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Comparison of Immunomodulatory factors produced by Mesenchymal Stem Cells in Multiple Sclerosis patients and Healthy Controls

Sarah Thevathas BSc

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Declaration

I declare that the thesis is the result of my own work which has been mainly undertaken during my period of registration for this degree at The University of Nottingham. Any results arising from collaborative work, or from working alongside others is acknowledged accordingly.

Signature: Date:

Acknowledgements

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To my family and my close friends, thank you for having faith in me and offering kind words of encouragement.

Abstract

Mesenchymal Stem Cells (MSCs) have been identified as playing a vital role in the modulation of the immune system (Rivera and Algnier, 2012). Here we aimed to characterise the role of MSC as immunomodulators of peripheral blood mononuclear cells (PBMC) and to measure the immunomodulatory effects of MSCs from MS patients and determine whether they are less pronounced than those from healthy controls (HC). **Methods:** MSCs from secondary progressive MS patients and HCs were isolated by bone marrow aspiration using Ficoll density centrifugation. Autologous PBMC were activated alone and together with MSCs using 1µg anti-CD3 and 1µg/ml anti-CD28. After 72hrs activation and 5hrs of re-activation with PMA-ionomycin-brefeldin A, they were stained intracellularly for the inflammatory cytokines IL-17, IFN-γ, and GM-CSF. Cytokine expression was then quantified using flow cytometry. Immunomodulatory functions in MSCs are known to be exerted through cytokines so cytokine profiling of MSCs from MS patients and HCs was also carried out, where cytokines and chemokines with known immune function were measured. **Results:** Activated PBMCs from MS patients in culture with autologous MSCs significantly ($p=0.01$) increased the percentage of IL-17. The percentage of IL-17+ cells was significantly ($p=0.046$) decreased in PBMC from HCs co-cultured with autologous MSC. The expression of IFN-γ by PBMC of healthy volunteers ($n=7$) was numerically but not statistically significantly higher than that of PBMC ($n=5$) from the volunteers with SPMS participating in this study. Cytokine profiling showed decreased IL-8 and IL-10 levels in MS-MSC supernatants compared to controls ($p=0.03$). No differences were seen in the proportion of IL-6, IL-1β, TNF-α, and GM-CSF between HC or MS patients. **Conclusion:** MSCs show great promise in therapeutic use in MS. However, this study shows large variability in the results. There is a possibility that MSCs from MS patients could be defective in their immunoregulatory ability, in regards to the Th17 subset. The translation to therapeutic clinical trials is feasible, but it will require caution and monitoring.

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Abbreviations

APC	Antigen presenting cell	MSC	Mesenchymal Stem Cells
BBB	Blood brain barrier	NK	Natural Killer cells
B _{reg}	Regulatory B cells	PBS	Phosphate buffered saline
CD	Cluster of differentiation	PLP	Proteolipin protein
CIS	Clinically Isolated Syndrome	PPMS	Primary progressive MS
CNS	Central nervous system	RPMI	Roswell Park Memorial Institute medium
CSF	Cerebrospinal fluid	RRMS	Relapsing remitting MS
EAE	Experimental autoimmune encephalomyelitis	SEM	Standard error of mean
EBV	Epstein-Barr Virus	SNP	Single nucleotide polymorphism
EDTA	Ethylenediaminetetraacetic acid	SPMS	Secondary progressive MS
FMO	Fluorescence minus one	Th	T helper cells
FOXP3	Forkhead transcription factor	TNF- α	Tumour necrosis factor-alpha
GM-CSF	Granulocyte-macrophage colony-stimulating factor	T _{reg}	Regulatory T cells
GWAS	Genome-wide association Studies		
HC	Healthy Control		
HLA	Human Leukocyte antigen		
IFN- γ	Interferon-gamma		
IL-	Interleukin-		
MBP	Myelin basic protein		
MHC	Major Histocompatibility Complex		
MS	Multiple Sclerosis		

Introduction

Multiple sclerosis

Multiple Sclerosis (MS) is one of the most common yet complex neurological diseases affecting approximately two and a half million people in the world. It is a disease of presumed autoimmune aetiology that targets the central nervous system (CNS) affecting the way in which nerve impulses are conducted as a result of damage caused to neurons, oligodendrocytes and microglial cells (Koriem, 2016). The main pathological characteristics of this immune-mediated disease are inflammation, demyelination, axonal damage, and gliosis, which combined cause loss of neurological function, including motor and sensory function (Bai *et al.*, 2012; Brownlee *et al.*, 2016).

The first documented cases of MS may date back to 1421 where 16-year old Saint Lidwina of Schiedan (1380-1433) presented symptoms that were potentially consistent with MS after she took a fall and damaged a rib whilst ice-skating. Sir Augustus d'Este (1794-1848) minutiously documented his own symptoms in detail, consistent with MS in his diaries over decades. Robert Carswell (1793-1857) illustrated the clinical features of MS however, he was unable to recognise it as a separate disease. Jean Cruveilhier (1791-1874) reviewed a case of a woman presenting with visual disturbances and weakness in her limbs. The first diagnosis of MS, then named “brain sclerosis” was probably made in 1849 by Friedrich von Frerichs. In 1868, MS was finally made a coherent entity by Jean Martin Charcot. Charcot recognized the symptoms and pathological features associated with MS and came to the conclusion that the disease was a distinct entity. He then went on to publish these in lectures and clinical presentations (Milo and Miller, 2014).

Clinical features

The symptoms of MS can vary from one individual to the next and the nature of the symptoms is highly dependent upon the localisation of the lesions (Diego, 2004).

The multifocal nature of the disease manifests clinically with symptoms that range from cerebellar, cognitive, neuropsychiatric, sensorimotor, sphincteric and also of a visual nature (Lim and Constantinescu, 2010).

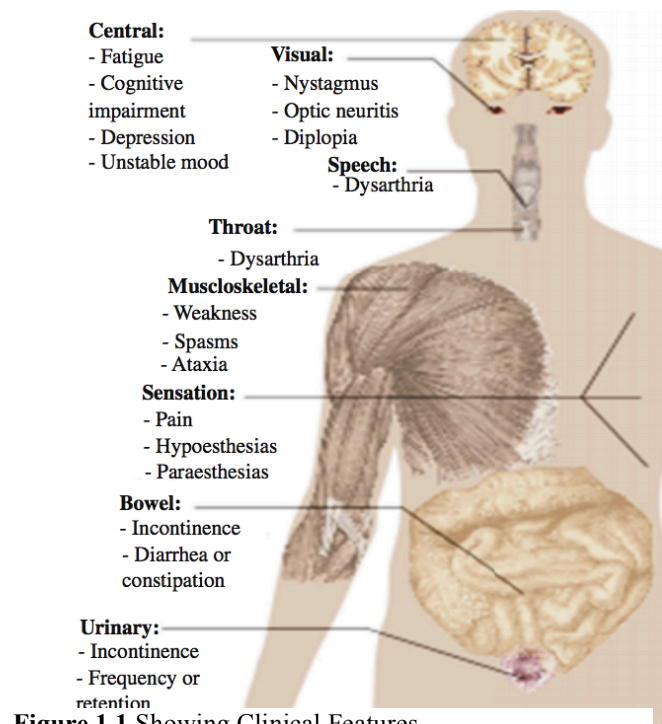


Figure 1.1 Showing Clinical Features

Taken from: (Koriem, 2016)

The main clinical features as shown in figure 1.1 are visual impairment, complete or partial focal sensory loss, weakness in limbs, autonomic motor abnormalities include bowel, bladder and also sexual dysfunction (Weissert, 2013), and loss of balance.

Amongst all of this the following may also arise: painful muscle spasms, depression and fatigue. The patient may also experience subtle cognitive difficulties and psychiatric disturbances.

Clinical subtypes (RR, SP, PP, CIS)

MS is presented in four clinical forms: Relapsing-remitting MS (RRMS), secondary-progressive MS (SPMS), and primary-progressive MS (PPMS). Clinically isolated syndrome (CIS) represents a single clinical attack of demyelination, which can convert into RRMS after a second attack.

Occurring in approximately 87% of patients, RRMS is the most common presentation. This form is characterised by clearly defined acute attacks, which can either lead to full or partial recovery for the patient (Sospedra and Martin, 2005; Loma, 2011). The patient may develop new or recurrent symptoms. RRMS patients go on to later develop the SPMS form. There are two progressive forms PPMS and SPMS. PPMS affects approximately 10-15% targeting mainly the older generation. It has steady progression from onset with an insidious approach. SPMS affects approximately 50% of RRMS patients after 10years of the initial diagnosis and 90% will develop this form after 25years. Relapses and minor remissions may occur in this stage. However, this form of MS will continue to progress developing more permanent disability (Milo and Miller, 2014; Weissert, 2013).

Epidemiology

In 2010, approximately 2.5million people across the globe were suffering with MS where 80/100000 were to be found in Europe alone. High risk areas are considered to be in central Europe, Southern Australia and North America and low risk areas are eastern Asia and Africa, shown in figure 1.2, suggesting that the prevalence of MS increases along a latitudinal gradient (Milo and Miller, 2014). MS is more frequent in females than in males. However, after the age of 50 the female to male ratio starts to become more balanced. In England, it has been estimated that the prevalence of MS has increased by 2.4% per year between 1990 and 2010 (Koriem, 2016).

MS is more frequent in young adults. Patients' ages usually range from 15-55 at the time of initial presentation (Weissert, 2013).

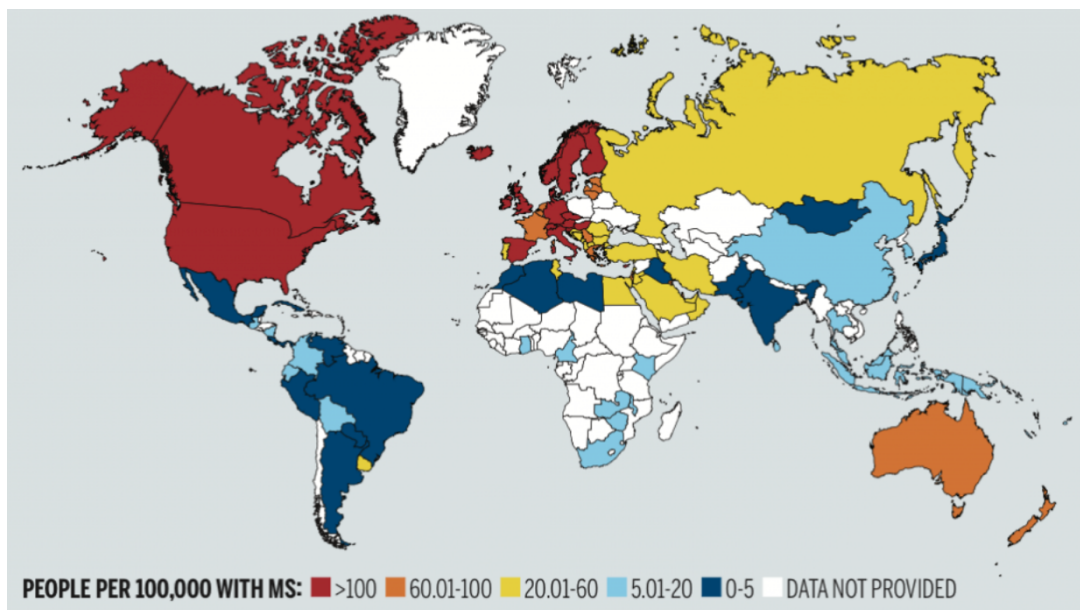


Figure 1.2. MS prevalence by country

Taken from: (Kingston, 2015) <http://www.macleans.ca/society/health/could-canada-cause-multiple-sclerosis/>

MS is the most common and a continuously growing reason to be causing neurological disability in young adults (Koriem, 2016).

Environmental Factors

Multiple Sclerosis is most prevalent in populations found in Northern Europe and this is thought to be associated with the lack of Vitamin D from reduced sunlight, combined with shared genetic factors (Koriem, 2016).

The geographic influences in MS are thought to be a combination of genetic predisposition, migration, and environmental factors. There is a global latitude gradient in the prevalence of MS in both the Northern and the Southern hemisphere. Together with studies that show that migration before the age of 15 results in acquiring the risk of the area where the patients have migrated to, these studies confirm the major role played by environmental factors in the causation of MS. Environmental factors combine with genetic factors to determine the risk of MS. (Compston and Coles, 2008).

Infectious Factors

Studies have repeatedly shown that there is a higher chance of a relapse occurring after a patient has had a viral infection (Sospedra and Martin, 2005). From this frequently reported association between infectious diseases and MS two hypotheses were proposed as a way of providing a plausible reason for the epidemiology of MS.

(1) “Prevalence Hypothesis”

Suggesting that MS is caused by a pathogen which is most likely to be found in areas of high MS prevalence. The pathogen can cause an asymptomatic persistent infection in most individuals.

(2) “Hygiene Hypothesis”

Suggesting that early exposure of infectious agents protects an individual against MS.

Epstein-Barr virus (EBV) is considered to play a major role in the susceptibility of MS. The infection has been associated with what seems to be a dramatic increase in the risk of developing MS and a significant increase for the risk for MS has been noted among individuals who have presented with a history of infectious mononucleosis. This is most commonly found in adults and is defined as a clinical manifestation of the EBV infection. Development of EBV at a later age increases the chances of MS developing (Ascherio and Munger, 2007 (a)).

Non-Infectious Factors

The two non-infectious factors that are extensively studied include:

(1) Sunlight exposure/ Vitamin D

Ecological studies noted an inverse correlation between sunlight/ vitamin D levels and MS prevalence. The prevalence of MS is thought to increase across a latitudinal gradient and the strongest correlate of latitude is sunlight. Injections of the active form of Vitamin D- 1,25(OH)₂D, were given to mice with the animal form of MS, experimental autoimmune encephalomyelitis (EAE) and both the pathological and clinical signs of the disease were completely prevented suggesting an association in between sunlight/vitamin D exposure and the epidemiology of MS (Ascherio and Munger, 2007 (a)).

(2) Smoking

The effect of smoking does not provide us with an explanation or connection with latitudinal gradient. However, it has been associated with an increased risk of MS, and an accelerated transition from the relapsing-remitting phase to the secondary progressive phase. The mechanisms behind it are not fully understood (Ascherio and Munger, 2007 (b)).

Genetics

Familial aggregation is presented in MS, where the risk of having MS is significantly higher among relatives of patients with MS (Milo and Miller, 2014). The recurrence rate in a family of MS is 20% (Compston and Coles, 2008).

The correlation between alleles in the major histocompatibility complex (MHC) and MS was first established in the early 1970s with the strongest affiliation pointing towards the human leukocyte antigen (HLA) HLA-DRB1 allele, which is of a class II region. There has been no evidence to suggest any genetic association within the course of the disease however, susceptibility to MS has now been refined as DQ6, DR15 and Dw2 with DRB1*1501, DRB5*0101, DQA1*0102 and DQB2*0602 representing the corresponding

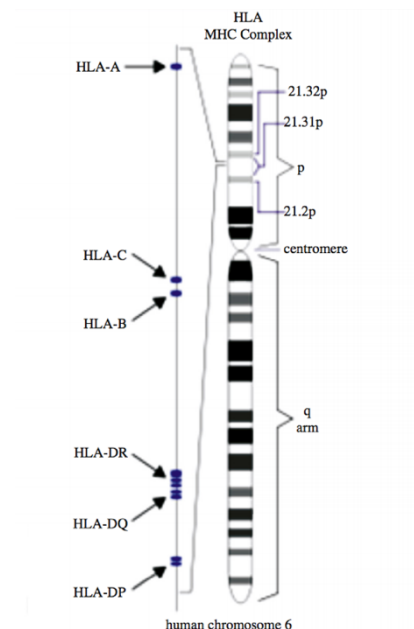


Figure 1.3. HLA region from chromosome 6. Specific HLA genes and haplotypes are associated with susceptibility to MS (see text)

Taken from: (Koriem, 2016)

genotypes (Compston and Coles, 2008; Milo and Miller, 2014). Figure 1.3 represents the HLA regions in chromosome 6.

The greatest individual effect on the genetic risk has been confirmed to be the variations present within the Major Histocompatibility Complex (MHC). The Genome-Wide Association Studies (GWAS) have identified genetic variants associated with the most complex diseases implicating approximately 150 genes in association with MS. In addition to the known role of the HLA, the great majority of these 150 genes that showed single nucleotide polymorphisms, some of which were shared with other autoimmune diseases, are genes involved in the immune system (Sawcer *et al.*, 2011).

Pathology

The processes of the disease are extremely complex. Pathologically MS is confirmed by an uneven distribution of multifocal inflammatory demyelinated plaques (Dutta and Trapp, 2011; Cohen, 2013). Sclerotic plaques are the hallmark of MS forming almost anywhere in the CNS a few examples being: grey matter, white matter, regions of spinal cord and brainstem and around the ventricles (Compston and Coles, 2008). The lesions can be divided into three groups: acute, chronic silent and chronic active (Milo and Miller, 2014).

The four key pathological features of MS are demyelination and remyelination, inflammation, axonal loss and gliosis. The plaques represent the final stages of these features (Cohen, 2013). The combination of damage to the myelin sheath, death of oligodendrocytes and irreversible axonal loss together fuel clinical disability (Constantinescu *et al.*, 2011).

Demyelination

As previously mentioned, demyelination is the hallmark of MS, which is caused by chronic inflammation to the CNS and damage to oligodendrocytes and the myelin sheath as seen in figure 1.4 (Lassmann *et al.*, 2012). The demyelination of the axon sheath stimulates inflammation by activating immune cells and increasing the production of cytokines and antibodies. This increase in immune cell inflammatory factor production causes more damage to the blood-brain barrier (BBB), which in turn increases the activation of even more cytokines and also macrophages (Koriem, 2016). Active demyelination tends to occur at sites of microglia activation (Lassmann, 2013).

Remyelination

Remyelination does take place providing hope for future therapies whose primary aim is to enhance endogenous repair mechanisms. However, over time the process of remyelination remains incomplete (Constantinescu *et al.*, 2011). There is a possibility that partial remyelination may successfully occur however this is only if the inflammatory process ceases at an early stage (Xiao *et al.*, 2015).

Inflammation

The events that lead to the tissue damage within the CNS are thought to be triggered by inflammation. Disruption to the BBB and inflammation are the cause of relapses in patients. Clinical relapses coincide with the infiltration of inflammatory cells within the CNS. The acute form of plaques is identified by the combination of demyelination and inflammatory infiltrates. Inflammatory infiltrates are composed of various immune cells that are located around small veins and are associated with ongoing disease activity.

As the loss of myelin increases, the lesion becomes more chronic and there is a decrease in inflammatory infiltrates and gliosis. This is what causes the appearance of plaques (Compston *et al.*, 1998; Constantinescu *et al.*, 2011).

The transfer of information within the CNS is decreased as the level of inflammation increases. This is due to an increased production of cytokines and antibodies leading to further destruction of the myelin sheath and also complete axonal damage (Koriem, 2016).

Inflammatory mechanisms are predominantly identified in the early stages of the disorder. The gradual decline seen in more progressive forms is thought to be caused by the neurodegeneration (Cohen, 2013).

Axonal Loss

Axonal loss is most actively seen at active inflammatory sites; however, it is chronic plaques that display the most severe forms of axonal loss. Progression in MS and the formation of more permanent, long-term disability stems from irreversible axonal loss (Constantinescu *et al.*, 2011).

Gliosis

During the acute phase of MS, mononuclear cells are activated and destroy myelin. These cells include: lymphocytes, macrophages, microglia and also oligodendrocytes. Over a period of time, gliosis develops and the plaques formed eventually reach a burn-out stage where they consist of demyelinated axons traversing glial scar tissue. As a response to demyelination, oligodendrocyte precursor cells (OPCs) begin to vigorously proliferate however, they fail to

differentiate terminally which prevents the possibility of effective remyelination. These events are all associated with axonal transection which leads to more permanent neurological deficits in the patient (Xiao *et al.*, 2015).

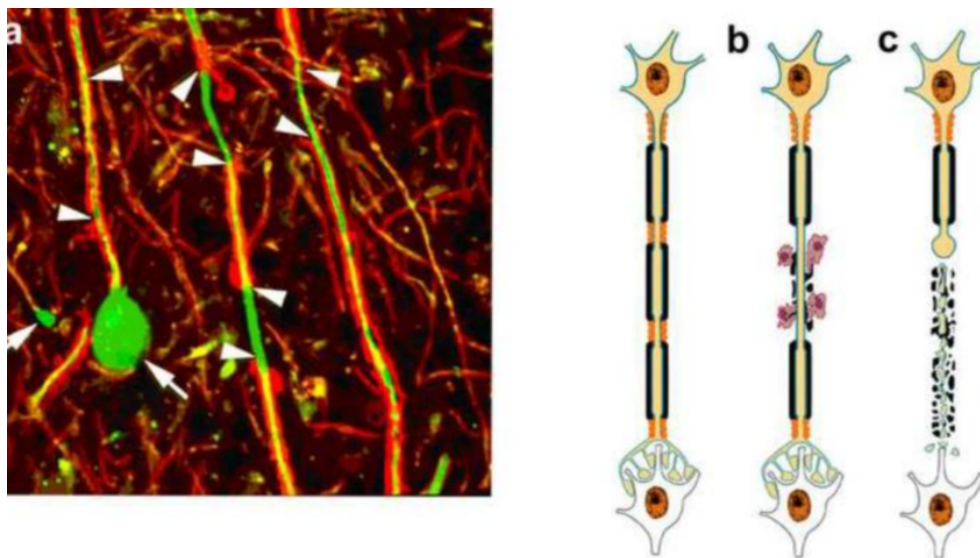


Figure 1.4. Acute axonal damage in MS (left panel) and damage to myelin (right panel b,c). The myelin sheath (red) on axons (green) is being stripped by the inflammatory process. The right panel shows a normal axon surrounded by the myelin sheath (a). Focal demyelination with macrophages ingesting myelin are shown in (b). Nerve degeneration with loss of the synapse is shown in (c). Image taken from (Dutta and Trapp, 2011).

Pathogenesis

The pathogenesis of MS is extremely complex where both the innate and adaptive immune systems are coupled and contribute to inflammation and neurodegeneration (Milo and Miller, 2014). There are vast amounts of evidence to support the hypothesis that MS is an autoimmune disease mediated by autoreactive T cells. The self-recognition aspect to it occurs first in the peripheral lymphatic tissue as a response to brain antigens leaking from the CNS (Lassmann *et al.*, 2012). MS is thought to be initiated by an increased level of activation and

migration of autoreactive lymphocytes across the BBB. Failure of regulatory mechanisms initiate the pathological cascade at random sites. Cell infiltrates form plaques that are unevenly clustered throughout the brain and spinal cord. Both CD4+ and CD8+ T lymphocytes are found in active plaques with CD8+ T cells being the dominant population (Compston *et al.*, 1998; Compston and Coles, 2008; Milo and Miller, 2014).

The activation and differentiation of CD4+ autoreactive T cells into T-helper 1 (Th1) phenotype is one of the most crucial events when attempting to understand the pathogenesis of MS (Sospedra and Martin, 2005).

The Immune System in MS

The activation of a cascade of chemicals and cytokines within the periphery triggers an immune response, which causes the degradation of the myelin sheath. The degrading myelin sheath destroys the insulation of the axons which interferes with signals being conducted efficiently (Bai *et al.*, 2012). MS is a disabling neurological disease caused by abnormal immune responses in the CNS (Diego, 2004).

The two most important events that need to occur in the immune system in order to protect the body against foreign agents are (1) recognition of the specific pathogen and (2) effective protection and defence against antigens (Todd and Spickett, 2010). Self recognition within the immune system is essential. The immune system during development becomes able to distinguish self from non-self. Failure to recognise self initiates a cascade of destructive

events. In MS and other autoimmune diseases, there is failure of self-recognition, and the immune system recognises self antigen as foreign (Compston *et al.*, 1998).

Over time and with extensive research a framework has been created from results of studies to somewhat understand just how immune responses in MS commence. A large part of this understanding was created from studies from animal models of MS, experimental autoimmune encephalomyelitis (EAE). These studies have shown that the peripheral activation of CD4⁺ T cells that have the potential to react against self-antigens in the CNS, are in fact the triggers for relapses in MS (Darlington and Bar-Or, 2011).

In the propagation of MS, auto-reactive T cells are activated in the periphery either by (a) molecular mimicry or (b) bystander activation.

(a) Molecular Mimicry

George Snell, Nobel Prize winner in 1980, reasoned that foreign agents have the ability to remain in an organism as they are capable of producing antigenic structures that are indistinguishable from self-determinants. Thus making them invisible to self tolerant immune cells (Compston *et al.*, 1998). Molecular mimicry is the main theory invoked when looking into how T and B cells react with antigenic determinants that are shared by both self and infectious antigens. Immune cells (e.g. T cells) may recognise foreign antigens such as viral proteins that bear structural resemblance to self antigens including myelin proteins. When the anti-viral immune cells encounter the self antigen mimic, they recognise these as foreign and initiate an immune response against them (Sospedra and Martin, 2005). The homology may be at the primary

structure level (sharing of aminoacids) but can be at the structural level, even without sharing of aminoacid sequences. This is illustrated in Figure 1.5.

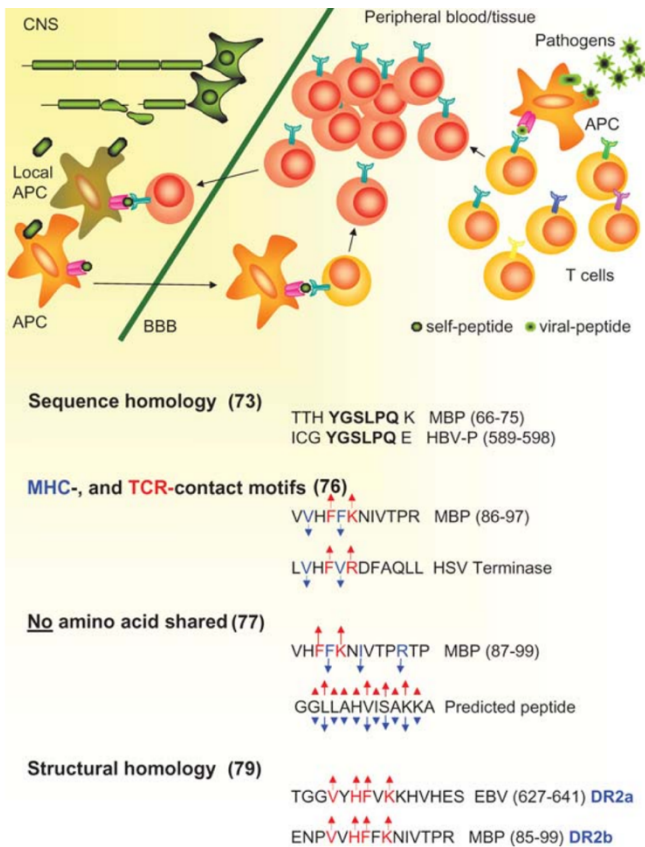


Figure 1.5. The concept of molecular mimicry.

In the periphery, T cells reactive to a viral antigen cross the blood brain barrier and encounter an antigen with aminoacid sequence homology, shared MHC or T cell receptor contact motifs, or structural homology despite no shared aminoacids, presented by local antigen presenting cells. This leads to their activation and subsequent CNS damage. Examples here are between peptides of myelin basic protein (MBP) and viral peptides from HBV, HSV, and EBV. Image taken from (Sospedra and Martin, 2005).

(b) Bystander activation

The mechanism involved in bystander activation can be divided into two groups (1) Activation of autoreactive T cells by inflammatory cytokines and others (2) revealing host antigens and the effect of foreign antigens on antigen-presenting cells (APCs). During infection cells of the body produces various chemokines and pro-inflammatory cytokines, both of which have been considered to be the activators of cytotoxic CD8+ T cells needed for protection against the infection. This may non-specifically activate dormant autoreactive T cells, thus generating autoimmune responses. The second

mechanism behind bystander activation is the revealing of host antigens as an outcome of viral tissue damage. Specific T cells approach the infected area by using viral epitopes as a method of detection. Here they kill not only infected cells but also self-tissue releasing auto-antigens. Auto-reactive T cells are activated as a response (Sospedra and Martin, 2005).

Cells involved in Immune Response in MS: production of IL17, IFN- γ and GM-CSF

The immune pathogenesis of MS is considered to be heterogenous. From recent research it is clear to see the crucial involvement of B cells and antibodies and also the various subsets of T cells. Despite the exact aetiology remaining unknown, it is speculated that under the influence of genetic susceptibility, MS stems from the loss of immune regulation, which in turn leads to the breakdown of immune tolerance (Lim and Constantinescu, 2010).

MS is initiated by increased traffic of autoreactive lymphocytes attempting to cross the BBB. The BBB guards the body dividing capillary blood from the cerebral tissue (Diego, 2004). It is thought that the various forms of MS that can possibly be presented depend on the different subsets of T helper (Th) cells present, the proportion of inflammation and finally whether interleukin-17 (IL-17), hallmark cytokine of T helper 17 (Th17) cells, or interferon-gamma (IFN- γ), hallmark cytokine of T helper 1 (Th1) cells, dominates (Jadidi-Niaragh and Mirshafiey, 2011). This however remains controversial in EAE, and even more so in MS. The main cells involved in the pathogenesis of MS are type 17- Th17, Th1, regulatory T cells, B cells and macrophages (Xiao *et al.*, 2015).

For many years it was thought that MS was a disease mediated by T helper type 1 (Th1) cells. More recently, T helper 17 cells are thought to play an important role, and even be the main pathogenic T cells in MS. Questions have been raised on the relevance of myelin-specific T cells as they have been found in both healthy controls and also in MS patients. An observation was made that these myelin-specific T cells had a greater likelihood of possessing a Th1 phenotype giving rise to and validating the hypothesis that these cells could potentially be the pathogenic cells in MS. Studies that have been carried out have shown that myelin-specific T cells that are found in healthy controls are naïve cells; whereas myelin-specific T cells from MS patients are more activated and also had memory myelin specific T cells. The exact roles of Th1 and Th17 cells in the development of MS lesions are still rather unclear however, both T cell populations have been associated with the formation of demyelinating lesions and also inflammation to the CNS (Constantinescu and Gran, 2014).

When looking closely at the animal model of MS, EAE, Th1 and Th17 cells have been shown to be able to transfer the disease. IL-23 appears to be crucial for the development of EAE . IL-23 controls not just the differentiation and activation of Th17 cells, but also induces the production of granulocyte-macrophage-colony-stimulating factor, GM-CSF. GM-CSF is a hematopoietic growth factor (Peelen et al., 2015) but also a pro-inflammatory cytokine. T cell derived GM-CSF may play an important role in EAE and its role in MS is emerging.

Rafei *et al.*, (2009) suggested that the immunomodulatory effects of MSCs are in fact related to the blockade of the inflammation driven by IL-17 alongside the cellular infiltration of the CNS (Rafei *et al.*, 2009).

A key determinant of the immune processes that occur in MS, is the differentiation of naïve T helper cells (Th0) into different phenotypes: Th1, Th2 and Th17 and the cytokines they produce. Cytokines are vital in the regulation of inflammatory responses as they reflect disease activity. The identification of Th17 cells and their corresponding cytokine, IL-17, broke the dichotomy of the Th1-Th2 axis in the pathogenesis of MS. Th17 cells are better adapted to attaching to the endothelial cells in the brain as a result of CD146 being present. CD146 cell adhesion molecule is associated with Th17 effector functions. In MS, higher frequencies of CD146 are found to be expressed in Th17 cells rather than Th1 (Wu *et al.*, 2015).

By producing IL-17, autoreactive Th17 cells have the ability to migrate through the BBB through down regulation of occludin, disrupting not only the endothelial cells but also tight junction proteins within the CNS. This down-regulation is seen to promote the migration of monocytes and lymphocytes. This process also increases IL-6, IL-8 and chemokine ligand 2 (CCL2) (Larochelle *et al.*, 2011) released by activated monocyte/macrophages, and by T cells activated in an autocrine manner. This results in increased recruitment of neutrophils and death of neurons revealing a highly potent pro-inflammatory function. RRMS patients display increased IL-17 levels during active phases, which decrease during remission (Tawfik *et al.*, 2016).

Th1 and Interferon-gamma (IFN- γ)

In MS, IFN- γ is the hallmark of Th1 cells increasing the drive for autoimmunity and inflammation. IFN- γ has a key role in orchestrating homeostasis within the immune system (Arellano *et al.*, 2015).

From initial clinical studies in EAE and MS, a positive correlation was shown between increased IFN- γ levels and demyelination within the CNS. A study carried out on EAE mice revealed that mice that were deficient in T-bet, the transcription factor required to drive differentiation of Th1 cells, were in fact protected from the development of EAE and mice that were immunised with Th1 cells producing IFN- γ were capable of developing EAE. In a pilot study on RRMS patients were treated with IFN- γ , seven of these patients displayed exacerbations of their MS, associated with evidence of systemic immune activation (Arellano *et al.*, 2015).

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Decades ago, the discovery of Granulocyte-macrophage colony-stimulating factor (GM-CSF) was made. GM-CSF was first discovered in mouse lung tissue with its ability to generate macrophages and granulocytes in addition to stimulating the proliferation of bone marrow cells in mice. Amongst the immune cells, potent producers of GM-CSF are Th1 and Th17 cells (Shi *et al.*, 2006); but a subset of cells produce only GM-CSF or GM-CSF plus IL-3, and little or no IL-17 or IFN- γ . These cells have been coined ThGM and shown to be pathogenic in EAE. Work in our laboratory has also recently identified these cells in humans, and shown them to be more prevalent in MS. These results are currently unpublished observations.

Studies have shown that the over expression of GM-CSF by T cells of a pathogenic nature or administration of GM-CSF in EAE caused disease progression. It is thought that GM-CSF affects EAE by activating Antigen presenting cells (APCs) which include microglial cells in the brain and spinal cord and other immune cells. Together with an increase in infiltrated macrophage cells in the CNS, microglial cells were found to be activated within the CNS

before the initial onset of EAE. Clinical data has revealed that EAE reactive T cells cause the activation of microglial cells by secreting GM-CSF (Bhattacharya *et al.*, 2015).

EAE as a model

EAE is viewed as the animal model of MS. Over time EAE has become a well characterised model for autoimmune diseases that are organ specific. The initial discovery of EAE was over 80 years ago and the model still remains popular. Novel functions, include the discovery of ROR- γ as a transcription factor for the development of Th17 cells, were first discovered and published in an EAE model (Constantinescu *et al.*, 2011).

EAE has since been induced in a multitude of species, their main targets being monkeys and rodents. The active form of EAE can be induced by (1) adoptive transfer of *ex vivo* primed CD4⁺ T cells or (2) immunisation *in vivo* with myelin peptides (Constantinescu *et al.*, 2011; Cohen, 2013).

Major damage of EAE is caused by CD4 cells

The neurological paralytic symptoms presented in patients with MS is mimicked in EAE mice. Like MS, EAE is mediated by CD4 T cells (Dittel, 2008). The adoptive transfer of EAE has been instrumental in understanding the key role played by myelin-reactive T cells in the pathogenesis of the disease. Researchers have been able to focus on factors which affect the disease during its ‘effector phase’ and ways to bypass the induction.

Table 1. McDonald criteria used for diagnosis of MS. Modified from: (Polman *et al.*, 2011).

Clinical Presentation	Additional requirements to confirm diagnosis
One lesion in addition to a history of relapses; presence of two or more lesions; Two+ relapses	No other requirements
Clinical evidence showing one lesion; Two + attacks	MRI to detect lesions; await on a relapse to show activity in another area of CNS
CIS presentation- one lesion; one attack	MRI to detect lesions; Await a new relapse
Clinical evidence showing two lesions; One attack	MRI with evidence of a new lesion since last scan; Await new relapse
Insidious progression suggesting MS	Ongoing observation; positive test from cerebrospinal fluid (CSF)

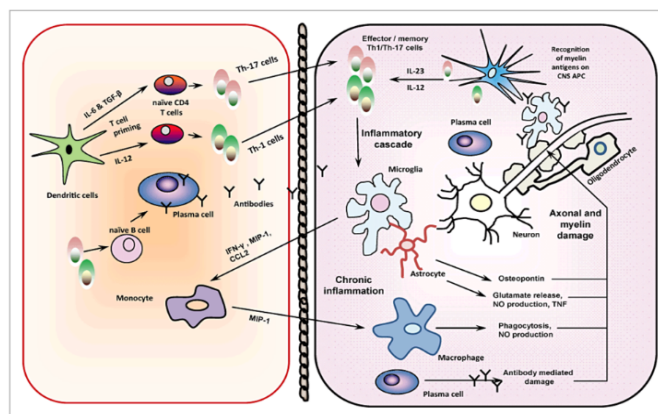


Figure 1.6. Pathogenesis of EAE. Schematic diagram of some of the key pathological features of EAE pathogenesis. Activated Th1 cells and Th17 cells are thought to be the main culprit in EAE and MS. Th1 are IFN-γ producing, and Th17 are IL-17 producing T lymphocytes. They are primed outside the CNS by dendritic cells, then cross the blood–brain barrier and encounter CNS antigen-presenting cells. They produce inflammatory products and cytokines that damage the myelin and axons. They also activate the resident microglia and produce factors that attract further inflammatory cells to the CNS and perpetuate the inflammatory cascade. Antibodies and B cells can also enter the CNS, and plasma cells produce antibodies within the CNS. Antibody mediated damage contributes to the inflammatory demyelination and neurodegeneration

Diagnosis

In 2001, the McDonald criteria was published to assist in the diagnosis of MS.

Table 1. McDonald criteria used for diagnosis of MS. Modified from: (Polman *et al.*, 2011).

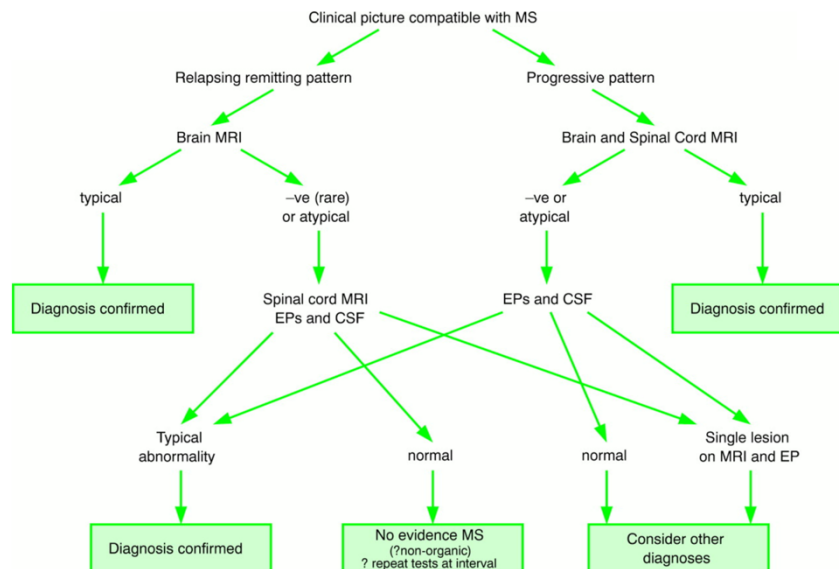


Figure 1.7. Diagnostic Algorithm for MS

Classically requires two or more attacks involving two or more parts of the CNS. With the advent of MRI, the diagnosis can now be made with a single attack, provided there is MRI evidence for dissemination in time and space. The diagnosis is also strengthened by MRI and by the finding of oligoclonal bands (immunoglobulins) in the CSF.

When a patient presents signs and symptoms typical of MS a diagnostic test is carried out to confirm. There is a possibility of three diagnostic tests being carried out to confirm the diagnosis: magnetic resonance imaging (MRI), 80% of the time an abnormal brain MRI confirms MS. Carrying out an MRI also allows identification of other abnormalities which can lead to an alternative diagnosis. Analysis of cerebrospinal fluid (CSF) for the presence of oligoclonal bands (OCB) can support evidence from MRI findings to confirm diagnosis. An increase in two or more OCBs and immunoglobulin concentration is detected in 90% of MS

patients. Neurophysiological testing of evoked potential studies in somatosensory auditory and visual pathways can potentially point out clinically silent lesions. Blood tests can also be used to exclude other diseases that could potentially mimic MS (Brownlee *et al.*, 2016; Milo and Miller, 2014).

Treatment

MS is an unpredictable disease; therefore, it is seen as challenging to treat.

Immunosuppressants have been used to treat MS, examples being azathioprine and cyclophosphamide, and although there have been some benefits, there is a high risk of infections in addition to adverse side effects (Lim and Constantinescu, 2010).

Immunomodulatory agents were produced with the aim to prevent relapses, minimise disease progression and reduce overall disability. These agents first became available in the early 1990s. First line therapy for MS patients are interferon beta-1a, beta-1b (IFN- β 1a, 1b) and also glatiramer acetate. In more progressive and severe forms, cytotoxic agent mitoxantrone and monoclonal antibody treatment natalizumab are used (Lim and Constantinescu, 2010). Natalizumab works against VLA-4 integrin, which from EAE studies, is required by T cells for entry into the CNS. It has been shown to reduce relapse rate by approximately 70% (Constantinescu *et al.*, 2011).

New treatments are constantly being trialled in various MS centres across the globe. These include oral agents and monoclonal antibodies in the hope to prevent relapses and disease progression (Lim and Constantinescu, 2010).

Oral Agents

(1) Dimethyl Fumarate (Tecfidera)

One mechanism of action shown by researchers has been Dimethyl Fumarate's abilities as an immunomodulator to promote a Th1 to Th2 shift promoting differentiation of anti-inflammatory dendritic cells whilst exerting neuroprotective functions in the process (Linker and Haghikia, 2016).

(2) Teriflunomide (Aubagio)

A metabolite that inhibits the action of dihydroorotate-dehydrogenase (DHODH). DHODH is a mitochondrial enzyme involved in synthesising pyrimidines present in T and B lymphocytes that are rapidly proliferating, attenuating an auto-immune response (Vermersch, 2017).

(3) Fingolimod (Gilenya)

Fingolimod is a structural analog of sphingosine. Fingolimod modulates sphingosine-1-phosphate (S1P) receptors and exerts its therapeutic effects. Expression of S1P causes down regulation of T cells therefore there is reduced entry of T lymphocytes into the CNS (Chung and Hartung, 2010).

Intravenous Antibodies

(4) Natalizumab

Restricted to patients who have rapid progression of MS, RRMS (Chung and Hartung, 2010). Natalizumab is known as a selective adhesion molecule (SAM) inhibitor where its mechanism of action is the prevention of the more specific inflammatory events

from developing to form lesions (Hutchinson, 2007) by preventing migration of inflammatory T cells and B cells expressing VLA-4 into the central nervous system.

(5) Alemtuzumab

A humanised antibody targeting the CD52 protein. This oral antibody is mainly used in RRMS. It has a rapid mode of action however; it is long lasting. Mechanism of action is by depletion of CD52-bearing B and T cells (Ruck *et al.*, 2015).

(6) Ocrelizumab

A humanised antibody targeting CD20 that selectively targets B cells expressing CD20 (Sorensen and Blinkenberg, 2016).

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent cells that are currently of high importance and being explored as a new therapeutic for treating various immune-mediated and degenerative diseases. MSCs were first studied in skeletal tissue repair as a regenerative form of therapy. From recent studies, MSCs have been shown to modulate endogenous immune cells and tissues (Figure 1.8). These studies that have been carried out reveal the mechanisms of action of MSCs and suggest they are short term and may be dependent on the interaction between the host cell and the MSCs. There has been a growing interest in these cells and their use in therapeutics (Parekkadan and Milwid, 2010).

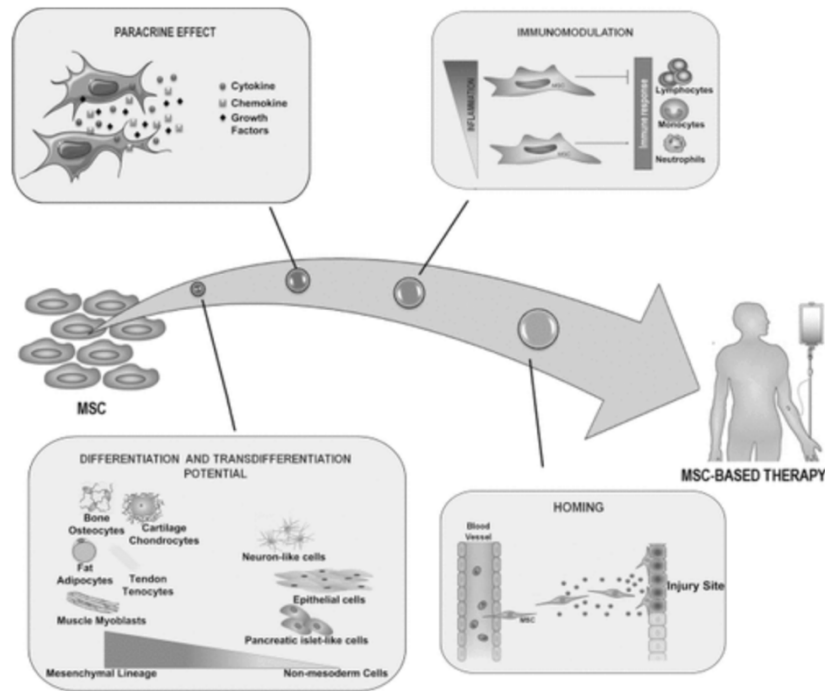


Figure 1.8. Biological and clinical properties of MSCs. (Squillaro *et al.*, 2016).

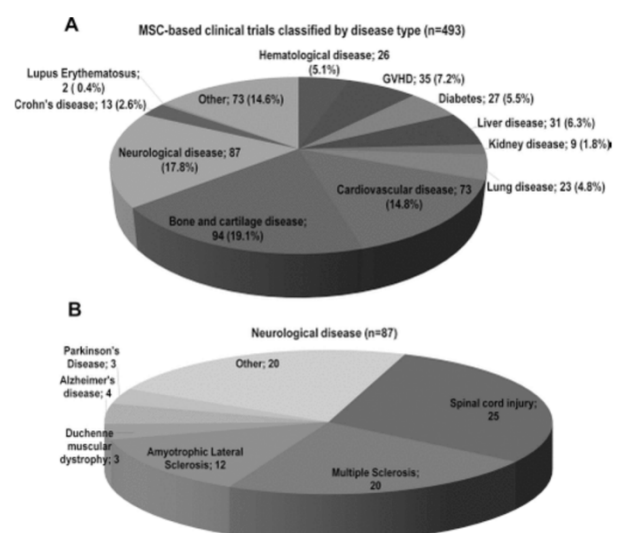
The top left panel illustrates the paracrine effects of MSC, which by producing cytokines, growth factors, and chemokines, influence the biology of neighbouring cells. They also have the potential for differentiating into other cell types such as bone or cartilage, neural cells, adipocytes (bottom left panel). They have immunomodulatory functions, in that they influence the activity of immune cells such as lymphocytes and monocytes (top right panel). MSC can home to sites of injury or inflammation, which can be used therapeutically (bottom right panel).

Friedenstein *et al.*, were first to isolate and characterise MSCs in 1968. MSCs are fibroblastic cells with a spindle-fibre shape. The actual term ‘MSC’ was proposed by Caplan *et al.* as MSCs were found to be able to be induced *in vivo* and *in vitro* and differentiate into various cell lines, including connective stromal cells, osteocytes, chondrocytes and adipocytes (Caplan, 1991). MSCs can be isolated from different sources, the most studied being the bone marrow. Positive expression markers for MSCs include CD73, CD90 and CD105 and negative expression markers include CD11a, CD19, CD34, CD45 and human leukocyte antigen-DR (HLA-DR) (Xiao *et al.*, 2015).

MSCs have been considered to be a promising form of treatment for MS. They are found in abundance in the bone marrow. They are pluripotent precursor cells that interact closely with hematopoietic stem cells (HSCs). Preclinical studies have explored the potential uses of MSCs in various disease settings. The main focus for MS is to test whether MSCs are capable of restoring damaged tissues as they are able to engraft within the CNS (Uccelli *et al.*, 2012).

MSCs are viewed as a great choice for all therapy for a number of reasons. These include the fact that MSCs can be accessed easily; the culture of these cells is simple, the isolation process has no complications and the cells can be expanded in a short time frame. Human trials using MSCs have been limited yet effective and patients have shown no serious adverse effects to autologous vs allogenic MSCs transplants. The transplantation of MSCs has been widely tested in various clinical trials, statistics shown in figure 1.9, and has provided successful and encouraging results. These clinical trials include immunological diseases, neurological disorders and also cardiovascular diseases (Parekkadan and Milwid, 2010).

Figure 1.9. (A) Statistics for the use of MSCs in clinical trials (B) Sub-classification showing clinical trials specifically in neurological diseases. Image taken from Squillaro et al, 2016



EAE Results

A hypothesis was created suggesting that MSCs have the ability to inhibit the autoimmune response against myelin antigens. In order to test this hypothesis, MSCs have been trialled in studies with EAE as well as in other neurological disorders and results have shown that the neuroprotective, neurogenerative and immunomodulatory effects of MSCs *in vitro* can be translated *in vivo*. Looking closely at EAE results from studies it can be seen that MSCs have induced peripheral tolerance and migrated to sites of injury promoting survival of damaged cells and reducing the release of pro-inflammatory cytokines. In one of the first studies carried out, MSCs were administered to EAE mice. Zappia *et al.*, revealed a reduction in demyelination and a decreased infiltration of B cells, T cells and macrophages in the CNS (Zappia *et al.*, 2005). EAE models of MS with both chronic and RRMS-like forms were induced with proteolipid protein (PLP) encephalitogenic peptides of the myelin oligodendrocyte glycoprotein (MOG) and treated with MSCs. MSCs were able to improve the clinical course of the disease by decreasing demyelination and also promoting tissue repair (Bai *et al.*, 2012; Bai *et al.*, 2009; Constantinescu *et al.*, 2010; Gerdoni *et al.*, 2007; Gordon *et al.*, 2008; Rafei *et al.*, 2009; Zappia *et al.*, 2005; Zhang *et al.*, 2006).

Mechanisms for immune tolerance were induced, which in turn caused the release of molecules, which foster tissue repair. It has been discovered that in order to achieve the maximum immunosuppressive phenotype from MSCs, they need to be primed by inflammatory cues. When MSCs cross paths with inflammatory signals they mediate their therapeutic effects by releasing neuroprotective and immunomodulatory molecules into the bloodstream (Uccelli *et al.*, 2012).

MS results

Autologous MSCs were given to patients who had a secondary progressive form of the disease and results revealed evidence of physiological, structural and functional improvement. A pilot study was carried out and the molecular mechanism of the effects of the infusion of MSCs for MS treatment was investigated by Mohajeri *et al.* The findings were that the expression of FoxP3 after an intrathecal injection of MSCs was significantly higher than the levels before treatment. (FoxP3) Forkhead transcription factor, is considered to be the master regulator in the development and functioning of regulatory T cells. Increased levels of expression of FOXP3 usually indicates greater clinical stability (Zhao *et al.*, 2016).

According to Gharibi *et al.*, 2015, clinical studies have been carried out using a co-culture of MSCs and peripheral blood mononuclear cells (PBMC) and results have shown increased levels of the generation of regulatory T cells (Gharibi *et al.*, 2015).

MSCs as a form of treatment

A form of treatment that would be considered as ideal and successful would have to (1) have the ability to induce some level of tissue regeneration and protection within the CNS and also (2) be capable of attenuating detrimental inflammatory responses. The mechanisms behind the amelioration in EAE is still under review. A key mechanism that has been studied closely is the inhibition of tissue destruction by the immune system by MSCs. Results and data collated from various clinical studies support the idea that MSCs may be able to access the CNS to provide protection against tissue damage whilst simultaneously facilitating tissue regeneration by producing the required growth factors.

The number of immunosuppressive and immunomodulatory therapies created to use in a clinical setting as a form of treatment for MS, is continuously growing. Many have been successful however, it is the progressive forms of MS that are seen to be neglected in the production of treatments. This is a cause for concern as there is still an unmet need for MS therapy that has the potential to abrogate immune-mediated injury and protect by remyelination (Darlington *et al.*, 2011). The integration of MSCs therapy, biology and engineering into future therapeutics will simply usher in successful treatment methods (Parekkadan and Milwid, 2010).

MSC display anti-proliferative, anti-inflammatory as well as anti-apoptotic characteristics which highlights their potential as a good method of treatment. All of this coupled with their ability to differentiate and their plasticity, makes them rather extraordinary (Bonab *et al.*, 2013). They have immunomodulatory properties which allow them to regulate immune cells by cell-to-cell interactions, chemokine attraction and production of soluble mediators. A tolerogenic environment is produced by T_{reg} and B_{reg} cells that have been induced by MSCs. This kind of environment leads to enhanced levels of immunosuppression against inflammation (Ma and Chan, 2016).

When comparing the use of MSCs to that of embryonic stem cells (ESCs), MSCs are seen as more favourable as evidence has shown a significant decrease in oncological transformation in therapies using MSCs. MSCs have been shown to inhibit apoptosis, gliosis along with preventing scar formation. Their immunomodulatory properties stem from the fact that they are able to produce regulatory cytokines, which lead to the inhibition of T and B lymphocytes proliferation (Odinak *et al.*, 2012).

Therapies that have been produced in order to treat MS are thought to be directed towards the suppression of the immune response. However, functional recovery that is considered to be long term is dependent on the CNS' ability to repair the damaged myelin and also replace damaged or lost oligodendrocytes. The CNS retains a population of more multipotent neural cells in addition to oligodendrocyte precursors. This realisation has encouraged therapies to be orientated more towards the repair of myelin (Bai *et al.*, 2012), including MSC.

Clinical Trials of MSCs as a treatment of MS

Clinical trials have been carried out on various neurological diseases and have shown MSCs to be safe allowing larger phase 2 trials to be carried out addressing the markers responsible for disease activity and the effect of MSCs on clinical outcomes (Uccelli *et al.*, 2011).

There have been a few studies that have been carried out and reported results as seen in Figure 1.10. Phase 1 studies that have been carried out on MS patients using autologous

	Study design	Source of MSCs	Route of delivery	Number of patients treated	Age range of patients (years)	Dose	Main outcome measures	Main results	Comments
Multiple sclerosis									
Mohyeddin et al ³²	Phase 1, open, safety	Autologous	Intrathecal	Ten	22–40	Mean of 8.73×10^4 cells per patient (range 2.5×10^4 to 18.0×10^4); one patient received two injections	Side-effects, efficacy	Safety: iatrogenic meningitis (n=2); headache (n=9) Efficacy: EDSS unchanged (n=4), worsened (n=5), or improved (n=1); MRI showed no change (n=7), increased lesion load (n=2), or decreased lesion load (n=1); mean follow-up of 19 months	Progressive multiple sclerosis with baseline EDSS <6.0
Yamout et al ³³	Phase 1, open, safety	Autologous	Intrathecal	Seven of ten treated; three of ten had inadequate number of MSCs	34–56	32×10^4 to 100×10^4 cells per patient	Side-effects, efficacy	Safety: transient encephalopathy and seizures in the patient receiving the highest dose of MSCs Efficacy: clinical improvement in treated patients (n=6); reported worsening of MRI outcomes	EDSS 4.0–7.5
Karussis et al ³⁴	Phase 1–2, open, safety	Autologous	Intrathecal in ten patients; intrathecal plus intravenous in five patients	15	25–65	Mean of 63.2×10^4 cells per patient (SD 2.5×10^4) for intrathecal infusion; 24.5×10^4 cells per patient (2.5×10^4) for intravenous infusion	Side-effects, efficacy	Safety: fever (n=10); headache (n=10); aseptic meningitis (n=1); other minor side-effects; no major adverse events Efficacy: reported reduction of EDSS	Active multiple sclerosis that did not respond to available treatments
Amyotrophic lateral sclerosis									
Mazzini et al ³⁵	Phase 1, open, safety	Autologous	Intra-spinal cord	Nine	21–75	Mean of 57×10^4 cells per patient	Side-effects	Transient pain (n=4); transient sensory disturbances (n=6)	--
Karussis et al ³⁴	Phase 1–2, open, safety	Autologous	Intrathecal in ten patients; intrathecal plus intravenous in nine patients	19	25–65	Mean of 54.7×10^4 cells per patient (SD 17.4×10^4) for intrathecal infusion; 23.4×10^4 cells per patient (6.0×10^4) for intravenous infusion	Side-effects, efficacy	Safety: fever (n=11); headache (n=5); dyspnoea (n=1); no major adverse events Efficacy: neurological disorders unchanged during 6 months of follow-up	--
Mazzini et al ³⁶	Phase 1, open, safety	Autologous	Intra-spinal cord	Ten	20–61	11.4×10^4 to 120×10^4 cells per patient; two–five injections	Side-effects	Pain (n=7); localised sensory impairment (n=5); localised tingling sensation (n=1)	Sporadic disease
Cerebral ischaemia									
Bang et al ³⁷	Phase 1–2 randomised, controlled	Autologous	Intravenous	Five patients in active treatment group; 25 patients in control group	54–72	5×10^7 cells per patient in two doses	Side-effects, efficacy	Safety: no death, stroke recurrence, or serious adverse events after 1 year; cellulitis of a foot (n=1) Efficacy: higher ventricular dilatation at 12 months in control group; trend towards increased functional recovery in MSC group	Middle cerebral artery infarcts
Genetic disorders									
Koc et al ³⁸	Phase 1–2, safety, efficacy	Allogeneic	Intravenous	Five with Hurler syndrome; six with metachromatic leukodystrophy	5–25	2×10^4 to 10×10^4 cells per kg	Side-effects, efficacy on nerve conduction velocities, bone mineral density	Safety: grade 1 fever (n=4); grade 2 phlebitis (n=1) Efficacy: increase in nerve conduction velocities in group with metachromatic leukodystrophy (n=4); bone mineral density unchanged	Patients previously treated with allogeneic haemopoietic stem-cell transplantation
Spinal cord injury									
Pal et al ³⁹	Pilot phase 1, open, safety	Autologous	Intrathecal	30	17–56	1×10^4 cells per kg	Safety, efficacy	Safety: no serious adverse events Efficacy: no change in ASIA scale; improvement in Barthel's index score in patients with thoracic injury occurred within 6 months before treatment	Patients with clinically complete spinal cord injury at either cervical or thoracic level
Ra et al ⁴⁰	Phase 1, open, safety	Autologous	Intravenous	Eight	23–54	4×10^4 cells per patient	Safety	No serious adverse events	MSCs derived from adipose tissue
MSCs=mesenchymal stem cells. EDSS=expanded disability status scale. ASIA=American Spinal Injury Association.									
Table: Clinical experience with MSCs in neurological diseases									

Figure 1.10. A table showing all the clinical studies on different neurological diseases carried out using MSCs. The top trials in the figure are in MS. Image taken from (Uccelli *et al.*, 2011).

MSCs were either given intracathecally or intravenously have been tolerated well showing some preliminary evidence of efficacy (Uccelli *et al.*, 2011).

On the basis of evidence presented that MSCs do have a beneficial effect in both acute and chronic models of MS, Connick and colleagues carried out an open-label phase 2a study, which looked closely at visual impairments, to assess how safe and efficient MSCs are when using them as a potential neuroprotective treatment for SPMS. The trial was conducted between November 2007 and August 2010 and the participants that were involved were from North London and also East Anglia. Criteria for eligibility included that participants should be between the ages of 18-65, show clinical evidence of abnormal visual potentials that are expected with demyelination also show the presence of a T2 lesion on the optic nerve on the MRI scan, amongst others. The use of disease modifying treatments 6 months prior to the trial immediately excluded a participant. Patients must have the capacity to provide consent then written consent and approval is gained (Connick *et al.*, 2012).

Participants with SPMS received an intravenous infusion of autologous bone-marrow derived mesenchymal stem cells. Hypersensitivity was reduced by pre-medications with chlorpheniramine, hydrocortisone and metoclopramide were given before administration of cells. The primary objective of this trial was measure safety and also feasibility comparing the adverse effects presented 20 months before the trial and then 10 months after the infusion. The second objective looked closely at efficacy in order to assess the anterior visual pathway and use that as a model of the disease as a whole. Participants were assessed at 3-6 month intervals and each assessment involved neurological and medical history with recording any adverse effects (Connick *et al.*, 2012).

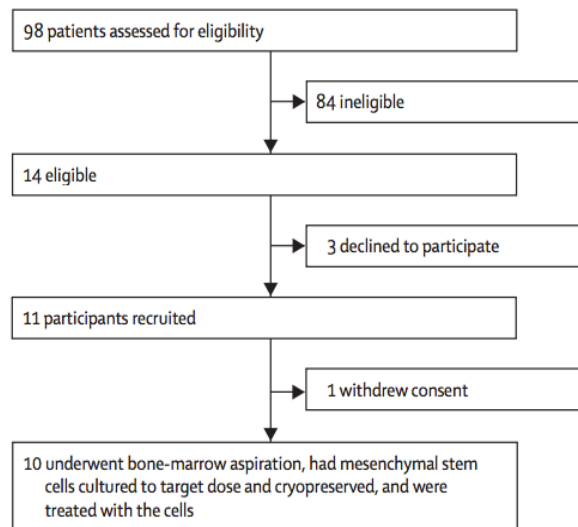


Figure 1.11 Showing the study profile. Image taken from (Connick *et al.*, 2012).

Figure 1.11 shows the final number of participants was 10 patients. 1.6×10^6 cells were administered as the mean dose depending on cells per kg of bodyweight. The findings showed no serious adverse effects in any of the participants. There was an improvement in the visual acuity after treatment with MSCs in addition to visual evoked response latency. No significant changes were seen on visual fields, retinal nerve fibre thickness or colour vision (Connick *et al.*, 2012).

Immunomodulatory therapies are continuing to advance, however, there are currently still no forms of treatment that can reverse the accumulation of the fixed disability presented in SPMS patients. Assessing different forms of neuroprotective therapies provides some level of hope, whilst presenting a challenge as the disease variability from one patient to another is completely unknown (Connick *et al.*, 2012).

Objectives

The underlying mechanisms of action of the use of MSCs as a form of therapy is only partially understood so it is extremely important that further studies are carried out so there is a more refined understanding of the functions of MSCs in the bone marrow. This will give us a greater insight into their exact mode of action allowing us to understand what makes them so important (Parekkadan and Milwid, 2010).

Hypothesis

Very few studies have investigated the interactions of MSC with immune cells from the same donor. Moreover, it is not known whether these interactions differ between MS patients and healthy donors.

Our hypothesis is that MSC from people with secondary progressive MS, having been exposed in vivo to an inflammatory environment for a long period of time, have a reduced immunomodulatory capacity compared to those from healthy volunteers. This may be due to reduced production of immunomodulatory substances or due to enhanced inflammatory state of immune cells from MS patients, or both.

Aims

The aim of this study is to (1) characterise the role of MSC as immunomodulators of peripheral blood mononuclear cells (PBMC) and (2) to measure the immunomodulatory effects of MSCs from MS patients and determine whether they are less pronounced than those from healthy controls.

Materials and Methods

All participants from this study provided informed consent as approved by the ethics review board (NRES reference:12/EM/0263) Informed consent forms were signed and obtained from all donors prior to the collection of the samples.

This research project ‘Comparison of Immunomodulatory factors produced by MSCs in MS patients and Healthy Controls’ was conducted at the University of Nottingham, and samples were collected with consent from Queens’ Medical Centre under the project ‘Phenotypic characteristics, Immune interactions and ultra-high field imaging of mesenchymal stem cells from MS patients and controls’ approved as above.

Table 2.1 and 2.2 presents donor information collected and used in this research project.

Table 2.1 Donor information for HCs

Healthy Control (HC)	Gender	Age
C004	M	58
C009	F	56
C012	F	26
C014	F	47
C015	F	41
C016	M	29
C020	F	49

Table 2.2. Donor information for MS patients

Patient No.	Age	Sex	Type of MS	Duration of MS	Duration of progressive disease	EDSS
P005	58	F	SPMS	14	8	6
P006	52	M	SPMS	29	4	5
P017	40	F	SPMS	15	12	7
P018	53	M	SPMS	19	8	6.5
P019	55	F	SPMS	10	7	3.5

For this research project I carried out the following methods, which were led and supervised by Nanci Frakich, Senior Technician/ Safety Officer.

Bone Marrow Aspiration

A bone marrow aspiration involves the removal of soft tissue from inside the posterior iliac crest bone. This procedure was carried out by the haematologist at Queens Medical Centre, Nottingham. The sample is collected, approximately 5ml, using a syringe loaded with 3000 units of heparin, approximately 1ml, to prevent clotting as seen in Figure 2.1. It is received in a sodium-heparin tube. It is also important to immediately invert the tube after collection to avoid clotting formation.

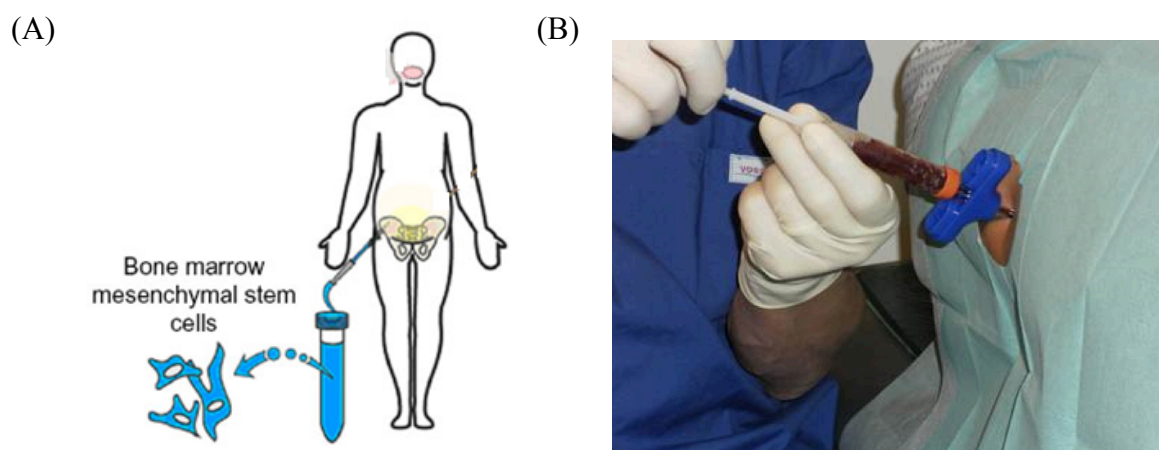


Figure 2.1. (A) Showing the basic outline of the steps required to obtain MSCs. (B) Bone Marrow Aspiration technique.

PBMC Isolation

Peripheral blood mononuclear cells (PBMC) were isolated from 30ml of blood using Ficoll density centrifugation. Equal volumes of Ficoll-Histopaque, Histopaque-1077 (Sigma-Aldrich), to the amount of blood was used. Blood was carefully layered on top of histopaque. The remaining blood was diluted with Phosphate buffered solution (PBS, Sigma-Aldrich) so no cells were lost from the sample of blood. The sample was then centrifuged at $800 \times g$ ($\sim 2000\text{rpm}$) for 20 minutes without brake. The PBMC ring layer was then removed and washed twice with PBS. The pellet of cells was then re-suspended in 10ml of PBS and a cell count was carried out. In order to count the cells $10\mu\text{l}$ of cell suspension and $90\mu\text{l}$ of Trypan Blue, Sigma-Aldrich, were mixed in an Eppendorf tube. $10\mu\text{l}$ of this suspension was then transferred into a Neubauer hemocytometer and a cell count was carried out. The number of cells reported were calculated as number of cells/ml.

$$\text{Total \# of viable cells} = 10 \text{ ml} \times \# \text{ of viable cell /ml} (\# \text{ cell} \times \text{dilution factor} (1/10) \times 10000)$$

Freeze down of PBMC

After counting the cell suspension, cells are centrifuged at 250 x g for 10 minutes.

Supernatant was discarded and cells were re-suspended in freezing media (RPMI-1640 Sigma R0883 Complete media containing 10% Dimethyl Sulfoxide DMSO) containing 0.5-1million cells/ml, frozen down using CoolCell alcohol-free cell freezing container, this ensures the standardized controlled-rate -1°C/minute cell freezing in a -80°C freezer, and stored in liquid nitrogen.

MSC Isolation and Passage

Isolation

Culture medium, Lonza: MSCGM Bullet Kit (PT-3001) including bullet kit and media, was warmed at 37°C in a water bath. The bone marrow sample was layered on top of the same volume of Histopaque-1077. The sample was then centrifuged at 800 x g for 20 minutes without brake. The buffy layer cells were re-suspended in culture medium and centrifuged at 400 x g for 10 minutes to wash. This step was repeated. The cells were then re-suspended into complete Lonza medium. The volume of medium used is dependent on the number of cells therefore a count was carried out with a 1 in 10 dilution to Trypan blue, which is used to assess the cell viability- 10µl of cell suspension and 90µl of Trypan Blue.

A T75 or T25 tissue culture flask (Corning) was seeded as appropriate (taking into consideration the number of cells) at a density of 4×10^5 cells/cm², and the cells were grown in 37°C with 5% CO₂.

After 24-48 hrs, non-adherent cells were discarded, the adherent cells were washed twice in PBS, fresh medium added and changed every two days.

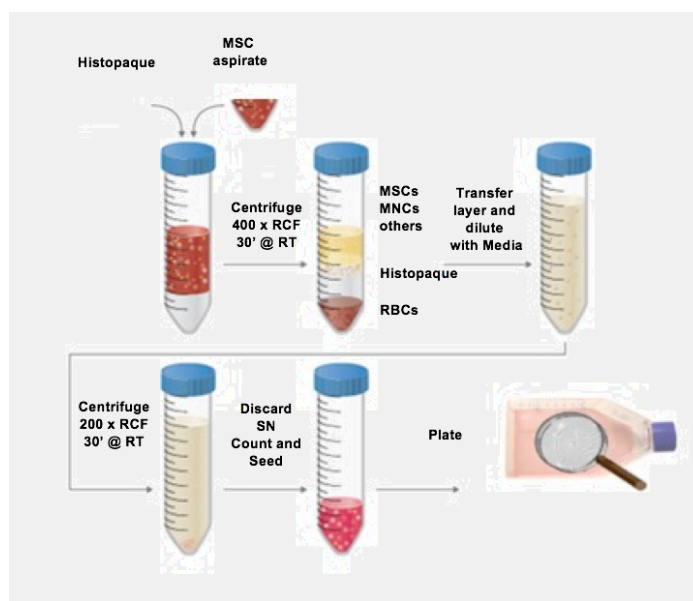


Figure 2.2. Step by step process of MSC Isolation.

Sub-culturing

The cells were harvested at approximately 80% confluence and then passaged to avoid differentiation. Old media was aseptically removed and discarded from the flask. The attached cell layer was washed with PBS and rinsed by gently rocking the flask back and forth several times. It was then aseptically removed and the wash solution discarded. An aliquot of 3ml of Trypsin /EDTA (Sigma T3924) added to each flask and incubated at 37°C for approximately 3 minutes, then observed under the microscope to ensure the cells had successfully detached. The Trypsin/EDTA solution was then neutralised with complete medium (10ml for T75 flask was used or 5ml for T25 flask). The cell suspension was then centrifuged at 1500rpm/ 400g for 5 minutes. The cells were re-suspended in minimal medium and counted with a haemocytometer. Cell suspension was either re-suspended in fresh medium and re-seeded based on the recommended seeding density for human mesenchymal stem cells (5,000-6,000 cells per cm²) or cryovials were prepared using freezing medium.

(Lonza MSCGM complete media containing 10% Dimethyl Sulfoxide DMSO) containing 0.5-1million cells/ml, froze down and stored in liquid nitrogen.

Sub-culture was performed until passage number 5.

Mesenchymal Stem cells (MSC) and autologous peripheral blood mononuclear cell (PBMCs) Co-culture

MSCs were thawed at passage n° 3 or 4 and cultured until ready for co-culture. To thaw the cells, the cryovial with cells was removed from liquid nitrogen and thawed in 37°C water bath for approximately 1min. The cells were then quickly diluted into 10ml culture medium and centrifuged 1500rpm/ 400g for 5 minutes. The cells were re-suspended into 1/2ml medium and a count was carried out using Trypan blue. After the count, the dilution was adjusted to ensure there was 250,000 cells per ml. MSCs were seeded into 2 wells of a 24-well plate at 1ml cell suspension containing 250,000 cells per well and incubated at 37°C.

PBMC were thawed on the same day the MSCs were seeded. The cryovial containing frozen PBMC was quickly thawed in a 37°C water bath and then re-suspended into 5/10ml of complete RPMI culture medium (RPMI-1640 Sigma R0883+10% Fetal bovine serum heat inactivated FBS (SIGMA) + Pen/Strep +L-glutamine 2mM). The cell suspension was then centrifuged at 1500rpm/ 400g for 5 minutes. The cells were washed twice with complete RPMI culture medium. After washing the cells were re-suspended in 10ml RPMI culture medium and counted. The cells were re-suspended at approx. $1-2 \times 10^6$ PBMCs/ml and incubated at 37°C, 5% CO₂ overnight.

After overnight incubation PBMC were washed with PBS and another cell count was performed. Based on previous co-culture experiments (e.g. Rozenburg *et al.*, Stem Cells

Transl Med 2016; and Nyirenda M, personal communications), we selected the ratio of MSC/PBMC to be approximately 1:4 therefore 1×10^6 PBMCs were seeded on top of the 24 wells containing MSC. Extra wells were seeded with 1×10^6 PBMCs alone as controls.

One well with MSC+PBMC and 1 well of PBMC alone were activated with $1 \mu\text{g}$ anti-CD3, (BD Pharmingen- 555329) and $1 \mu\text{g}$ anti-CD28 (BD Pharmingen- 555725). Extra PBMC wells were activated to use in single colours and fluorescence minus one (FMO) controls. The cells were incubated for 3 days and then reactivated with cell stimulation cocktail (cocktail of phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin (eBioscience- 00-4975, $2 \mu\text{g}/\text{ml}$)) for extra 5 hours at 37°C . After the incubation period, the PBMC were recovered and washed with PBS. The cells were divided into the FACS tubes (Sarsted, Germany) at approximately 0.5×10^6 - 1×10^6 cells per tube to proceed to the staining protocol of surface and intracellular markers.

Live /Dead staining

Live/dead staining was performed using Live/Dead Fixable Blue Dead Cell Stain Kit (Invitrogen): $1 \mu\text{l}$ of reconstituted fluorescent reactive dye was added to all the tubes- $1 \mu\text{l}$ dye/ $1000 \mu\text{l}$ cell suspension. Cells were incubated for 30 minutes at room temp, protected from the light. After the 30 minute incubation period, the cells were washed with 2ml staining buffer (PBS +2% Fetal bovine serum FBS) and then centrifuged at 250g for 10 minutes. Supernatants were discarded and pellets re-suspended.

Extracellular flow cytometer staining

Extracellular flow cytometer staining was performed using APC Cy7 anti CD4 (BD Pharmingen- RPA-T4 (RUO) – 557871). $5 \mu\text{l}$ of anti-CD4 was added to its corresponding

tube as suggested by data sheet. After a 20 minute incubation at room temp, protected from the light, the cells were washed with 2 ml stain buffer, centrifuged at 250 x g for 10 min, and the wash buffer removed.

Intracellular flow cytometer staining cytokines

Finally, intracellular staining for PE anti IL-17 (BD Pharmingen- N49-653 (RUO) – 560486), FitC anti IFN- γ (Beckman Coulter, Inc.- 41116015), PerCP Cy5.5 anti GM CSF (BD Pharmingen- BVD2-21C11 (RUO) – 562258) was performed.

After extracellular staining the cells were fixed and permeabilised using Human FoxP3Buffer Set (BD 560098).

The cells were fixed with 2ml of Human FoxP3 Buffer A and incubated for 10 minutes at room temperature, light protected. After incubation, the cells were centrifuged at 500g for 5 minutes and then washed. The cells were then permeabilised by re-suspending in 0.5 ml of 1xBuffer C and incubated for 30 minutes at room temperature, light protected. The cells were washed twice with 2ml of staining buffer. Supernatants were discarded, pellets re-suspended and stained by adding the antibodies to their corresponding tubes and incubated for 30 minutes at room temperature.

After incubation, the cells were washed twice with staining buffer and then re-suspended in 300 μ l staining buffer.

The cells were analysed by Flow cytometry using LSRII flow cytometer (BD biosciences, USA) and KALUZA software.

Fluorescence minus one (FMO) samples were used to set the gating.

FMO controls

FMO controls are used to correctly interpret data collected from flow cytometry. They contain all the fluorochromes in a panel. Using Kaluza, the spread of fluorochromes into the particular channel of interest, e.g. PE, is correctly identified by showing the difference between the isotype bound and the FMO bound representing the spread of data which is due to fluorescence from the PE channel (Bushnell, T).

Phenotyping

The International Society for Cellular Therapy (ISCT) proposed a cell surface marker panel for the minimal identification of Human MSCs derived from bone marrow. Under this recommendation MSCs should be positive for CD73, CD90 and CD105, but be negative for CD34, CD45, CD11b, CD19 and HLA-DR.

To confirm proper surface marker expression, all lines of MSCs were assessed between passage 5 and 6 using the Human MSC Phenotyping Kit (Human MSC Analysis Kit- BD Stemflow- 562245). The kit provides a positive cocktail (FitC CD90, PerCP-Cy5.5 CD105 and APC CD73) and a negative cocktail in the PE channel (PE CD45, PE CD34, PE CD11b, PE CD19, and PE HLA-DR) as well as the correspondent isotype controls. The cells were analysed by Flow cytometry using LSRII flow cytometer (BD biosciences, USA) and KALUZA software.

Cytokine profiling of MSCs from patients with MS or HCs

The MILLIPLEX map Human High sensitivity Magnetic Bead Panel (from MILLIPORE) was used to evaluate the cytokine and growth factor production by MS-MSC and HC-MSC. This immunoassay, which is based on Luminex xMAP (multi-analyte profiling) technology,

enables simultaneous detection and quantification of multiple secreted proteins (cytokines, chemokines, growth factors, etc.)

1. Luminex uses proprietary techniques to internally colour-code microspheres with two fluorescence dyes. Through precise concentrations of these dyes, distinctly coloured bead sets of 80 6.45µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
2. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
3. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
4. The Luminex instrument has 2 lasers in which one is used to excite and detect a specific bead and the other to detect the PE bound to a reporter antibody.
5. Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals.

The capability of adding multiple conjugated beads to each sample in one well results in the ability to obtain multiple results from each sample.

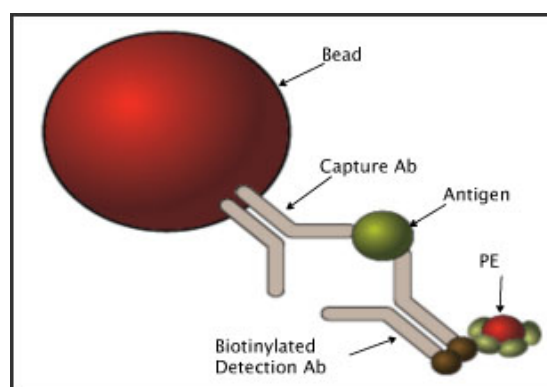


Figure 2.3. Bead label with a unique captive antibody

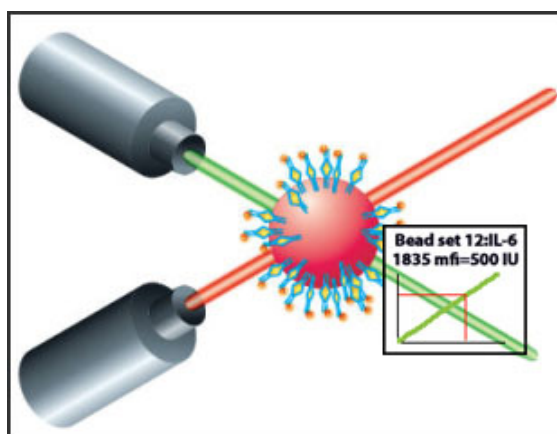


Figure 2.4. Detection of Antigen

The cytokines and growth factors investigated were:

1. IL-10
2. IL-12
3. IL-1 β
4. IL-6
5. IL-8
6. TNF- α
7. GM-CSF

250,000 MSCs passage 4-5 were cultivated for 24 hours in 1ml of Lonza culture medium in a 24-well plate. Culture supernatants were collected after 24 hours' incubation (culture medium). The conditioned medium was recovered and centrifuged in an Eppendorf at high speed for 10 minutes to remove and tissue debris or particles such as cells. The supernatant was aliquoted and stored at -80°C until the sample was processed.

The assay was performed following the manufacturer's instructions (Millipore Human High Sensitivity T cell magnetic bead panel CAT: HSTCMAG-28SK).

The culture medium was used as the solution for backgrounds and also for the reconstruction of standards and controls.

Processing the sample

Using a 96-well plate, 200µl wash buffer was added to each well and mixed on a plate shaker for 10 minutes. Wash buffer was removed and 50µl standard control was added to the appropriate wells. 25µl Assay buffer and 25µl of sample was added to the sample wells. Mixed beads were then added to the sample wells. The samples were then incubated overnight at 4°C, protected from light.

After 24hr incubation, contents of each well were discarded and the plate was washed 3 times using handheld magnet (EMD Millipore Catalog # 40-285). 50µl of Detection Antibodies were then added to each well and incubated for 1hr at room temperature, protected from the light. 50µl of Streptavidin-Phycoerythrin was then added to each well and incubated for 30 minutes at room temperature. Contents of wells were then discarded and the plate was washed 3 times. 150µl Sheath Fluid was then added to all wells. The beads were then re-suspended on a plate shaker for 5 minutes. The plate was then run on Luminex 200, HTS, FLEXMAP 3D software (100µL, 50 beads per bead set).

RNA extraction

RNA extraction was carried out using the High Pure RNA Isolation Kit, Roche. A full T75 flask was used to efficiently isolate high purify RNA. First the cells were detached using the standard protocol for sub-culturing. After cells were detached, they were washed twice with 10ml PBS. A second wash was carried out and the cells were re-suspended in 200µl PBS. 400µl of lysis/ binding buffer was then added and the sample vortexed for 15 seconds. The

sample was then transferred to the upper reservoir of a high pure filter tube and centrifuged for 15 seconds at 8000 x g. The filter tube was then removed from the collection tube and the flowthrough discarded. 90µl DNase I incubation buffer and 10µl DNase I were mixed in a sterile reaction tube, added in the upper reservoir of the filter tube and then incubated for 15 minutes, room temperature. 500µl wash buffer I was then added and centrifuged for 15 seconds at 8000 x g. The flowthrough was discarded and 200µl of wash buffer II was added and centrifuged for 2 minutes at 13,000 x g. The collection tube was then discarded and the filter tube was inserted into a clean 1.5ml microcentrifuge tube. 50-60µl elution buffer was added and centrifuged for 1 minute at 8000 x g. The purified RNA was removed and stored in an Eppendorf tube. The RNA concentration was determined using a NanoDrop Spectrophotometer.

RNA aliquots were kept for future research.

Statistics

GraphPad Prism 7 was used for all statistical analyses. Two-tailed T test was used for statistical comparisons of all data achieved. The graphs show the mean with standard error of the mean. For each figure all statistical tests are indicated in the corresponding legends. P values of ≤ 0.05 were considered to be statistically significant. The p values are only mentioned for the graphs that represent statistically significant data.

Results

MSC phenotyping was carried out to confirm the purity and expression levels of surface markers in MSCs from patients and HCs.

Figure 3.1 shows the characteristic morphology of MSC with regular, elongated fusiform shape. It also shows that the great majority of the cells (passage 4) are morphologically MSC,

with no contamination with fibroblasts or other cell types. For the experiments in this thesis, this was the typical appearance of the cells used, and all experiments described here were performed on cells at passage 4 to 6.

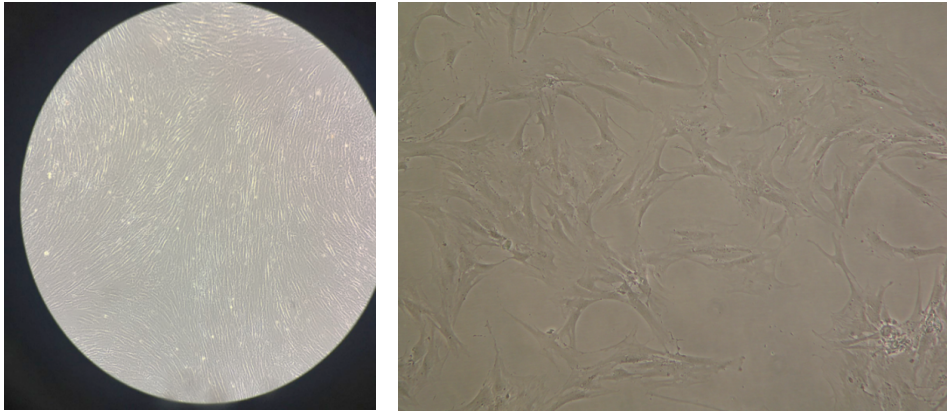


Figure 3.1. Typical MSC Morphology. Images show the morphology of MSCs under culture conditions. From left to right, representative images of cultures at high (10X magnification), and low confluence (40X magnification).

Figures 3.2 and 3.3 show a patient MS sample and a sample from HC that are both >95% pure revealing in Figure 3.1 that they have retained their stem cell characteristics. Both MSC samples stained positively for the MSC markers CD90, CD73 and CD105 and appropriately negative with the PE- negative cocktail (CKTL) (cocktail (PE CD45, PE CD34, PE CD11b, PE CD19 and PE HLA-DR), with isotype control. Phenotyping analysis was carried out using Kaluza.

The figures are illustrations of representative examples from one control and one patient with MS. The same phenotypic analysis was performed on all samples used in this experiment.

Patient P019-JT MSC –p5

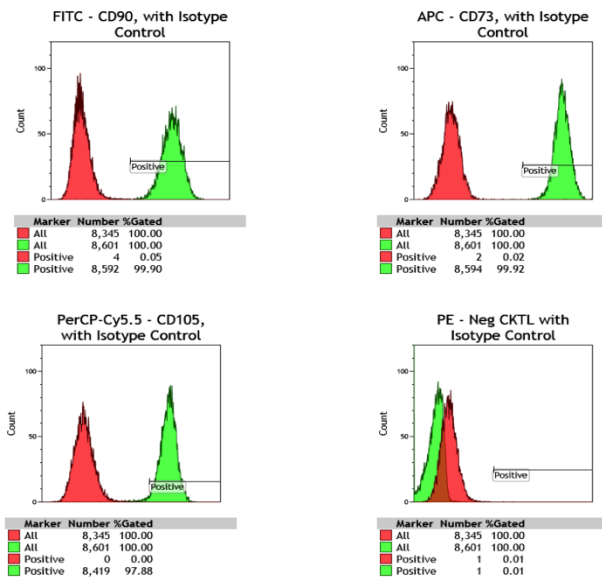


Figure 3.2. Phenotyping of MSCs in MS patient sample. Sample shows purity (%) and confirms positive lineage from markers tested in addition to showing less than 1% in isotype control

Control C012-CL MSC – p5

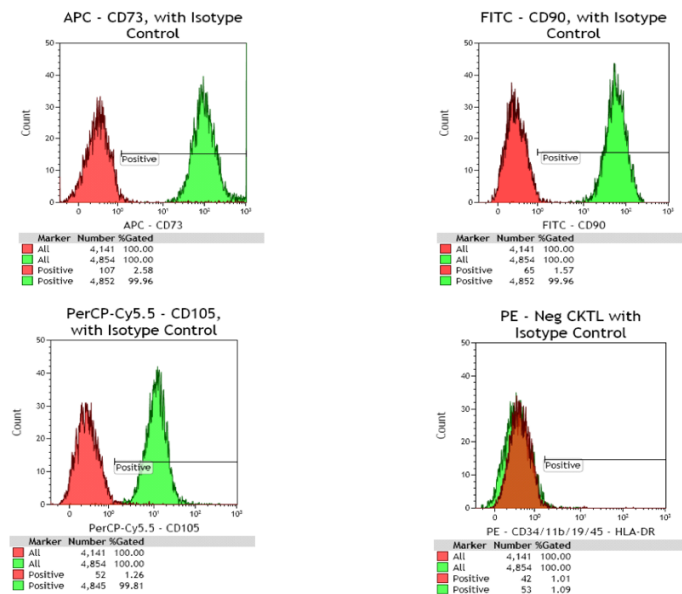


Figure 3.3. Phenotyping of MSCs in Healthy control. Sample shows purity (%) and confirms positive lineage from the markers tested in addition to showing less than 1% in isotype control

Co-culture results

Cytokine production by stimulated PBMC in the presence or absence of autologous MSC. Stimulated PBMC cultured alone or in co-culture with autologous MSC were stained intracellularly for the inflammatory cytokines IL-17, IFN- γ , and GM-CSF. Representative examples for one MS patient and one healthy control are shown below.

Results are from MSC and autologous PBMC co-culture, after 72hrs activation and 5hrs of re-activation with PMA-ionomycin-brefeldine A (stimulation cocktail). Figures 3.4, 3.5, 3.6 and 3.7 are a representative dot plot examples out of 5 patients and the control is a representative example out of 7.

Patient Sample- P019

Figures 3.4 and 3.5 show examples of flow cytometry analysis done on Kaluza software programme of patient sample P019. The viability (%) of the lymphocytes present in MSC + PBMC is shown to be 42.31%. Among these live cells, 26% were CD4+. 1.58% of the live cells were found to be IL-17 +, 0.15% GM-CSF +, 9.21%, and IFN- γ +. The patient sample with PBMC alone (Figure 3.5) has a slightly increased viability, in comparison with MSC + PBMC, of 53.33%. However, the percentage of CD4+ cells remained the same (25.2%). From these live cells 0.38% were IL-17 +, 0.40% GM-CSF +, 7.15% IFN- γ +. Figures 3.10, 3.11, 3.12 and 3.13 all show bar chart representation of the mean data collected from 5 different MS patients.

Healthy Control Sample- C012

Figures 3.6 and 3.7 show examples of the analysis done on Kaluza software programme of HC sample C012. The viability (%) of the lymphocytes present in MSC + PBMC alone is revealed as 91.43% where 0.30% were IL-17 +, 0.32% GM-CSF+, 10.28% IFN- γ + and 44.34% of the live cells were CD4+. The HC sample with PBMC alone (Figure 3.7) has a viability of 91.55%. Among these live cells, 0.34% were IL-17 +, 0.10% GM-CSF+, 11.54% IFN- γ + and 41.52% of the live cells were CD4 +. Figures 3.8, 3.9, 3.10 and 3.11 all show bar chart representation of the mean data collected from 7 different HCs.

For the scatterplots used in the experiments, we used “fluorescence minus one” as control for cytokine staining as described in Materials and Methods (Figures 3.8 and 3.9)

MSC+PBMC-Stimulated-Full staining- P019

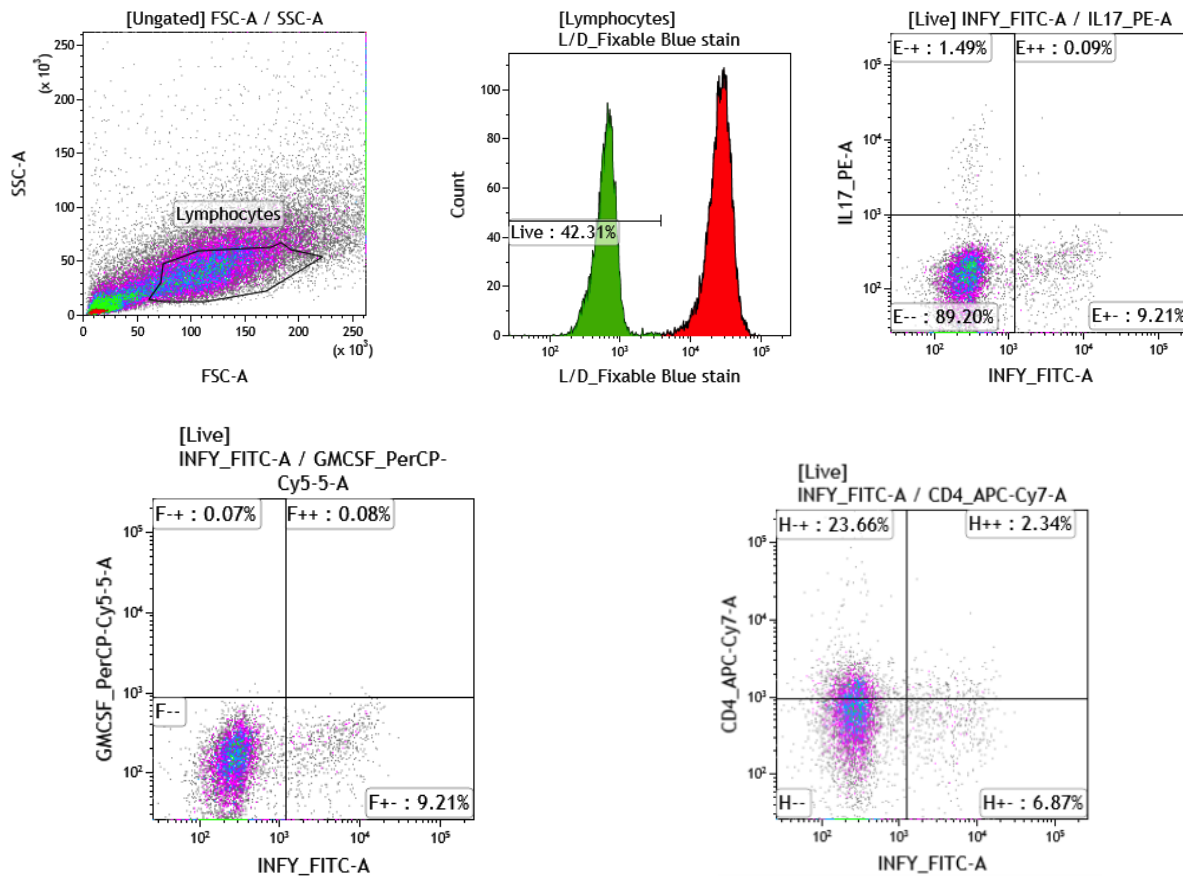


Figure 3.4: Representative flow cytometry analysis of live T cells from MS patients-PBMC and MSC. In panel A Lymphocytes are gated based on physical parameters side scatter (SSC) and forward scatter (FSC). Viability from Live/Dead stain is shown to be 42.31%. Using flow cytometry the activation of the cells were evaluated through the positive detection of IL-17, IFN- γ , GM-CSF and CD4 and their representative single colours.

Patient sample P019 shows 1.58% positive for IL-17, 0.15% positive for GM-CSF, 9.21% Positive for IFN- γ and 26% positive expression of CD4+ T cells.

In the above figure the distinction is difficult between positive and negative staining for CD4. This is because the gate for CD4 is set in the FMO (see page 66 below), and there is down-regulation of CD4 upon phorbol ester stimulation. See figure 3.4b below for unstimulated CD4 expression.

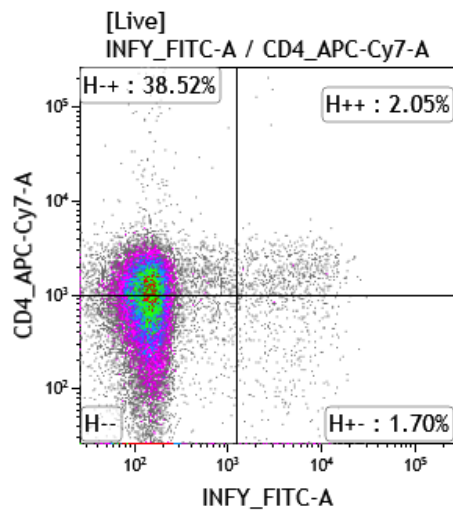


Figure 3.4b. The distinction between positive and negative staining for CD4 is based on the baseline from FMO (see below in thesis text). Compared to stimulated CD4, there is higher surface expression of CD4 in unstimulated cells (PMA down-regulates CD4 expression) (Petersen *et al.*, 1992).

PBMC-Stimulated-Full stain- P019

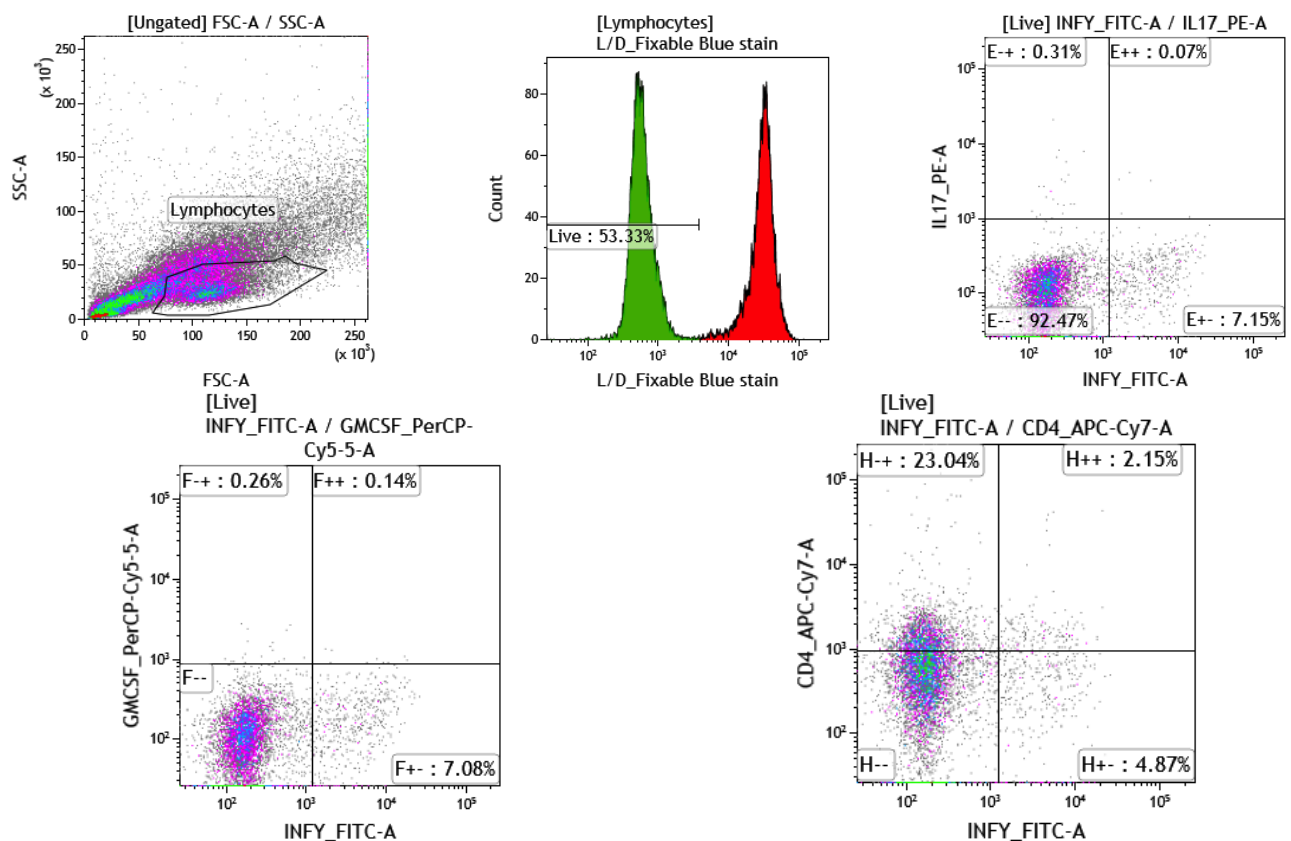


Figure 3.5: Representative flow cytometry analysis of live T cells from MS patients, PBMC alone. In panel A Lymphocytes are gated based on physical parameters side scatter (SSC) and forward scatter (FSC). Viability from Live/Dead stain is shown to be 53.33%.

Using flow cytometry the activation of the cells were evaluated through the detection of IL-17, IFN- γ , GM-CSF and CD4 and their representative single colours. Patient sample P019 shows 0.38% positive for IL-17, 0.40% positive for GM-CSF, 7.15% positive for IFN- γ and 25.19% positive expression of CD4+ T cells.

MSC+PBMC-Stimulated-Full stain- C012

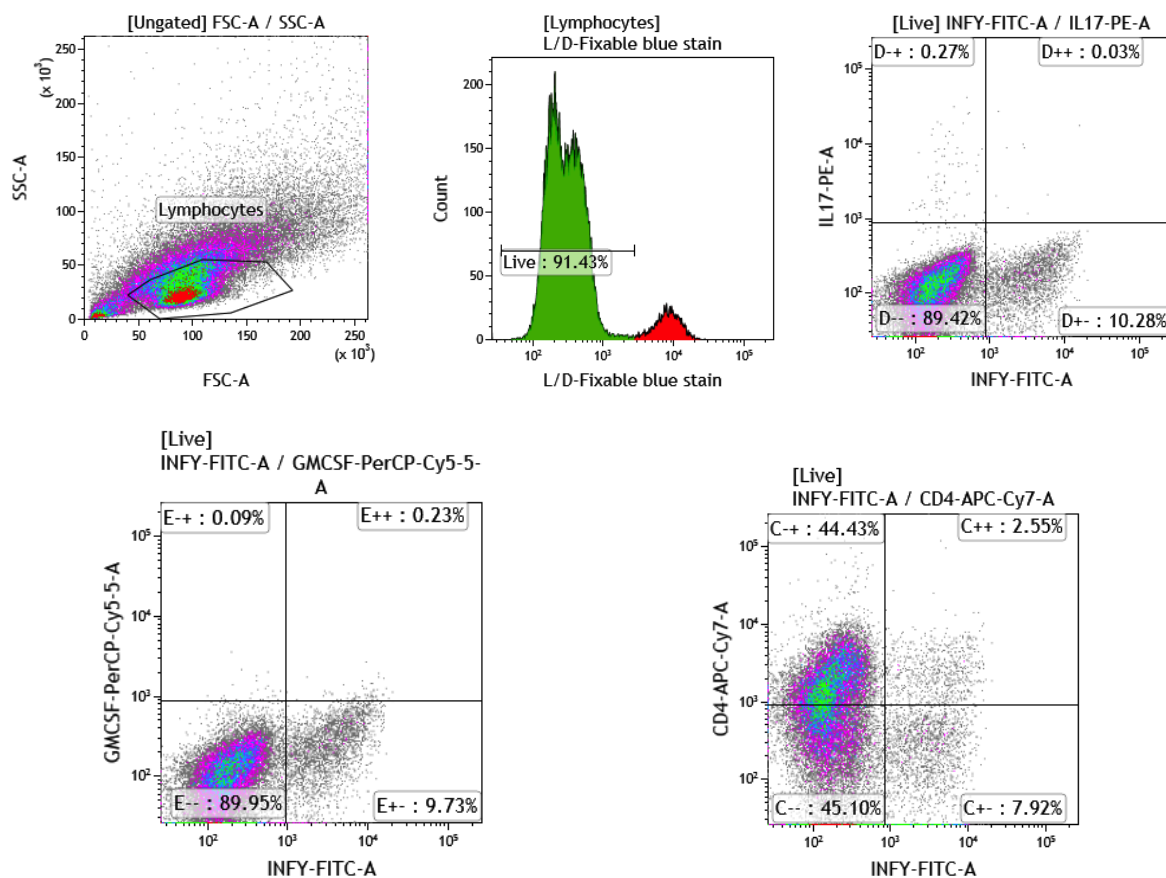


Figure 3.6: Representative flow cytometry analysis of live T cells from HC- MSC and PBMC. In panel A Lymphocytes are gated based on physical parameters side scatter (SSC) and forward scatter (FSC). Viability from Live/Dead stain is shown to be 91.43%. Using flow cytometry the activation of the cells were evaluated through the detection of IL-17, IFN- γ , GM-CSF and CD4 and their representative single colours. HC sample C012 shows 0.30% positive for IL-17, 0.32% positive for GM-CSF, 10.28% positive for IFN- γ and 44.43% positive expression of CD4+ T cells.

PBMC-Stimulated-Full stain- C012

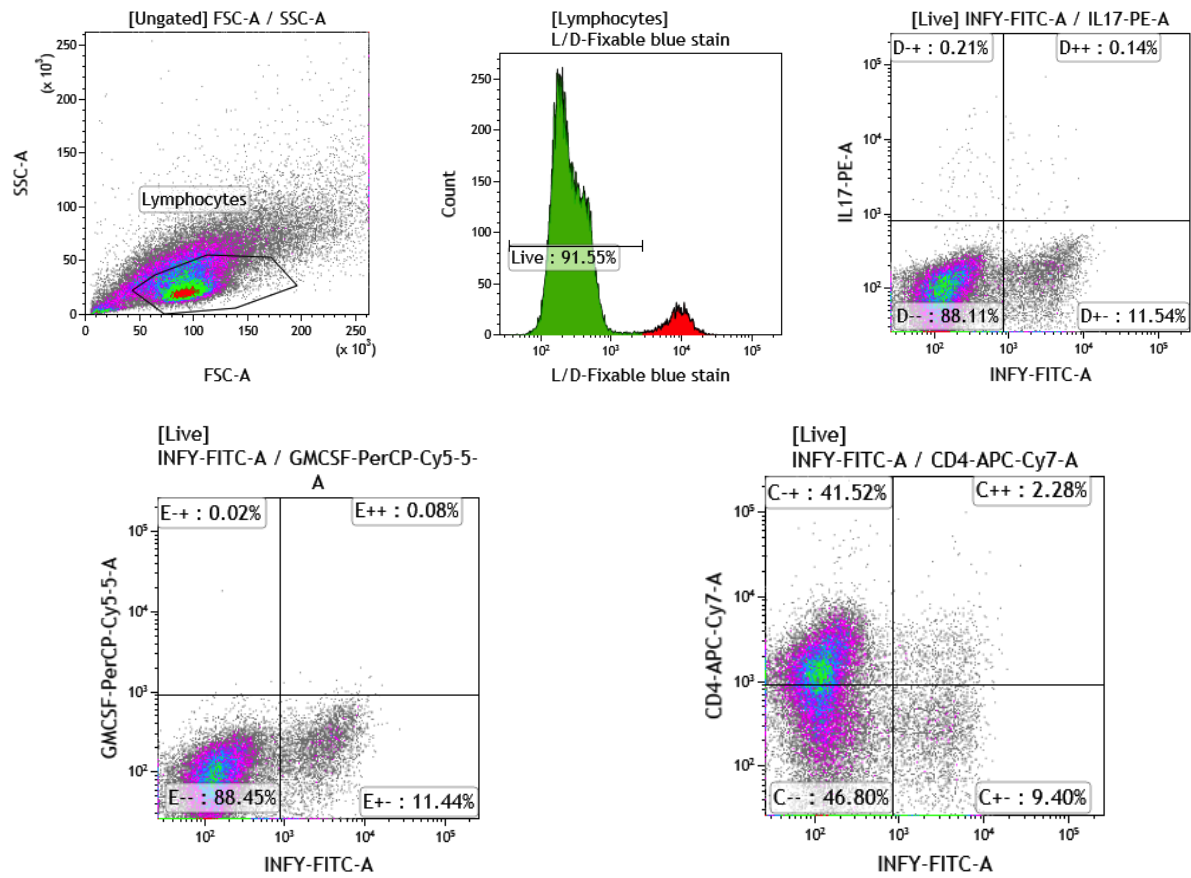


Figure 3.7: Representative flow cytometry analysis of live T cells from HC PBMC alone. In panel A Lymphocytes are gated based on physical parameters side scatter (SSC) and forward scatter (FSC). Viability from Live/Dead stain is shown to be 91.55%. Using flow cytometry the activation of the cells were evaluated through the detection of IL-17, IFN- γ , GM-CSF and CD4 and their representative single colours. HC sample C012 shows 0.34% positive for IL-17, 0.10% positive for GM-CSF, 11.54% positive for IFN- γ and 41.52% positive expression of CD4+ T cells.

Example of Flow Cytometer controls

Fluorescence minus one (FMO) controls were included to set the gating

Figure 3.8. below. Kaluza plot representative of FMO controls for PE-IL-17 and FitC- IFN- γ

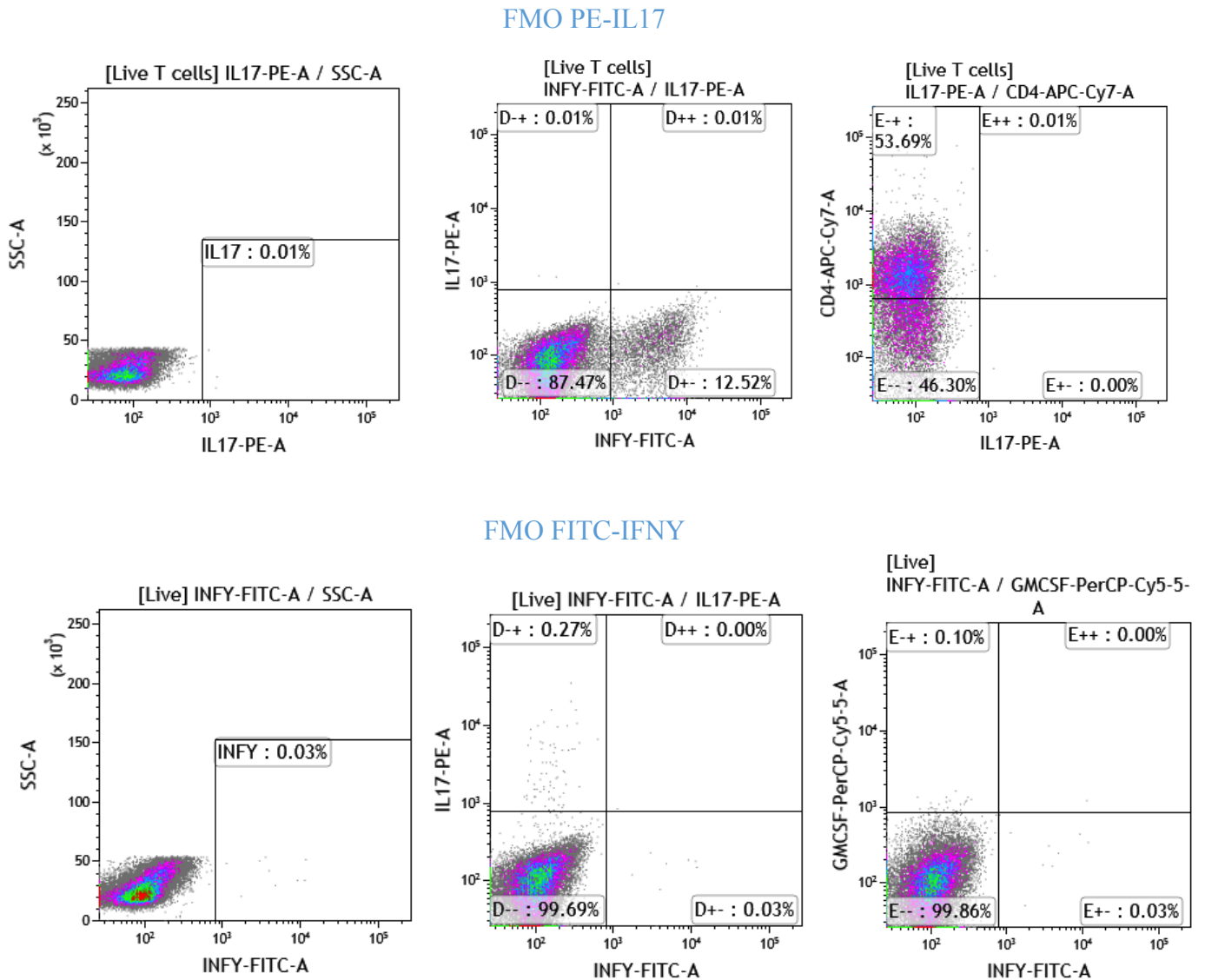
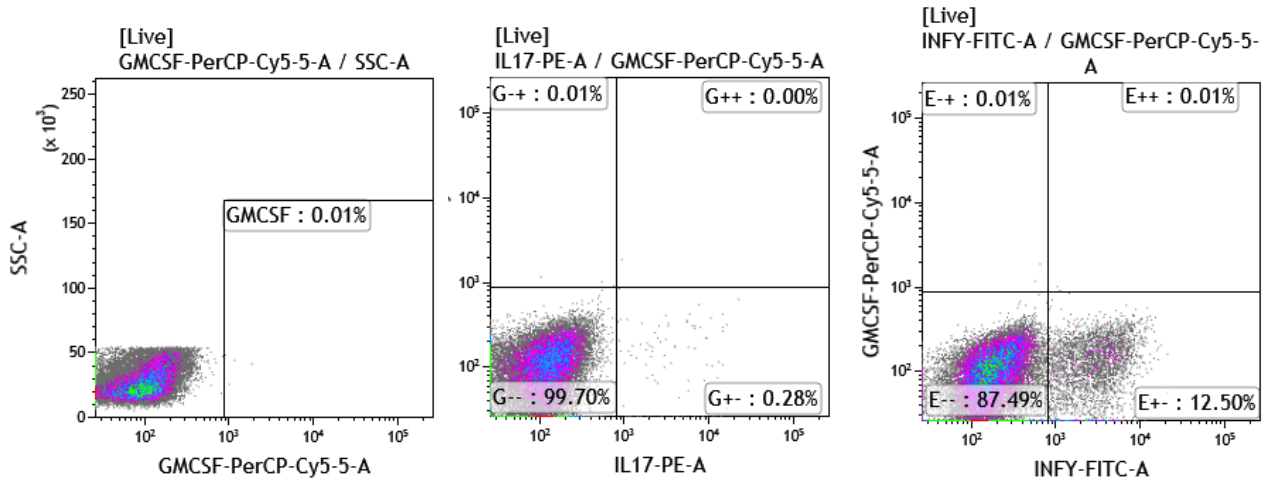
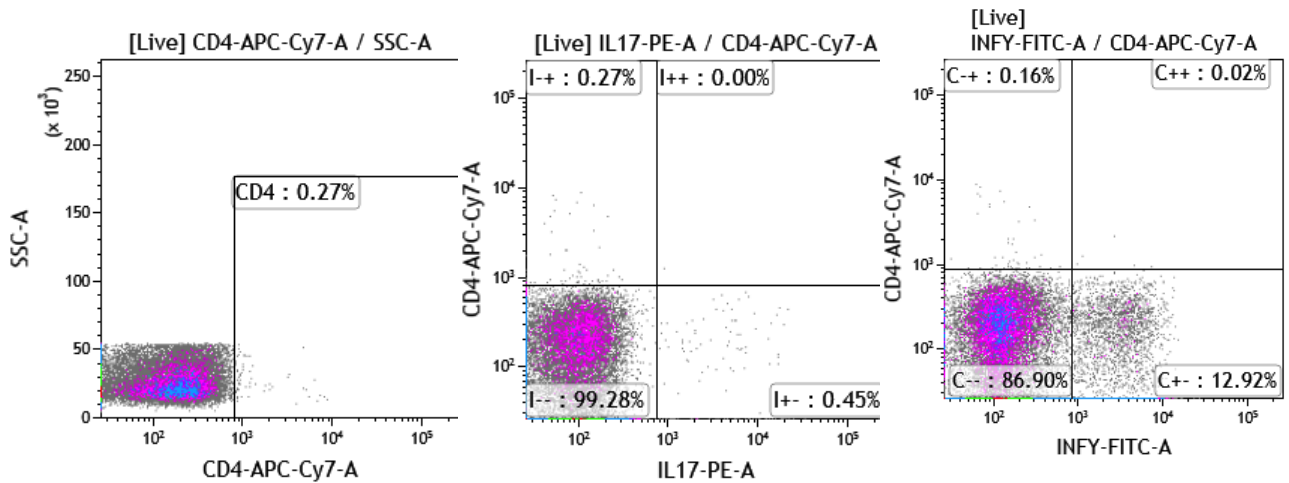


Figure 3.9 below. Kaluza plot representative of FMO controls for PerCP-Cy5.5-GM-CSF and APC- CD4

FMO PerCP-Cy5.5-GMCSF



FMO-APC-CD4



The above figures show representative examples of cytokine expression by stimulated PBMC from a healthy control and from an MS patient in the presence or absence of autologous MSC.

These data were collected from a total of 7 healthy controls and 5 MS patients. The percentages of cytokine positive cells were averaged. As there were wide differences in individual levels (within a relatively normal distribution) differences between stimulation results with and without MSC in co-culture for each inflammatory cytokine were also plotted.

Results from this study show that the activated PBMCs from MS patients in culture with autologous MSCs significantly ($p=0.011$) increased the percentage of IL-17 positive cells. whilst the percentage of IL-17+ cells was significantly ($p=0.045$) decreased in PBMC from healthy controls co-cultured with autologous MSC.

Interestingly, in this sample, the expression of IFN- γ by PBMC of healthy volunteers ($n=7$) was significantly higher than that of PBMC ($n=5$) from the volunteers with SPMS participating in this study ($p=0.016$). In co-culture with autologous MSC, the difference was maintained, with higher levels in controls than in MS patients ($p=0.02$), although both MS patients and controls had a numerical decrease in the percentage of IFN- γ + cells in the presence of MSC, which was not statistically significant. The differences between MS patients and controls in the proportion of IFN- γ + cells with and without MSC were not statistically significant (Figure 3.11)

There was a numerical reduction in the percentages of GM-CSF⁺ cells exposed to autologous MSC in both MS patients and healthy controls; however, the reduction did not reach statistical significance (Figure 3.12).

We also determined the effect of MSC-PBMC co-cultures on the percentages of cells positive for CD4, the universal marker of helper T cells that are important producers of the inflammatory cytokines studied. There were no statistically significant differences in the percentages of CD4⁺ T cells between MS patients and healthy controls either in the PBMC population alone or in the PBMC co-cultured to MSC.

Co – Culture (MSC+PBMC) Results

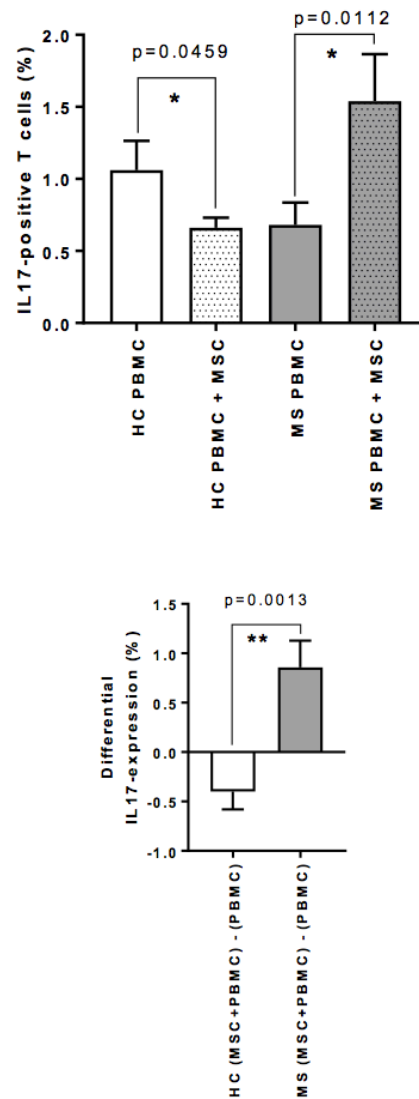


Figure 3.10. IL-17 Expression from MSC and autologous PBMC co-culture. Bar Chart representative of IL-17 expression shows significant increase ($p=0.012$) in the expression of IL-17 with the presence of MSCs in patient sample. IL-17 levels reduced in HCs with MSCs present. Differential IL-17 shows there is a significant difference ($p=0.001$) between HC and MS patients with MSCs present.

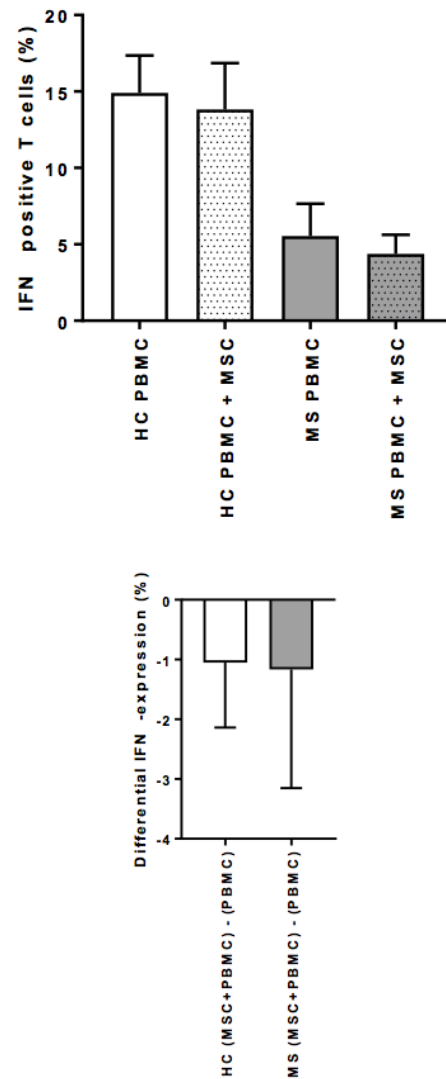


Figure 3.11. IFN- γ Expression from MSC and autologous PBMC co-culture. Bar Chart representative of IFN- γ expression shows reduced expression in patients with or without MSC. The differences in PBMC alone ($p=0.01$) and MSC + PBMC ($p=0.02$) are statistically significant.

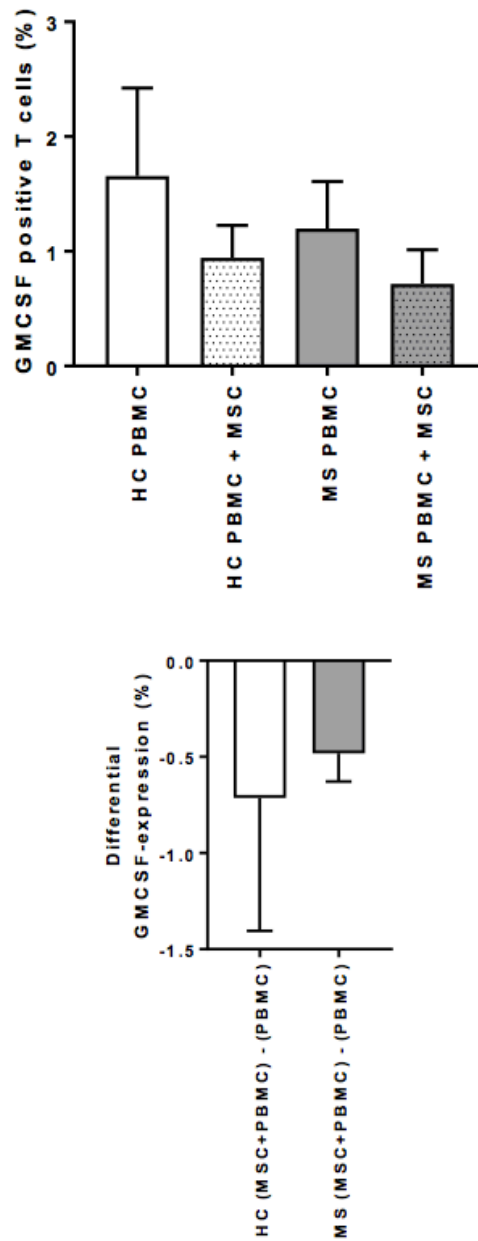


Figure 3.12. GM-CSF Expression from MSC and autologous PBMC co-culture. Bar Chart representative of GM-CSF expression shows no significant difference between HCs and MS patients with or without MSCs.

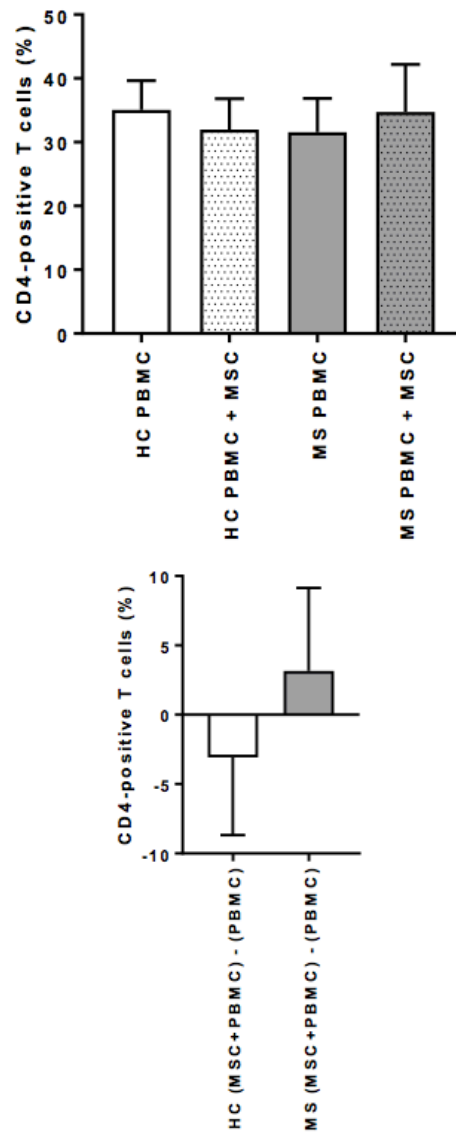


Figure 3.13. CD4 Expression from MSC and autologous PBMC co-culture. Bar Chart representative of CD4 expression shows no significant difference between HCs and MS patients with or without MSCs.

Cytokine profiling of Mesenchymal Stem Cells from patients with MS or healthy controls.

Since the immunomodulatory functions of MSCs are known to be exerted in substantial part by secretion of cytokines, growth factors, and chemokines, in addition to the direct cell-to-cell contact the investigation of the cytokine profile secreted by the MSC is of great interest.

We have applied a multiplex array based on the Luminex xMAP (multi-analyte profiling) technology, which enables the simultaneous detection of multiple secreted proteins.

We selected a number of cytokines and chemokines with known immune functions and known to be expressed by MSC and involved in the immunological effects of MSC.

We have selected an initial panel with the intention to investigate further markers in subsequent experiments that are not within the scope of this thesis.

Supernatants were tested after they were obtained from >95% pure MSC cultures from MS patients and controls at passages between 4 and 6. The cells were not stimulated prior to the collection of supernatants.

The cytokine/chemokine profile (figure 3.14) shows that the abundance of the anti-inflammatory, immunoregulatory cytokine, IL-10 was reduced in MSC culture supernatants from MS patients compared to controls ($p=0.02$).

There were also decreased IL-8 levels in MS MSC supernatants compared to controls ($p=0.03$). There were no differences in the proportion of IL-6, IL-1, TNF, and GM-CSF between HC or MS patients, despite an insignificant trend for the latter. IL-12 was measured as well, but the levels were not detectable in any of the unstimulated MSC.

Figure 3.14 shows bar graphs represented means and standard errors of the mean for the detectable cytokines from these supernatants.

Cytokines Profile from MSC

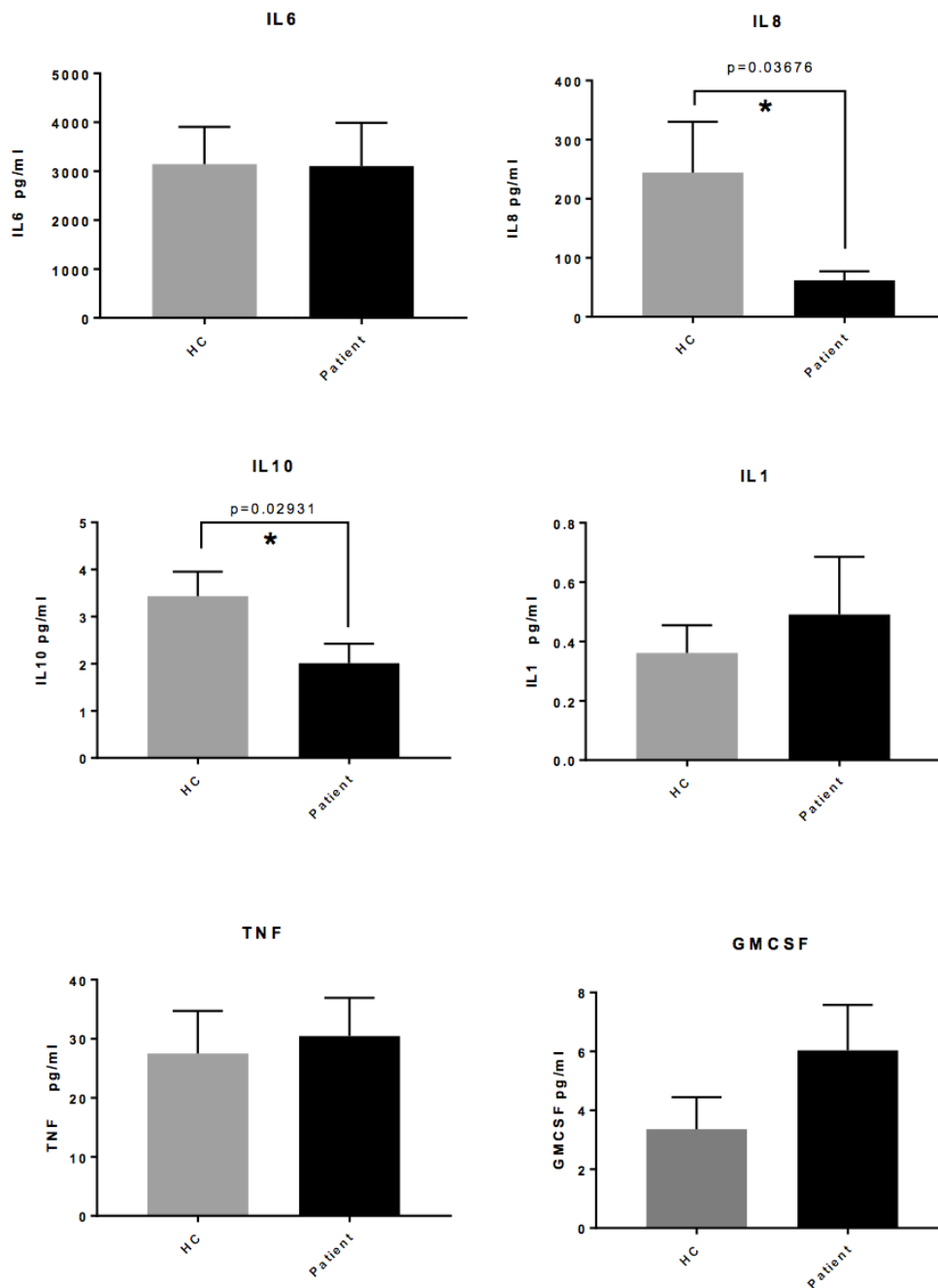


Figure 3.14. Comparison of cytokine profile from MS patients and HCs. Bar chart of 6 cytokines expression levels in MSC tissue culture supernatant from MS patients and HCs. Data is representative of 5 different MS-MSC supernatants and 7 different HC-MSC supernatants showing the mean and SEM for the expression of each cytokine. IL-8 and IL-10 showing a significant decrease in supernatants from MS patients when compared to HC.

RNA Extraction Results

Although RNA was not analysed as part of this thesis, I have performed the extraction of RNA as part of the MRes work. The RNA will be used in future experiments continuing this work, to obtain a quantitative estimate of differential gene expression of unstimulated MSC from MS patients and healthy controls. Initially a microarray panel will be tested following validation by quantitative real time polymerase chain reaction.

Table 3.1 RNA extraction levels from MS patients and HCs

	Sample ID	Nucleic Acid conc	Unit	Sample type	Factor
1	C012	9.6	ng/ μ l	RNA	40
2	C004-p5	58.6	ng/ μ l	RNA	40
3	P018-p7	49.2	ng/ μ l	RNA	40
4	P018-p4	125.9	ng/ μ l	RNA	40
5	P017-p4	125.7	ng/ μ l	RNA	40
6	C016-p4	176.2	ng/ μ l	RNA	40
7	C009-p4	359.4	ng/ μ l	RNA	40
8	C009-p2	166.6	ng/ μ l	RNA	40

High Pure RNA Isolation Kit, Roche and protocol was used to purify RNA from cultured MSC from MS patients and HCs. RNA extraction protocol was carried out when the cells (passage 4 to 6) were 80%+ confluent. Table 3.1 shows the sample ID and nucleic acid concentration for each sample.

Discussion

Substantial research has been carried out over the years investigating MSCs and their immunomodulatory properties. Data has underscored their anti-inflammatory effects increasing their potential use as a therapeutic form of treatment in MS (Rozenburg *et al.*, 2016).

Despite an increased interest in the therapeutic potential of MSC in a variety of conditions, and lately also, importantly, in MS, the actual data on the role of MSC in MS and their potential mechanisms of action remain scarce. In particular, there is little knowledge about how MSC from people with MS differ from those considered to be healthy controls without MS. Moreover, very few studies investigated the interaction of MSC with immune cells from the same donor. Therefore, we investigated whether MSC from MS patients have the same purported ability to immunomodulate as those from healthy people.

The hypothesis of this study was that MSCs from people with secondary progressive MS, having been exposed *in vivo* to an inflammatory environment for a long period of time, have a reduced immunomodulatory capacity compared to those from healthy controls. We had postulated that this may be due to reduced production of immunomodulatory substances or due to enhanced inflammatory state of immune cells from MS patients, or even both.

Contrary to previous results, largely derived from studies from the animal model EAE, emphasising the immunomodulatory effects of MSC, we noted that MSCs induce IL-17 expression, rather than reduce it, in MS patients, but it reduced slightly it in healthy controls. This is consistent with some of the previous studies. Darlington *et al.* (2010) showed that MSC derived from patients with MS suppressed Th1 but enhanced Th17 cells in co-culture

with heterologous T cells differentiated in vitro towards Th1 or Th17 phenotypes. When MSC were stimulated with IL-1 before the exposure to T cells, these effects were enhanced, and there was a reduction in the Th1/Th17 double expressing cells. In a further study, the same group Rozenberg *et al.*, (2016) confirmed this ability of MSC to enhance Th17 responses, inviting caution in future studies using MSC therapeutically in autoimmune diseases where Th17 or Th1/Th17 cells are thought to be important, including MS. Our presumption is that in this second study, MSC were also from people with MS and the T cells, differentiated into Th1 and Th17, were again, obtained from healthy controls. This is not specified in the paper, but the source of MSC is an MS centre conducting an MS trial (Mellen Center, Cleveland Clinic), and the T cells are obtained from donors from the McGill University community, in Montreal.

Our study explores, to our knowledge for the first time, the interactions of MSC with autologous immune cells from the same donors, and is compared alongside the MSC from patients with SPMS to those from healthy controls. The differential effect on Th17 cells, with the enhancement of Th17 in MS patients and reduction in healthy controls is noted, while the MS data is consistent with previous study, it indicates that MSC from healthy controls, without MS, have a higher immunomodulatory potential with respect to Th17.

With regard to IFN- γ , the effects of MSC were slightly reduced for both MS patients and healthy controls, but insufficient to reach significance. Thus, contrary to previous results (Rozenberg et al 2016, Darlington et al 2010), MSC did not significantly decrease Th1 cells. The role of IFN- γ and Th1 cells in MS remains unclear, but it is generally thought that IL-17 and Th17 cells are more prominent and more disease relevant. Indeed, depletion of Th17 but not Th1 cells underlines the dramatic effect of haematopoietic stem cell transplantation in MS

(Darlington et al 2013). Therefore, the ability of MSC from MS patients to enhance autologous Th17 cells could possibly outweigh their effect on Th1 cells.

The Th1/Th17 subset is a small subset that co-expresses IFN- γ and IL-17 and are thought to be pro-inflammatory. We have shown an up-regulation in MS relapses (Edwards *et al.*, 2010). The percentages of these cells in our studies shown here was too small to warrant any sort of analysis, but future studies will be important to determine whether and how these cells are modulated by MSCs. In the study carried out by Darlington and colleagues (2010) Th1/Th17 cells were suppressed by MSCs, after pre-exposure of MSCs to IL-1. Of note, these studies were performed on MS-MSCs in co-culture with T cells that had already differentiated into Th17, Th1, or Th1/Th17 cells, in contrast to our study which used PBMC with a slightly less than half of the live cells being T helper cells. We think, however, that the use of PBMC unseparated into cellular subsets reflects the mixture of cellularity encountered in inflammatory conditions.

GM-CSF is emerging as a critical cytokine for autoimmunity, particularly in MS. Studies parallel to this one from our laboratory have shown a dysregulation of GM-CSF in MS. This data is currently unpublished (Hartmann et al 2014). Here, we show a numerical down-regulation of GM-CSF expressing cells by MSCs in both MS and controls, but this is not statistically significant. Also PBMC alone from MS patients tend to express more GM-CSF than those from healthy controls, but this is not significant in this small sample. Future studies may need to address this question more systematically.

Our results suggest that the effects of MSCs co-culture on cytokine, which are primarily T cell cytokines, expression by PBMC are due to a real modulation either by cell contact or by soluble mediators, and not by an overall decrease in number of T cells. This is shown by the fact that exposure of PBMC to MSCs did not significantly affect the proportion of CD4⁺ cells, either in MS patients or in healthy controls. This is also reassuring in estimating the proportion of cytokine positive cells that is represented by CD4 T cells, in view of the theoretical consideration that stimulation of T cells with phorbol esters down-regulates surface CD4 (Petersen *et al.*, 1992).

A significant finding in our multiplex cytokine array was that unstimulated MSCs from MS patients contains less IL-10 than healthy controls. This is important as it underscores a reduced immunomodulatory potential of MSCs from MS patients, and is congruent with the findings of an enhanced Th17 response. Although our study failed to show differences in IL-6, an inducer of IL-17, in the MSCs supernatants from MS patients and healthy controls, the increased IL-17 in MS patients exposed to autologous MSC could possibly reflect the inability of these MSCs to regulate Th17 cells in MS, due to reduced ability to secrete IL-10. IL-10 is a known suppressor of inflammatory T cells, including Th17 cells. Our findings are also consistent with findings of Darlington and colleagues (2010) who found that conditioned media from the same MSCs, from MS patients, that enhanced Th17 cells, also induced less IL-10 in T cells.

Our study revealed higher levels of the chemokine IL-8 in healthy controls compared to MS patients. This finding is potentially paradoxical and quite difficult to explain.

IL-8 is a chemoattractant cytokine that is produced by various blood cells and tissues (Bickel, 1995). It is a cytokine responsible for the attraction of neutrophils. Although neutrophils and

IL-8 are not thought to play a major role in MS pathogenesis, a higher production of this proinflammatory cytokine would have appeared more plausible in MSC from MS patients. MSC are known to reduce neutrophil recruitment to inflamed endothelium (though not trans-endothelial migration), but possibly this effect may require co-culture with endothelial cells. Also, the soluble mediator responsible for MSC-induced inhibition of neutrophil recruitment is not IL-8 but IL-6 (Munir et al 2016). This is another seemingly paradoxical, anti-inflammatory action of a proinflammatory cytokine.

In this study, we did not pre-stimulate MSCs with inflammatory molecules before co-culture or before collection of conditioned media. This is because we intended to assess the immunomodulatory properties of MSCs without prior manipulation. The assumption was that MSCs from patients with SPMS had already been exposed in vivo to an environment of chronic inflammation, and they may carry this signature ex vivo. This differs from the previous studies cited above, where MSCs were pre-exposed to IL-1b. The lack of detection of some cytokines measured and low levels of others may be attributed to the use of unstimulated cells, but we think the results may reflect better the intrinsic characteristics of the MSCs. On the other hand, the fact that some results were consistent with those of other studies using pre-stimulation of MSCs, validates our findings.

The strengths of our study are that it uniquely compares, side to side, immune effects of MSCs in autologous immune cells from MS patients and non-MS healthy controls. Thus the reciprocal regulation of Th1 and Th17 by MSCs seems to hold primarily for MSCs from MS patients. The study confirms that future therapeutic trials on MSCs in MS need to proceed with significant caution, and with careful immune monitoring. Another strength of our study is that it allows a first glimpse into mechanisms of the reduced immunoregulation of Th17

cells by MSCs in MS. The fact that, in this small sample, there were no differences in the proportions of Th17 cells expressed by PBMC without MSCs, suggested that the deficiency of immunoregulation by MS MSCs is not due to having to deal with more Th17 cells, but that they are less capable of suppressing equivalent numbers of Th17 cells. This indicated that MSCs from MS patients have decreased immunoregulatory molecules. In the limited time for the experiments, we identify one of the deficient immunomodulatory factors as IL-10. Deficient IL-10 production by MSCs may underline the reduced immune regulation by MS-MSCs. Other factors which could contribute include TGF- β , and this will be explored in the future. In addition, the RNA extraction during these experiments will allow a more unbiased search for an array of reduced immunoregulatory or pro-inflammatory factors in MS-MSCs compared to healthy control, MSCs. Preliminary western blot studies on the MSCs in this study also show a reduction in immunomodulatory indolamine deoxygenase (IDO) immunoreactivity in MS MSCs compared to MSCs from controls, leading to another potential mechanism (data not shown).

There were some weaknesses of the study, the first being the number of samples from both MS patients and healthy controls was low. This was due to the difficult recruitment of altruistic bone marrow donors with or without MS, the former with the knowledge that this is not a therapeutic, but simply a research study. Also, among the recruited donors, some cells failed to grow and proliferate, and MSCs could not be obtained, particularly from MS patients. Despite this, the small sample size yielded results that were consistent with other studies, but also showed the level of variability between individuals, which needs to be taken into account in future studies. Some differences, e.g. GM-CSF, may have been significant in a larger sample.

The short experimental time allocated to this research project allowed the investigation of only a small number of cytokines, chemokines and growth factors in the conditioned media of MSCs. Other molecules of interest, known or suspected to be produced by MSCs, have not been investigated, but some of these are part of a second multiplex assay to be done soon. Microarrays will also be performed on the RNA.

One potential limitation was that we looked at cytokine expressing cells rather than cytokine levels. However, from previous studies e.g. Jung *et al.*, 1993, it is known that quantifying cytokine expressing cells correlated with levels detected by ELISA, for example. Another weakness was that we did not look at the effects of MSCs on anti-inflammatory cytokine expression by immune cells in co-culture or at foxp3 expression, a marker of regulatory T_{regs}. This will be part of future studies.

Among the soluble factors that have not been investigated, although I have learned the necessary techniques, are exosomes of MSCs. Supernatants have also been collected for the study of exosomes, and again this is part of future studies.

Future Directions

Future studies will need to explore more the immunoregulatory deficiencies of MSCs in MS as outlined above. Parallel studies in the laboratory have explored the imaging potential of the MSCs prior to transplantation, and, despite the potentially insufficient immune modulation achieved by these cells from MS patients, their neuroregenerative potential may be retained, and need to be explored. Their ability to travel to sites of nerve damage, e.g. the CNS, needs to be investigated.

Conclusion

MSCs show great promise in therapeutic use in MS. However, there has been large variability in the results from this study. There is a possibility that MSCs from MS patients could be defective in their immunoregulatory ability, in particular with regard to the Th17 subset. The translation to therapeutic clinical trials is feasible, but it will require caution and close monitoring.

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