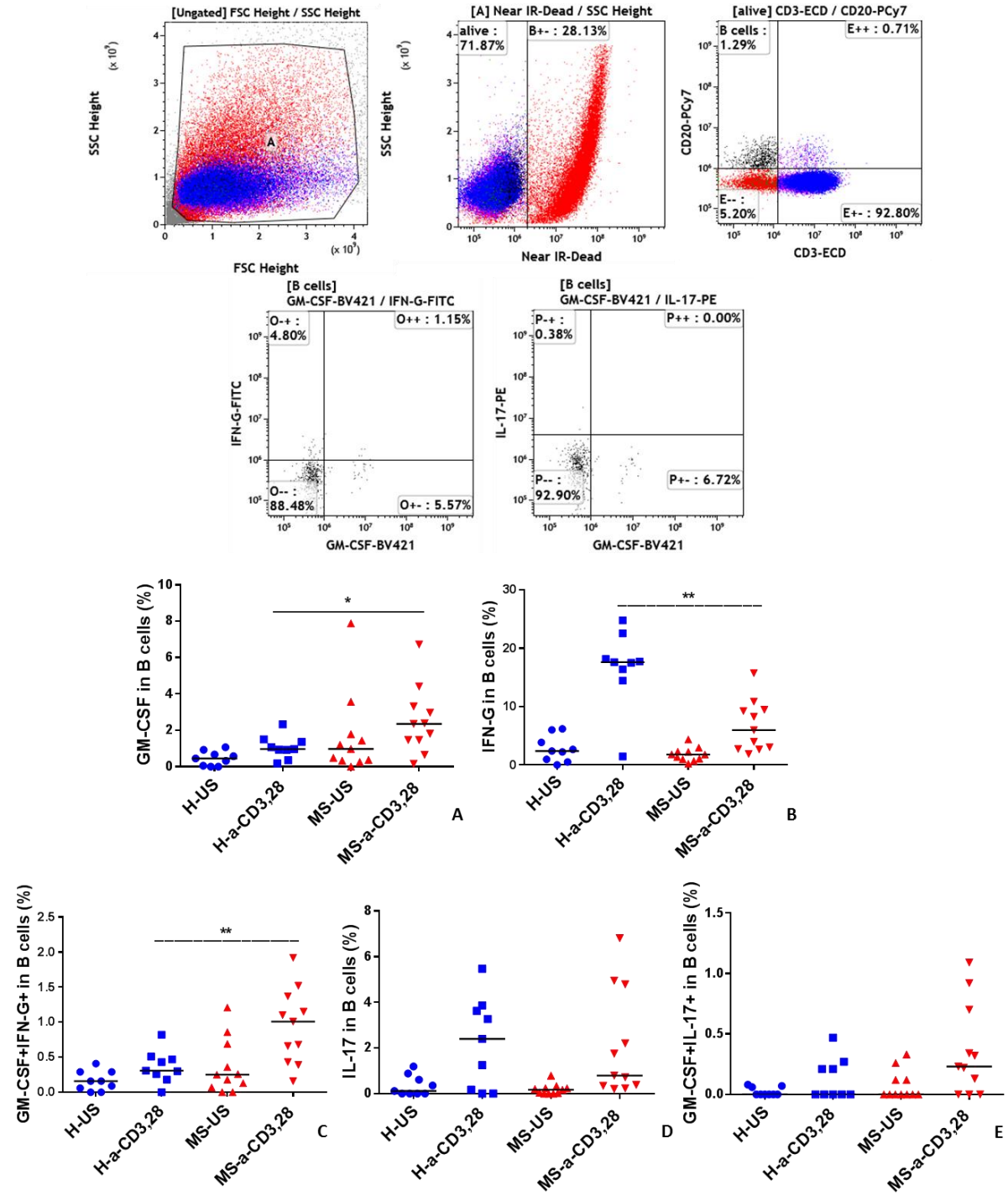


Figure 5-2: Expression of GM-CSF, IFN-γ, and IL-17 in B cells.

Frozen/thawed PBMCs were stimulated following anti-CD3+anti-CD28 protocol in healthy controls (n=9) and MS patients (n=11). Live CD3-CD20+ cells were gated (black-coloured population in flow cytometry plots). H-US: unstimulated samples from healthy controls; H-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from healthy controls; MS-US: unstimulated samples from MS patients; MS-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from MS patients. * $p<0.05$; ** $p<0.01$. Horizontal lines are medians

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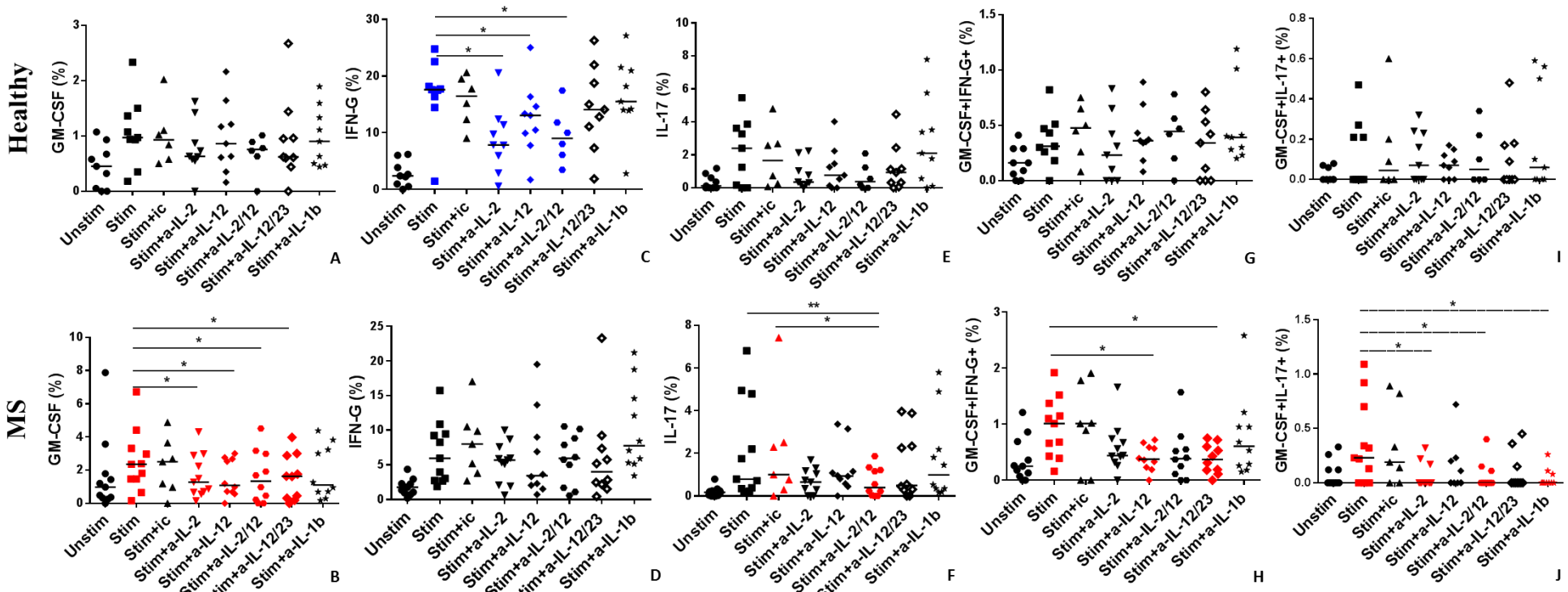


Figure 5-3: Effects of anti-cytokine antibodies on the frequency of B cells expressing GM-CSF, IFN-γ, IL-17, GM-CSF/IFN-γ, and GM-CSF/IL-17.

Frozen/thawed cultured PBMCs in healthy controls (H) (n=11) and MS patients (n=12). PBMCs were cultured either unstimulated (Unstim) or stimulated (Stim) with anti-CD3+anti-CD28 for 5 days. This was done with or without adding one of the antibodies to block the following cytokine (a-cytokine: cytokine antibody): IL-2, IL-12, IL-2+IL-12 (IL-2/IL-12), IL-12+IL-23 (IL-12/IL-23), and IL-1β. In addition, cells were cultured with antibody isotype control (ic) as controls. Only live CD3-CD20+ cells were selected and analysed for the above-listed parameters. Upper row represents healthy control results, lower row represents MS patient results * $p<0.05$. Horizontal lines represent medians.

5.3.5 No differences in Frequency of GMR-Expressing B Cells in Healthy Controls and MS Patients

Isolated and Stimulated PBMCs (anti-CD3+anti-CD28 protocol) from healthy controls and MS patients were stained for GMR. There were no significant differences between the two groups in CD20+ cells (HC n=7, MS n=9, Mann-Whitney test p=0.46) (Figure 5-4 A). In addition, there was no change in GMR expression upon stimulation in healthy controls (Wilcoxon test HC n=7, p=0.68), but its expression increased in a statistically significant manner in the MS group (paired t-test MS n=9, p=0.04). On the other hand, the frequency of GMR-expressing B cells was higher when B cells were isolated and stimulated with CD40L+ anti-IgG/IgM for 24 hours (median around 20% in non EBV infected cells). In this set of results, the frequency of GMR-Expressing B cells was similar in healthy controls and MS patients (Figure 5-5). In addition, none of the cytokine antibodies affected GMR expression in B cells in healthy controls and untreated RRMS patients in remission stage.

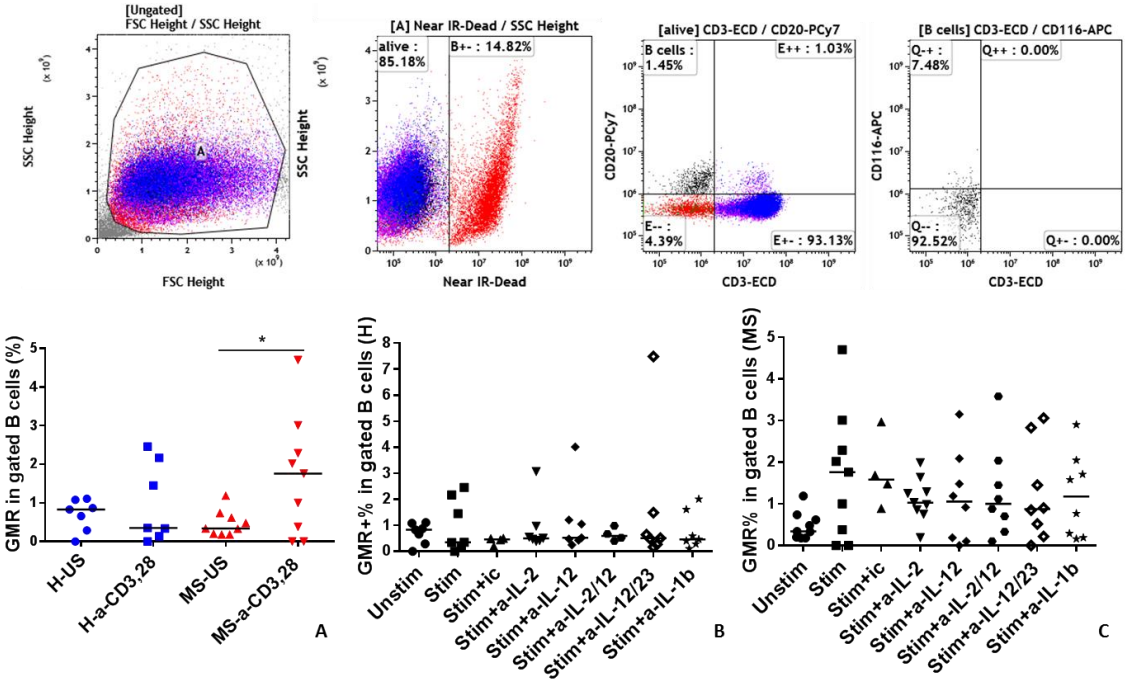


Figure 5-4: GMR expression in B cells under different stimulation/blocking conditions.

Upper panel: a representative example of flow cytometry plots to illustrate how the gating was done: after excluding cell debris (first plot), only live cells were selected (second plot). From these cells, only CD3-CD20+ cells were selected and examined for GMR (CD116) expression. A: GMR expression in B cells of healthy controls and MS patients. B: GMR expression by NK cells in healthy controls. C: GMR expression by NK cells in MS patients. PBMCs were cultured either unstimulated (Unstim) or stimulated (Stim) with anti-CD3+anti-CD28 for 5 days. This was done with or without adding antibody isotype control (ic) or one of the antibodies to block the following cytokine (a-cytokine: cytokine antibody): IL-2, IL-12, IL-2+IL-12 (IL-2/IL-12), IL-12+IL-23 (IL-12/IL-23), and IL-1β. Only live CD3-CD20+ cells were examined (black-coloured population). H-US: unstimulated samples from healthy controls; H-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from healthy controls; MS-US: unstimulated samples from MS patients; MS-a-CD3,28: anti-CD3+anti-CD28-stimulated samples from MS patients. * $p<0.05$. Horizontal lines are medians.

5.3.6 EBV infection Reduced Percentage of GMR-Expressing B Cells

Isolated B cells from healthy controls and MS patients were either left uninfected or infected with EBV. EBV infected (MS n=3, HC n=9) or non-infected (MS n=6, HC n=6) B cells were stimulated with CD40L and anti-IgG/IgM for 24 hours. EBV infected B cells were taken from different MS patients, not the same patients who gave blood samples for

non-EBV infected B cell experiment for GMR expression detection. On the other hand, all the six samples of non-EBV-infected B cells from healthy volunteers were part of the nine samples who participated in EBV-infected B cell experiment for GMR expression. Flow cytometry staining showed a significant reduction in frequency of GMR-expressing B cells after EBV infection in healthy controls (EBV-, meaning non-EBV infected n=6, EBV+ n=9, Wilcoxon test p=0.03), but not in MS patients (EBV- n=6, EBV+ n=3, Wilcoxon test p=0.25) (Figure 5-5).

5.3.7 Blocking GM-CSF in Non EBV Infected and EBV Infected B cells

Isolated B cells from healthy controls and MS patients were stimulated with CD40L+ anti-IgG/IgM for 4 days with or without adding GM-CSF and anti-GM-CSF antibody (see methods section 5.2.2.1). EBV infection was performed on B cells from healthy controls and not from MS patients in this experiment, as in section 5.2.2.2. Blocking GM-CSF revealed no change in the percentage of apoptotic cells (Figure 5-6 A, D), dead cells (Figure 5-6 B, E), live cells (Figure 5-6 C, F), and in the GMR expression in live cells (Figure 5-7 A, B) in healthy controls and MS patients. For all the results mentioned above, Wilcoxon test was used for comparison.

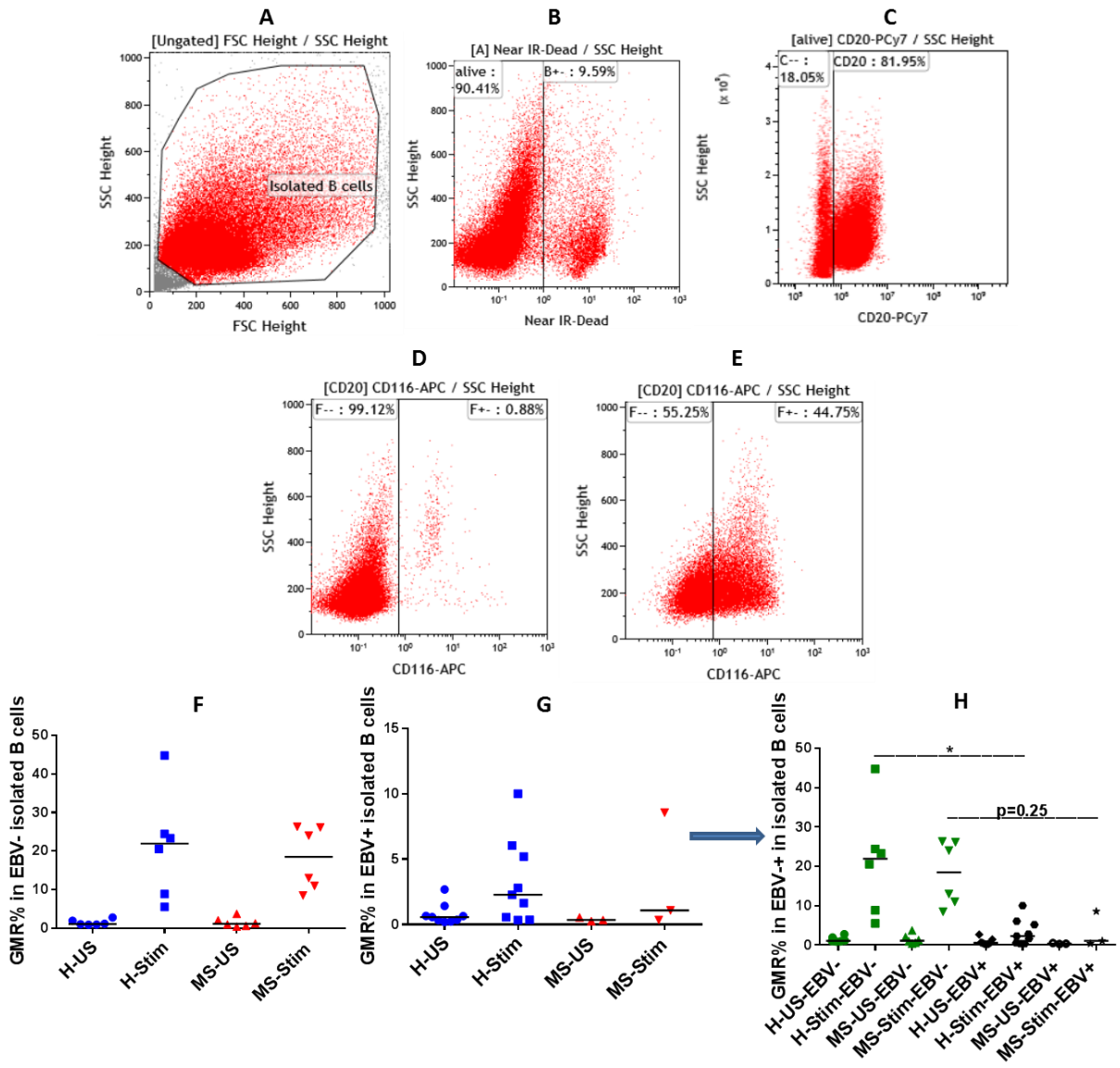


Figure 5-5: GMR expression in isolated B cells with/without EBV infection.

B cells with/without EBV infection were either left unstimulated, or stimulated with CD40L+anti-IgG/IgM for 24hr in healthy controls and MS patients. A-E: representative flow cytometry plots of B cells without EBV infection, illustrating how the gating was done: after excluding cell debris (A), only live cells were selected (B). From these cells, only CD20+ cells were selected (C) and examined for GMR (CD116) expression in unstimulated (E) and stimulated (F) cells. Wilcoxon test was used for comparison of GMR expression between B cells with and without EBV infection. H-US (H-MS): unstimulated cells from healthy controls (or MS patients), H-Stim (MS-Stim): stimulated cells from healthy controls (or MS patients), EBV-: without EBV infection, EBV+: with EBV infection. * $p<0.05$. Horizontal lines are medians.

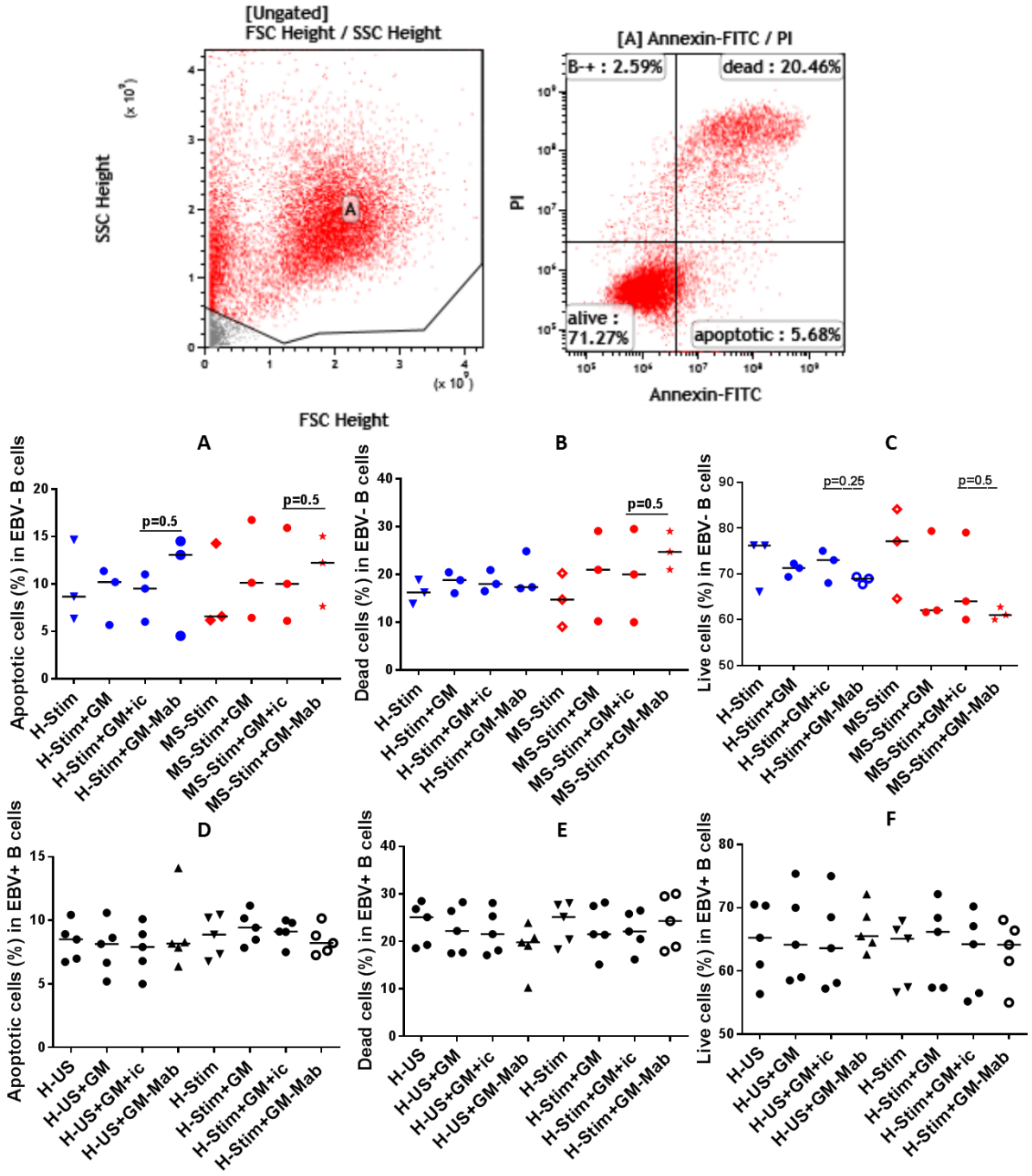


Figure 5-6: Frequency of apoptotic, dead and live cells in B cells with/without EBV infection in MS patients and healthy controls.

B cells with/without EBV infection were either left unstimulated (US), or stimulated (Stim) with CD40L+anti-IgG/IgM for 4 days in healthy controls (H) and MS patients, with/without GM-CSF (GM), antibody isotype control (ic), and GM-CSF neutralizing antibody (GM-Mab). EBV-: without EBV, EBV+: with EBV. Upper panel: representative flow cytometry plots showing how cells were gated. Apoptotic cells are Annexin+ propidium iodide (PI)-, dead cells are Annexin-PI+, while live cells were Annexin-PI-. A, B, and C: percentage of apoptotic, dead, and live EBV-uninfected B cells, respectively. D, E, and F: percentage of apoptotic, dead, and live EBV-infected B cells, respectively. Healthy controls (H) (EBV- n=3, EBV+ n=5), and MS patients (EBV- n=3, no EBV+ MS patients). Horizontal lines are medians.

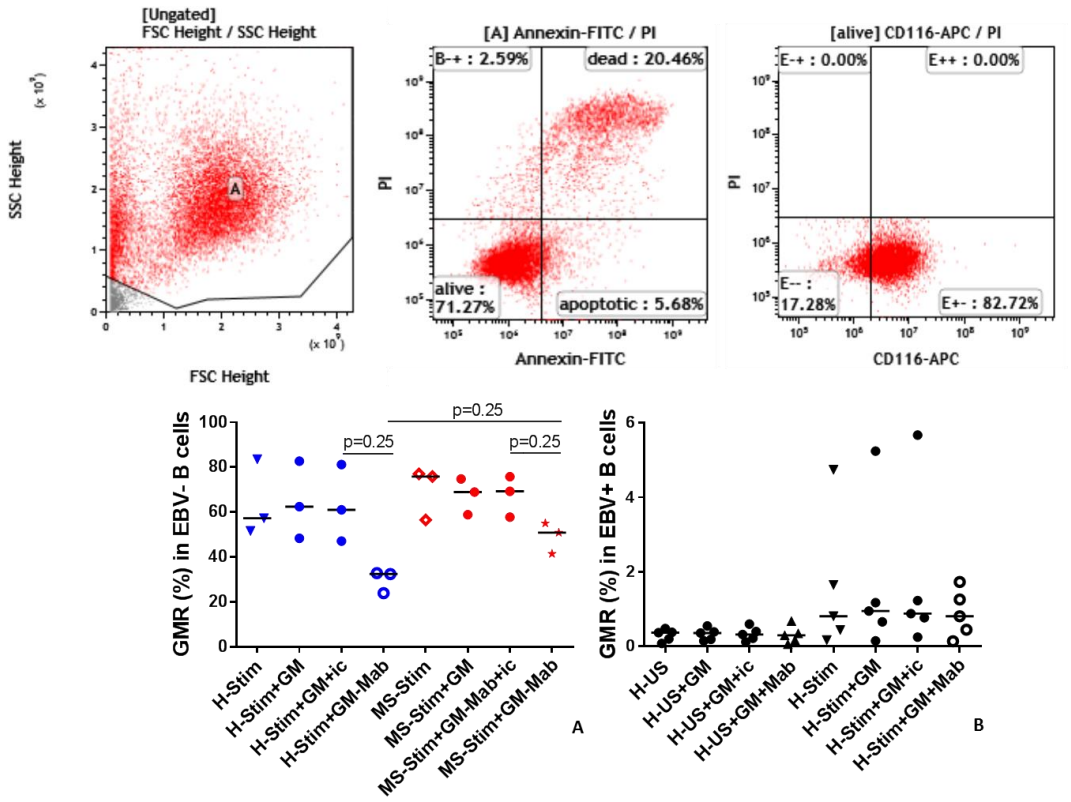


Figure 5-7: GMR expression in isolated B cells with or without EBV infection in healthy controls and MS cases.

B cells with/without EBV infection were either left unstimulated (US), or stimulated (Stim) with CD40L+anti-IgG/IgM for 4 days in healthy controls and MS patients, with/without GM-CSF (GM), antibody isotype control (ic), and GM-CSF neutralizing antibody (GM-Mab). **A:** GMR expression in non-EBV infected B cells; **B:** GMR expression in EBV-infected B cells. EBV-: without EBV, EBV+: with EBV. Representative flow cytometry plots showing how cells were selected. Apoptotic cells are Annexin+ propidium iodide (PI)-, dead cells are Annexin-PI+, while live cells were Annexin-PI-. GMR (CD116) expression was calculated in live B cells. Healthy controls (H) (EBV- n=3, EBV+ n=5), and MS patients (EBV- n=3, no EBV+ MS patients). Horizontal lines are medians.

Table 5-5 below illustrates a summary of the significant findings in this chapter that will be discussed in the next section.

Table 5-5: Main findings of work illustrated in chapter four

Result (compared to healthy controls)	Significance test	P value
Increased frequency of B cells expressing GM-CSF in MS patients	Unpaired t-test	0.03
Increased frequency of B cells co-expressing GM-CSF and IFN-γ in MS patients	Unpaired t-test	0.007
Blocking IL-2, IL-12, IL-2/12, and IL-12/23 Reduces the Frequency of B Cells Expressing GM-CSF in MS Patients	Wilcoxon test	Table 5-3

5.4 Discussion

Our results show a significant increase in the percentage of B cells expressing GM-CSF in untreated RRMS patients when compared to healthy controls. This was shown after stimulation of PBMCs with anti-CD3+anti-CD28 for 5 days and PMA/I for the last 5 hours. This may indicate that B cells are more activated in MS patients in remission stage, and this may be due to higher T cell activation with increased CD40L expression that may bind to CD40 expressed on B cells (Trinchieri, 2003). f(ab) anti-IgM/IgG (reproduces B cell receptor binding antigen), accompanied by CD40-CD40L interaction (reproduced by anti-CD40 or CD40L) is an efficient B cell activation pathway. This is consistent with the findings of another recent study where isolated B cells were stimulated with either PMA/I or CD40L+ anti-IgM (Li *et al*., 2015) (published after these experiments were completed). Our results are showing for the first time that more B cells produce GM-CSF in untreated RRMS patients within the mixed PBMC population that is meant to favour T cell expansion.

It has been shown that GM-CSF production pathways in B cells via STAT5/6 were enhanced in MS patients, and B cell depleting therapies have been reported to normalize these pathways in B cells and reduce their GM-CSF production (Li *et al*., 2015). Therefore, attenuating autoimmune response in MS after B cell depleting therapy may be in part due to depletion of B cell-derived GM-CSF.

Higher frequency of GM-CSF-expressing B cells was accompanied by a reduction in frequency of IFN-γ-expressing B cells in untreated RRMS patients when compared to healthy controls. There is no explanation for this finding so far. Previous studies have demonstrated that B cells produce IFN-γ, which may aggravate autoimmune pathology (Harris *et*

al., 2005a, Ballesteros-Tato *et al*., 2014, Bao *et al*., 2014). B cell-derived IFN-γ has recently shown a pathogenic role in a mouse model of arthritis. Higher levels of B cell-derived IFN-γ were detected, acting via different mechanisms. These included an autocrine action on B cells to induce their antigen presentation function, acting on monocytes/macrophages to stimulate them and induce their antigen presentation function, and acting on T cells to stimulate their autoreactive functions (Bao *et al*., 2014, Fillatreau, 2015, Olalekan *et al*., 2015).

Therefore, we hypothesized that IFN-γ expression by B cells may be higher in MS patients in remission than healthy controls. This is also because B cell-derived IFN-γ requires the presence of Th1 or IL-12/IL-18 (Harris *et al*., 2005a), and IL-12 was shown to be significantly higher in MS patients in our results (chapter 3 section 3.3.7). IFN-γ production pathway in B cells was shown to be entirely dependent on IFN-γ-IFN-γ receptor-T-bet loop (Harris *et al*., 2005a). Nothing was found in the literature with regard to change in IFN-γ expression by B cells in MS. In addition, no change in T-bet expression was shown by previous research in MS patients during remission and healthy controls (Frisullo *et al*., 2006, Frisullo *et al*., 2008, Iorio *et al*., 2009). On the other hand, there may be suggested pathways which may explain this finding. IL-4 was demonstrated to be a B cell-stimulating cytokine which induces B cell differentiation to so-called B2 or IL-4- (and not IFN-γ-) producing B cells (Harris *et al*., 2005b). In addition, it was shown that IL-4 induced GM-CSF expression by B cells (Li *et al*., 2015). IL-4 was not measured in culture supernatants in our study. However, previous studies have found higher IL-4 levels in peripheral blood during the remission stage of MS (Soderstrom *et al*., 1995, Obradovic *et al*., 2012).

We also show that the blockade of IL-2, IL-12, and IL-12/23p40 significantly reduced GM-CSF-expressing B cells in untreated RRMS patients but not in healthy controls (Figure 5-3 A and B). IL-2R-α (CD25) was shown to be expressed on immature (Boyman and Sprent, 2012) and mature (Amu and Brisslert, 2011) B cells. When IL-2 binds to its receptor, it induces B cell proliferation and differentiation into plasma cells and increases antibody secretion (Mingari *et al*., 1984, Nakagawa *et al*., 1985, Miyawaki *et al*., 1987, Le Gallou *et al*., 2012). However, no reports have yet been published with regard to IL-2 effects on GM-CSF expression in B cells. On the other hand, higher cytokine expression is a sign of activated lymphocytes in general (Ai *et al*., 2013). Therefore, inhibited activation and proliferation of B cells (mediated by blocking IL-2) has resulted in reduced GM-CSF expression. This effect became statistically significant in untreated RRMS patients, possibly because of higher IL-2 levels in culture supernatants of MS patients. It was shown that GM-CSF may exert an autocrine effect on increasing B cell survival (Harris *et al*., 2000), which may have a pathogenic role in MS and may aggravate the disease. We show here that higher IL-2 levels mediated higher GM-CSF expression by B cells in untreated RRMS patients than in healthy controls (Figure 5-3 A and B). Therefore, reducing B cell-derived GM-CSF may be one of explanations for the efficacy of treatments blocking IL-2 in MS.

Regarding the effects of IL-12 on B cells, it was shown to induce B cell differentiation into a distinct subset that is similar to Th1 cells (Durali *et al*., 2003). However, our results show less IFN-γ expression by untreated RRMS patients despite the higher level of IL-12 in culture supernatants of the MS group. This may be due to other pathways that reduced IFN-γ

expression by B cells. The effects of IL-12 on the expression of GM-CSF in B cells have not been investigated so far.

We hypothesized that blocking IL-12 will reduce GM-CSF expression by B cells, and our results were consistent with our hypothesis. IL-12 was shown to induce GM-CSF expression in T cells (Trinchieri, 1994); however, this was not explored thoroughly in B cells. A previous study has shown IL-12 to induce B cell differentiation into a distinct subset similar to Th1 cells (Durali *et al.*, 2003). They used exogenous IL-12 in a concentration of 100 ng/ml for 3 days in B cell culture, which is more than what we have detected in PBMC supernatants (1-10 pg/ml) after 5 days of stimulation. However, we have found a significant difference in IL-12 levels between HCs and MS patients, being higher in MS patients. Our results show for the first time that IL-12 is non-redundant for GM-CSF expression by B cells in untreated RRMS patients when compared to healthy controls. This may be explained by concentration-related effects of IL-12 on GM-CSF expression by B cells in MS. There may be also other factors other than the concentration of IL-12 itself that affect GM-CSF expression by B cells in MS, such as IL-12 polymorphism which is one of the genetic risk factors of MS (Shokrgozar *et al.*, 2009, Javan *et al.*, 2017). However, the link of IL-12 polymorphism and GM-CSF expression has not been investigated as yet.

Blocking IL-2 and IL-12 together had a similar effect on blocking either IL-2 or IL-12 alone in reducing the frequency of GM-CSF-expressing B cells in MS patients. This was despite the fact that both act on different receptors and have different signalling pathways. This finding may suggest that there may be other molecules participating in GM-CSF expression during blocking of either IL-2 or IL-12 signalling pathways. A recent study in EAE has shown that STAT4 signalling induces GM-

CSF expression by Th1 and Th17 cells via unknown molecules but not IL-12 or IL-23 (McWilliams *et al*., 2015).

The IL-12 and IL-23 blocking antibody that we used binds to the common p40 subunit that is found in IL-12 and IL-23 and prevents both cytokines from binding to their receptors (Figure 5-8). Our results show that IL-23 levels were not different in the MS patients and healthy controls. We also show that IL-12 and IL-23 were specifically detected in culture supernatants in a way that there was no cross-linking detection (different and specific beads were used for each cytokine). Despite this, we used IL-12/23p40. Our results show that blocking IL-12 and IL-23 together resulted in significant reduction in the frequency of GM-CSF-expressing B cells in MS patients but not in healthy controls. This is similar to what we found after blocking IL-12p70. This indicates these effects may be due to blocking IL-12.

We also looked at cytokine blocking effects on IFN-γ expression by B cells in cultured PBMCs. Unexpectedly, only healthy volunteers but not MS patients showed reduction in the frequency of IFN-γ-expressing B cells after blocking IL-2, IL-12, and both together. The reasons behind this were not investigated in this study. However, a possible explanation may be that the B cell subtype in MS that is producing GM-CSF is not IFN-γ producing (i.e., not B1 (IFN-g-producing) but B2 (IL-4-producing)); however, this is not studied yet in MS. Another possible explanation for this finding may be that inflammatory B cells from MS patients are more resistant and less amenable to immunomodulation than B cells from controls. No reports have been published with regards to IL-2 effects on IFN-γ expression in normal B cells. However, higher IFN-γ expression was found in human B cell lines after treatment with IL-2 (Pang *et al*., 1992). IL-12 was shown to induce IFN-γ expression in

B cells (Harris *et al*., 2005a). Further work is needed to clarify this finding.

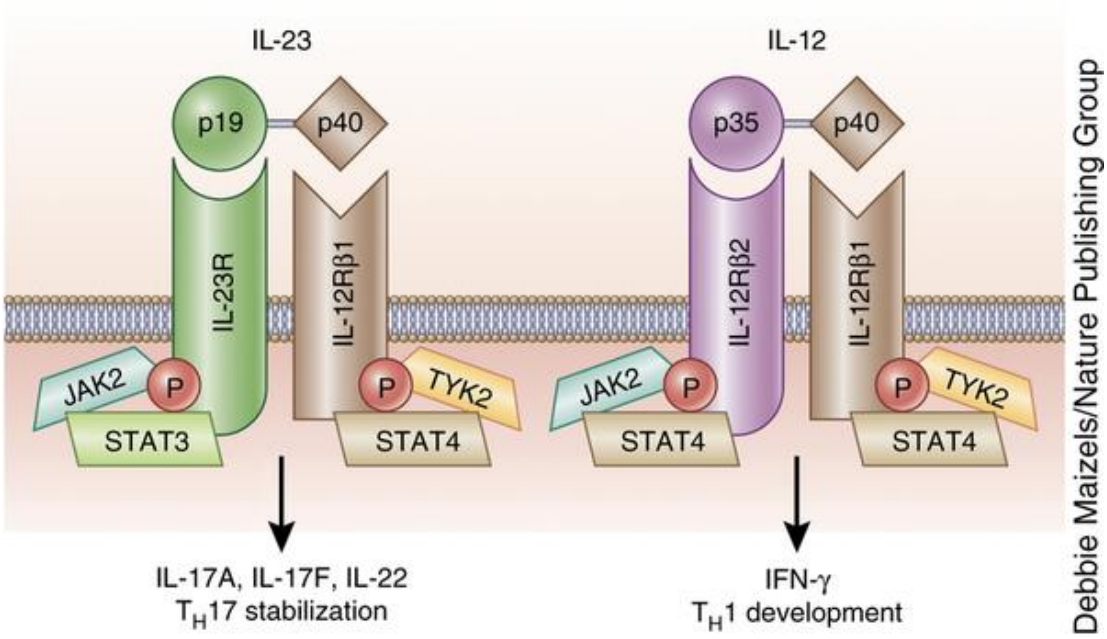


Figure 5-8: IL-12 and IL-23 subunits with their receptors and main signalling pathways

IL-23 consists of two subunits: p19 and p40. IL-23 receptor consists of two subunits: IL23R which binds to p19, and IL-12Rβ1 which binds to p40. IL-12 consists of two subunits: p35 and p40. IL-12 receptor consists of two subunits: IL-12Rβ2 which binds to p35, and IL-12Rβ1 which binds to p40. Binding of IL-23 to its receptor will induce signalling pathways by activating of janus kinase 2 (JAK2), tyrosine kinase (TYK2), and signal transducer of activation and transcription (STAT3 and STAT4) via phosphorylation (P). This will lead to the expression of IL-17A, IL-17F, IL-22, and Th17 stabilization. Binding of IL-12 to its receptor will induce signalling pathways by activating JAK2, TYK2, and STAT4. This will lead to IFN-γ production and Th1 development. IL-12 and IL-23 share a common p40 subunit that trigger similar signalling pathways. (From Teng *et al*., 2015), reproduced with permission.

We also show that the percentage of IL-17-expressing B cells was reduced after IL-2 and IL-12 blocking in PBMCs of MS patients but not in healthy controls. It was shown that B cell-derived IL-17 was found in RA patients and contributed to total IL-17 by lymphocytes. However, RA patients and healthy controls have shown similar IL-17 levels (Schlegel *et al*., 2013). With regard to EAE, no IL-17 was expressed by B cells (Komiyama *et al*., 2006). There are no reports in the literature directly linking IL-2 or IL-12 with IL-17 expression in B cells.

Therefore, this work is the first to show that IL-2 and IL-12 together may have a role in regulating B cell-derived IL-17. However, no change was detected after IL-12/23p40 blocking, which may suggest that IL-23 signalling is not involved significantly in IL-17 expression by B cells. The reason behind this finding is unknown and was not further explored, as our main aim was to look at GM-CSF regulation. There are no published reports to clarify possible causes for this finding. However, there may be factors that render IL-23 redundant in IL-17 production by B cell, such as parasite infection (Leon and Lund, 2013). This may not be a reasonable explanation for our finding. More work is needed to clarify possible causes of IL-23 redundancy in IL-17 production by B cells.

Our results also show that blocking IL-12 and IL-12/IL-23p40 reduced the frequency of B cells expressing GM-CSF+IFN-γ+ in MS patients but not in healthy controls. GM-CSF expression in B cells was significantly reduced after blocking IL-2, IL-12, IL-2 and IL-12, and IL-12/23p40. We also show no change in IFN-γ expression by B cells in MS patients after blocking the above-mentioned cytokines in PBMCs. With regard to GM-CSF+IL-17+ B cells, our results demonstrate that blocking IL-2, IL-2+IL-12, and IL-1β significantly reduced these cells in MS patients but not in healthy controls. The role of these cells is not specified yet in MS or EAE. However, B cells co-expressing GM-CSF with either IFN-γ or IL-17 may aggravate autoimmune inflammation by being a source of two pro-inflammatory cytokines.

Blocking IL-1β show an inhibitory effect on the percentage of GM-CSF+IL-17+ B cells in MS patients. IL-1β blockade was performed by neutralizing IL-1β and IL-1 receptor I and II (IL-1RI and IL-1RII) isotypes. IL-1R I and II blockers competitively inhibit IL-1α and IL-1β

binding to their receptors. Receptor neutralization was performed to exclude any IL-1α-related effects which are generally similar to those of IL-1β. IL-1 induces B cell proliferation and activation (Falkoff *et al*., 1983, Lipsky *et al*., 1983, Pike and Nossal, 1985). The exact explanation behind this finding are still unclear, but increased cytokine expression is a sign of activated lymphocytes in general (Ai *et al*., 2013).

With regard to B cells expressing GMR, isolated and CD40L+anti-IgM-stimulated B cells for 24 hrs show similar frequency of these cells in healthy controls and MS patients. However, GMR frequency was approximately 10-fold higher than B cells in cultured and stimulated PBMCs (anti-CD3+anti-CD28 protocol). GMR expression in B cells has not yet been explored in autoimmune diseases; however, this may be of a great importance in the pathologic course of MS. This may be because autocrine GM-CSF was shown to induce IgM production by innate response activator (IRA) B cells (Rauch *et al*., 2012, Weber *et al*., 2014); and antibody secretion by B cells is among the main pro-inflammatory pathways exerted by B cells. Moreover, GM-CSF was shown to act (in an autocrine manner) on B cells to increase their survival, but exogenous GM-CSF had no effect on these cells (Harris *et al*., 2000). This was not explored in MS; therefore, we hypothesized that GM-CSF may induce B cell survival in MS. We show no change in apoptotic, dead, and live cell frequency in isolated B cells between MS patients and healthy controls after blocking GM-CSF. Similarly, there was no change in GMR expression in both groups. These findings were performed on a small number of samples to test the hypothesis that GM-CSF affects B cell survival in MS; further experiments to clarify this are planned.

We also aimed to find the effects of GM-CSF on EBV-infected B cells. EBV immortalises B cells and should therefore reduce apoptosis.

Therefore, no comparison was necessary between the viability of B cells with/without EBV. GMCSF was reported to be a growth and survival factor for B cells. Therefore, the hypothesis was whether EBV immortalises B cells VIA GMCSF. No difference was noticed after adding or blocking GM-CSF on EBV-infected B cell viability. Different patients participated in EBV-infected B cell experiment from those who participated in non EBV-infected B cell experiment, because EBV-transformed B cells from different MS patients were already available. In addition, there were not enough PBMCs available to isolate B cells and leave for EBV infection. It is unlikely that using EBV+ B cells from the same or from different patients changed the results, as there was a general reduction in GMR expression after EBV infection of B cells regardless if they were from healthy donors or MS patients. A study has shown that adding GM-CSF to EBV-infected B cell lines induced their proliferation (Paul and Baumann, 1990). In that study, exogenous GM-CSF induced EBV-infected B cell proliferation only after 4 and 5 days after stimulation. However, the same protocol in our studies presented here did not show this effect, for unclear reasons. GM-CSF and its receptor may not be major survival factors for EBV-infected B cells.

In conclusion, we showed that more B cells produce GM-CSF in MS patients than in healthy controls. In addition, we showed that IL-2 and IL-12 may induce GM-CSF expression by B cells. Finally, GM-CSF may not have major effects in survival and proliferation of EBV-infected B cells.

In the next chapter, we presented work done to identify a distinct Th cells, mainly producing GM-CSF. In addition, we looked at their frequency in MS.

Chapter 6. Identification of a Distinct GM-CSF-Producing Th Cell Subset in MS

6.1 Introduction

In the previous chapters, GM-CSF and its receptor's expression and regulation in PBMC subtypes (T cells, monocytes, NK cells, and B cells) were investigated with relevance to MS. The T cell subtypes that were investigated were phenotyped to include Th1 (IFN-γ-producing) and Th17 (IL-17-producing). However, another Th cell phenotype was recently identified in mice and was suggested to be functionally distinct from the other Th cell subtypes due to its distinct cytokine production profile, which were mainly GM-CSF and IL-3, but not IFN-γ or IL-17 (Sheng *et al*., 2014). Therefore, these cells were called Th-GM, and the researchers have found that these cells originate from naïve CD4 T cells and differentiate into Th-GM in the presence of IL-7. It was also shown that they are essential for autoimmune neuro-inflammation (Figure 6-1).

Moreover, another recent study has identified a distinct Th cell subset in humans with distinct chemokine receptor profile on their surface which is different from Th1, Th2, and Th17. This makes them have different migration properties (Noster *et al*., 2014). This is because they have a distinct combination of chemokine receptor expression ($CXCR3^{-}CCR4^{+}CCR6^{-}CCR10^{+}$) that differs from the other identified T cell subsets. They also found that IL-2 induces their production. They also suggested the involvement of all GM-CSF-producing Th cells in MS.

Therefore, we investigated the presence of these cells in humans, in healthy controls and MS patients. We also aimed to demonstrate naïve CD4 T cell differentiation pathway, which leads to the development of Th-GM. The hypothesis was that there is an increase in Th-GM

prevalence in MS patients, because they were found to have an important role in inducing autoimmune neuro-inflammation in EAE mice. In addition, IL-2 and IL-7 receptor polymorphisms are among the genetic risk factors for MS (Sawcer *et al*., 2011); hence, when they bind to their ligands they may induce different signalling pathways favouring more production of GM-CSF-producing Th cells in MS. This was investigated with regard to IL-2 receptor polymorphism in MS, in work to which we contributed (Hartmann *et al*., 2014). However, it was not determined whether the GM-CSF-producing Th cells are a distinct subset from Th1 or Th17. The work in this chapter aims to determine whether the Th-GM cells can be found in humans, and whether they are more prevalent in MS.

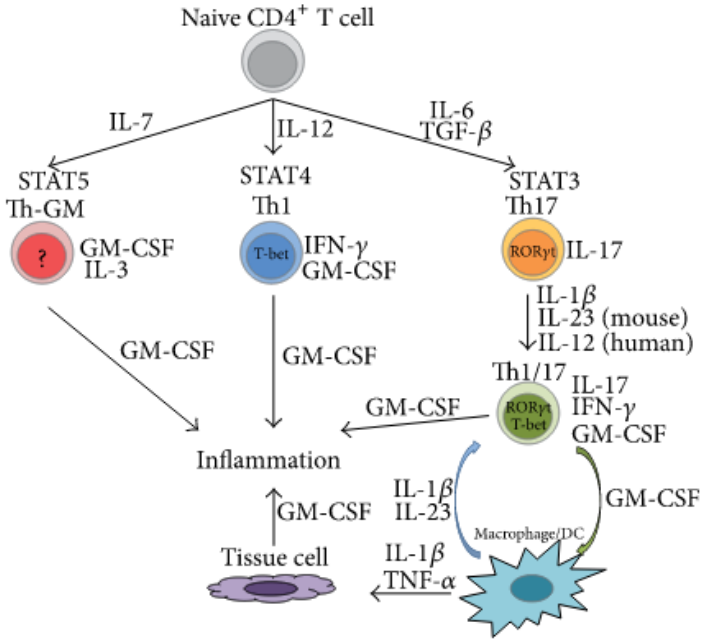


Figure 6-1: Naïve CD4 differentiation pathways into GM-CSF-producing T cells.

Naïve CD4 T cells can differentiate into different GM-CSF-producing T cell subsets, depending on the cytokines present in the cell milieu. IL-7 is proposed to activate STAT5 signalling pathway in naïve CD4 T cells and induce their differentiation into Th-GM which produce GM-CSF and IL-3. IL-12 will activate STAT4 signalling pathways and induce naïve CD4 T cell differentiation into Th1. IL-6 and TGF-β will induce naïve CD4 T cell differentiation into Th17 which, in the presence of IL-1β and IL-23 (mice) or IL-12 (human) will develop into Th1/17 T cell. GM-CSF can activate macrophages/dendritic cells (DC) to produce cytokines that play a role in Th1/Th17 formation and aggravating inflammation. (From Shiomi and Usui, 2015), reproduced with permission.

6.2 Subjects and Methods

6.2.1 Subjects (Inclusion/Exclusion Criteria)

Participants were healthy controls and MS patients during relapse stage. Patients' inclusion criteria are being ≥ 18 years old, having current relapse and EDSS range of ≥ 2 to ≤ 6.5. They were also included even if they were receiving DMT treatment. Exclusion criteria for healthy controls and MS patients were as in Chapter 3 section 3.2.1 above. All the important details of the subjects and the experiment(s) each subject has participated in are listed in appendix I.

6.2.2 Methods

6.2.2.1 Naïve CD4+ Isolation and Stimulation

Venous blood (30-40 ml) was taken into EDTA tubes from participants. PBMCs were isolated and counted (section 2.1). Naïve CD4 cells were isolated using magnetic Naïve CD4+ T Cell Isolation Kit II (Miltenyi Biotech), following manufacturer's instructions. It is done by depleting magnetically-labelled non T-helper cells and memory T cells. Purity check was done and samples were ≥ 90% CD4+CD45RA+ (Figure 6-2).

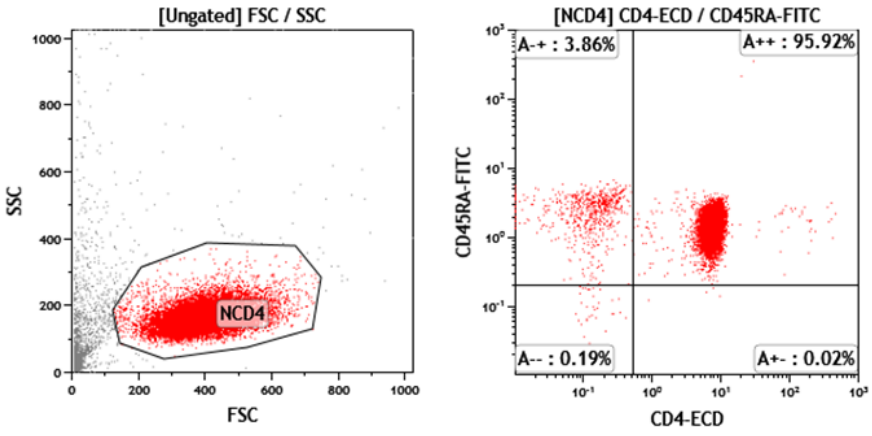


Figure 6-2: Naïve CD4+ purity check from an MS patient sample.

After magnetic isolation of naïve CD4 (NCD4) cells from fresh PBMCs, cells were stained with CD4 and CD45RA, and run on the flow cytometer to examine their purity. Samples were included when purity was ≥ 90%.

Cells were distributed in 24-well plate and divided into five wells (1 x 10^6 cells/ml/well) left either unstimulated, or stimulated with soluble anti-CD3 (3 μg/ml) and anti-CD28 (1 μg/ml) (both from BD Biosciences). Stimulated wells were treated with or without either of the following: IL-2 (10 ng/ml), IL-7 (20 ng/ml), or their combination (both from Peprotech). A preliminary experiment was performed in one healthy control (results not shown) to find out the time point with the best outcome. Cells were incubated at 37°C with 5% CO_2 for seven days, then re-stimulated with PMA (50 ng/ml) + I (0.5 μg/ml) in presence of Brefeldin A (10 μg/ml) in the last five hours of stimulation.

6.2.2.2 Flow Cytometry and Data Analysis

Flow cytometry and data analysis was performed as sections 2.5 and 2.7 above.

6.3 Results

6.3.1 Demographic and Clinical Characteristics of the Participants

Table 6-1 below demonstrates the demographics and clinical characteristics of all participants recruited in this experiment.

Table 6-1: Demographic and clinical characteristics of subjects who contributed samples to the studies of Naïve CD4 stimulation experiment.

Demographics	Healthy Controls	MS Patients during relapse
Total no. of participants	5	3
Age mean (y) (SD)	47.8 (10.21)	42 (15)
Female no. (%)	4 (80)	3 (100)
Ethnicity:		
White (%)	4 (80)	3 (100)
Other (%)	1 (20)	-
Time since MS diagnosis (y)	-	3.6 (0.57)
EDSS mean (SD)	-	2.2 (0.35)
Smoking status:		
Smoker (%)	0	1 (33.3)
Ex-smoker (%)	1 (20)	0

6.3.2 IL-2 + IL-7 Stimulate Naïve CD4+ Cells to Express GM-CSF+IL-3+ but not IFN-γ and IL-17 in MS Patients

After naïve CD4+ T cell isolation and stimulation with anti-CD3+anti-CD28 along with IL-2, IL-7, or both for 7 days, the cells were re-stimulated with PMA/I in the last 5 hours. Flow cytometry staining and data analysis show a statistically significant increase in live CD4+GM-CSF+IL-3+IFN-γ-IL-17- levels in MS patients (n=3) compared with healthy controls (n=5) (Mann-Whitney test p=0.03) (Figure 6-3 B). This significant increase was at the expense of the significant decrease in CD4+GM-CSF+IL-3+IFN-γ+IL-17- cells in MS patients compared to the healthy group (Figure 6-3 C) (Mann-Whitney p=0.03).

6.3.3 Identification of CD4+ Cells that are GM-CSF+ and Negative for the Other Cytokines

The above procedure also resulted in identification of CD4+ cells that, after stimulation with the conditions mentioned above, were GM-CSF+, but were not expressing either of IFN-γ, IL-17, or IL-3(Figure 6-4 B). These cells were found in healthy controls and MS patients, and there was no statistical significance between the two groups (healthy n=5, MS n=3, Mann-Whitney test, p=0.78).

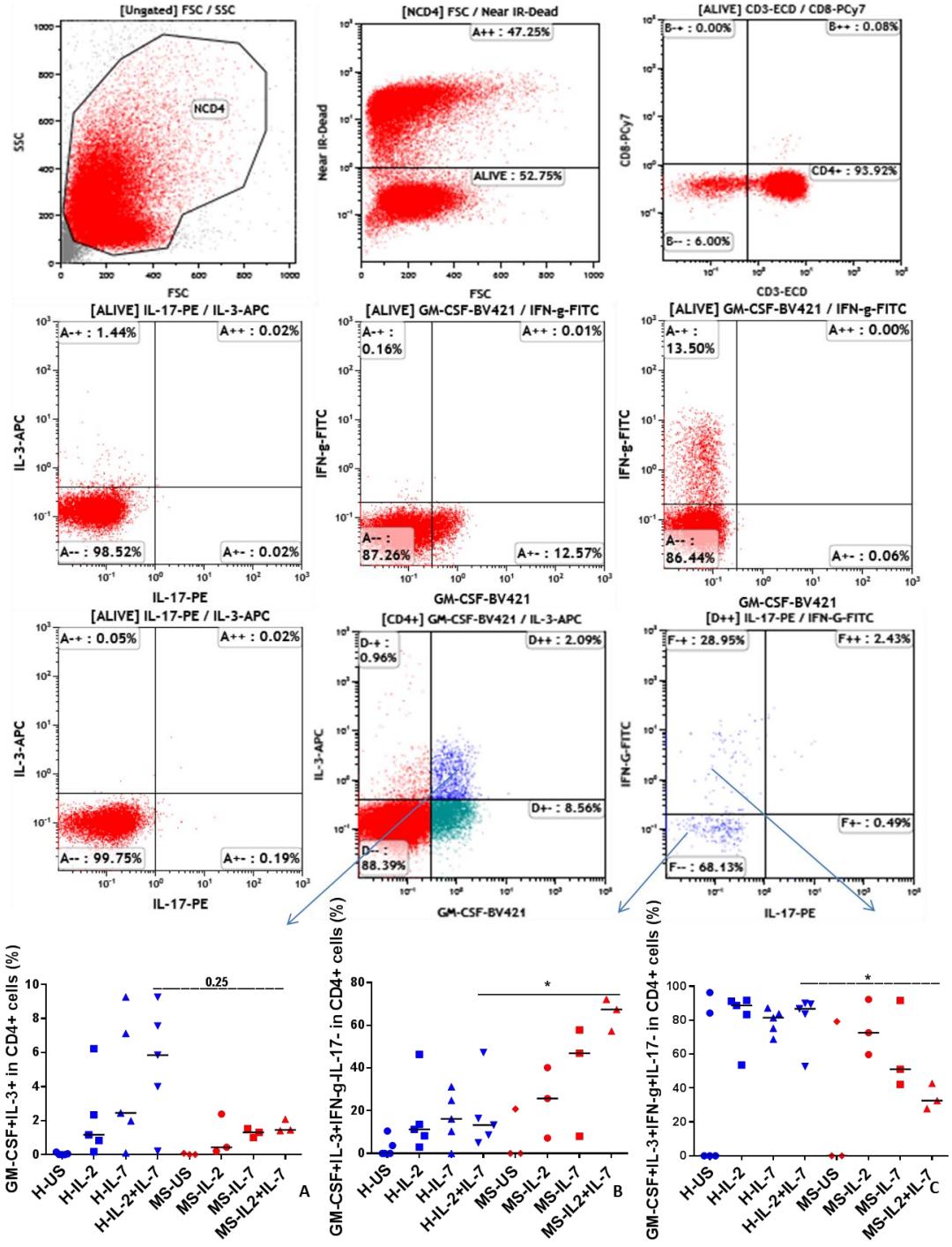


Figure 6-3: Differentiation of naïve CD4+ cells to Th-GM.

Naïve CD4+ T cells were isolated and stimulated with anti-CD3+anti-CD28 and IL-2 and/or IL-7 for 7 days. Flow cytometry plots show only live cells were selected for analysis. Middle and lower left panel plots show how quadrants were set based on FMO controls for IL-17, IFN-γ, GM-CSF, and IL-3, respectively. Only GM-CSF+IL-3+ (blue-coloured population) was selected and out of this population, only IL-17-IFN-γ- population was calculated. Cumulative results: GM-CSF+IL-3+ CD4+ T cells (blue population) **(A)** were further gated to select cells which are mainly not Th1 cells or Th17 (IFN-γ- and IL-17-) **(B)**. Healthy controls (n=5) and MS patients (n=3) were enrolled. H-US (MS-US): unstimulated cells from healthy controls (MS patients); H-IL-2 (MS-IL-2): stimulated cells from healthy controls (MS patients) with IL-2; same labelling was used with adding IL-7 or IL-2+IL-7. * $p<0.05$. Horizontal lines are medians.

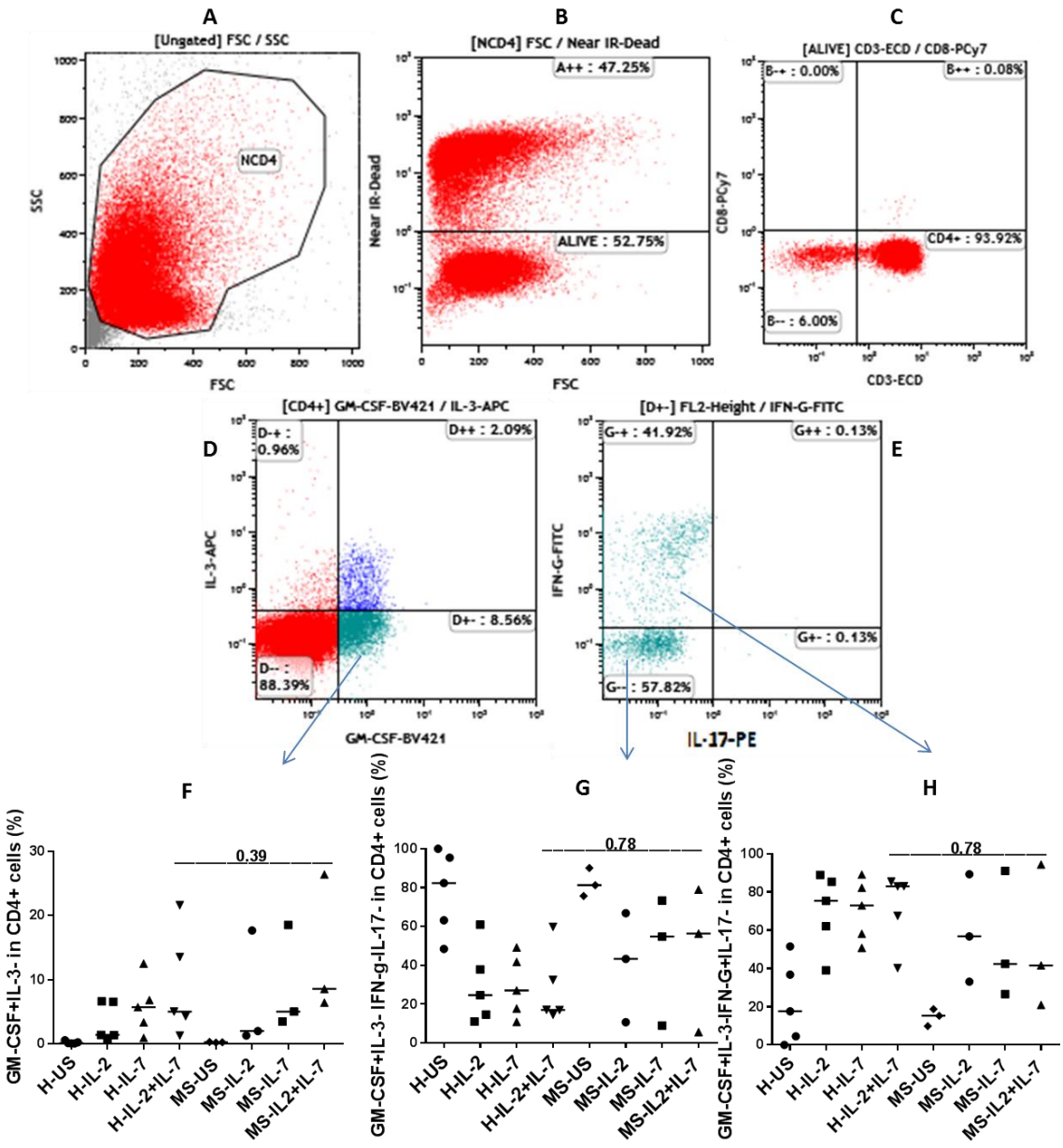


Figure 6-4: Differentiation of naïve CD4+ cells to GM-CSF-producing non Th1, non Th17, and non Th-GM (not expressing IL-3) cells.

Naïve CD4+ T cells were isolated and stimulated with anti-CD3+anti-CD28 and IL-2 and/or IL-7 for 7 days. **A-C:** representative flow cytometry plots show only live CD3+CD8- cells were gated and selected to examine GM-CSF and IL-3 expression **(D)**. Quadrants were set as in Figure 6-3 based on FMO controls for IL-17, IFN-γ, GM-CSF, and IL-3. **D:** only GM-CSF+IL-3- (green-coloured population) was selected and out of this population, only IL-17-IFN-γ- population was calculated **(E)**. Cumulative results: GM-CSF+IL-3- CD4+ T cells (green population) **(F)** were further gated to select cells which are mainly non Th1 non Th17 (IFN-γ- and IL-17-), and non Th-GM (IL-3-) GM-CSF-only producing cells **(G)**. The rest were mainly IFN-γ-producing (GM-CSF-producing Th1), their cumulative results are presented in graph **(H)**. Healthy controls (n=5) and MS patients (n=3) were enrolled. H-US (MS-US): unstimulated cells from healthy controls (MS patients); H-IL-2 (MS-IL-2): stimulated cells from healthy controls (MS patients) with IL-2; same labelling was used with adding IL-7 or IL-2+IL-7. * $p<0.05$. Horizontal lines are medians.

6.4 Discussion

Our experimental work in this chapter was aimed to determine whether the Th-GM cells differentiated from naïve CD4 T cells as identified in mice (Sheng *et al*., 2014) are also present in humans. These cells were identified as CD4+GM-CSF+ IL-3+IL-17-IFN-γ-. We were unable to identify these cells in human healthy controls (n=5) and MS patients (n=4) using exactly the same stimulation procedure of naïve CD4 T cells as used in mice (results not shown). In addition, after using IL-7 concentration of 2 ng/ml, we could not detect Th-GM. Instead, 20 ng/ml was used according to other protocols to stimulate PBMCs or cord blood in mice and humans (Michel *et al*., 2012), including a University of Nottingham MSc dissertation (Lin, 2008). These protocols used IL-7 for IL-17 expression; however, IL-7 did not work well alone in GM-CSF or Th-GM detection in our experiment. Therefore, IL-2 was added alone or with IL-7. This is because it has been found that adding IL-2 (10 ng/ml) to CD4 memory T cells for 5 days significantly increased GM-CSF-producing Th cells without affecting IFN-γ or IL-17 expression (Noster *et al*., 2014). GM-CSF and IL-3 expression was checked at the following time points during the experiment setup (3, 4, 5, 6 and 7 days of stimulation). Their expression was the highest after 7 days of stimulation.

Our results show that combined IL-2 and IL-7 stimulation of naïve CD4 T cells results in the higher induction of GM-CSF than stimulation with each cytokine alone. Moreover, it appears that IL-2 and IL-7 are having additive effects on each other (the sum effect of both cytokines' separate effects) and not synergistic effects (greater than the sum effect of both cytokines' separate effects). Therefore, as a next step, we are interested in doing IL-2/IL-7 titrations alone or in combination and using higher concentration of each cytokine alone to detect if they exert the same

effect on Th-GM expression. Moreover, cytokine antibodies may be added to detect which are having role in their regulation. IL-7 was reported to bind to its receptor on T cells and act via STAT5 activation to induce GM-CSF in CD4 T cells (Herndler-Brandstetter and Flavell, 2014). In addition, a recent study has shown that Th-GM cells were not expressing T-bet and RORγt, but having distinct gene expressions (Sheng *et al*., 2014). Therefore, more experiments are required to identify the genotype of these cells in humans. We show that these cells were increased in frequency in MS patients. This was at the expense of reduction in the frequency of GM-CSF+IFN-γ+IL-17-IL-3+ CD4+ T cells (or GM-CSF+ Th1 cells). More investigation is needed about the role of these cells in MS pathogenesis in relapse and remission stages.

We also identified another phenotype of stimulated CD4+ T cells which produce GM-CSF only, but not IFN-γ, IL-17, or IL-3. These cells were detected in healthy controls and MS patients. More investigation is needed to clarify what are these cells and whether they have any relevance to MS. They may be any Th cell subset that stopped producing the above-mentioned cytokines for certain reasons. Their expression of T-bet, RORC, and chemokine receptor profile should be examined.

In conclusion, we identified a distinct Th subset that mainly express GM-CSF and IL-3 in humans (called Th-GM). We also found that the frequency of Th-GM cells was elevated in MS patients. Finally, another Th subset was also identified in humans, which expressed GM-CSF only. However, no difference in the frequency of these cells was detected between healthy controls and MS patients. Based on our results (presented in chapter 3, 4, 5, and 6) detecting high GM-CSF-expressing immune cells in MS, we investigated the immune-related effects of *in vivo* neutralization of GM-CSF in a phase I clinical trial in MS.

Chapter 7. Phase I, Randomised, Double-Blind, Placebo-Controlled Clinical Trial of GM-CSF Neutralization in MS: Immunological Effects

7.1 Introduction

In the previous chapters, we investigated GM-CSF expression and regulation by immune cells, with relevance to MS. We have found high frequency of immune cells (Th, Tc, monocytes, NK cells, and B cells) expressing GM-CSF in MS. We have also identified a distinct Th cell subset, mainly producing GM-CSF. This subset was in higher frequency in MS patients during relapse. In this chapter, we presented work done to neutralize GM-CSF *in vivo*, as part of a phase I clinical trial in MS patients. The aim of work presented in this chapter is to investigate immune-related effects of GM-CSF neutralization, which was done for the first time in MS.

It has been shown that GM-CSF is a pro-inflammatory cytokine that, in high levels, plays a critical role in the pathogenesis of inflammatory and autoimmune diseases, such as pulmonary fibrosis (Johnson *et al*., 1989, Worgall *et al*., 1999) rheumatoid arthritis, SLE (Xu *et al*., 1989, Fiehn *et al*., 1992) and allergy/asthma (Nouri-Aria *et al*., 1998, Willart *et al*., 2012).

With regard to EAE, reported evidence from animal research has shown that GM-CSF-knockout mice are resistant to the development of EAE compared to wild type mice, suggesting a crucial role for GM-CSF in developing the autoimmune reaction in the CNS (McQualter *et al*., 2001, Ponomarev *et al*., 2007). In addition, it was shown that Th1 and Th17 cells that lack GM-CSF lose their ability to transfer EAE (Codarri *et al*., 2011, El-Behi *et al*., 2011). As with MS, some studies have found higher

GM-CSF levels in the CSF of MS patients (Perrella *et al*., 1993, Carrieri *et al*., 1998); while recent studies have shown increased GM-CSF expression by peripheral immune cells in MS (Hartmann *et al*., 2014, Li *et al*., 2015, Rasouli *et al*., 2015). It was also noted that GM-CSF levels were reduced by conventional MS therapies (Mellergard *et al*., 2010, Li *et al*., 2015, Rasouli *et al*., 2015), suggesting another mechanism of action of these therapies.

The exact pathogenic mechanisms of GM-CSF in MS are not fully understood yet. However, it is suggested that the resident microglia, infiltrating monocytes/macrophages, and encephalitogenic T cells in the CNS are the major causes of initiating and maintaining autoimmunity. This is achieved by being a source of GM-CSF and other pro-inflammatory cytokines that activate microglia. These microglia will further secrete GM-CSF with other pro-inflammatory cytokines, and differentiate to M1-like pro-inflammatory macrophage phenotype. The latter will act as APC, presenting myelin peptides to T cells to activate them. This results in increased levels of glutamate, reactive oxygen (ROS) and nitrogen (RNS) species that will collectively participate in axonal demyelination and damage, as illustrated in Figure 1-9 (Bogie *et al*., 2014, Gonzalez and Pacheco, 2014). GM-CSF is also associated with pain by acting on nociceptors expressed on sensory nerves (van Nieuwenhuijze *et al*., 2013). It has been shown that GM-CSF located at the site of inflammation (e.g., in RA) and tumour can induce pain via sensory afferent neurons (Cook *et al*., 2013). In addition, studies have revealed that GM-CSF-mediated recruitment of peripheral myeloid cells and infiltration to the CNS is involved in disruption of BBB and BSCB integrity (King *et al*., 2009, Aube *et al*., 2014).

All the above mentioned results suggest a pathogenic role of GM-CSF in MS. More importantly, our results in chapters 3-6 show that GM-CSF is highly expressed by PBMC subsets in MS. Therefore, these results were the main rationale for using GM-CSF neutralization in this chapter in a clinical trial as a therapy for MS. With regard to autoimmune diseases, research and clinical trials were carried out (and still ongoing) using different GM-CSF or GMR monoclonal antibodies (Table 7-1).

Morphosys 103® (MOR103) (manufactured by Morphosys, Germany) is the first anti-GM-CSF neutralising monoclonal antibody that has been used in a clinical trial as a therapy for MS. It is a recombinant human monoclonal IgG1 antibody with high affinity against GM-CSF, binding to it and preventing its interaction with its receptor (Steidl *et al*., 2008). It was tested for safety and efficacy in RA and positive results were seen (Behrens *et al*., 2015). Human IgG antibodies have the ability to cross the BBB in small amounts; however, in MS (where the BBB is disrupted) (Minagar and Alexander, 2003), it was thought that more MOR103 may cross the BBB. This chapter will focus on the immune-related outcomes of GM-CSF neutralization in MS patients after MOR103 monoclonal antibody therapy, assessing T cell subsets and their expression of GM-CSF, IL-17, and IFN-γ before and after MOR103 treatment, with comparison to a group of healthy controls.

Table 7-1: Clinical trials targeting GM-CSF and its receptor.

Target	Drug	Disease	Phase	Status	Reference
GM-CSFR	Mavrilimumab (CAM-3001)	RA	I	Completed	(Burmester *et al*., 2011)
		RA	IIa	Completed	(Burmester *et al*., 2013)
		RA	IIb	Completed	(Burmester *et al*., 2014)
		RA	IIb	Active, not recruiting	(Burmester *et al*., 2015)
		RA	II	Completed	https://clinicaltrials.gov/ct2/show/study/NCT01715896
GM-CSF	MOR103	RA	Ib/IIa	Completed	(Behrens *et al*., 2015)
		MS	Ib	Completed	(Constantinescu *et al*., 2015)
GM-CSF	Namilumab (MT203)	RA	Ib	Completed	https://clinicaltrials.gov/ct2/show/study/NCT01317797
		RA	II	Recruiting	https://clinicaltrials.gov/ct2/show/NCT02393378
		RA	II	Recruiting	https://clinicaltrials.gov/ct2/show/NCT02379091
		Psoriasis	II	Recruiting	https://clinicaltrials.gov/ct2/show/NCT02129777
GM-CSF	KB003	RA	II	Terminated	https://clinicaltrials.gov/ct2/show/results/NCT00995449
		Asthma	II	Completed	https://clinicaltrials.gov/ct2/show/NCT01603277
GM-CSF	MORAb-022	RA	I	Completed	https://clinicaltrials.gov/ct2/show/NCT01357759

7.2 Subjects (Inclusion/Exclusion Criteria)

All the below mentioned sample collection and experimental work was approved by Nottingham Research Ethics Committee, as shown in Appendix II. This chapter presents a part of the results of a randomised, double blinded, placebo controlled multi-centre clinical trial of GM-CSF neutralisation. It was conducted in five centres in the United Kingdom, Poland, and Germany, following the Declaration of Helsinki (2008) and Good Clinical Practice Guidelines. Patients' randomization and the cohorts included in this trial are shown in Figure 7-1. Six patients out of 31 were enrolled at the Queen's Medical Centre, Nottingham and their immunological results are presented in this chapter (all were receiving MOR103, not placebo). In addition, six healthy controls were enrolled, they were matched with the MS patients according to age, gender, and ethnic group.

Untreated RRMS and SPMS patients were randomized to receive either placebo or one of the three MOR103 doses (0.5, 1, and 2 mg/kg) in a double blinded manner (Figure 7-1). Doses were given every 2 weeks at weeks zero, 2, 4, 6, 8, and 10. Peripheral venous blood samples (20 ml) were collected from MS patients at baseline (before treatment) and after 20 weeks (after treatment), while blood samples (20-30 ml) from healthy controls were collected only once around the same time as the trial (January 2013-January 2014).

Patients had to be ambulatory, with EDSS range of ≥ 2 to ≤ 6.5. Exclusion criteria were: having PPMS, having exacerbation of disease within 1 month of the trial start, being pregnant or breast-feeding, treated with immuno-modulatory therapies within 1-12 months (depending on the type of therapy) of trial start or having a disease that needs to be

treated with such medications, having serious infections or conditions (hepatic, renal, psychiatric, addiction, pulmonary, cardiac, or malignancy), having vaccination within 6 months of trial start, being treated with B or T cell-depleting medications, stem cells, cytotoxic agents, or having undergone total lymphoid irradiation or bone marrow transplantation. All the important details of the subjects and the experiment(s) each subject has participated in are listed in appendix I.

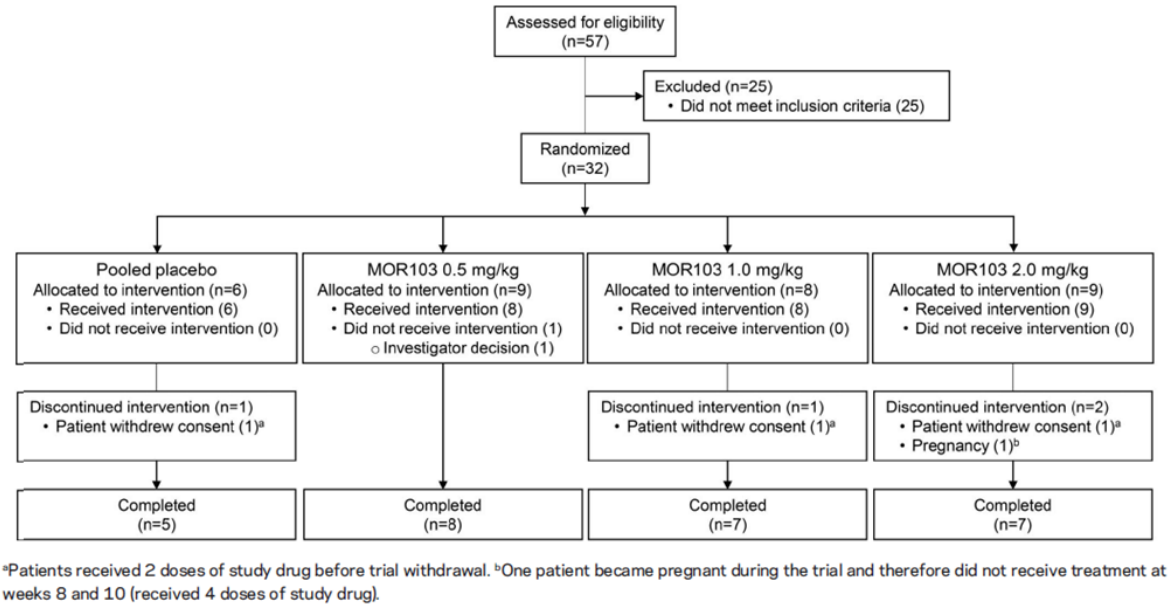


Figure 7-1: Patient randomisation in MOR103 clinical trial.

Six patients out of the final number participated in this trial (n=27) were collected in Nottingham. Two of them were randomised to receive 1 mg/kg, and the remaining four were randomised to receive 2 mg/kg of MOR103. Both patients who received 1 mg/kg dose were having SPMS. With regard to the remaining four patients receiving 2 mg/kg, two of them had RRMS and the other two had SPMS. (From Constantinescu *et al*., 2015), reproduced with permission.

7.3 Methods

All the below mentioned sample collection and experimental work was approved by Nottingham Research Ethics Committee, as shown in Appendix II. PBMC isolation and counting was done as in chapter 2 section 2.1. Th17 stimulation with PDB/I was performed as in section 2.3. Th17 and Treg staining was done as in section 2.5. Flow

cytometry and data analysis was performed as in section 2.7. Mann-Whitney test was applied to compare between the results of control subjects and MS patients at baseline or after treatment. The Wilcoxon signed rank test was applied to compare between patients' data before and after treatment.

7.4 Results

7.4.1 Demographic and Clinical Characteristics of the Participants

Table 7-2 below demonstrates the demographics and clinical characteristics of all participants recruited in this clinical trial.

Table 7-2: Demographic and clinical characteristics of patients and healthy controls enrolled in MOR103 clinical trial.

BMI: body mass index.

Demographics	**Healthy Controls**	**MS (MOR103 1.0 mg/Kg)**	**MS (MOR103 2.0 mg/Kg)**	**Overall MS patients**
Total no. of participants	6	2	4	6
Age mean (y) (SD)	51.1 (5.03)	47.5 (7.77)	53 (2.94)	51.1 (5.03)
Female no. (%)	5 (83.3)	2 (100)	3 (75)	5 (83.3)
Ethnicity (%)	White (100)	White (100)	White (100)	White (100)
Time since MS diagnosis (y)	-	7 (1.4)	11 (5.9)	9.6 (5.08)
MS type:				
RRMS no. (%)	-	0 (0)	2 (50)	2 (33.3)
SPMS no. (%)	-	2 (100)	2 (50)	4 (66.6)
EDSS mean (SD)	-	6.25 (0.35)	5.375 (1.60)	5.66 (1.33)
Smoking status:				
Smoker (%)	1 (16.6)	0	0	0
Ex-smoker (%)	1 (16.6)	0	0	0

7.4.2 Clinical and MRI findings of this trial

This study show that MOR103 is safe and tolerable (no immunogenicity detected) in the doses used (maximum 2 mg/kg), with linear blood levels after each dose and no accumulation of the drug over time (Constantinescu *et al*., 2015). Only mild to moderate nasopharyngitis was the most common respiratory adverse effect of placebo and the

lowest MOR103 dose (0.5 mg/kg) but not the higher doses (Table 7-3). No change in pulmonary function and no lung toxicity was detected in all MOR103 doses.

This study also shows no change in the number of brain lesions detected by MRI among patients taking placebo and different MO103 doses. However, MS relapse was not detected in MS patients taking the highest MOR103 doses (1 and 2 mg/kg) and was detected in placebo and 0.5 m/kg MOR103 study group (Table 7-3). Of note, this phase I study was not powered to demonstrate efficacy of the drugs, the primary outcome measure being safety, tolerability, and immunogenicity.

Table 7-3: MOR103-related adverse effects.

This table shows the adverse effects (AE) during the trial. (From Constantinescu *et al.*, 2015), reproduced with permission.

		MOR103			
Treatment-emergent AE related to study drug[a]	Placebo (n = 6)	0.5 mg/kg (n = 8)	1.0 mg/kg (n = 8)	2.0 mg/kg (n = 9)	Total active (n = 25)
Patients with ≥1 treatment-emergent AE related to study drug,[a] n (%)	5 (83.3)	6 (75.0)	4 (50.0)	3 (33.3)	13 (52.0)
No. of treatment-emergent AEs related to study drug[a]					
Total	21	35	8	7	50
Events/patient	3.5	4.4	1.0	0.8	2.0
Patients with ≥1 treatment-emergent AE related to study drug,[a] n (%)					
Nasopharyngitis	3 (50.0)	2 (25.0)	1 (12.5)	0 (0.0)	3 (12.0)
Multiple sclerosis exacerbation[b]	2 (33.3)	2 (25.0)	0 (0.0)	0 (0.0)	2 (8.0)
Fatigue	1 (16.7)	1 (12.5)	0 (0.0)	1 (11.1)	2 (8.0)
Increased C-reactive protein	0 (0.0)	0 (0.0)	1 (12.5)	1 (11.1)	2 (8.0)
Extremity pain	0 (0.0)	1 (12.5)	1 (12.5)	0 (0.0)	2 (8.0)
Headache	2 (33.3)	1 (12.5)	0 (0.0)	0 (0.0)	1 (4.0)
Paresthesia	1 (16.7)	0 (0.0)	0 (0.0)	1 (11.1)	1 (4.0)

[a] Includes AEs considered to be possibly, probably, and definitely related to study drug.
[b] Worsening of existing multiple sclerosis.

7.4.3 Reduction in total CD8+ lymphocytes after MOR103 Therapy

Flow cytometry staining of PBMCs show a significant reduction in the frequency of total CD8+ lymphocytes after MOR103 treatment (p=0.03) compared with before treatment (Figure 7-2). Further gating on these cells based on their CD3 expression revealed that CD3+CD8+ and CD3-CD8+ were numerically reduced after MOR103 treatment. However, none reached statistically significant reduction (Figure 7-2). No significant changes between the healthy controls and patient groups (either before or after treatment) were seen.

7.4.4 Reduction in Naïve Treg Frequency after MOR103 Treatment

Treg staining and data acquisition via flow cytometry showed a statistically significant decrease in the frequency of naïve Tregs (CD4+CD45RA+CD25++ lymphocytes) in MS patients after MOR103 therapy (P=0.03), being close to control levels. This reduction was corresponding to a statistically significant increase in the frequency of total Tregs (CD4+CD25+foxp3+ lymphocytes) (Figure 7-3). No significant changes were seen between healthy controls and patient groups in both Treg phenotypes.

7.4.5 No Change in Expression of CD3, CD4, CD154, IL-17, IFN-γ, and GM-CSF in Healthy Controls and MS Patients

After PBMCs were cultured and stimulated with PDB/I for 16 hours, they were stained to detect the expression of surface markers and intracellular cytokines mentioned above. Our results show no significant changes in the frequency of CD3, CD4, CD145 lymphocytes among healthy controls and patients groups. There was also no significant changes in the percentage of CD3, CD4, CD8, and CD145 lymphocytes expressing IL-17, IFN-γ, and GM-CSF among tested groups (Figure 7-4).

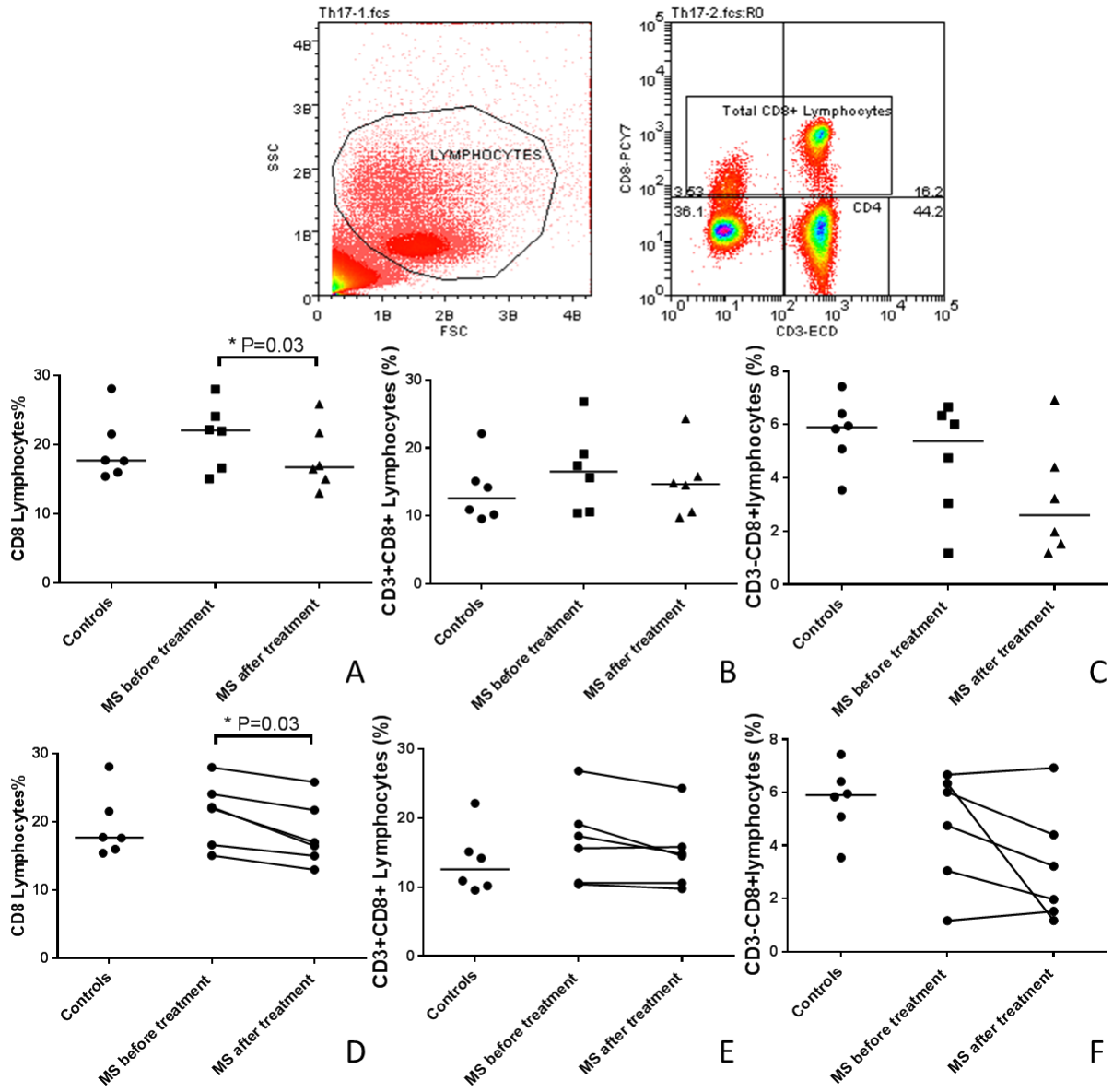


Figure 7-2: Expression of CD8 lymphocytes in healthy controls and MS patients before and after MOR103 treatment.

Flow cytometry plots are representative of CD3 and CD8 expression and gating in lymphocytes. A, B, and C: collective values of CD8 lymphocyte expression, with or without CD3 expression in healthy controls and MS patients, showing medians as horizontal lines. D, E, and F: collective values of CD8 expression, with or without CD3 expression in healthy controls and MS patients, showing relation of values before-after MOR103 therapy.

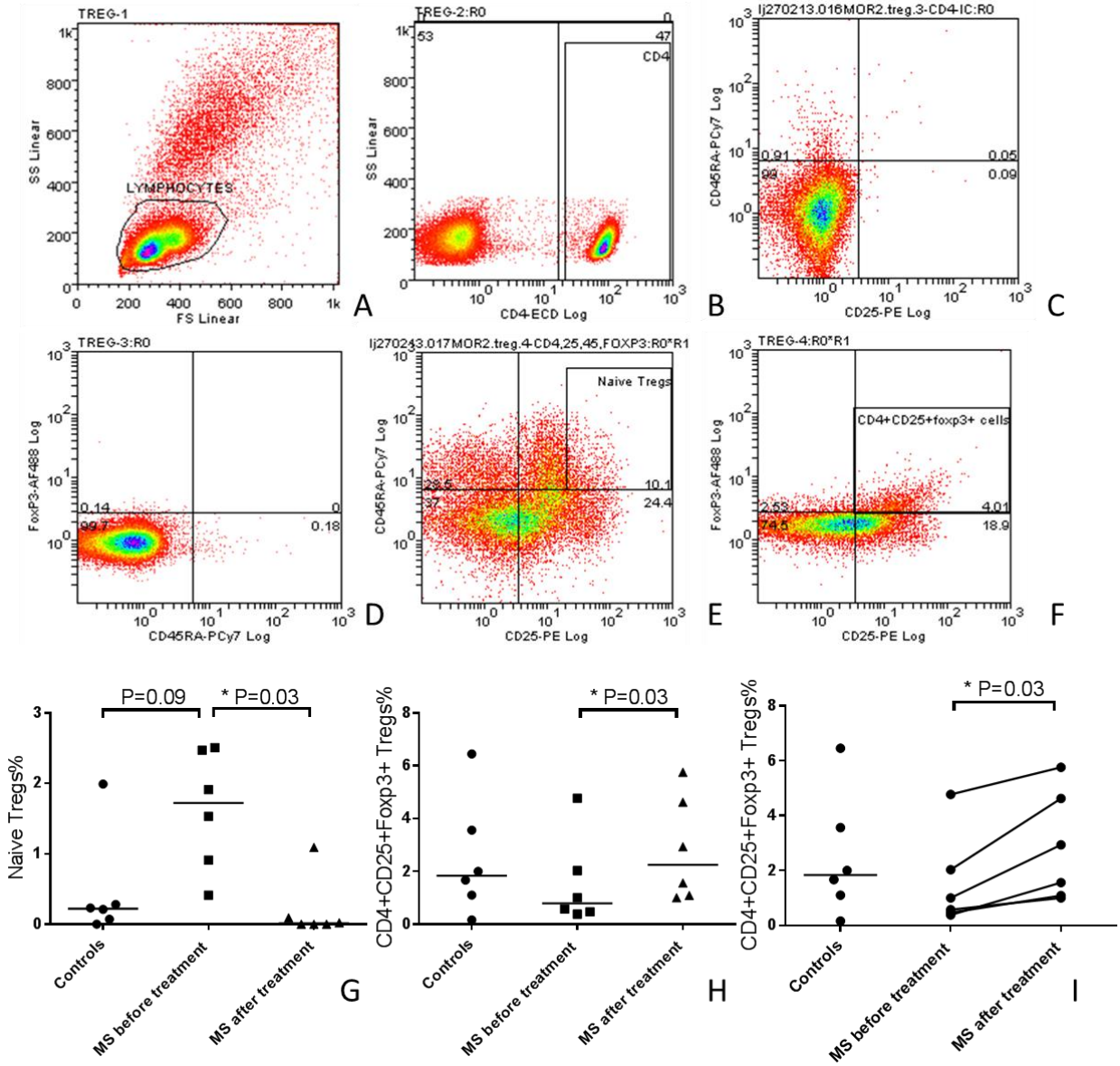


Figure 7-3: Reduction in Naïve Tregs after MOR103 therapy.

Representative flow cytometry plots showing gating of CD4 lymphocytes (A and B), setting quadrants of CD25, CD45RA, and foxp3 based on isotype controls (C and D). All the selected cells were CD4+. Naïve Tregs were identified as CD4+CD45RA+CD25++ (E) and total Tregs were identified as CD4+CD25+foxp3 (F). Lower panel plots illustrate collective data from healthy controls and MS patients before and after MOR103 treatment showing percentage of naïve Tregs (G) and total Tregs with horizontal lines representing medians (H) and linking values before-after MOR103 therapy (I).

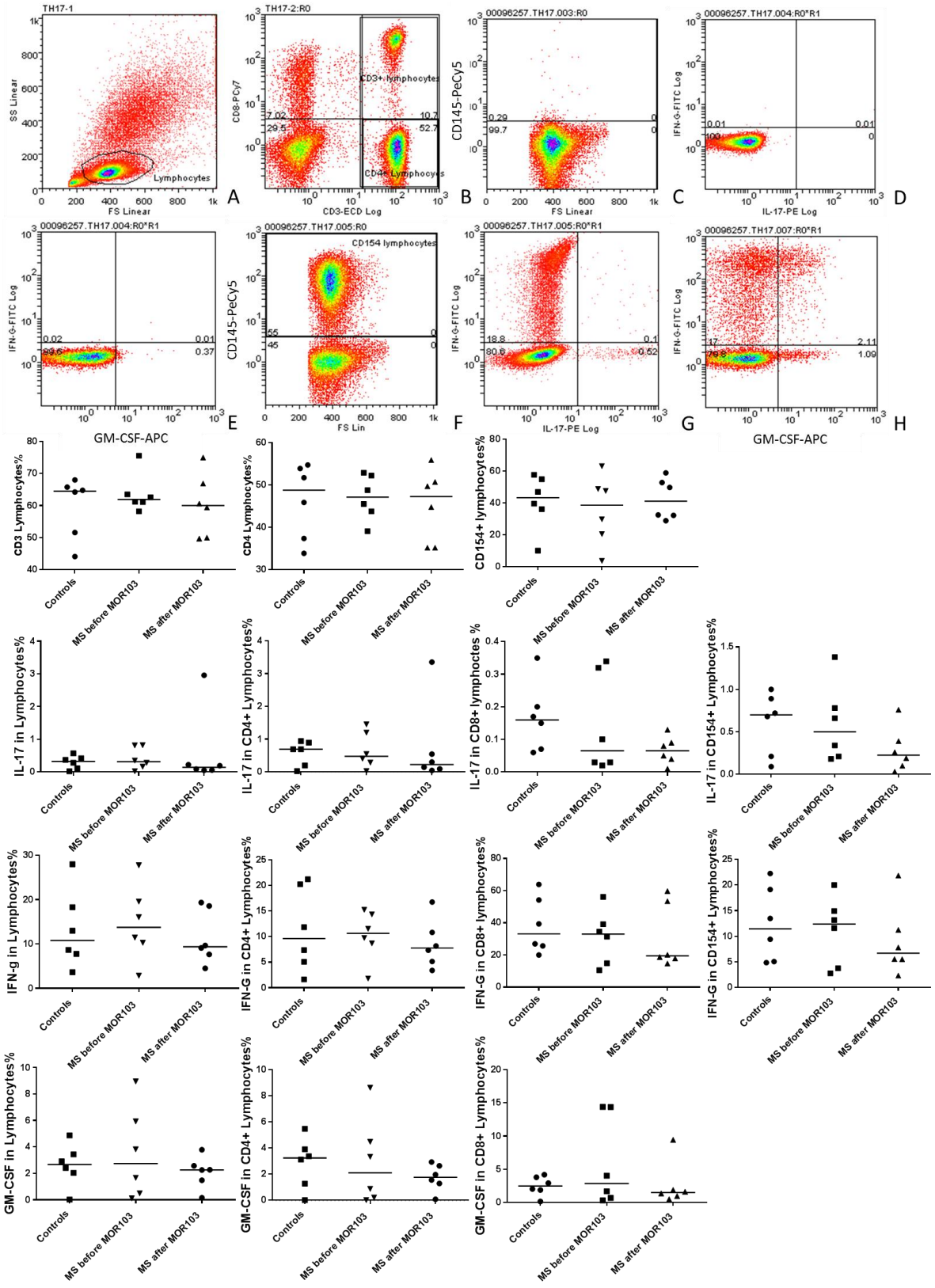


Figure 7-4: Expression of CD4, CD8, CD154 lymphocytes and their production of IL-17, IFN-γ, and GM-CSF in healthy controls and MS patients before and after MOR103 therapy.

Upper panel: representative flow cytometry plots showing gating of lymphocytes (R0) (A), CD4+ cells (R1) (B), and CD3+ cells (B). Quadrants were set using isotype controls (C, D, and E). CD145 and cytokine expression is also shown (F, G, and H). Lower plots illustrate collective data from participants. Horizontal lines are medians.

7.5 Discussion

The main objectives of this clinical trial were to determine the safety, pharmacokinetics, and immunogenicity of MOR103 as a recombinant fully human antibody against GM-CSF. This study shows that MOR103 is safe, tolerable, and without immunogenicity detected in the doses used (maximum 2 mg/kg), with linear blood levels after each dose. It was thought that GMCSF neutralisation needs to be considered with caution, as this was the first trial in MS. Also, GM-CSF deficiency (due to either genetic causes, or presence of autoantibodies against GM-CSF, or secondary to underlying diseases) can lead to reduced alveolar macrophage numbers and impaired functions. GM-CSF was also suggested to be involved in surfactant metabolism by the alveolar macrophages. GM-CSF deficiency sometimes leads to increased incidence of infections and accumulation of pulmonary surfactant in alveoli with various degrees of respiratory dysfunction, a syndrome known as pulmonary alveolar proteinosis (PAP) (Seymour and Presneill, 2002, Trapnell *et al*., 2003, Carey and Trapnell, 2010). Animal studies have also shown that GM-CSF knockout mice had abnormal lung histology similar to PAP, yet no infection was detected (Dranoff *et al*., 1994). With regard to this trial, mild to moderate nasopharyngitis was the most common respiratory adverse effect of placebo and the lowest MOR103 dose (0.5 mg/kg) but not the higher doses (Table 7-3). The reason is unclear. It may be a chance finding, which, in view of the small numbers, may not be clinically significant. The proportion of patients with naspharyngitis in the low dose group was lower than the placebo group (Table 7-3), suggesting that the treatment did not increase susceptibility to nasopharyngitis. No change in pulmonary function and

lung toxicity was not detected at all MOR103 doses. Surfactant protein levels were also measured and shown not to change significantly.

This study also shows no significant change in the number of brain lesions detected by MRI among patients taking placebo and different MO103 doses. However, MS relapse was not detected in MS patients taking the highest MOR103 doses (1 and 2 mg/kg) and was detected in placebo and 0.5 m/kg MOR103 study group (Table 7-3). It is unknown whether the reduced the number of relapses after GM-CSF neutralization in this trial was due to coincidence or it was therapy-related. One has to take into account that the absence of relapses within a 6 month period is not uncommon in MS, and that the small trial was not powered to assess the effect of the antibody on the frequency of relapses. Till now there is no full understanding of molecular mechanisms during relapse, yet it is thought that during relapse, a great influx of immune cells (mainly T and B cells) through the BBB into the brain takes place (Disanto *et al*., 2012, Irizar *et al*., 2014). This may be mediated by GM-CSF according to EAE and human studies, as GM-CSF was shown to increase BBB and BSCB permeability and monocytes' migration ability (King *et al.*, 2009, Aube *et al.*, 2014, Vogel *et al.*, 2015). In addition, previous studies have shown that GM-CSF levels were increased in the CSF during relapse stages of MS (Carrieri *et al*., 1998), and in MS in general when compared with other neurological diseases (Perrella *et al*., 1993). Besides, GM-CSF was found to be expressed in CD4+ and CD8+ T cells in MS brain lesions (Rasouli *et al*., 2015).

All these findings may support the pathologic role of GM-CSF in MS and may explain the results of no exacerbations of the disease after GM-CSF neutralization with the highest MOR103 doses in this trial. In addition, there is evidence that GM-CSF has central and peripheral

pathogenic effects in EAE models (King *et al*., 2009, Codarri *et al*., 2011, El-Behi *et al*., 2011). It is not yet clarified whether central or peripheral pathways of GM-CSF neutralization being mainly involved in disease amelioration. However, no conclusive statements can be made, as the number of patients was too small in each treatment group, and the duration of treatment was limited. All the above mentioned clinical findings are promising to proceed with studies looking at underlying mechanisms of GM-CSF neutralization.

GM-CSF neutralization by MOR103 has shown effectiveness in rheumatoid arthritis patients (Behrens *et al*., 2015). Therefore, it was suggested to be an effective therapy for autoimmune inflammation in MS, and the results we show here are promising. GM-CSF receptor is normally expressed on murine astrocytes, microglia, neurons, and oligodendrocytes (Sawada *et al*., 1993), suggesting a regulatory role of GM-CSF on these cells. GM-CSF that can be detected in healthy human and murine CNS is produced mainly by astrocytes (Malipiero *et al*., 1990, Dame *et al*., 1999), and may be essential for the normal functioning of microglial cells (Malipiero *et al*., 1990). Some studies have found that GM-CSF can be expressed by astrocytes and microglia upon stimulation in humans and mice (Lee *et al*., 1994, Fischer and Reichmann, 2001). With regard to EAE, invading encephalitogenic T cells were found to be the main GM-CSF source that has led to microglial activation and onset of EAE (Ponomarev *et al*., 2007). Taken together, it is clear that GM-CSF plays a non-redundant role in EAE induction and severity. However, no reports available yet about whether the centrally-produced or the invading cell-produced GM-CSF (or both) play the major role in autoimmune inflammation in MS.

This trial shows a significant reduction in percentage of CD8+ T cells after MOR103 therapy compared to before treatment levels. This may be a chance finding of no biological significance, as the total number of CD8 cells remained within the normal range. The normal range of CD8+ T cells varies from one lab to another, but usually it may range between 0.39-0.6 x 10^6/Litre of peripheral blood (Elling *et al.*, 1996). Therefore, the decrease was only in the proportion of CD8+ cells, but it was unlikely to bring the total number out of the normal range. Immunohistochemistry, fluorescence microscopy and flow cytometry techniques in many studies have shown that the majority of T cells infiltrated into MS lesions (site of inflammation) were CD8+ regardless of disease activity (Gay *et al*., 1997, Babbe *et al*., 2000, Lassmann *et al*., 2007, Frischer *et al*., 2009, Larochelle *et al*., 2016). This suggests an essential role of these cells in MS pathogenesis.

Further evidence was reported to support a key role of CD8+ T cells in the pathogenesis of MS, these cells were seen interacting with APCs at margins of active and chronic MS lesions (Serafini *et al*., 2006). This interaction was via MHC-I molecules that are poorly expressed normally but highly expressed in oligodendrocytes, neurons, and astrocytes in MS lesions (Traugott *et al*., 1983, Wong *et al*., 1984, Neumann *et al*., 2002). There is still no direct evidence on the causes of the preferential accumulation of CD8+ T cells over CD4+ T cells in MS brains, knowing that both were seen expressing GM-CSF in MS lesions (Rasouli *et al*., 2015).

All the above mentioned favours the key role of GM-CSF and CD8+ T cells in MS pathology. According to the literature, GM-CSF primes macrophages (centrally and peripherally) and microglial cells to become effective APCs (Aloisi *et al*., 1998, Hamilton, 2008); i.e. present myelin

peptides to T cells that leads to the latter cells activation, expansion, and trafficking to the site of inflammation (CNS). There is a report suggesting higher CD4+ T cell frequency in peripheral blood, with no change in CD8+ T cell frequency in untreated MS patients compared to normal subjects (Michalowska-Wender *et al*., 2003). Upon GM-CSF neutralization, APC activity may be reduced; hence, proliferation of T and B cells in general is proposed to decrease, and it is still unknown why only CD8+ T cell frequency was reduced in the blood. However, to confirm this finding and look for underlying causes, future studies are needed.

The decrease in naïve Tregs noticed after MOR103 treatment could was accompanied by an increase in the percentage of total Tregs (Figure 1-4). Quadrants were set for naïve Tregs and cytokines in this chapter based on isotype controls and not FMO controls. This is because this experiment was performed during my first year work (before I started using FMO). Tregs are known immuno-regulatory cells that were targeted to be increased in some MS therapies, such as IFN-β-1a (de Andres *et al*., 2007). They play a beneficial role in MS by secreting anti-inflammatory cytokines and direct cell-cell contact (Takahashi *et al*., 1998). Direct cell-cell contact is mediated by molecules expressed on the Treg cell surface (such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)), which binds to CD80/CD86 expressed on APCs and effector T cells. This leads to transmission of suppressive signals to APCs and effector T cells (Cools *et al.*, 2007). According to this trial's results, patients before MOR103 therapy group were not statistically different from healthy control group in terms of total Treg frequencies, which is consistent with other studies findings (Viglietta *et al*., 2004, Haas *et al*., 2005, Feger *et al*., 2007). However, GM-CSF neutralization with

MOR103 statistically increased total (CD4+CD25+foxp3+) Treg frequency in peripheral blood. T cells, in general, do not express GM-CSF receptor (Park *et al*., 1986, Miyamoto *et al*., 2002); however, GM-CSF may act by an indirect pathway. It has been demonstrated that GM-CSF induces DC maturation and antigen presentation activity (Fischer and Reichmann, 2001, Kelly *et al*., 2001). This leads to the assumption that GM-CSF neutralization in this trial may have reduced DC maturation and antigen presentation, which was shown to induce foxp3+ Tregs formation from foxp3- T cells (Kretschmer *et al*., 2005). This may need be confirmed in larger studies designed to address these questions.

CD154 (or CD40L) is a membrane protein that serves as a marker for T cell activation. It is suggested that CD154 mediates an important co-stimulatory pathway in MS via binding with CD40 expressed on B cells and myeloid cells (APCs), leading to APC, B and T cell stimulation and production of pro-inflammatory cytokines such as IL-12 (Grewal *et al*., 1996, Cook, 2006). CD154 was measured during this trial to determine if there is an indirect effect of GM-CSF on the frequency of peripheral activated T cells. This trial did not show any differences the frequency of CD154+ T cells between healthy and MS patients before MOR103 therapy. This finding was consistent with another study (Bilinska *et al*., 2006). However, it was inconsistent with other studies' findings (Jensen *et al*., 2001, Sanchooli *et al*., 2014). The exact reason behind this difference is not clear, but may be due to blood handling, and the stage of the MS. There was no difference in activated T cell proportions before and after MOR103 therapy.

In contrast to the results in chapter 3, there were no differences in the frequencies of GM-CSF-expressing T cells between MS patients and healthy controls. The study was not powered to look at such differences

and the samples were drawn from different populations than those in chapter 3. In addition, PBMC stimulation methods differed between these populations. GM-CSF neutralization with MOR103 did not change frequencies of GM-CSF-producing CD4+ and CD8+ T cells in peripheral blood. Up to date, there is no data in the literature showing the effects of GM-CSF neutralization on its own production by immune cells. It was expected that GM-CSF neutralization would reduce its own production, since GM-CSF was suggested to have a positive feedback mechanism on itself. This is via acting on myeloid cells to induce their maturation, priming, and production of IL-1β and IL-23, which act on T cells to increase their GM-CSF production (Li *et al*., 2013, Shiomi and Usui, 2015). GM-CSF expression was measured by peripherally activated T cell populations with no differences shown. Again, this may be because this study was not designed to detect differences between healthy controls and MS patients. However, the most important biological outcome of this therapy is the neutralization of secreted GM-CSF at the site of inflammation.

This trial has achieved the purpose it was designed for. However, there were a number of limitations that made it difficult to clearly interpret immunological findings: low number of patients, limited duration of therapy, limited techniques and antibodies approved for use, and using this therapy for the first time in MS patients. However, the promising results of this trial are encouraging for starting phase II, in an attempt to determine MOR103 efficacy, minimize the limitations, and examine the underlying mechanisms. In conclusion, we show that neutralization of GM-CSF with MOR103 may have effects on reducing the frequency of total CD8 lymphocytes and naïve Tregs. It may also have an inducing effect on total Tregs. This needs further experiments to be confirmed.

Chapter 8. Main Discussion and Conclusions

8.1 Discussion

Despite extensive studies, the pro-inflammatory pathways involved in MS pathogenesis are not fully understood yet. GM-CSF was shown to play a role in regulating functional activities of myeloid cells and maturation of DCs (Hamilton, 2008). In addition, it was shown to act in an autocrine manner on B cells to increase their survival (Harris *et al*., 2000). Others have shown indirect functions of GM-CSF on T cell proliferation and survival via APCs (Sonderegger *et al*., 2008).

The main rationale for accomplishing this work arises from the importance of GM-CSF in EAE induction. GM-CSF knock-out mice were shown to be resistant to EAE (McQualter *et al*., 2001); and over-expression of GM-CSF has led to more severe EAE by increased influx of inflammatory cells to the CNS (Marusic *et al*., 2002). In addition, myelin peptide-reactive Th1 and Th17 cells that lack GM-CSF, failed to transfer EAE (Codarri *et al*., 2011, El-Behi *et al*., 2011). With regard to the role of GM-CSF in MS, no direct evidence was shown yet; however, GM-CSF levels in the CSF were shown to be elevated in the active stage of MS (Carrieri *et al*., 1998). Moreover, a recent study has identified a distinct Th subtype that mainly express GM-CSF and IL-3 and is essential for EAE pathogenesis (Sheng *et al*., 2014).

Therefore, our hypothesis was that in MS, there is increased GM-CSF expression by immune cells. This is a result of imbalance between pro- and anti-inflammatory pathways, favouring the former in MS. We also hypothesized that the distinct Th-GM subset can be identified in MS, and their frequency is higher in MS. Finally, we hypothesized that GM-

CSF neutralization *in vivo* will be beneficial in MS. Hence, our work aimed to investigate GM-CSF expression by PBMC subsets and its regulation with relevance to MS. In the next paragraphs, I will discuss how/to what extent we could answer the research questions/hypothesis in each chapter, what the main findings are, how our results fit in the context of existing literature, and whether the experimental model we used is relevant in a biological context of MS. Our hypotheses were tested mainly by PBMC stimulation and detection of GM-CSF expression by different PBMC subsets. In almost all of the experiments, we used flow cytometry as a technique to detect GM-CSF. Flow cytometry is the only technique that can measure the intensity of up to 14 protein markers (in our university facility) in every single cell within a cell population. Moreover, flow cytometry can measure spontaneously produced cytokines by a single cell type within a cell population. In our experiments, flow cytometry was preferred to ELISA or mRNA based assays, as the last two assays do not identify the cells containing the protein of interest or its transcripts (Sachdeva and Asthana, 2007). In addition, many factors can repress DNA conversion into proteins, such as DNA methylation and mRNA deadenylation (Baker and Coller, 2006).

To present flow cytometry results, either dot plot or MFI can be used. The dot plots were chosen to present this work because they are more informative in terms of changes in the percentage of cells expressing the cytokines of interest, which sometimes cannot be detected using MFI. MFI detects if there are changes in the fluorescence intensity of the cytokines of interest in the cells expressing them but not the proportion of these cells. Therefore, a difference can be seen when using MFI if the cell population shifts from dim to brighter location in the dot plot, and vice versa. In general, such changes were not seen in these experiments,

where the selected cell populations were in the same location in the dot plot (expressing similar MFI) but different percentages with different conditions or donors.

In the work reported in chapter 3, we tested the hypothesis that activated Th1 and Th17 cells express GM-CSF in humans as in mice. These GM-CSF-expressing cells were hypothesized to be higher in frequency in MS patients. We found that activated Th1 and Th17 cells express GM-CSF in the human immune system. Furthermore, we found that the frequency of GM-CSF-expressing Th1 cells was higher in MS patients than healthy controls. This may be due to higher IL-12 levels in MS that we found, as discussed below, as IL-12 is the main cytokine in the Th1 differentiation pathway. In addition, higher IL-2 levels detected in our work (chapter 3), and IL-2R polymorphisms reported in MS (Hartmann *et al*., 2014) may contribute to this finding.

We also hypothesized a significant increase in GM-CSF-expressing Th and Tc cells in MS patients. This is because T cells were hypothesized to be activated *in vivo* in MS patients and capable of being re-activated *in vitro*, which may lead to higher GM-CSF expression. We also investigated IFN-γ and IL-17 and we hypothesized that their expression will be higher in MS patients as well. Our results show that GM-CSF (but not IFN-γ and IL-17) was expressed in significantly higher frequency by Th and Tc cells in MS patients, when compared to healthy controls. This is consistent with the findings of recent studies (Hartmann *et al*., 2014, Rasouli *et al*., 2015) and may indicate that GM-CSF may have a crucial role in the autoimmune inflammation cascade.

Our study also showed that the frequency of Th cells co-expressing GM-CSF and IFN-γ (but not GM-CSF and IL-17) is higher in MS patients

than healthy controls. This is consistent with the findings of a recent study (Rasouli *et al*., 2015). It is also supported by the findings of another study showing the Th1-GM-CSF pathway being more activated than Th-17-GM-CSF pathway (Noster *et al*., 2014). In addition, Noster and colleagues showed higher co-expression of GM-CSF and IFN-γ (and not GM-CSF and IL-17) by Th cells in the CSF of recently diagnosed untreated RRMS patients compared to healthy controls.

We also tested the hypothesis that monocytes express more GM-CSF in MS patients. This is because monocytes and macrophages in MS generally produce higher levels of pro-inflammatory cytokines (Filion *et al*., 2003a, Filion *et al*., 2003b). We found that GM-CSF is expressed significantly more by monocytes in the mixed PBMC population in MS patients, which was not shown before. This may indicate that more monocytes can be differentiated to M1 pro-inflammatory macrophages in the presence of GM-CSF in MS, and more antigen (myelin peptides) presentation to T cells.

In the results shown in chapter 3, we also measured the concentrations in culture supernatants, of main cytokines that are involved in MS pathogenesis. We were also interested to look at IL-7, which was shown recently to be crucial for Th-GM differentiation in mice (Sheng *et al*., 2014). We could not hypothesize which cytokines exactly that would be in significantly higher levels in stimulated cultured PBMC supernatants of MS patients, because of the complex interaction between the PBMC subtypes. However, we were expecting that cytokines essential for Th1 differentiation (IL-12, IFN-γ), in addition to GM-CSF, will be higher in the MS group than healthy controls. Our findings show that levels of GM-CSF, IL-2, IL-12, and IL-1β were significantly higher levels in stimulated cultured PBMCs in MS patients than healthy controls. These

cytokines have been shown to be able to induce GM-CSF in various immune cell types. In addition, significantly lower TGF-β levels were measured in MS patients when compared to healthy controls. These were interesting outcomes for the next experiment to investigate the effects of blocking each or a combination of the cytokines that were elevated in MS on GM-CSF (and also IFN-γ and IL-17) expression by PBMC subtypes in untreated RRMS patients.

The aim of doing cytokine blocking experiments was to identify which cytokine pathway is linked with increased GM-CSF expression. We chose to measure the cytokines that were known to regulate GM-CSF in T and NK cells mainly. Then, we chose to block the cytokines that we showed to be increased in the PBMC culture supernatants of MS patients. This is because we hypothesized that higher concentration of one or more of these cytokines may result in higher GM-CSF expression by immune cells in MS.

The main outcome of cytokine blocking in T cells was that blocking IL-12 significantly reduced the frequency of Tc cells expressing GM-CSF in MS patients but not in healthy controls. IL-12 is involved in the generation of Tc cells. It works in synergy with IL-2 to increase GM-CSF expression (Trinchieri, 1995). The reason behind this difference in healthy controls and MS patients was not investigated in this study. It was shown that IL-12A and IL-12B genetic polymorphism is one of the genetic risk factors for MS (Sawcer *et al*., 2011), which may lead to increased IL-12-induced GM-CSF expression. However, the exact role of IL-12 polymorphism on GM-CSF expression by CD8+ T cells in MS is not yet investigated.

The cytokines that we found to be up-regulated in MS are involved in T cell stimulation as well as NK cell stimulation. Therefore, our work shown in chapter four was performed to test the hypothesis that NK cells expressing GM-CSF present in higher frequencies in MS patients, which is something not investigated yet. We found a significantly higher frequency of NK cells expressing GM-CSF in MS patients when compared to healthy controls. To our knowledge, there are no studies about GM-CSF secretion profile of NK cells in MS. A previous study has found an increase in NK cells in PBMCs during remission stage of MS, which were NK2 subtype exerting anti-inflammatory actions including suppressing Th1 responses (Takahashi *et al*., 2001). It was reported that peripheral NK2 and NK1 cells produce similar amounts of GM-CSF. Production of IFN-γ by NK2 is lower than that of NK1 (Peritt *et al*., 1998). The NK cells investigated in our study were expressing the pro-inflammatory cytokines GM-CSF, IFN-γ, and some IL-17, suggesting they play a pro-inflammatory role. They were elevated in MS patients, indicating a potential pathogenic role. This also suggests that even during the remission stages of MS, NK cells, like other immune cells, respond more vigorously to inflammatory stimuli. To our knowledge, this study is the first to show increased GMCSF in NK cells from MS patients. The role of NK cells and NK-cell derived GMCSF needs to be investigated further.

Indirect stimulation of NK cells with a T cell stimulus (anti-CD3+anti-CD28) induced higher frequency of GM-CSF-expressing NK cells in MS. Therefore, we wanted to look at GM-CSF expressing NK cell frequency in more depth. We did address this by investigating GM-CSF expression in isolated NK cells, using stimuli that were shown to induce high GM-CSF concentrations in NK cells (Cooper *et al.*, 2001c). We

hypothesized that isolated NK cells will still express higher GM-CSF in MS patients compared to healthy controls. However, our results have revealed similar GM-CSF expression in MS patients and healthy controls. This may indicate that there may be factor(s) that were more abundant in the mixed PBMC population but not in isolated NK cells in the samples of MS patients. These factor(s) were involved in higher GM-CSF and IFN-γ expression. As mentioned above, our work showed GM-CSF, IL-2, IL-12, and IL-1β were significantly elevated in the culture supernatants of MS patients.

Then, we hypothesized that blocking one or more of the cytokines highly secreted in MS may abrogate the higher GM-CSF expression in NK cells in the mixed PBMC population. Our results revealed that blocking both IL-2 and IL-12 significantly reduced the frequency of CD3-CD56+ cells expressing GM-CSF, GM-CSF+IFN-γ, and GM-CSF+IL-17+ in MS patients but not in healthy controls. We also showed in the previous experiment on isolated NK cells that adding IL-2 and IL-12 in the same concentration to the healthy controls and MS patients resulted in high but similar GM-CSF expression in both groups. This may indicate that higher levels of IL-2 and IL-12 in the PBMC culture of MS patients were involved in higher GM-CSF expression by NK cells in the mixed PBMC population.

Our results are consistent with previous reports which have shown that both IL-2 and IL-12 induced NK cell cytotoxicity and GM-CSF and IFN-γ production (Lehmann *et al*., 2001, Parihar *et al*., 2002, Leong *et al*., 2014). However, our results did not show any significant effects of blocking IL-2 or IL-12 on IFN-γ expression by CD3-CD56+ PBMCs. This may indicate there are other pathways (other than IL-2 and IL-12) involved in the expression of IFN-γ by NK cells in our PBMC

population mixtures, such as IL-1 and IL-21 (Cooper *et al.*, 2001b, Roda *et al.*, 2006). The exact role of NK cells co-expressing GM-CSF/IFN-γ and GM-CSF/IL-17 in MS is not determined yet. We show for the first time that IL-2 and/or IL-12 blocking significantly reduces these two populations of NK cells in the mixed PBMC population of MS patients but not healthy controls. More experiments are needed to evaluate the role of these cells in MS pathogenesis and progression of inflammation.

The work presented in chapter four was done to test the hypothesis that GMR is up-regulated in NK cells when GM-CSF expression is reduced, and vice versa. This is due to the fact that GMR was reported to be expressed by NK cells (Kubista *et al*., 2003), it may be up-regulated when GM-CSF levels are reduced, and vice versa, as shown in monocytes and neutrophils (Cannistra *et al*., 1990, Brizzi *et al*., 1991). Our results show that the decline in the frequency of GM-CSF-expressing CD3-CD56+ cells was accompanied by an increase in the frequency of GMR-expressing CD3-CD56+ cells after IL-2 and IL-12 blocking in the mixtures of PBMC population of MS patients. Our results indicate that the NK cells may contribute to the higher overall GM-CSF expression in MS patients. On the other hand, our results also suggest that direct effects of GM-CSF on NK cells in MS may be minor. It is interesting to look at any changes in GMR expression or sensitivity after disease-modifying therapy in MS patients, and whether this may alter NK cell activity.

In chapter five, the presented work was to test the hypothesis that the frequency of GM-CSF-expressing B cells are higher in PBMC population mixtures of MS patients. We have also hypothesized an increase in the pro-inflammatory cytokines IFN-γ and IL-17. This was something not investigated at the time of starting the experiment and

may be as a result of over-activation of immune cells in MS. Our results demonstrate higher GM-CSF-expressing B cells in MS patients. This is consistent with the findings of a recent study, performed on B cells after their isolation and stimulation with either PMA/I or CD40L+ anti-IgM (Li *et al*., 2015). However, our results are showing for the first time that more B cells produce GM-CSF in MS patients within mixed PBMC population in which T cells were primarily stimulated.

Possible mechanisms may implicate the effects of one or more of the cytokines elevated in PBMC culture supernatants of MS patients. Therefore, we hypothesized that blocking one or more of these cytokines will regulate GM-CSF-expressing B cell frequency. Our results show a decline in GM-CSF-expressing B cells after blocking IL-2, IL-12, IL-2+IL-12, and IL-12/IL-23. It was shown that IL-2 induces B cell proliferation and differentiation into plasma cells and increases antibody secretion (Mingari *et al*., 1984, Nakagawa *et al*., 1985, Miyawaki *et al*., 1987, Le Gallou *et al*., 2012). With regard to IL-12 effects on B cells, it was mainly shown to induce B cells differentiation into a distinct subset that is similar to Th1 pattern (Durali *et al*., 2003). With regard to IL-12 effects on GM-CSF expression in B cells, it is not clearly shown in the literature so far. However, we hypothesized that blocking IL-12 will reduce GM-CSF expression by B cells, and our results were consistent with our hypothesis. Our results show for the first time that IL-12 is non-redundant for GM-CSF expression by B cells in MS patients. It is unknown whether this effect of IL-12 on GM-CSF expression by B cells is by direct or indirect pathways. It is important to investigate the signalling pathways for IL-12 in B cells that lead to GM-CSF expression, and IRA B cells (which mainly produce GM-CSF and IL-3) in MS.

Our results also show that blocking p40 subunit which is common in IL-12 and IL-23 resulted in reduction of GM-CSF-expressing B cells in MS patients but not in healthy controls. This is similar to what we found after blocking IL-12p70. This may indicate that IL-23 may not be involved in GM-CSF expression in B cells. Our findings show for the first time that IL-12 may be involved in GM-CSF expression in B cells in MS patients. Table 8-1 below is a summary of the main results of cytokine blocking experiments in the PBMC subsets examined in this work.

Table 8-1: Summarised main effects of cytokine blocking on PBMC subsets

Cytokine(s) blocked	Cell type	Effects of blocking
IL-12	Tc cells	Lower frequency of GM-CSF-expressing Tc cells in MS
IL-1β	Th cells	Lower frequency of IL-17-expressing Th cells in healthy controls
IL-2+IL-12	NK cells	Lower frequency of GM-CSF-expressing NK cells in MS
IL-2	NK cells	Higher frequency of GMR-expressing NK cells in MS
IL-2, IL-12, IL-2+IL-12, IL-12+IL-23	B cells	Lower frequency of B Cells Expressing GM-CSF in MS

We also hypothesized that the frequency of GMR-expressing B cells will be reduced in MS patients when compared with healthy controls. This may be due to increased frequency of GM-CSF-expressing B cells when they are stimulated. No reports are published yet studying the effects of GM-CSF on its receptor's expression in B cells. However, it was shown that GM-CSF has down-regulated GMR expression on monocytes, myeloid progenitor cells, and neutrophils (Cannistra *et al*., 1990, Brizzi *et al*., 1991). Our results show no difference in GMR expressing B cells between MS patients and healthy controls. GMR expression in B cells has not yet been explored in autoimmune diseases; however, this may be

of a big importance in the pathologic course of MS. This is because autocrine GM-CSF was shown to induce IgM production by IRA B cells (Rauch *et al*., 2012, Weber *et al*., 2014). Antibody secretion by B cells (mainly IgG, with some IgM) is among the main pro-inflammatory pathways exerted by B cells in MS (Reiber *et al*., 1998, Villar *et al*., 2005). Moreover, GM-CSF may act (in an autocrine manner) on B cells to increase their survival, but exogenous GM-CSF had no effect on these cells (Harris *et al*., 2000). This was not explored in MS; therefore, we studied the effects of adding and blocking GM-CSF on the viability of isolated B cells in MS patients during relapse compared to healthy controls. In this small exploratory sample we found no statistically significant differences in the frequency of apoptotic, dead, and live cells amongst isolated B cells after blocking GM-CSF. Similarly, there was no difference in the frequency of GMR-expressing B cells in healthy controls and MS patients after blocking GM-CSF. This was done because we hypothesized that GM-CSF neutralization may have therapeutic outcomes via reducing B cell survival, as a previous study had identified GM-CSF as an autocrine survival factor for B cells (Harris *et al.*, 2000). With this exploratory experiment and with the sample size used in this work, this hypothesis could not be proved. The importance of B cells in MS is confirmed by the efficacy of B cell depletion therapies observed in MS patients (Disanto *et al*., 2012, Lehmann-Horn *et al*., 2013).

We have also aimed to determine the effects of EBV transformation on GM-CSF-mediated effects exerted by GMR and on B cell viability. EBV infection of B cells reduced GMR expression when compared to uninfected B cells in isolated and CD40L/anti-IgM-stimulated B cells after 1 day of stimulation. To confirm this finding and examine if there

are any GM-CSF effects on EBV-infected B cell viability, we added GM-CSF or neutralising anti-GMCSF antibody to EBV infected B cell lines from healthy controls. Our results have shown no effects of either CD40L/anti-IgM antibody, GM-CSF, or anti-GM-CSF antibody on the viability of EBV-infected B cells. In addition, GMR was measured again after 4 days stimulation and its frequency was low as well. This may confirm our finding of reduced GMR upon EBV infection of B cells. There is a study which has shown that adding GM-CSF to EBV-infected B cell lines induced their proliferation (Paul and Baumann, 1990). Our results suggest that EBV infected B cells depend on other mechanisms for their survival other than GM-CSF pathway.

After looking at GM-CSF and GMR expression and regulation by the main PBMC subsets (T cells, NK cells, and B cells), it was important to look at the distinct GM-CSF-producing Th cells (Th-GM). These cells were recently identified in EAE but not yet detected in MS. Therefore, our work presented in chapter six was done to test the hypothesis that we Th-GM cells can be identified in humans as was done recently in mice, and they may be in higher frequency in MS patients during relapse. These cells were shown to be distinct from Th1 or Th17 (Sheng *et al*., 2014); therefore, we have identified/phenotyped these cells as CD4+GM-CSF+IL-3+IFN-γ-IL-17- lymphocytes. We were able to identify these cells and show that they were increased in MS patients during relapse. This was at the expense of reduction in CD4+GM-CSF+IL-3+IFN-γ+IL-17- lymphocytes. More investigation is needed about the role of these cells in MS pathogenesis in relapse and remission stages. Our results show that addition of IL-2 and IL-7 together to naïve CD4 T cells resulted in the highest frequency of Th-GM cells than adding each cytokine alone. This is different from mouse cells where IL-

7 alone was enough to detect these cells in comparable frequency to ours after IL-2+IL-7 stimulation (Sheng *et al*., 2014). Moreover, it appears that IL-2 and IL-7 are having additive, but perhaps not synergistic effects. In future experiments IL-2/IL-7 titrations will determine which stimuli or combinations result in the highest Th-GM expression.

In view of the above-mentioned results showing that GM-CSF expression is induced in MS, it is interesting to explore the possibility of *in vivo* GM-CSF neutralisation. This was performed in a phase Ib clinical trial. Before analysing the effects and mechanisms of action of a neutralising monoclonal antibody against GM-CSF, the important aim is to ensure its safety. Therefore, this trial was designed mainly to determine the safety of the drug (MOR103). The second outcome measures were to assess the pharmacokinetics and the immunogenicity of the antibody. We performed a sub-study of a small cohort of participants recruited from our centre, to explore a number of immunological changes. We could not predict if GM-CSF expression will be changed or not, as different mechanisms may control its expression, including a compensatory up-regulation of its expression after being neutralized, or reduced GM-CSF expression due to inhibition of GM-CSF-dependent positive feedback loop on its own expression after being neutralized (Li *et al*., 2013, Shiomi and Usui, 2015).

Our results show a reduction in CD8+ T cells and naïve Tregs, accompanied by an increase in total Tregs after MOR103 treatment when compared to before treatment. On the other hand, no differences were detected in the frequency of CD3+, CD4+ T cells or in lymphocyte activation. In addition, no differences were detected in GM-CSF expression by lymphocytes after MOR103 therapy. Upon GM-CSF neutralization, APC activity may be reduced; hence, proliferation of T

and B cells in general is proposed to decrease, and it is still unknown why only CD8+ T cell frequency was reduced peripherally. This may be a chance finding of no biological significance, as the total number of CD8 cells remained within normal range. It may be due to the small number of samples tested, because the study was not designed to look at these parameters mainly.

The decrease in naïve Tregs noticed after MOR103 treatment was accompanied by an increase in the percentage of total Tregs (Figure 7-3). Tregs are cells that suppress autoreactive effector T cells. Tregs are targeted to be increased in some MS therapies such as IFN-β1a (de Andres *et al*., 2007). They play a beneficial role in MS by secreting anti-inflammatory cytokines and direct cell-cell contact (Takahashi *et al*., 1998). According to the results of this trial, we show no significant differences in total Treg frequencies between patients before MOR103 therapy and healthy controls. This is consistent with the findings of other studies (Viglietta *et al*., 2004, Haas *et al*., 2005, Feger *et al*., 2007). However, GM-CSF neutralization with MOR103 increased total Treg frequency in peripheral blood. T cells, in general, do not express GM-CSF receptor (Park *et al*., 1986, Miyamoto *et al*., 2002); however, GM-CSF may have effects on T cells by indirect pathway(s).

MS is an autoimmune disease, in which there is an imbalance between the pro-inflammatory and the anti-inflammatory pathways, favouring the former. Based on our *in vitro* results (chapter 3), the levels of TGF-β (one of the key cytokines that induces Treg differentiation) were significantly reduced in MS. This may indicate a dysregulation in the Treg pathways (homeostasis and/or functioning) in MS, as reported by many studies (Schneider-Hohendorf *et al.*, 2010, Venken *et al.*, 2010, Nyirenda *et al.*, 2015). Reduced immune regulation may be one of the

mechanisms leading to increased percentage of T cells expressing GM-CSF in MS that this work has shown *in vitro*. Tregs were shown to reduce the percentage of GM-CSF-expressing CD4 T cells *in vitro* (Peelen *et al.*, 2015).

Moreover, it has been demonstrated that GM-CSF induces DC maturation and antigen presentation activity (Fischer and Reichmann, 2001, Kelly *et al*., 2001). This leads to the assumption that GM-CSF neutralization in this trial may have reduced DC maturation and antigen presentation. Suboptimal DC activation may reduce pro-inflammatory cytokines that supress Treg function; or may induce foxp3+ Treg formation from foxp3- T cells as previously shown (Kretschmer *et al*., 2005). This can be confirmed by our *in vitro* findings in chapter 3, in which high levels of GM-CSF were accompanied by low levels of TGF-β in MS. This means more DC maturation, which stimulates the differentiation of naïve T cells into pro-inflammatory T cell subsets. This pathway may be enhanced by low TGF-β levels, favouring less Treg differentiation in MS. Larger patient numbers and more experiments are needed to clarify underlying mechanisms behind elevated total Tregs after blocking GM-CSF *in vivo*. The trial has shown that *in vivo* blockade of GM-CSF using human neutralising antibodies is safe and feasible (Constantinescu et al., 2015). Larger trials are thus warranted.

8.2 Limitations of the study

This study has a number of limitations, including difficulties in recruiting MS patients who fulfil the inclusion criteria. Another limitation in our work is that we did not use other techniques than flow

cytometry to corroborate our findings, such as mRNA to detect the regulation of GM-CSF gene.

In most of our experiments, we used a mixed PBMC population and not sorted or isolated cell types to detect GM-CSF expression. However, this may also be reliable, as we do gate the cells based on surface and intracellular markers (e.g., foxp3). In addition, this model may be closer to *in vivo* conditions, as all cell types are mixed together. This allows communication between these cells that may influence GM-CSF expression. This was clearly shown in our results, when a higher frequency of NK cells in the mixed PBMC population expressed GM-CSF in MS patients, while similar frequencies of isolated NK cells produced GM-CSF in healthy controls and MS patients. This supported our hypothesis that the mixed PBMC population contains factors that regulate GM-CSF in MS patients in a different way from healthy controls. However, another limitation for this study was not measuring the effects of cytokine antibodies on the cytotoxicity of Tc and NK cells.

With regard to sample size in each of our experiments, we did not calculate it or use pre-determined numbers. Instead, exploratory numbers were estimated, largely based on other published related studies (Hartmann *et al*., 2014, Noster *et al*., 2014, Rasouli *et al*., 2015). In addition, statistics were usually performed for exploratory purposes, to get ideas of differences and trends between groups; therefore, p value was not corrected for multiple comparisons. In addition, it is likely that many of the results are collinear and interdependent, whereas correction for multiple comparisons requires independence of the variables measured. In GM-CSF neutralization experiments with MOR103, the number of samples enrolled in Nottingham was small, because the study was not designed to compare immune-related parameters. However, we

only wanted to look at trends as this was the first time to use this drug in MS patients. Therefore, results need to be interpreted with caution and no definitive conclusions about the immune effects of the GMCSF neutralisation should be made.

Another limitation of this study was the inability to use fresh PBMCs in all of the experiments, as it was difficult to collect blood samples from MS patients and process it at the same time. On the other hand, there were ready to use frozen PBMCs from MS patients and healthy controls. There was a lower cytokine (especially GM-CSF) expression in frozen/thawed PBMCs compared to fresh ones. However, statistical significance was still achieved in most cases after comparing GM-CSF expression in immune cell types between healthy controls and MS patients when frozen/thawed cell data were combined.

In terms of comparing GM-CSF and GMR expression during relapse and remission stages (or before/after IFN-β therapy), the limitation was the availability of RNA samples only. This RNA was extracted from unstimulated lysed blood cells, which have low cytokine (in particular GM-CSF) expression. Upon stimulation, cytokine expression increases in general, and differences can be detected. Therefore, fresh PBMCs may be the ideal sample to detect GM-CSF and GMR expression in the same patient during relapse and remission stages or before/after DMTs. This advantage has to be weighed however against the practicability challenges when dealing with longitudinal clinical samples or with those obtained during clinical trials, and against the variability between assays performed at multiple time points.

In this study, we have not demonstrated how effective the neutralisation of the cytokines measured was after using anti-cytokine antibodies. This

has been stated as a future plan in section 8.5. However, some neutralisation is likely, as using an isotype control antibody had no effect. It was added as another condition to cultured and anti-CD3+anti-CD28-stimulated PBMCs. In all the parameters tested, there was no change between stimulated versus stimulated with isotype control antibody. In addition, the manufacturer provided data on effective cytokine neutralisation at the dilutions used in our experiments.

With regard to GM-CSF regulation in MS, the limitation was not investigating the molecular mechanisms behind the dysregulation of GM-CSF seen in our work. We have examined the cytokines that, according to the literature, affect GM-CSF expression. Then, the cytokines which were found to be elevated in culture supernatants in MS patients were blocked to detect their effects on GM-CSF expression. Blocking the effects of some cytokines reduced GM-CSF expression in MS. However, the molecular mechanisms behind the effects of these cytokines on GM-CSF expression were not investigated in this work. This is discussed in the future directions section 8.5.

8.3 Conclusions

The main conclusions of this work are summarized as below:

1. The frequencies of GM-CSF-expressing Th cells, Tc cells, NK cells, B cells, and monocytes were statistically higher in MS patients than healthy controls. However, isolated and stimulated NK cells had no difference in GM-CSF production between MS patients and healthy controls.

2. Both Th1 and Th17 cells expressed GM-CSF in MS patients and healthy controls. However, the frequency of GM-CSF-expressing Th1 cells was higher in MS patients than in healthy controls.

3. IL-2 and IL-12 may have an important role in inducing GM-CSF expression by Tc, NK, and B cells in MS patients and not in healthy controls.

4. A distinct Th cell subset is identified in humans *in vitro*, which were not expressing IFN-γ (Th1) or IL-17 (Th17). Instead, they were expressing GM-CSF and IL-3. Based on a recent EAE study, they were called Th-GM. Higher frequency of Th-GM was detected in MS patients during relapse after IL-2+IL-7 stimulation of naïve CD4+ T cells.

5. GM-CSF neutralization has led to reduced total CD8+ T cell frequency, reduced naïve Tregs, and increased total Tregs. However, the study was not designed to look at differences in immune-related parameters mainly. This may underlie the promising results obtained in phase I clinical trial, but this requires further investigation.

8.4 Implications of This Work

The implications of this study are based on the main conclusions mentioned above. We showed that almost all PBMC subtypes are high producers of GM-CSF in MS when compared to healthy controls. Therefore, any approved medication which inhibits one or more of these PBMC subsets (e.g., B cell depletion therapies, glatiramer acetate, fingolimod) may repress GM-CSF expression and/or action. This may be one of the mechanisms involved in the effectiveness of these medications in MS. In addition, the effectiveness of medications that target/block IL-2 pathway via blocking IL-2R-α (Daclizumab) may be

(in part) via its blocking to IL-2-mediated GM-CSF expression by CD8+, NK and B cells, which we showed to be increased *in vitro*, adding another potential mechanism of effectiveness. Moreover, GM-CSF neutralization *in vivo* has resulted in promising outcomes in MS which encourages proceeding with a phase II trial.

We also concluded that both Th1 and Th17 lymphocytes express GM-CSF in MS, and the frequency of GM-CSF-expressing Th1 is higher in MS patients than in healthy controls. This may suggest that Th1 differentiation pathways can result in more GM-CSF expressing cells in MS. Therefore, blocking Th1 differentiation pathway, or shifting it towards Th2 pathway (e.g., by using glatiramer acetate) may be therapeutically effective via reduction in GM-CSF expression. In addition, we concluded that IL-12 may be involved in GM-CSF expression by Tc, NK (with IL-2), and B cells in MS. A previous study has shown effectiveness of anti-IL-12/23p40 antibody in RRMS and SPMS (Vollmer *et al*., 2011). However, another study has shown the IL-12/23 p40 monoclonal antibody ustekinumab to be ineffective in a phase II clinical trial in untreated RRMS (Segal *et al*., 2008). Reducing GM-CSF expression by the above-mentioned PBMC subsets may be one of the mechanisms behind the effectiveness of blocking IL-12 in MS.

We also concluded that GM-CSF may have a role in enhancing B cell survival, and stimulated B cells express high levels of GMR in healthy controls and MS patients. Therefore, blocking GM-CSF may reduce B cell survival, knowing that B cells play a crucial role in autoimmune inflammation in MS.

With regard to Th-GM identification, we have found higher expression of these cells in MS patients during relapse when compared to healthy

controls, which is a novel finding and a novel identification of these cells in humans. The recent animal study which identified these cells has shown that they are essential for autoimmune inflammation in EAE (Sheng *et al*., 2014). Therefore, they may exert an essential role in MS pathogenesis, which should be further investigated. IL-2 and IL-7 were shown to induce GM-CSF expression via activating STAT5 (Noster *et al*., 2014, Sheng *et al*., 2014). Therefore, blocking either STAT5 or Th-GM may have beneficial therapeutic outcomes in MS.

8.5 Future Directions

In addition to GM-CSF expression and regulation in PBMCs in MS, it is important to explore its expression and regulation in neutrophils in terms of MS stages. Studies have shown high neutrophil count with enhanced survival in RRMS patients (Lund *et al*., 2004, Naegele *et al*., 2012). Neutrophils are one of major blood cell types that express GM-CSF and GMR. A study on healthy neutrophils has shown that GM-CSF induced their expression of TLR2 and IL-8 (Kurt-Jones *et al.*, 2002). However, the expression of GM-CSF by neutrophils and the role of GM-CSF on neutrophils have not been examined in MS. Therefore, GM-CSF expression, GM-CSF or GMR blocking on neutrophils may help understand the role of GM-CSF in functioning of these cells in MS.

It is important to determine whether there are longitudinal changes in the same MS patient (e.g., during relapse and remission, or before and after initiation of DMT). Moreover, to have a more clear view about the role of GM-CSF in autoimmune inflammation in MS, it is important to look at its expression by different PBMC subsets in progressive MS, in CIS, in recently diagnosed MS, and in paediatric MS.

Moreover, it is essential to detect the *in vivo* effects of immune-modulating therapies on GM-CSF expression by immune cells, which may be one of the underlying mechanisms behind their effectiveness.

It is also important to demonstrate how effective the neutralisation of the cytokines measured was after using anti-cytokine antibodies. This is performed by examining the down-stream signalling pathways of the tested cytokines after blocking them. Similarly, it is essential to define the molecular mechanisms behind IL-2- and IL-12-related GM-CSF production by Tc, NK, and B cells. This may be detected mainly by blocking STAT5 and/or STAT4. With regard to B cells, it is important to determine if there is any difference in externally added and autocrine GM-CSF on B cell viability and proliferation in MS. In addition, these GM-CSF effects can be detected in EBV seronegative compared with EBV seropositive healthy controls and MS patients.

More work is required to examine the role of IL-2 and IL-7 in Th-GM differentiation, the role and genotyping of Th-GM in MS in terms of different disease stages, and in the immune system in general. With regard to GM-CSF neutralization *in vivo*, it is essential to procced with phase II clinical trial, based on the promising results obtained in RA and phase I in MS.

This thesis did not study the mechanisms of action and cellular targets of GM-CSF in MS, although it has started towards this, by looking at GMR expression by subpopulations of immune cells. GM-CSF neutralization trial with MOR103 was not sufficient to provide clear conclusions in terms of mechanisms of action. Therefore, future studies including blocking GM-CSF or GMR are important to examine the effect of GM-CSF on the activity and migration ability of granulocytes, macrophages,

B cells, and other GMR-expressing cells to the CNS in MS. In addition, effects of GM-CSF or GMR blockade on BBB permeability in MS may further contribute to the understanding of the pathophysiology of GM-CSF in MS.

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Appendix I: Overall Subject Details

1. Overall Healthy Control Details and the Experiments they participated in

Subject ID	Age (yrs)	Gender	Experiment(s) participated in *
HC1	47	F	1. PDB/I stimulation 16h/PBMCs 2. q-PCR (whole blood) 3. MOR103 experiment
HC2	50	M	1. PDB/I stimulation 16h/PBMCs 2. q-PCR (whole blood) 3. Isolated B cell experiment (with and without EBV)
HC3	42	M	PDB/I stimulation 16h/PBMCs
HC4	37	F	1. PDB/I stimulation PBMCs. 2. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 3. Multiplex bead assay 4. Isolated NK cell experiment
HC5	28	M	PDB/I stimulation 16h/PBMCs
HC6	31	F	1. PDB/I stimulation PBMCs. 2. anti-CD3+anti-CD28 stimulation PBMCs (fresh and frozen) 3. Multiplex bead assay 4. q-PCR (cultured PBMCs and whole blood) 5. Th-GM experiment
HC7	23	F	1. PDB/I stimulation 16h/PBMCs 2. anti-CD3+anti-CD28 stimulation PBMCs (frozen) 3. q-PCR (whole blood) 4. Isolated B cell experiment (with EBV, apoptosis)
HC8	31	M	1. PDB/I stimulation 16h/PBMCs 2. Isolated B cell experiment (with EBV)

HC9	38	F	1. PDB/I stimulation 16h/PBMCs 2. q-PCR (whole blood)
HC10	44	M	PDB/I stimulation 16h/PBMCs
HC11	28	M	1. PDB/I stimulation PBMCs. 2. anti-CD3+anti-CD28 stimulation PBMCs (fresh and frozen) 3. Multiplex bead assay
HC12	36	F	PDB/I stimulation 16h/PBMCs
HC13	59	F	PDB/I stimulation 16h/PBMCs
HC14	33	M	PDB/I stimulation 16h/PBMCs
HC15	48	F	PDB/I stimulation 16h/PBMCs
HC16	43	F	1. PDB/I stimulation 16h/PBMCs 2. anti-CD3+anti-CD28 stimulation PBMCs (frozen)
HC17	22	M	PDB/I stimulation 16h/PBMCs
HC18	55	F	1. PDB/I stimulation 16h/PBMCs 2. anti-CD3+anti-CD28 stimulation PBMCs (frozen) 3. Isolated B cell experiment (with EBV, apoptosis) 4. MOR103 experiment
HC19	46	F	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh and frozen) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs) 4. Isolated B cell experiment (with and without EBV) 5. Th-GM experiment
HC20	44	M	1. anti-CD3+anti-CD28 stimulation/ PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs) 4. Isolated NK cell experiment
HC21	27	M	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs) 4. Isolated NK cell experiment

HC22	48	F	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs) 4. Isolated NK cell experiment 5. Th-GM experiment
HC23	52	F	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs and whole blood) 4. Isolated NK cell experiment 5. Isolated B cell experiment (with and without EBV, apoptosis) 6. Th-GM experiment 7. MOR103 experiment
HC24	45	F	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh and frozen) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs and whole blood) 4. Isolated B cell experiment (with and without EBV) 5. MOR103 experiment
HC25	41	F	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh and frozen) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs)
HC26	22	M	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
HC27	27	F	1. anti-CD3+anti-CD28 stimulation PBMCs (frozen) 2. q-PCR (whole blood) 3. Isolated B cell experiment (with and without EBV, apoptosis)
HC28	32	F	1. anti-CD3+anti-CD28 stimulation PBMCs (frozen) 2. q-PCR (cultured PBMCs)
HC29	32	M	q-PCR (whole blood)
HC30	44	F	q-PCR (whole blood)
HC31	42	M	q-PCR (whole blood)

HC32	47	M	q-PCR (whole blood)
HC33	44	M	q-PCR (whole blood)
HC34	44	M	q-PCR (whole blood)
HC35	53	F	q-PCR (whole blood)
HC36	28	F	Isolated B cell experiment (with EBV, apoptosis)
HC37	60	M	Th-GM experiment
HC38	56	F	MOR103 experiment
HC39	54	M	MOR103 experiment

*****when a control subject is shown to be participated in more than one experiment, different blood samples were taken on different occasions around the same time of collecting the samples from patients in different experiments.

2. Overall MS patient Details and the Experiments they participated in

Subject ID	**Age**	**Gender**	**MS subtype**	**Experiment(s) participated in**
MS1	36	M	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS2	34	M	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS3	33	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS4	30	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS5	55	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS6	45	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS7	32	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS8	69	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS9	49	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS10	58	M	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS11	53	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS12	50	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS13	40	M	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS14	64	M	Untreated RRMS	PDB/I stimulation 16h/PBMCs

MS15	50	M	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS16	39	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS17	49	F	Untreated RRMS	PDB/I stimulation 16h/PBMCs
MS18	45	F	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay
MS19	33	F	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs) 4. Isolated NK cell experiment
MS20	44	M	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs)
MS21	44	M	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs)
MS22	34	F	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs)
MS23	39	F	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs)
MS24	56	F	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs)

MS25	49	M	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay 3. q-PCR (cultured PBMCs) 4. Isolated NK cell experiment
MS26	51	F	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (fresh) 2. Multiplex bead assay
MS27	38	F	Untreated RRMS	1. Multiplex bead assay 2. Isolated NK cell experiment
MS28	18	M	Untreated RRMS	Multiplex bead assay
MS29	54	F	Untreated RRMS	1. Multiplex bead assay 2. Isolated NK cell experiment
MS30	49	F	Untreated RRMS	Multiplex bead assay
MS31	51	M	Untreated RRMS	1. Multiplex bead assay 2. q-PCR (cultured PBMCs) 3. Isolated NK cell experiment
MS32	41	M	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS33	33	F	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS34	27	F	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS35	23	F	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS36	34	F	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS37	33	F	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS38	42	F	Untreated RRMS	1. anti-CD3+anti-CD28 stimulation PBMCs (frozen) 2. Isolated B cell experiment (without EBV+ apoptosis)

MS39	30	F	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS40	34	F	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS41	45	F	Untreated RRMS	anti-CD3+anti-CD28 stimulation PBMCs (frozen)
MS42	52	F	MS in relapse (Rx: IFN-β)*	q-PCR (whole blood)
MS43	45	F	MS in relapse	q-PCR (whole blood)
MS44	46	M	MS in relapse	q-PCR (whole blood)
MS45	37	F	MS in relapse (Rx: GA)	q-PCR (whole blood)
MS46	34	M	MS in relapse	q-PCR (whole blood)
MS47	36	F	MS in relapse	q-PCR (whole blood)
MS48	46	M	MS in relapse	q-PCR (whole blood)
MS49	50	M	MS in relapse	q-PCR (whole blood)
MS50	17	F	MS in relapse	q-PCR (whole blood)
MS51	31	F	MS in relapse	q-PCR (whole blood)
MS52	45	F	MS in relapse	q-PCR (whole blood)
MS53	27	F	RRMS remission (Rx: IFN-β)	q-PCR (whole blood)
MS54	49	F	RRMS remission (Rx: IFN-β)	q-PCR (whole blood)
MS55	53	M	RRMS remission (Rx: IFN-β)	q-PCR (whole blood)
MS56	46	F	RRMS remission (Rx: IFN-β)	q-PCR (whole blood)
MS57	42	M	RRMS remission (Rx: IFN-β)	q-PCR (whole blood)
MS58	49	F	RRMS remission (Rx: IFN-β)	q-PCR (whole blood)
MS59	31	F	RRMS remission (Rx: IFN-β)	q-PCR (whole blood)
MS60	58	F	Untreated RRMS	Isolated NK cell experiment

MS61	31	F	Untreated RRMS	Isolated NK cell experiment
MS62	60	F	MS in relapse	Isolated B cell experiment (without EBV)
MS63	30	F	MS in relapse	Isolated B cell experiment (without EBV+ apoptosis)
MS64	55	F	MS in relapse	Isolated B cell experiment (without EBV+ apoptosis)
MS65	42	F	MS in relapse	Isolated B cell experiment (without EBV+ apoptosis)
MS66	55	F	MS in relapse	Isolated B cell experiment (without EBV+ apoptosis)
MS67	41	M	MS in relapse	Isolated B cell experiment (with EBV)
MS68	62	F	MS in relapse	Isolated B cell experiment (with EBV)
MS69	47	F	MS in relapse	Isolated B cell experiment (with EBV)
MS70	27	F	MS in relapse (Rx IFN-β)	Th-GM experiment
MS71	42	F	MS in relapse	Th-GM experiment
MS72	57	F	MS in relapse	Th-GM experiment
MS73	42	F	Untreated SPMS	MOR103 clinical trial
MS74	53	F	Untreated SPMS	MOR103 clinical trial
MS75	56	F	Untreated RRMS	MOR103 clinical trial
MS76	49	F	Untreated SPMS	MOR103 clinical trial
MS77	53	F	Untreated SPMS	MOR103 clinical trial
MS78	54	M	Untreated RRMS	MOR103 clinical trial

Appendix II: Ethics Approval Letters for Work Conducted in this Thesis

1. Research Ethics Committee Approval for the work conducted in chapter 3, 4, 5, and 6 in this thesis.

NHS
National Research Ethics Service
Nottingham Research Ethics Committee 2
1 Standard Court
Park Row
Nottingham
NG1 6GN

Telephone: 0115 8839368
Facsimile: 0115 9123300

05 January 2009

Dr Bruno Gran
Clinical Associate Professor and Honorary Consultant Neurologist
University of Nottingham
Division of Clinical Neurology
Room B31, Medical School
QMC, Derby Road
Nottingham
NG7 2UH

Dear Dr Gran,

Full title of study: **Role of the innate immune system in Multiple Sclerosis**
REC reference number: **08/H0408/167**

Thank you for your letter of 23 December 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

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

Confirmation of ethical opinion

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

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). The favourable opinion for the study applies to all sites involved in the research. There is no requirement for other Local Research Ethics Committees to be informed or SSA to be carried out at each site.

Conditions of the favourable opinion

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

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

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

This Research Ethics Committee is an advisory committee to East Midlands Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

WPH 1370

Approved documents

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

Document	*Version*	*Date*
Investigator CV		26 September 2008
Application	12208/14135/1/324	10 November 2008
Letter of invitation to participant	1.0	26 September 2008
Evidence of Insurance		05 August 2008
Letter from Sponsor		25 September 2008
Letter from Funder		17 December 2007
Peer Review		
EU Funding		17 October 2006
Peer Review		
Advertisement	1.0	26 September 2008
Response to Request for Further Information		23 December 2008
Participant Consent Form: Patients giving a blood and CSF sample	2.0	23 December 2008
Participant Consent Form: Participants giving a blood sample	2.0	23 December 2008
Participant Information Sheet: Patient controls giving a blood sample	2.0	23 December 2008
Participant Information Sheet: MS patients giving a blood cerebralspinal fluid sample	2.0	23 December 2008
Participant Information Sheet: Healthy Controls giving a blood sample	2.0	23 December 2008
Participant Information Sheet: MS Patients giving a blood sample	2.0	23 December 2008
Participant Information Sheet: Patient Controls giving a blood and cerebrospinal fluid sample	2.0	23 December 2008
Letter of invitation to participant	Patient control - 1.0	23 December 2008
Protocol	2.0	23 December 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

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

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

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

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

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0408/167	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely,

Dr Martin Hewitt / Ms Linda Ellis
Chair / Committee Coordinator

Email: linda.ellis@nottspct.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Paul Cartledge - University of Nottingham*
R&D office for NHS care organisation at lead site - NUH (via email)

2. Research Ethics Committee Approval for MOR103 phase Ib clinical trial, including the work presented in chapter 7 of this thesis.

NHS
National Research Ethics Service
NRES Committee Yorkshire & The Humber - Leeds East
Yorkshire and Humber REC Office
First Floor, Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Telephone: 0113 3050108
Facsimile:

07 October 2011

Cris Constantinescu
Queen's Medical Centre
Derby Road
Nottingham
NG7 2UH

Dear Mr Constantinescu

Study title:	A Randomized, Double-Blind, Placebo-Controlled Phase 1B Study to Evaluate the Safety and Pharmacokinetics of MOR103, A Human Antibody to GM-CSF, in Patients with Mutliple Sclerosis
REC reference:	11/YH/0337
Protocol number:	MOR103C103
EudraCT number:	2011-001064-22

Thank you for your letter of 29 September 2011, responding to the Committee s request for further information on the above research and submitting revised documentation.

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

Confirmation of ethical opinion

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

Ethical review of research sites

NHS sites

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

Non-NHS sites

Notification(s) of no objection have been received from local assessors for the non-NHS site(s) listed in the table below, following site-specific assessment (SSA).

I am pleased to confirm that the favourable opinion applies to the following research site(s), subject to site management permission being obtained prior to the start of the study at the site (see under 'Conditions of the favourable opinion below').

This Research Ethics Committee is an advisory committee to the Yorkshire and The Humber Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Research Site	Principal Investigator / Local Collaborator
MAC Clinical Research.	Dr Stuart Ratcliffe

Plans to include any new sites in the study in addition to those listed in the application should be notified to the Committee as a substantial amendment. The study should not start at the new site until ethical approval and site management permission is obtained.

Conditions of the favourable opinion

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

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

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

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

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

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

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

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

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

Approved documents

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

Document	*Version*	*Date*
Covering Letter		11 August 2011
Evidence of insurance or indemnity		29 July 2011
GP/Consultant Information Sheets	1	10 August 2011
GP/Consultant Information Sheets	2.0	19 September 2011
Investigator CV		20 June 2011
Investigator's Brochure	5	27 June 2011
Other: Patient appointment card	1	21 July 2011

Other: Physician referral letter	1	10 August 2011
Other: Letter of authorization enabling the applicant to act on behalf of the sponsor	1	31 May 2011
Other: Summary of the scientific advice provided by the Paul-Ehrlich-Institute on the manufacturing process of MOR103	1	18 August 2009
Other: Summary of product characteristic for the placebo	1	01 November 2010
Participant Consent Form: Main study	1	10 August 2011
Participant Consent Form	2.0	20 September 2011
Participant Information Sheet	1	10 August 2011
Participant Information Sheet	2.0	20 September 2011
Protocol	1	20 July 2011
REC application		10 August 2011
Response to Request for Further Information		29 September 2011
Sample Diary/Patient Card		21 July 2011

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

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

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

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

Feedback

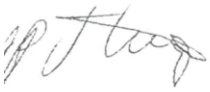
You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

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

11/YH/0337	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



Dr Carol Chu
Chair

Email: jade.thorpe@nhs.net

Copy to: *Mr Sheldon Greenwood*
Dr Jetta Holmes, Nottingham Integrated Clinical Research Centre