Immunomodulatory therapies for autoimmune diseases

By

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Declaration of authorship

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Jamal Sulaimani
Abstract

Multiple sclerosis (MS) and psoriasis are both T cell mediated autoimmune diseases that are triggered in genetically susceptible individuals in response to environmental factors. Th17 cells are strongly implicated in the pathogenesis of both MS and psoriasis, whereas Treg cells suppress effector T cell responses and prevent autoimmunity, however, their function has been shown to be impaired in both diseases. Therefore, targeting the Treg:Th17 axis is of great therapeutic interest in such autoimmune diseases. Therapies for both MS and psoriasis include a range of biologics or small molecules, some of which have very specific mechanisms of action whereas others have broader and less well understood mechanisms of action. Unfortunately most of these therapies can be associated with undesirable side effects and not all patients respond to treatment. Therefore, there is a need for safer therapies and a better understanding of the mechanisms of action of existing therapies.

It has been established that vitamin D can exert anti-inflammatory effects in vitro and in vivo in the animal model for MS, prompting the idea of testing oral vitamin D3 supplementation as a therapy for MS. Vitamin D3 is an attractive therapy as it is safe and taken orally, however its efficacy in MS has not yet been established. A double-blind placebo-controlled randomised trial was conducted to examine the immunomodulatory effects of vitamin D3 in healthy controls (HC) (n=38) and clinically isolated syndrome (CIS) patients (n=29). CIS patients, who are those who have experienced only a single clinical neurological episode, were selected since they do not qualify for standard therapies, allowing for the effects of vitamin D3 supplementation to be assessed in isolation. However, although vitamin D3 supplementation increased serum 25(OH)D from baseline in both HC and CIS patients, no clinical or immunomodulatory effects on T cell subsets were observed and the trial did not meet its endpoints.

Dimethyl fumarate (DMF) is the active ingredient in Fumaderm™, an oral drug which is used for the treatment of psoriasis, and DMF is now also used to treat MS. However, there is a need to better understand the mechanism of action of DMF both in vitro, and in vivo in psoriasis patients since it is poorly understood. Thus, the immunomodulatory effects of both vitamin D3 and DMF were examined in this study. In vitro studies using DMF revealed that the oxidative stress induced by DMF provided a relative advantage to Treg cells via their increased ability to resist oxidative stress. Furthermore, an increased frequency of Treg cells was also observed in psoriasis patients who had been treated with Fumaderm™, and this was associated with a significant decrease in memory CD4+ T cells and more specifically Th17 lineage cells.

In summary, this study has provided useful information on the safety and dosage of vitamin D3 supplementation, but could not demonstrate clinical efficacy or immunomodulatory effects on T cell subsets. However this study has provided novel insights into the mechanism of action of DMF in vitro and in vivo, demonstrating that DMF modulated the Treg:Th17 axis in favour of Treg cells both in vitro and in vivo in psoriasis patients treated with Fumaderm™.
Acknowledgements

I would like to express my deepest gratitude and appreciation to Prof. Jean Fletcher. You have been the most wonderful supervisor any PhD student could ask for. You have been the most inspiring person I have ever come across, and you have taught me so much, not only about science but about myself. You are a book of knowledge and wisdom, and every day I appreciate how lucky I am to have had you as my friend and supervisor for the duration of this project. I will never be able to repay you for your endless patience and encouragement. I am thankful for all the time you made for all of my questions. I am also very thankful that you introduced me to meditation and got me to seek help when I needed it most, it really changed my perspective on life. You are the kindest person and I am blessed to have gained the knowledge that I have today. I would also like to thank the Saudi Cultural Bureau for funding me for the four years, this wouldn’t have been possible without this funding.

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"إذا كان عقلك فارغًا فهو دائمًا جاهز لأي شيء؛ إنها مفتوحة على كل شيء. في عقل المبتدئين هناك العديد من الاحتمالات؛ ولكن في عقل الخبراء هناك عدد قليل"
- شونرو زو زكي

“If your mind is empty, it is always ready for anything; it is open to everything. In the beginners mind there are many possibilities; in the experts mind there are few”
- Shunryu Suzuki
**List of abbreviations**

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<thead>
<tr>
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<th>Definition</th>
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<tr>
<td>1,25(OH)(_2)D(_3)</td>
<td>1, 25-dihydroxyvitamin D(_3)</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-hydroxyvitamin D</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response elements</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<td>BMDC</td>
<td>bone marrow derived dendritic cell</td>
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<td>Bregs</td>
<td>regulatory B cells</td>
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<tr>
<td>BSA</td>
<td>body surface area</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>calcium</td>
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<tr>
<td>CCL</td>
<td>chemokine ligand</td>
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<td>CCR</td>
<td>chemokine receptor</td>
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<tr>
<td>CIS</td>
<td>clinically isolated syndrome</td>
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<tr>
<td>CM</td>
<td>central memory T cell</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>cRPMI</td>
<td>complete RPMI</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
</tr>
<tr>
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<td>celltrace violet</td>
</tr>
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<td>CYP</td>
<td>cytochrome p450</td>
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<td>DAMPS</td>
<td>danger associated molecular patterns</td>
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<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl fumarate</td>
</tr>
<tr>
<td>DMT</td>
<td>disease modifying therapies</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBV</td>
<td>epstein-barr virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immune sorbent assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>EM</td>
<td>effector memory T cell</td>
</tr>
<tr>
<td>EMA</td>
<td>European medicine agency</td>
</tr>
<tr>
<td>EPRE</td>
<td>Electrophile response elements</td>
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<td>ETC</td>
<td>Electron transport chain</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FAE</td>
<td>Fumaric acid esters</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
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<tr>
<td>Foxp3</td>
<td>Factor forkhead box p3</td>
</tr>
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<td>GA</td>
<td>Glatiramer acetate</td>
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<tr>
<td>GADPH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GlyCAM-1</td>
<td>Glycosylation dependent cell adhesion molecule-1</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>GS-DMS</td>
<td>S-(1,2-dimethoxycarbonylethyl) glutathione</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy controls</td>
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<tr>
<td>HCA2</td>
<td>Hydroxycarboxylic acid receptor</td>
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<td>HEV</td>
<td>High endothelial venule</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor 1 alpha</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HLB</td>
<td>Hydrophilic lipophilic balance</td>
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<td>HO-1</td>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour</td>
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<tr>
<td>HRP</td>
<td>Horseradish-peroxide</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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</tr>
<tr>
<td>IL-2RA</td>
<td>interleukin-2 receptor-a</td>
</tr>
<tr>
<td>IM</td>
<td>infectious mononucleosis</td>
</tr>
<tr>
<td>ITP</td>
<td>idiopathic thrombocytopenic purpura</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>JCV</td>
<td>John Cunningham virus</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function associated antigen 1</td>
</tr>
<tr>
<td>MACS</td>
<td>magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>mDC</td>
<td>myeloid dendritic cells</td>
</tr>
<tr>
<td>MFI</td>
<td>median fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Min</td>
<td>minutes</td>
</tr>
<tr>
<td>MMF</td>
<td>monomethyl fumarate</td>
</tr>
<tr>
<td>mPM</td>
<td>mice peritoneal macrophages</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NET</td>
<td>neutrophil extracellular traps</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NF-AT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear factor E related transcription factor</td>
</tr>
<tr>
<td>PAD</td>
<td>protein arginine deiminase</td>
</tr>
<tr>
<td>PAMPS</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PASI</td>
<td>psoriasis activity and severity index</td>
</tr>
</tbody>
</table>
PBS  phosphate buffered saline
PHA  phytohaemagglutinin
PKCθ  protein kinase c theta
PMA  phorbol 12 myristate 13 acetate
PML  progressive multifocal leukoencephalopathy
PPMS  primary progressive multiple sclerosis
PRR  pathogen recognition receptors
PSORIS1  psoriasis susceptibility 1
PTH  parathyroid hormone
PUVA  psoralen-ultraviolet A
RLR  RIG-I-like receptors
RNA  ribonucleic acid
RNS  reactive nitrogen species
ROS  reactive oxygen species
RPMI  roswell park memorial institute
RRMS  relapsing remitting multiple sclerosis
RXR  retinoid X receptor
slanDC  6-sulfo LacNAc DC
SNP  single nucleotide polymorphisms
SPMS  secondary progressive multiple sclerosis
STAT  signal transducer and activator of transcription
TCR  T cell receptor
Tconv  T conventional cells
Th  helper T cell
TLR  toll-like receptors
TNF  tumor necrosis factor
TQD  triple quadruple mass
Treg  regulatory T cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TT</td>
<td>tetanus toxoid</td>
</tr>
<tr>
<td>UVB</td>
<td>ultraviolet B</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>vitamin D response elements</td>
</tr>
<tr>
<td>Wk</td>
<td>weeks</td>
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Chapter 1:

Introduction
1.1 Inflammation and the immune system

Inflammation is a process that is normally initiated to combat infection and is a rapid response that involves molecular and cellular mediators. At the site of infection, cells are recruited to eliminate foreign pathogens, activating an immune response. There are five clinical signs of inflammation: redness, heat, loss of function, swelling, and pain. During inflammation, the first lines of defence are innate cells, including neutrophils, macrophages, dendritic cells (DC) and NK cells. These innate cells will subsequently activate the adaptive immune response which consists of B and T lymphocytes. In addition to its role in resolving infection however, inflammation can also lead to tissue damage as a result of chronic inflammation.

1.1.1 The innate immune response

The immune system as a whole is responsible for defending against pathogens, without causing harm to the body, by distinguishing between self and non-self. The innate immune response, which is the first line of defence, is rapid and immediate, but does not lead to lasting immunity. It is broadly specific in its response to detecting and eliminating a range of microbes. Innate immunity includes external barriers such as the skin, and mucosal membranes that line the digestive system, respiratory, and genitourinary tracts that prevent the entry of harmful microbes. The innate immune system relies on receptors that can identify features that are common to pathogens. These receptors can recognise gram positive and negative bacteria and ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) from viruses, fungi and protozoa (Creagh and O’Neill, 2006, O’Neill, 2005). These receptors are known as pathogen recognition receptors (PRR), and include toll-like receptors (TLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR). These PRRs identify pathogen-associated molecular patterns (PAMPs) expressed by pathogens, or endogenous danger associated molecular patterns (DAMPs). PRRs can recognise different PAMP or DAMP: for example, TLR3, TLR7, TLR8, and TLR9 and RLRs can detect viral DNA or RNA, whereas TLR2, TLR4 and NLRs such as NLRP1, NLRP3 and Ipaf can detect bacterial products. When a PRR recognises a PAMP from an invading pathogen, intracellular signalling pathways are activated which perpetuate inflammation via production of cytokines and chemokines (Murphy and Weaver, 2016, Creagh and O’Neill, 2006, O’Neill, 2005).

PRRs can be divided into four main groups depending on their localisation and function within the cell. These include membrane bound free receptors, phagocytic receptors, membrane bound signalling receptors, and cytoplasmic signalling receptors. Once a PRR is ligated, it activates professional antigen presenting cells (APC) such as DC, macrophages,
and monocytes to ingest a pathogen. Once these pathogens have been phagocytosed, they are processed into peptides that will be presented on major histocompatibility complex (MHC) molecules on the cell surface to T cells. Recognition of these cognate antigens by lymphocytes, together with the ligation of costimulatory molecules and the production of cytokines by APC, activates the adaptive immune response. Antigens presented by MHC class II can activate CD4+ T cells, whereas those presented by MHC class I molecules activate CD8+ T cells. Thus, the innate immune system has a major role in shaping the adaptive immune system (Murphy and Weaver, 2016, Creagh and O’Neill, 2006, O’Neill, 2005).

1.1.1 DC

The most important APC in the activation of T cells are DC. The important function of DC is to ingest and present antigen. These DC are a crucial link between the innate and adaptive response, they do so by presenting antigens to the appropriate naïve T cell, which induces a T cell immune response. DC are derived from pluripotent hematopoietic stem cells before they reside in specific locations in the body. Immature DC migrate through the blood from the bone marrow to enter the tissue. Once they encounter a pathogen they mature and activate and migrate to the lymph nodes where they encounter T cells. The immature phenotype of DC are associated with low expression of MHC proteins and CD80/CD86 (B7.1/7.2) or ICAM co stimulatory molecules. Meaning they are not yet equipped to activate naïve T cells, however, they are very active at taking up antigen either by pinocytosis or phagocytosis. These immature DC can recognise pathogens by using TLRs that can recognise PAMPs, this engagement results in the upregulation of co-stimulatory molecules which enhances antigen presentation. Following from antigen uptake the DC becomes activated and the TLR signalling induces chemokine receptor 7 (CCR7) and it also enhances the processing of the pathogen derived antigen. CCR7 directs the migration of DC into the lymphoid tissues and increases the expression of co stimulatory molecules and MHC molecules. Once in the draining lymph node the mature DC loses its ability to phagocytose and become phenotypically APC (Murphy and Weaver, 2016).

1.1.2 The adaptive immune response

The induction of the adaptive immune response is essential for long-term immunity and follows the initial innate response. The fundamental features of specificity, diversity, and immunological memory define the adaptive response. The adaptive immune system consists of T cells and B cells, which have the ability to recognise an infinite array of pathogens using antigen receptors. Their ability to generate such a large repertoire of antigen receptors is due
to somatic recombination of gene segments. Adaptive immunity involves both humoral and cell-mediated immunity. Humoral responses involve B cells, which produce antibodies to target pathogens. On the other hand, T cells mediate cell-mediated immunity via secretion of cytokines and cytotoxicity. Both of these mechanisms contribute to pathogen clearance by directly killing the invading pathogen or enhancing other immune responses. B and T cells are derived from bone marrow pluripotent hematopoietic stem cells, which give rise to common lymphoid progenitors that develop into the lymphoid lineage of antigen-specific lymphocytes. These lymphocytes are inactive, or naïve, until they have been primed with a specific antigen. Once activation by their cognate antigen has occurred and they have undergone proliferation and differentiation, they become fully functional effector lymphocytes. During an immune response, lymphocytes will also differentiate into memory cells. Upon second exposure to their specific antigen, these memory cells rapidly become effector cells in order to target infection more quickly and effectively. Memory cells are long lived whereas most effector cells will die by apoptosis after an immune response has been resolved. During their development in the primary lymphoid organs, the reactivity of these lymphocytes towards self-molecules are tested and strongly self-reactive lymphocytes are deleted, in a process known as clonal deletion. However, some auto-reactive lymphocytes can escape this process, which can lead to autoimmunity (Murphy and Weaver, 2016, Wakim and Bevan, 2010).

1.1.2.1 T cells
Lymphoid progenitors in the bone marrow migrate to the thymus, where they develop into T cells after undergoing both positive and negative selection and lineage engagement. The selection of these T cells is based on the T cell receptor (TCR) signal strength, which has profound effects on the development of T cells. TCRs most commonly consist of α and β chains, however some T cells convey equivalent receptors that consist of a different pair of polypeptide chains, γ and δ. Initially, thymocytes or pro-T cells are double negative for CD4, CD8 and negative for the CD3 complex and it is at this point that TCRβ gene recombination occurs. TCRβ is expressed once VDJ recombination is completed; at this point the TCRβ chain becomes expressed on the cell surface alongside an invariant protein called pre-Tα after VDJ recombination is also completed, which forms the pre-TCR complex of pre-T cells. The pre-TCR complex promotes intracellular signals that can promote expression of the TCRα. Once the TCR complex is completed, the pre-T cell becomes an immature double positive T cell that expresses both CD4 and CD8. During thymic selection, MHC molecules on thymic epithelial cells will present self-peptides to double positive immature T cells.
Positive selection is the rescue of double positive T cells from programmed cell death and the maturation of either CD4 or CD8 T cells. While negative selection is where double positive T cells react too strongly to self-MHC undergo apoptosis, which eliminates self-reactive T cells. During positive and negative selection, cells that react appropriately to self-MHC are passed on to mature, while cells that react with high affinity to self-MHC are eliminated by apoptosis; this process is negative selection. Immature T cells that recognise MHC I or MHC II molecules through their TCR determine the lineage of the T cell. A successful MHC I interaction directs the development of the double positive pre-T cell into CD8+ T cells, while MHC II interaction leads to the development of a CD4+ T cell. Double positive T cells that fail to recognise MHC molecule do not undergo positive selection which causes these double positive T cells to undergo apoptosis death by neglect. The elimination of these self-reactive thymocytes is important in preventing autoimmunity, however in some circumstances, high affinity self-reactive T cells can escape thymic selection undetected, thereby contributing to autoimmunity. Once selected for survival and maturation, mature but naïve T cells migrate to the peripheral lymphoid organs such as lymph nodes and spleen, where they anticipate an interaction by an activated APC. This process is regulated by chemokines and adhesion molecules. The first step is the binding of L-selectin on naïve T cells to glycosylation dependent cell adhesion molecule-1 (GlyCAM-1) and CD34 on high endothelial venule (HEV) which allows for the rolling interaction. Secondly chemokines such as CCL21 on HEV stimulate chemokine receptor CCR7 which ultimately activates lymphocyte function associated antigen 1 (LFA-1). Finally, LFA-1 activation allows the T cell to tightly bind to intercellular adhesion molecule 1 (ICAM-1), which allows the naïve T cell to migrate into the lymphoid organ. For a naïve T cell to be activated by an APC, it must undergo antigen recognition and signal transduction (signal 1) and costimulation via CD80 and CD86 on the APC, which are recognised by CD28 on the naïve T cell. If a TCR is activated but does not receive the correct co-stimulation, the naïve T cell becomes anergic. In addition, depending on the manner in which the APC is activated, it secretes different cytokine profiles which can differentiate or polarise naïve T cells into particular subsets with specialised functions. Once a T cell has been correctly stimulated and differentiated, it can migrate to the site of infection and exert its effector functions via exiting the lymph nodes in response to sphingosine-1-phosphate (S1P) signalling. (Murphy and Weaver, 2016).

1.1.2.2 T helper cell differentiation

It was shown in the 1980s that CD4+ T cell subsets can be distinguished by the cytokines they produce, however since then additional subsets have been described (Mosmann et al.,
In 1986, Mosmann and Coffman described helper T cell (Th)1 and Th2 subsets of CD4+ T cells, which were characterised by their cytokine production (Mosmann et al., 1986). Th1 cells produced the signature cytokine interferon (IFN)γ, whereas Th2 cells produced the signature cytokine Interleukin (IL)-4 (Abbas et al., 2012). Activation of DC by pathogens results in the production of different cytokine profiles, dependant on the pathogen (Abbas et al., 2012). These cytokines then skew the differentiation of naïve T cells into different subsets, which have specialised effector functions in order to target the particular pathogen.

IL-12 and IFNγ can promote the differentiation of naïve CD4+ T cells into the Th1 phenotype. This is achieved via activation of the janus kinase (JAK) - signal transducer and activator of transcription (STAT) pathway of signalling proteins by IL-12 and/or IFNγ. This in turn activates the expression of the transcription factor T box transcription factor (T-bet), which directs the function of Th1 cells including their production of the cytokines IFNγ, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2. In addition, Th1 cells can also inhibit the differentiation of Th2 cells (Murphy and Weaver, 2016). Th1 cells primarily defend against intracellular microbes. IFNγ produced by Th1 cells helps to activate macrophages to kill intracellular bacteria and Th1 cells also provide help to B cells for antibody production that targets viruses. Th1 cells also play a major role in autoimmune disease, and tissue damage in association with chronic infection via their cytokine production (Cosmi et al., 2008).

Th2 cells are induced by the presence of IL-4 and are distinguished by the transcription factor GATA3 and play a role in protection against viruses and parasites, which is achieved with the production of cytokines IL-4, IL-5, IL-10 and IL-13 (Abbas et al., 2012). Th2 cells also play a key role in stimulating the production of immunoglobulin E (IgE) and to allergic disease (Abbas et al., 2012). Another subset of Th cells has more recently been identified as primarily producing IL-17; these cells are known as Th17 cells and they play a role in fighting against extracellular bacteria and fungi, but have also been described as playing a pathogenic role in autoimmunity and inflammatory diseases. Despite the classifications described above, it is important to note that CD4+ Th cells can produce multiple cytokines, and cannot always be easily classified into distinct subsets. There can be plasticity within these subpopulations, which means a subset can convert into another under certain conditions (Abbas et al., 2012).
**Th17 cells**

Th17 cells are a subset of T cells, defined by a distinctive cytokine and transcription factor, neither of which are found in Th1 or Th2 responses. Th17 cells produce the cytokines IL-17A, IL-17F, and GM-CSF, tumor necrosis factor (TNF), IL-21, IL-22, and IL-9. IL-17 binds to its receptor which is widely expressed, inducing the expression of chemokines such as IL-8, which attracts neutrophils to the site of infection (Murphy and Weaver, 2016). Thus Th17 cells are responsible for targeting extracellular pathogens such as bacteria and fungi, however they also play an important pathogenic role in mediating autoimmunity and inflammatory diseases (Murphy and Weaver, 2016). Several studies have shown that murine Th17 cells differentiate from naïve CD4+ T cells in the presence of IL-6 and TGF-β, and are then amplified or stabilised by IL-23 and IL-21 (Cosmi et al., 2008, Bettelli et al., 2006, Deenick and Tangye, 2007). In contrast, polarisation of human Th17 cells occurs in response to the combined activity of IL-1β, IL-6, IL-21 and IL-23 (Stockinger, 2007). STAT3 is induced by Th17 polarising cytokines such as IL-6, resulting in the induction of RORγt which is the master regulator of Th17 cell function. In comparison to Th1 and Th2 cells, which are considered to be stable lineages, Th17 cells have the ability to convert to other lineage subsets (Guéry and Hugues, 2015). Studies have produced evidence that shows Th17 cells switching into Th1-like cells (Lexberg et al., 2008, Lee et al., 2009). These Th1-like cells are also known as exTh17 cells, lose expression of IL-17 and begin to express IFNγ which makes them similar to classical Th1 cells, and these exTh17 cells are more proinflammatory in inflamed tissue (Sundrud and Trivigno, 2013). Although most studies have defined Th17 cells by their IL-17 production, it has been suggested that Th17 cells are better categorised by their expression of RORγt, CCR6, and CD161, which are all virtually absent in classical Th1 cells (Cosmi et al., 2008). CD161 expression is considered a hallmark for identifying Th17 cells and the expression of CD161 and RORγt in Th17 cells has been linked to IL-17 secretion in IL-17+CD4+ T cells (Cosmi et al., 2008).

**Regulatory T (Treg) cells**

The immune system must be controlled to prevent damage to self-tissues, and Treg cells help to suppress the immune system after the elimination of a pathogen. Treg cells also maintain immunological self-tolerance by suppressing self-reactive cells. Treg cells were first described in 1995, and these CD4+ T cells were identified as expressing the interleukin-2 receptor-α (IL-2RA or CD25) chain and defined as mediating immune tolerance (Sakaguchi et al., 1995). It was later shown that CD4+ T cells expressing the transcription factor forkhead box p3 (Foxp3) had a positive role in the treatment and prevention of autoimmune disease
after adoptive transfer in rats (Hillebrands et al., 2006). Foxp3 is an exclusive intracellular marker for Treg cells, and is crucial for maintaining their development and immunosuppressive ability (Hori et al., 2003). There are two main groups of Treg cells; one of the subsets is called thymic Treg cells which become regulatory during development in the thymus. tTreg cells can develop and mature in the thymus under the influence of IL-2 (Pacholczyk and Kern, 2008). The other subset is peripheral Treg cells, which are derived from naïve T cells in the periphery; this induction is achieved when the cognate antigen is presented in the presence of TGF-β and IL-2, and in the absence of IL-6. Treg cell selection in the thymus is thought to be different from auto reactive T cells that have high affinity, which are deleted, or non-self-reactive T cells, which are positively selected. Usually Treg cells that are selected in the thymus have intermediate TCR affinity with self-peptide in the MHC complex (Jordan et al., 2001). Studies have shown that thymocytes that expressed a lower TCR affinity could not differentiate into Treg cells, which indicates that a high affinity interaction is essential for Treg cell development. This positive selection of Treg cells is thought to be due to their expression of Foxp3 which allows them to resist negative selection, while conventional T cells of similar affinity for self-antigens would be selected for deletion. (Pacholczyk and Kern, 2008, van Santen et al., 2004, Liston et al., 2003). Both subsets of Treg cells play a role in the suppression of proliferation and effector function of activated T cells. Treg cells can constrain the activation and expansion of naïve and effector CD4+, CD8+ T cells, NK cells, and APC. Treg cells have various ways to mediate its suppressive capability which include; the release of immunosuppressive regulatory cytokines such as IL-10 and TGFβ (Taylor et al., 2006). Treg cells can also release perforin and granzyme, which causes cytolysis (Shevach, 2009). Treg cells can restrict extracellular cysteine from DC that is needed for naïve T cell activation (Yan et al., 2010b). Treg cells can also cause suppression via CTLA-4 and induce the enzyme indoleamine 2, 3-dioxygenase, in DC which facilitates the catabolism of tryptophan into immunosuppressive metabolites (Boasso et al., 2005). Lymphocyte activation gene 3 are highly expressed on Treg cells and can bind to MHCII with higher affinity than CD4 which impairs TCR mediated activation of CD4+ T cells (Huard et al., 1995). Treg cells are not easy to identify, as they do not have a unique cell surface marker to distinguish them, thus a combination of markers including CD4, CD25, CD127, and Foxp3 is used. The population of Treg cells is defined as CD4+ CD25+ CD127lo Foxp3+. The main problem with Treg cell markers, particularly in humans, is that both CD25 and FoxP3 can also be expressed on activated effector cells. Another marker that has been added to the panel to identify Treg cells is the IL-7 receptor chain (CD127) expression which is an IL-7 receptor chain, this marker is downregulated in Treg cells relative to effector cells,
and has been added to the panel of markers to define Treg cells (Liu et al., 2006b). The current consensus is that using a combination of markers to identify Treg cells is the most accurate way to distinguish Treg cells from other T cell subsets (Seddiki et al., 2006).

1.1.3 Dysregulation of the immune system and autoimmunity

Autoimmunity is the reaction of the immune system to self-antigens as a result of the failure of self-tolerance. There are a number of tolerance mechanisms, which help to prevent autoimmunity. These include the deletion of self-reactive lymphocytes (central tolerance) in addition to peripheral mechanisms such as antigen segregation, anergy induction, and Treg cells. The breakdown of self-tolerance is due to self-reactive lymphocytes escaping deletion during thymic selection, in addition to the failure of peripheral tolerance mechanisms. When these self-reactive lymphocytes are subsequently activated, this triggers autoimmune disease (Murphy and Weaver, 2016). There are a number of factors that can contribute to the activation of self-reactive T cells, which include molecular mimicry, whereby a self-antigen shares similar sequence or structures with foreign antigens and danger signals from endogenous or infectious sources that activates the innate response that in turn promotes activation of autoreactive T cells (Murphy and Weaver, 2016, Atkins et al., 2012, Abbas et al., 2012).

1.2 Multiple sclerosis (MS)

MS is an autoimmune and neurodegenerative disease that affects the central nervous system (CNS), including the brain, spinal cord, and optic nerves, and results in damage to myelin and axons. MS has three main clinical forms: relapsing remitting (RRMS), primary progressive (PPMS), and secondary progressive (SPMS). RRMS is the most common form of MS, however, over time, many patients will progress to SPMS. RRMS accounts for approximately 85% of cases and is characterised by intermittent clinical episodes that can be severe, interspersed with periods of remission. In contrast, the progressive forms of MS are characterised by neurodegeneration, which occurs mostly in the absence of relapses. MS usually presents in adults from 20-40 years of age. The clinical signs are dependent on the location of inflammation within the CNS and include cognitive impairment, unilateral painful loss of vision, clumsiness, poor balance, diplopia, impaired speech, emotional lability, stiffness and painful spasms, and fatigue (Atkins et al., 2012, Jones et al., 2016, Loma and Heyman, 2011, Rostami and Ciric, 2013, Frohman et al., 2006).
1.2.1 Pathogenesis

In the case of RRMS, the disease is thought to be triggered when an unknown trigger activates myelin-specific CD4$^+$ T cells. Myelin specific T cells can usually also be found in healthy controls (HC) which show that the presence of these cells per se does not cause the onset of disease, but rather these myelin-specific T cells are inappropriately activated or ineffectively regulated in MS patients (Ota et al., 1990, Chou et al., 1991, Sun et al., 1991). Once these activated T cells leave the lymph node, they enter the CNS through the blood brain barrier (BBB), during which the BBB becomes permeable facilitating a further influx of T cells and other immune cells. The infiltrating auto-reactive T cells encounter their cognate myelin antigens and are reactivated by APC, which include local microglial cells and infiltrating peripheral APC. The reactivated T cells expand and secrete inflammatory cytokines and chemokines, which recruit other immune cells to the site of inflammation. Activated cells such as microglial cells and other recruited immune cells produce proteases, glutamate and reactive oxygen species (ROS), which in turn promote myelin destruction. Although CD4$^+$ T cells are important in the initiation of MS, they do not account for the majority of cells found within inflammatory lesions (Frohman et al., 2006, Rostami and Ciric, 2013, Atkins et al., 2012).

1.2.2 Risk factors

The risk factors associated with MS include both genetic and environmental risk factors such as viral infection, smoking, poor diet and lack of ultraviolet B (UVB) exposure/vitamin D deficiency.

1.2.2.1 Genetics

In studying genetic susceptibility as a risk factor, it seems that MS is more prevalent in lighter skin individuals that are ethnically Caucasian over other ethnic groups. Human leukocyte antigen HLA-DRB1*15:01 is associated with disease susceptibility in northern Europeans while HLA-DRB1*15:03 seems to be linked to increased susceptibility to disease in African Americans (Oksenberg et al., 2004). The HLA-DRB1*15:01 allele which is associated with higher risk of MS, has a significantly increased risk in smokers (Hedstrom et al., 2011), or patients that were infected with EBV (Sundqvist et al., 2012), or adolescents with obesity (Hedstrom et al., 2014). Interestingly, it has been shown that HLA-DRB1*15:01 is an MS risk allele in northern Europeans and that vitamin D can influence the expression of HLA-DRB1*15:01, thereby possibly altering thymic selection in utero, however, it is not known how reduced vitamin D and HLA-DRB1*1501 contribute to risk of MS (Ramagopalan et al., 2009). Reduced vitamin D and HLA-DRB1*1501 expression
might contribute to the selection of autoreactive myelin specific T cells (Hayes et al., 2011). However, even though these myelin specific T cells are found in healthy subjects, studies have shown that they are increased or altered in MS patients (Bielekova et al., 2004, Ota et al., 1990). Alleles of the IL-2 receptor alpha gene, IL-7 receptor alpha gene, and several alleles in the HLA locus are identified as genetically inheritable risk factors for MS (Consortium, 2007). MS is also more common in women than men with a female: male ratio of 2:1 in France and 3:1 in Canada, and a 2.7:1 in Ireland (Orton et al., 2011, Orton et al., 2006, O’Connell et al., 2017).

1.2.2.2 Viral infection
Viral infections have been implicated in the etiology of MS. Several viruses have been associated, including the measles virus, rubella virus, Epstein-Barr virus (EBV), and varicella-zoster virus. It has been hypothesised that a viral infection in adolescence could trigger the onset of MS, as a result of the combination of environmental and genetic factors. However, the idea that a virus is involved in the etiology of MS has not been proven, and can only be hypothesised; for example, a virus such as EBV may have structural similarities with self-antigens that could activate autoreactive T cells resulting in MS. It has been suggested that the sequence homology of the EBV virus is similar to antigenic structures in the CNS; this type of sequence similarity is called molecular mimicry (Quaratino et al., 1995). There are epidemiological studies that show that the risk factor for MS are nearly close to zero in individuals who are EBV negative, and intermediate when infected with EBV in early childhood without infectious mononucleosis (IM), and the risk factor is highest when individuals are infected with EBV in adolescence and present with IM (Thacker et al., 2006).

1.2.2.3 Smoking
Another environmental risk factor associated with MS in epidemiological studies is cigarette smoking (Hernán et al., 1999, Hernán et al., 2005, Thorogood and Hannaford, 1998). Smokers have a 40-80% increased risk of MS compared to non-smokers, however these studies were restricted to females (Villard-Mackintosh and Vessey, 1993). In a study by Hernan et al., 2005 it was estimated that the risk of developing SPMS was more than three times higher in smokers vs non-smokers (Hernán et al., 2005). As cigarette smoke contains nitric oxide (NO), it has been suggested that cigarette smoke increased NO plasma levels, and exposure to NO has been shown to cause axonal degeneration or block axonal conduction (Smith et al., 2001, Kapoor et al., 2003). High levels of NO metabolites within the CSF have also been associated with MS progression (Rejdak et al., 2004). Smoking can
also initiate the citrullination process by cell death which increases cytoplasmic concentration of calcium (Ca\(^{2+}\)), which is then catalysed by protein binding proteins called peptidylarginine deiminases. The disruption of Ca\(^{2+}\) and activation of protein arginine deiminase (PAD) have been suggested to lead to myelin basic protein (MBP) citrullination which generated neo-epitopes, which could then trigger the immune system of MS patients (Berer and Krishnamoorothy, 2014, Utz et al., 2000).

### 1.2.2.4 Diet

It has been hypothesised that diet might play a role in the risk of MS; an epidemiological study showed that there was a high prevalence of MS in populations with high intake of saturated fats and low vitamin D or sunlight (Ebers, 2008). The high prevalence of MS which is seen in western countries and in areas distant from the equator, suggest that there may be a link to regional dietary habits (Organization, 2008). A study by Argonoff et al., 1974 showed that the consumption of dairy fats and red meats was associated with an increased risk of MS; they also found that omega-3 and omega-6 polyunsaturated fatty acids, which are found in vegetables, nuts, and fish, were associated with a reduced risk of MS (Agranoff and Goldberg, 1974). Other studies have supported the increased risk of MS with saturated animal fat, and similarly to the Argonoff study, polyunsaturated fatty acids were inversely associated with the risk of MS (Alter et al., 1974, Lauer, 1994, Esparza et al., 1995).

### 1.2.2.5 Vitamin D/sunlight

**Latitude gradient of MS**

It has been noted that the prevalence of MS has a striking geographical variance, where the prevalence increases with increasing latitude in both hemispheres corresponding to the amount and duration of UVB and serum 25-hydroxyvitamin D (25(OH)D) concentrations (Hayes et al., 1997, Acheson et al., 1960). While the risk of MS decreases with lower latitudes, there has been an increase in MS prevalence in the lower latitudes, which may be due to the changes in lifestyles as a result of skin cancer awareness, and cosmetic concerns. A study by S-M Orton et al., 2011 showed that there was a correlation between the prevalence of MS and regional UVB radiation, which supports the hypothesis of reduced sunlight exposure and risk of MS. Higher UVB radiation was correlated with lower risk of MS in the French population, and MS prevalence increased when UVB exposure was low, and was higher in females than in males (Orton et al., 2011). Another study in New Zealand confirmed that there was a correlation between the prevalence of MS and latitude gradient, and the gradient was mostly seen in European females with RRMS/SPMS (Orton et al., 2011, Taylor et al., 2010). In summary, latitude plays an important role in exposure to UVB, which
ultimately determines vitamin D levels. However, the latitude gradient may also be influenced by several other factors such as gender, ethnicity, and individuals who are genetically susceptible to the risk of MS.

A study investigated the relationship between skin pigmentation and the ability to produce vitamin D from sun exposure. Interestingly, they found that lighter skinned individuals made twice the amount of serum concentration in comparison to darker pigmented individuals (Armas et al., 2007). The correlation between serum 25(OH)D and latitude has been shown in several studies, however studies have also shown that dark pigmentation is associated with lower increase of 25(OH)D after UVB exposure in latitude areas that contained high UVB (Ross et al., 2011, Loomis, 1967, Clemens et al., 1982, Jablonski and Chaplin, 2012, Goldberg et al., 1986).

**Month of birth studies in MS**

Several studies have suggested that birth in the spring months is a risk factor for MS, and this is related to vitamin D levels during pregnancy. Dobson et al., 2013 concluded that month of birth has a significant effect on the risk of MS, and is due to reduced UVB exposure and maternal vitamin D during winter (Dobson et al., 2013). The month of birth effect relates to individuals who are born in winter having a reduced MS risk, however, individuals born in the spring have an increased risk of MS (Ebers, 2008). A number of studies measuring the amount of UVB at higher latitudes have indicated that insufficient UVB light can reach the skin in order to synthesise vitamin D₃ between the months of October and March, thus babies born in spring are exposed to low maternal vitamin D throughout gestation (Willer et al., 2005, Saastamoinen et al., 2012, Menni et al., 2012, Disanto et al., 2012). It is important to note that the month of birth effect would only be significant at higher latitudes and not at lower latitudes, and has been suggested to only apply to RRMS and not PPMS (Sadovnick et al., 2007). In addition, in utero studies showed that vitamin D deficiency has a significant effect on developing the immune system (Weiss and Litonjua, 2011).

Recent evidence suggests that some of the above mentioned genetic and environmental factors can actually interact with each other to affect the risk of MS. For example, expression of the MHC II risk allele HLA-DRB1, which is the major locus determining genetic susceptibility in northern Europeans, is regulated by vitamin D via a direct interaction of the vitamin D receptor (VDR) with a vitamin D response element within the HLA-DRB1 promoter region (Ramagopalan et al., 2009).
1.2.3 Diagnosis

Most MS patients present with an acute episode affecting one or several sites, which includes sites such as the cerebrum, optic nerve, cerebellum, brain stem, and spinal cord. The symptoms associated with the affected sites can increase in number and severity over time. Magnetic resonance imaging (MRI) to detect lesions in the CNS is the mainstay of MS diagnosis, however electrophoresis of the cerebral spinal fluid (CSF) to identify immunoglobulin oligoclonal bands is also used as a diagnostic (Atkins et al., 2012). The presence of immunoglobulin oligoclonal bands in serum or CSF is an indicator for high risk for MS (McDonald et al., 2001). In order to provide a diagnosis of MS, the McDonald criteria must be fulfilled. The McDonald criteria are solely based on clinical symptoms; if clinical evidence is lacking, an MRI is carried out to detect lesions that are disseminated in space and time, which is an episode of damage to the CNS at different times and in different locations. The criteria are specific: an attack/relapse must last for at least 24 hours (hr), and the duration between the onset the first attack/relapse to any subsequent attack/relapse must be at least 30 days for it to count as a separate attack/relapse (McDonald et al., 2001).

1.2.4 Clinically isolated syndrome (CIS)

CIS is a term used to describe the first clinical episode with features suggestive of MS. About 70 to 80 % of patients that present with CIS go on to develop MS. In some cases, CIS patients can recover from their first neurological episode and not become MS definite. This is termed monophasic; however, the term CIS is commonly understood to refer to patients who will go on to develop MS (Miller et al., 2012). Interestingly, in some studies on CIS in relation to MS, the conversion from CIS to MS was up to 85% when CIS patients presented with optic neuritis. The overall likelihood of developing MS is generally high across all types of CIS when presenting with clinical features such as brainstem, or spinal cord syndromes (Fisniku et al., 2008, Tintore et al., 2010, Young et al., 2009, Miller et al., 2012).

1.2.5 Immunomodulatory therapies for MS

A number of different immunomodulatory therapies are used for the treatment of MS, and these can be divided into first line and second line therapies. Interferon beta (IFNβ) (Avonex, Rebif and Betaseron) is a first-line therapy for RRMS, which is known for both its antiviral and immunomodulatory effects. IFNβ has various immunomodulatory effects, such as maintaining the integrity of the BBB and has also been reported to skew the T cells cytokine response towards a less inflammatory profile. It has also been reported to inhibit the development and pathogenesis of Th17 cells (Atkins et al., 2012, Sweeney et al., 2011). This
effect was exerted directly upon Th17 cells, or via APC, where it inhibited the production of IL-1 and IL-23 and induced IL-27 (Sweeney et al., 2011). However, it has been suggested by other studies that IFNβ therapy actually aggravates Th17 cell-mediated disease, since IFNβ therapy exacerbates neuromyelitis optica (NMO) which is thought to be Th17 driven (Li et al., 2011). IFNβ is administered by injection, with some patients experiencing undesired side effects such as flu like symptoms and depression.

Another first line therapy is glatiramer acetate (GA; Copaxone), which was designed to mimic MBP and to aggravate disease in the experimental autoimmune encephalomyelitis (EAE) model, however it had unexpected immunomodulatory effects, and was later developed as a therapy for MS (Teitelbaum et al., 1971). GA has various immunomodulatory effects, such as random binding to MHC class II, which prevents activation of autoantigen-specific T cells, and skewing towards an anti-inflammatory Th response via the APC. GA also can induce regulatory responses including CD8 suppressor cells, Treg cells, regulatory B cells (Breg cells), and the induction of neurotrophic factor (Comi et al., 2001). GA has a good side effect profile, however, similarly to IFNβ it is administered by injection. The most common side effect of GA is adverse reaction at the site of injection which can cause erythema. There have also been reports of non-fatal anaphylactic reactions in patients, however these reports were rare (Rauschka et al., 2005).

Teriflunomide (Aubagio), is now used as a first-line oral therapy. Teriflunomide inhibits pyrimidine synthesis in rapidly dividing lymphocytes such as B cells and T cells, thereby exerting immunomodulatory effects. The most common side effects were nasopharyngitis, elevated alanine aminotransferase levels, and pancreatic fibrosis. There has been no reports of progressive multifocal leukoencephalopathy (PML), which is inflammation of the white matter caused by the John Cunningham virus (JCV) (He et al., 2012).

Dimethyl fumarate (DMF) (Tecfidera), is now used as a first-line oral therapy. It has immunomodulatory and neuroprotective effects, which are thought to be mediated via the activation of nuclear factor E related transcription factor (Nrf2), a transcription factor that regulates the expression of antioxidant proteins and cellular reduction-oxidation homeostasis (Linker et al., 2011). DMF-induced increase in antioxidants could provide neural protection from oxidative metabolic stress, and may also exert anti-inflammatory effects by inhibiting nuclear factor kappa B (NF-κB) and modulating APC function (Mrowietz and Asadullah, 2005). It has been shown that DMF can cause lymphocyte apoptosis, which may account for the lymphopenia that has been observed in some patients (Mrowietz and Asadullah, 2005).
Natalizumab (Tysabri) is a second-line therapy for the treatment of RRMS. Natalizumab is a humanised monoclonal antibody, specific for the integrin VLA-4 (α4β1), which inhibits the ability of T cells to cross the BBB and reduces relapse rates by up to 68% (Polman et al., 2006). This therapy was initially used as a first line disease modifying therapies (DMT) but was restricted to second line due as a result of some patients developing PML. The drug is now licensed as a second-line therapy and is administrated when first-line therapy fails or as a first line therapy in cases of highly active RRMS.

Fingolimod (Gilenya) is a first-line therapy in the USA but is second line in the EU. Fingolimod is an S1P antagonist which acts by binding to S1P receptors on T cells, causing their internalisation. Fingolimod thereby traps naïve and CCR7+ central memory T cells (CM) within the secondary lymphoid organs while CCR7- effector memory T cells (EM) retain the ability to move from the secondary lymphoid organs to peripheral blood (Mehling et al., 2010). Fingolimod has also been shown to have important direct effects on the CNS which include improved survival of oligodendrocytes, myelin repair, and neuroprotective effects (Pelletier and Hafler, 2012). With this, however, it also has many side effects, such as bradycardia, reduction of peripheral blood lymphocytes, elevated liver enzymes, and an increase in risk of skin cancer and viral infections (Pelletier and Hafler, 2012).

Alemtuzumab (Lemtrada), is a humanised monoclonal antibody specific for CD52, which depletes CD52-expressing immune cells, which are mainly T and B cells. Depleting these specific cells prevents their trafficking to the CNS. Alemtuzumab is administered by intravenous infusion once per year and results in long lasting depletion, after which lymphocytes slowly reconstitute. It has been shown to be more effective than IFNβ, however, it has potentially serious side effects. These include autoimmune disorders such as idiopathic thrombocytopenic purpura (ITP) clotting and autoimmune thyroid disease.

Ocrelizumab is an infusible humanized mAb that selectively depletes CD20+ B cells, and was recently approved by the US FDA and the EMA for the treatment of RRMS and PPMS (Food and Administration, Mulero et al., 2018, Mayer et al., 2019). Ocrelizumab reduced clinical and MRI activity compared with IFNβ and placebo, and most common adverse event with Ocrelizumab was infusion related reactions in all three phases of the trials. (Hauser et al., 2017, Montalban et al., 2017).
1.3 Vitamin D

1.3.1 Sources of vitamin D

The majority of vitamin D in humans comes from skin exposure to UVB from the sun. The amount of UVB radiation received and therefore subsequent vitamin D\textsubscript{3} synthesis is dependent on latitude, season and duration of exposure. A study on the relationship of skin pigmentation and vitamin D\textsubscript{3} synthesis suggested that individuals with light skin pigmentation generated double the amount of serum 25(OH)D in comparison with individuals with darker pigmentation (Armas et al., 2007). Another study found that serum 25(OH)D levels were consistently lower in darker pigmented individuals (Looker et al., 2008, Ross et al., 2011). Concerns about the increased risk of skin cancer as a result of direct sun exposure have prompted many to avoid direct sunlight and use sunscreens, contributing to vitamin D deficiency. Thus, many people are now more dependent on dietary vitamin D\textsubscript{3}. Most diets are low in vitamin D, while some are vitamin D rich including diets that contain oily fish such as salmon, cod liver oil, and mackerel. However, even vitamin D rich food does not have enough vitamin D to maintain vitamin D sufficiency in the absence of UVB exposure. Consumption of vitamin D in our diet alongside vitamin D from UVB exposure is essential for a healthy vitamin D status (Nair and Maseeh, 2012).

1.3.2 Metabolism of vitamin D\textsubscript{3}

Vitamin D\textsubscript{3} is a biologically inactive prohormone, and for it to function it must be metabolised to its hormonal form. Upon exposure to UVB, vitamin D\textsubscript{3} (cholecalciferol) is synthesised from a cholesterol-like precursor (7-dehydrocholesterol) within the skin. Vitamin D\textsubscript{3} subsequently undergoes two-enzymatic hydroxylations, resulting in the active form of vitamin D\textsubscript{3}, namely 1, 25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}) (Ross et al., 2011) (Figure 1.1).

Once vitamin D\textsubscript{3} reaches the liver, the first hydroxylation step occurs converting vitamin D\textsubscript{3} to 25(OH)D; this is the form that is measured in serum. It has been suggested that the conversion of vitamin D\textsubscript{3} to serum 25(OH)D is most likely carried out by CYP2R1, which is part of a large and diverse group of enzymes called cytochrome p450 (CYP) (Cheng et al., 2003). Serum 25(OH)D is then further 1α-hydroxylated in the kidney to create its metabolically active form 1,25(OH)\textsubscript{2}D\textsubscript{3}, also known as calcitriol (Jones et al., 1998, Lips, 2006). 1,25(OH)\textsubscript{2}D\textsubscript{3} can induce its own destruction by stimulating the 24-hydroxylase enzyme known as CYP24A1 (Jones et al., 1998). CYP24A1 is found in all tissues and is usually induced by the interaction of 1,25(OH)\textsubscript{2}D\textsubscript{3} with VDR. CYP24A1 is responsible for
the clearance and degradation of the active $1,25(\text{OH})_2\text{D}_3$ and serum $25(\text{OH})\text{D}$ (Saito et al., 2005, Jones et al., 1998).

**Figure 1.1 Metabolism of Vitamin D$_3$**

Upon UVB exposure, the inactive form of vitamin D which is named vitamin D$_3$ or cholecalciferol, is synthesised from the cholesterol-like precursor 7-dehydrocholesterol in the skin. Vitamin D$_3$ can also be obtained from the diet or supplementation. Once vitamin D$_3$ reaches the liver it undergoes two hydroxylations to form $25(\text{OH})\text{D}$; once $25(\text{OH})\text{D}$ reaches the kidney it is further hydroxylated to its active form $1,25(\text{OH})_2\text{D}_3$.

**1.3.3 Measurement of vitamin D**

Serum $25(\text{OH})\text{D}$ is the metabolite used for determining vitamin D status. The status of $25(\text{OH})\text{D}$ is measured in serum and reported in either nmol/L or ng/ml concentrations. The nmol/L measure can be converted to ng/ml by dividing by a factor of 2.5. Vitamin D levels in diet are defined in international units (IU) or in µg amounts and 1 IU of vitamin D is equivalent to 0.025 µg. Vitamin D deficiency is defined as <30 nmol/L and vitamin D insufficiency is determined as 30-49.9 nmol/L; sufficient vitamin D is defined as ≥50 nmol/L (Ross et al., 2011). Although it would be more valuable and ideal to measure the active form of vitamin D, this is not feasible as $1,25(\text{OH})_2\text{D}_3$ has a short circulating half-life in comparison to $25(\text{OH})\text{D}$. However, it is not clearly established whether serum $25(\text{OH})\text{D}$ concentration serves as an accurate biomarker of its immunomodulatory effect. Interestingly the formation of the active form $1,25(\text{OH})_2\text{D}_3$ is not directly regulated by vitamin D intake;
other factors contribute to its formation such as serum parathyroid hormone (PTH) and calcium levels (Ross et al., 2011, Holick, 2009).

1.3.4 Role of vitamin D

1.3.4.1 VDR

1,25(OH)₂D₃ exerts its effects on cells by regulating gene expression through transcription, which is mediated by the binding of VDR. VDR is a ligand-dependant transcription regulator molecule of a superfamily of nuclear receptors, which are widely expressed throughout the body. Vitamin D is dependent on the VDR to regulate processes such as calcium-phosphate homeostasis. However, VDR is found in tissues that do not involve the homeostasis of serum calcium and phosphate, which suggests that 1,25(OH)₂D₃ may have a more general role, or that alternative ligands can activate the VDR (Adams and Hewison, 2008). VDR is mainly distributed in the cytoplasm in the absence of 1,25(OH)₂D₃. Once VDR interacts with its ligand 1,25(OH)₂D₃ it induces the formation of two independent protein interaction surfaces on the VDR. One of the independent interactions is that VDR interacts with retinoid X receptor (RXR) to form a heterodimer that can translocate to the nucleus and bind to specific DNA sequences called vitamin D response elements (VDRE) in vitamin D responsive genes (Fig 1.2). The VDR/RXR complex then recruits various co-activators and co-repressors that influence gene expression and alter cellular activity, such as protein synthesis and secretion, cellular proliferation and/or differentiation (Nagpal et al., 2005, Pike and Meyer, 2012). VDR seems to be present in the nucleus of many cells; for example, VDR expression has been described in epidermal keratinocytes, inactivated T cells, antigen-presenting cells, macrophages, and in monocytes and cytotoxic T cells. Thus, vitamin D deficiency can prevent VDR activation and its wide range of biological actions (Brot et al., 2001, Holick, 2007, Moan et al., 2008).
1,25(OH)₂D₃ interacts with its VDR (1). VDR then interacts with retinoid X receptor (2) (RXR) to form a heterodimer (3) that can translocate to the nucleus and bind to specific DNA sequences called vitamin D response elements (VDRE) (4). VDR/RXR complex then recruits various co-activators and co-repressors (4) in vitamin D responsive genes.

1.3.4.2 Vitamin D in calcium, phosphorus, and bone metabolism

It has been well established that 1,25(OH)₂D₃ regulates serum calcium and phosphate homeostasis, which are responsible for the maintenance and growth of healthy bone (Fig 1.3) (Suda et al., 2003). The interaction of 1,25(OH)₂D₃ with the VDR increases the efficiency of intestinal calcium absorption to 30-40%, with an increase in phosphorus absorption to 80% (Holick, 2007, Bouillon et al., 2001). Tubular reabsorption of calcium, and kidney stimulation to produce 1,25(OH)₂D₃, is enhanced by PTH, which can also activate osteoblasts which transform preosteoblasts into mature osteoclasts. PTH can cause phosphaturia, which results in low serum phosphorus level if there is not enough calcium-phosphorus production, leading to the classic signs of rickets, which are due to diminished mineralisation of collagen matrix. Vitamin D is important in reducing osteoporotic fractures. A study by Chapuy et al. reported that elderly woman that were given 1200 mg of calcium and 800 IU of vitamin D₃ for three years had a reduction in hip fracture of 43% and nonvertebral fracture of 32% (Chapuy et al., 1997).
Figure 1.3 Calcium metabolism and regulation

The parathyroid glands sense serum calcium level, and secrete PTH if calcium becomes too low. When calcium intake is low, PTH stimulates the activity of the 25(OH)D 1-alpha-hydroxylase enzyme in the kidney, which results in increased production of 1,25(OH)2D3. If serum calcium is too high, this will in turn prevent the secretion of PTH and therefore the conversion of 25(OH)D to its active form 1,25(OH)2D3.

1.3.4.3 Immunomodulatory effects of vitamin D

In addition to the well-described role of vitamin D in bone health, it has emerged that it may have important immunomodulatory effects and a role in other diseases. VDR has been shown to be widely expressed in immune cells throughout the body, including circulating monocytes, DC, and activated T cells, and also in high concentrations in immature immune cells within the thymus, but also in CD8 T cells regardless of their activation state (Jones et al., 1998, Stio et al., 2006).

It has been suggested that 1,25(OH)2D3 has immunomodulatory and anti-proliferative effects through autocrine and paracrine pathways (Adams and Hewison, 2008). It has also been suggested that 1,25(OH)2D3 can play a therapeutic or preventative role in different conditions; for example, in cancer it has been suggested that 1,25(OH)2D3 has anti-cancer potential (Masuda and Jones, 2006). In 1981, a study by Abe et al., 1981 found that 1,25(OH)2D3 inhibited proliferation of a variety of leukemic cell lines (Abe et al., 1981). In a cardiovascular disease study, patients with hypertension were exposed to UVB three times a week (wk) for three months, which increased their serum 25(OH)D levels and blood
pressure became normal. The cardiovascular study also determined a correlation between vitamin D deficiency and congestive heart failure (Zittermann, 2006). \(1,25(\text{OH})_2\text{D}_3\) has been shown to have potent immunomodulatory effects in infectious disease such as tuberculosis (Kawaura et al., 2006, Liu et al., 2007b). Another study demonstrated the potent immunomodulatory effects in Helicobacter pylori, where elderly women that were administered vitamin \(\text{D}_3\) as an antiosteoporosis agent had significantly lower rate of Helicobacter pylori infections in comparison to the untreated (Kawaura et al., 2006).

Vitamin D has other mechanisms that are involved in immunity to infection. For example, it has been shown that the conversion of \(25(\text{OH})\text{D}\) into \(1,25(\text{OH})_2\text{D}_3\) in monocytes and macrophages can produce cathelicidin, which is an antimicrobial peptide that is responsible for killing bacteria such as mycobacterium tuberculosis. Thus the production of cathelicidin in response to vitamin D promotes anti-bacterial immunity (Liu et al., 2006a). \(1,25(\text{OH})_2\text{D}_3\) has been shown to inhibit T cell proliferation and B cell immunoglobulin production, and promotes the proliferation of Treg cells and their accumulation at the site of inflammation (Penna et al., 2007). Studies are now comparing \textit{in vivo} and \textit{in vitro} results to understand the mechanism and immunomodulatory effects of vitamin \(\text{D}_3\) and \(1,25(\text{OH})_2\text{D}_3\) in different T cell subsets such as naïve T cells, Th1, Th17, and Treg cells (Bhargava et al., 2015, Ashtari et al., 2016, Sotirchos et al., 2016, Bhargava et al., 2016, Muris et al., 2016).

### 1.3.4.4 Innate immune cells

\(1,25(\text{OH})_2\text{D}_3\) exerts immunomodulatory effects on innate immune cells; in particular, it has a profound effect on APC. \textit{In vitro}, it has been suggested that \(1,25(\text{OH})_2\text{D}_3\) inhibits the differentiation of monocytes into DC and reduces the expression of co-stimulatory molecules, which in turn can hinder the stimulatory activity on T cells (Griffin et al., 2001, Penna and Adorini, 2000). These DC are tolerogenic, which has been suggested to promote an anti-inflammatory response and an induction of Treg cells (Penna et al., 2005). \(1,25(\text{OH})_2\text{D}_3\) can also stimulate phagocytosis by macrophages, but suppresses the antigen presenting ability of both macrophages and DC cells (Griffin et al., 2000). However, a study suggested that \(1,25(\text{OH})_2\text{D}_3\) can enhance the differentiation of monocytes into macrophages, and inhibit the secretion of inflammatory cytokines (Abu-Amer and Bar-Shavit, 1994). Other evidence also suggests that \(1,25(\text{OH})_2\text{D}_3\) can also enhance the differentiation of monocytes and mononuclear phagocytes (Provvedini et al., 1986, Miyaura et al., 1981, McCarthy et al., 1983). \(1,25(\text{OH})_2\text{D}_3\) has also been shown to block secretion of the pro-inflammatory cytokine IL-12 from macrophages (Griffin et al., 2000). \(1,25(\text{OH})_2\text{D}_3\) can inhibit monocyte production of inflammatory cytokines IL-1, IL-6, IL-8, IL-12 and TNF (Almerighi et al.,
2009). IL-12 inhibition is achieved by the direct binding of 1,25(OH)\textsubscript{2}D\textsubscript{3} to VDR, which has a direct effect on NF-kB-induced transcription of IL-12 (D'Ambrosio et al., 1998). 1,25(OH)\textsubscript{2}D\textsubscript{3} can inhibit DC maturation and differentiation with a decreased expression of MHC II, co stimulatory molecules and IL-12 (Szeles et al., 2009). Interestingly 1,25(OH)\textsubscript{2}D\textsubscript{3} can interfere with the stimulatory capacity of macrophages via IL-10, and this inhibition in stimulation was abrogated in IL-10 deficient mice (Korf et al., 2012). Vitamin D deficiency can alter the ability of a macrophage to mature; this can affect the macrophage-specific surface antigens, and also the inhibition of the production of lysosomal enzyme acid phosphatase, and the secretion of hydrogen peroxide, which is important in its function against microbes (Abu-Amer and Bar-Shavit, 1994). However, several studies had suggested that the expression of specific surface antigens and lysosomal enzyme acid phosphatase was in fact increased in macrophages when 1,25(OH)\textsubscript{2}D\textsubscript{3} was present (Mangelsdorf et al., 1984, Cannell et al., 2006). The immunomodulatory effects of vitamin D on innate cells does not seem to be fully understood and there is contradictory evidence on to the effects of vitamin D on innate cells in terms of whether or not it can suppress or promote differentiation. It is also not clear whether vitamin D inhibits or promotes production of lysosomal enzyme acid phosphatase and specific surface antigens.

1.3.4.5 T cells

As discussed above, 1,25(OH)\textsubscript{2}D\textsubscript{3} can suppress the proliferation and activation of T cells via DC-mediated effects and it can also directly inhibit T cell proliferation and cytokine production (Griffin et al., 2001, Penna and Adorini, 2000). However, the complexity of the role of vitamin D has been demonstrated as it is also essential for the activation of naïve T cells through signalling via VDR (von Essen et al., 2010). 1,25(OH)\textsubscript{2}D\textsubscript{3} exerts direct effects on T cells and it has been suggested that these effects are achieved via inhibition of transcription via the VDR. It has also been demonstrated 1,25(OH)\textsubscript{2}D\textsubscript{3} signalled T cells to induce CCR10 which allows T cells to migrate towards the skin via secretion of skin specific chemokine CCL27 by keratinocytes (Sigmundsdottir et al., 2007). by a study that 1,25(OH)\textsubscript{2}D\textsubscript{3} can inhibit Th1 cell proliferation and cytokine production, which is achieved by the ability of 1,25(OH)\textsubscript{2}D\textsubscript{3} to decrease IL-2 and IFN\textgreek{g} expression by CD4 T cells, and an increase of IL-5 and IL-10, which shifts the pro-inflammatory Th1 cells into a dominant Th2 phenotype. (Boonstra et al., 2001, van Etten and Mathieu, 2005). A study also demonstrated that 1,25(OH)\textsubscript{2}D\textsubscript{3} inhibited Th1 cytokines IFN\textgreek{g}, IL-2 and TNF\textgreek{a} (Palmer et al., 2011). There are controversial papers that suggest that IL-4 is upregulated in vivo by the addition of 1,25(OH)\textsubscript{2}D\textsubscript{3}, however other studies suggest that 1,25(OH)\textsubscript{2}D can downregulate both Th1
and Th2 cytokines including IL-4 (Cantorna et al., 1998, Staeva-Vieira and Freedman, 2002). Another cytokine IL-6 has also been shown to be inhibited by the addition of 1,25(OH)2D3 (Nonn et al., 2006), and is important in stimulating Th17 cells, which have a major role in autoimmune disease (Stockinger, 2007).

Th17 cells have been implicated in MS and Th17 related cytokines such as IL-17 and IL-22 have been described to be elevated before a clinical relapse (Rolla et al., 2014). In a study by da Costa et al., 2016 where PBMC from MS patients and HC were treated with 1,25(OH)2D3 for three days, this reduced the production of IL-17 in both groups. Interestingly, pro-inflammatory cytokines IL-1, IL-6, IL-17, IL-22 from the MS group were significantly higher than the control group when stimulated with PHA, however when treated with 1,25(OH)2D3 it was less potent on the reduction of proinflammatory cytokines from the MS group, this observation has been suggested to be due to low levels of VDR expression on T cells from MS patients (da Costa et al., 2016). Similarly, another study showed that the addition of 1,25(OH)2D3 downregulated IFNγ and IL-17 from CD4+ T cells in the context of both inflammatory bowel disease (IBD) and healthy individuals (Cantorna et al., 2014).

Similarly, another study showed that 1,25(OH)2D3 inhibited IL-17, IFNγ and IL-21 from activated T cells (Jefferi et al., 2009). A study by Correale et al., 2009 showed that the addition of 1,25(OH)2D3 reduced the proliferation of CD4+ T cells from RRMS patients, as well as IL-6 and IL-17 production when stimulated with myelin antigens such as MBP (Correale et al., 2009). Similarly, 1,25(OH)2D3 inhibited the proliferation of CD4+ T cells and MBP-specific T cells in vitro (Correale et al., 2011, Rigby et al., 1984, Mahon et al., 2003b). 1,25(OH)2D3 can directly influence T cells by reducing pro-inflammatory cytokines, however, it can also influence T cells indirectly. In a study by Wu et al., 2015 they demonstrated that 1,25(OH)2D3 induced a tolerogenic phenotype in DC from patients with lupus. These DC produced low levels of IL-12 and high levels of IL-10 which reduced the differentiation of naïve cells into Th1 and Th17 and increased Treg cells (Wu et al., 2015). A study also demonstrated that 1,25(OH)2D3 in vitro induced IL-10 producing Treg cells (Barrat et al., 2002). In summary, there is in vitro evidence to suggest that 1,25(OH)2D3 can modulate the immune system towards an anti-inflammatory response, and reduce the proportion of pathogenic T cells directly or indirectly via APC.

1.4 Vitamin D in MS
1.4.1 Vitamin D deficiency in MS

Many autoimmune diseases have been associated with vitamin D deficiency, including type1 diabetes, rheumatoid arthritis and MS, suggesting that vitamin D may play a role in the
regulation of the immune system (Adorini and Penna, 2008). There have been several studies that associate lower vitamin D levels with MS (Munger et al., 2004, Munger et al., 2006). Whereas high serum vitamin D levels in MS has been associated with lower relapses (Smolders et al., 2008, Simpson et al., 2010). However, causality cannot necessarily be assumed from these associations and various factors may contribute to the association between low vitamin D and MS. Individuals with poor health, can often avoid time outdoors, and this must be taken into consideration when associating disease with low vitamin D. Indeed, a study by Munger et al., 2006 suggested that low vitamin D levels are a result of sun avoidance by MS patients, and not a direct cause of the disease (Munger et al., 2006).

1.4.2 Effect of Vitamin D in the EAE model
EAE is an animal model of autoimmune disease of the CNS. It resembles MS in many ways, and is used to investigate MS. There have been several studies carried out on the therapeutic effects of vitamin D after the induction of EAE or on the role of vitamin D in preventing EAE. Hauser et al., 1984 showed the effect of UV in preventing EAE, where UV administration was effective in preventing EAE before the initial immunisation of EAE induction, but UV irradiation was ineffective in reducing ongoing EAE or in preventing relapses that was induced by re-immunisation (Hauser et al., 1984). A study showed that 1,25(OH)₂D₃ inhibited EAE in wild type mice, however it did not inhibit EAE in IL-10 deficient mice. This showed that the IL-10-IL-10R pathway is essential for the inhibition of EAE in mice (Spach et al., 2006). Another study using 1,25(OH)₂D₃ to treat EAE showed that the treatment of 1,25(OH)₂D₃ during the immunisation phase in mice, prevented the onset of EAE (Cantorna et al., 1996). When 1,25(OH)₂D₃ was administered after the appearance of clinical signs, the severity of the disease was decreased in mice and also in rats (Cantorna et al., 1996, Nataf et al., 1996). Cantorna et al., 1996 also demonstrated that a diet deficient in vitamin D resulted in an increase of EAE severity, and demonstrated that removing 1,25(OH)₂D₃ from the diet resumed the clinical signs of EAE, which showed the importance of vitamin D in regulating EAE (Cantorna et al., 1996).

1.4.3 Vitamin D trials in MS
Taken together, the epidemiological, in vitro and in vivo data in the EAE model suggests that vitamin D supplementation could be beneficial in MS, and this has led to a number of trials in MS patients. However, many of these studies have not provided conclusive evidence due to defects in the trial design, such as lack of a placebo control group or blinding. Supporting studies showed the benefit of vitamin D₃ supplementations in RRMS patients. A phase two clinical trial performed in 2010 by Burton et al. used both vitamin D₃ and
calcium to assess the therapeutic impact of vitamin D immunologically and clinically in MS patients, as well as tolerability of vitamin D. The trial was a 52-wk randomised controlled non-blinded trial, with 49 participants, 45 were RRMS while the other 4 were SPMS – 25 in the test group, and 24 controls, however there was no placebo control. Varying vitamin D₃ doses up to 40,000 IU per day were administered to the test group for the first 28 wk, followed by 10,000 IU per day for 12 wk, after which the dosage was titrated down further, eventually reaching 0 IU per day. Participants in the test group were also administered a dosage of calcium alongside the vitamin D₃, which was an invariable amount of 1200 mg per day. Conversely, the control group were administered 4,000 IU per day over the 52 wk, with a dosage of calcium if desired. The mean baseline for serum 25(OH)D was 78 nmol/l, and as expected serum 25(OH)D rose significantly from baseline at 413 nmol/l. The results from this trial concluded that patients in the test group had fewer relapses, with a reduction of T cell proliferation in comparison to controls; this was determined by a T cell score. However, the trial was not powered or blinded to assess clinical outcomes and no significance was found between groups (Burton et al., 2010).

Another randomised double blinded controlled trial studied the safety of 20,000 IU vitamin D₃ weekly as an add-on therapy to IFNβ in patients with RRMS. The 1-year trial was conducted with 66 RRMS patients, the main endpoint of which was T2 burden of disease on MRI scans. Secondary endpoints were on the number of enhancing T1 lesions and T2 lesions, as well as relapse rate. The mean baseline levels of 25(OH)D were 54 nmol/l and rose to 110 nmol/l. 84% of patients had increased serum 25(OH)D above >85 nmol/l. The results of vitamin D₃ as an add-on to IFNβ showed significantly reduced MRI disease activity in RRMS patients (Soilu-Hänninen et al., 2012).

A double-blinded placebo controlled clinical trial performed in 2016 studied the effect of high dose vitamin D₃ intake on the quality of life in RRMS. There were 94 RRMS patients randomised into two groups - one group was administered 50,000 IU every five days for three months, while the other group was administered placebo at the same frequency. In both cases, each group continued IFNβ treatment for the duration of the trial. The results demonstrated that the vitamin D₃ group experienced a significant improvement in quality of life, in comparison to placebo (Ashtari et al., 2016).

A double blinded, single centre randomised pilot study with no placebo control was carried out in 2016. 40 RRMS patients were recruited and randomly split into groups to receive either 10,400 IU or 800 IU daily for 6 months. The results showed that serum 25(OH)D was
significantly higher in the high dose group than in the low dose group, the mean change from baseline to the end of the study in the high dose group being 87 nmol/L and in the low dose group 17 nmol/L. They also concluded that IL-17+CD4+ T cells, CD161+CD4+ T cells, and T cells were reduced significantly in the high dose group. The results also indicated that there was a significant increase in CM CD4+ T cells and naïve CD4+ T cells with a significant reduction in EM T cells (Sotirchos et al., 2016).

A 12-wk trial with 15 RRMS patients that were treated with IFNβ and were supplemented with 20,000 IU of vitamin D3 daily. The trial showed a significant increase in vitamin D from 50 nmol/L to 380 nmol/L. high dose vitamin D was well tolerated and did not induce decompensation of calcium metabolism. The trial also demonstrated that there was an increase in the proportion of IL-10+CD4+ T cells and a decrease in the ratio of IFN-γ+CD4+ T cells/IL-4+CD4+ T cells (Smolders et al., 2011).

There are also contradicting studies that showed that vitamin D2 was of no benefit to RRMS. In a randomised double blinded placebo control trial, in 23 RRMS that were administered high dose 6000 IU or 1000 IU of vitamin D2 daily for 6 months. This trial concluded that there was no therapeutic effect seen with vitamin D2 supplementation. However, this result could have been due to the short duration and low dose vitamin D2 regimens administered (Stein et al., 2011), or could be due to the use of vitamin D2 over vitamin D3, as a study in all primates species showed that vitamin D3 is more potent than vitamin D2 (Houghton and Vieth, 2006).

Kampman et al. 2012 conducted a randomised double blinded placebo controlled trial in 35 RRMS patients that were administered 20,000 IU vitamin D3 weekly for 96 wk. However, no effect on annualised relapse rated was observed when treated with vitamin D in addition to immune modifying treatments. This study did not indicate the treatments of RRMS patients and it is also possible that the negative result is due to the low dose administered to patients (Kampman et al., 2012).

A trial by Shaygannerjad et al. 2012 in randomised double-blind placebo controlled phase II trial in 25 RRMS that were receiving 4000 IU of vitamin D3 for 2 wk and increased to 8000 IU, and they demonstrated that vitamin D3 had no effect on MRI outcomes (Shaygannejad et al., 2012). Similarly, another study that did not have any effect on lesions however, after 6 months of vitamin D treatment there was a significant decrease in the proliferation of lymphocytes that were stimulated with PHA compared to control. After 6 months of vitamin D treatment IL-10 and transforming growth factor-beta was significantly higher in the
supernatants of PBMC that were stimulated with PHA compared to control (Mosayebi et al., 2011).

A double-blinded placebo controlled clinical trial performed in 2003 studied the effect of vitamin D supplementation and cytokine profile of RRMS. 39 RRMS patients were recruited and separated into control group n=22 and vitamin D treated group n=17. The vitamin D group were supplemented with 1,000 IU of vitamin D daily for 26 wks. Serum vitamin D significantly increased to 70 nmol/L following 6 months of vitamin D supplementation compared to control group. The trial also showed a significant increase in serum TGFβ in treatment group compared to control group after 6 months (Mahon et al., 2003a).

Although the above trials indicate that there is contradicting evidence on the effect of vitamin D₃ supplementation in RRMS patients. Vitamin D may exert therapeutic effects in MS via immunomodulation, however as summarised in Table 1.1, most of the studies were not of optimal design and lacked either placebo control or blinding. However, two studies were of optimal design and included both placebo control and blinding (Mahon et al., 2003a, Mosayebi et al., 2011). Additional studies that were not mentioned above were summarised in Table 1.1.

Prior to running a clinical trial at our centre, a pilot study was carried out in order to test and optimise the immunological analysis after vitamin D₃ supplementation in HC (Allen et al., 2012). The pilot study examined the immunomodulatory effects of vitamin D₃ in 4 healthy individuals over the course of 15 wk. All 4 participants were supplemented with 5,000 IU of vitamin D₃ per day for 10 wk, with half of the group receiving an increased dosage of 10,000 IU per day for the final 5 wk. After the 15 wk of vitamin D₃ supplementation, serum 25(OH)D was measured and had a mean baseline level of 38 nmol/l, which is insufficient, and the maximum concentration achieved were 151 and 191 nmol/l, when supplemented with 5,000 IU. When further supplemented with 10,000 IU, maximum concentration of serum 25(OH)D reached 152 and 223 nmol/l. PBMC were stimulated for 3-5 days with polyclonal activators or recall antigens and supernatants were harvested and cytokines IL-10, IFN-γ and IL-17 were measured by enzyme linked immune sorbent assay (ELISA). PBMC were also re-stimulated with PHA and restimulated with phorbol 12 myristate 13 acetate (PMA)/ionomycin to measure intracellular IL-17 and IFN-γ production by CD4⁺ T cells using flow cytometry (Allen et al., 2012). Overall, the pilot study showed a significant increase in the production of IL-10 by PBMC, and demonstrated a significant reduction in the frequency of Th17 cells in response to vitamin D₃ supplementation.
After the pilot study was performed, a randomised double-blinded placebo-controlled trial of vitamin D₃ supplementation in CIS patients was conducted at St. Vincent’s University Hospital, which forms the basis of this thesis (O’Connell et al., 2013). The endpoint of the trial was the immunological effects of two doses of vitamin D₃ compared to placebo over 24 wk, in both HC participants and CIS patients. There were 39 HC and 45 CIS patients recruited. The doses of vitamin D₃ supplementation were 5,000 IU, 10,000 IU and placebo. The primary endpoint was the immunological effects of vitamin D₃ on the frequency of CD4⁺ T cell subsets, and cytokine responses. Secondary endpoints in CIS patients included relapse activity, and the number of T2 lesions and gadolinium-enhancing lesions by MRI. This was performed in both vitamin D₃-treated CIS groups and compared to the placebo CIS group over a period of 24 wk (O’Connell et al., 2013).
<table>
<thead>
<tr>
<th>Vitamin D</th>
<th>Dose/day</th>
<th>Other TX</th>
<th>Length (wk)</th>
<th>#Treated</th>
<th>#Con</th>
<th>Blinded</th>
<th>Adverse Events</th>
<th>Serum 25(OH)D nM</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VitD&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10,000-40,000 IU</td>
<td>Ca</td>
<td>52</td>
<td>25</td>
<td>24</td>
<td>No</td>
<td>None</td>
<td>413</td>
<td>Trend</td>
<td>(Burton et al., 2010)</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>300,000 IU monthly</td>
<td>IFNβ</td>
<td>26</td>
<td>28</td>
<td>24 placebo</td>
<td>Yes</td>
<td>None</td>
<td>140</td>
<td>None</td>
<td>(Mosayebi et al., 2011)</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;3&lt;/sub&gt;</td>
<td>4,000-40,000 IU</td>
<td>Ca</td>
<td>52</td>
<td>25</td>
<td>24</td>
<td>No</td>
<td>None</td>
<td>179</td>
<td>Yes</td>
<td>(Kimball et al., 2011)</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;4&lt;/sub&gt;</td>
<td>100 IU</td>
<td>Diet</td>
<td>48</td>
<td>15</td>
<td>None</td>
<td>No</td>
<td>Yes</td>
<td>N/A</td>
<td>Trend</td>
<td>(Wingerchuk et al., 2005)</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;5&lt;/sub&gt;</td>
<td>20,000 IU</td>
<td>IFNβ</td>
<td>12</td>
<td>15</td>
<td>None</td>
<td>No</td>
<td>None</td>
<td>380</td>
<td>Yes</td>
<td>(Smolders et al., 2010)</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;6&lt;/sub&gt;</td>
<td>1,000 IU</td>
<td>Ca</td>
<td>26</td>
<td>17</td>
<td>22 placebo</td>
<td>Yes</td>
<td>None</td>
<td>70</td>
<td>No</td>
<td>(Mahon et al., 2003a)</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;7&lt;/sub&gt;</td>
<td>4,000-40,000 IU</td>
<td>Ca</td>
<td>28</td>
<td>12</td>
<td>None</td>
<td>No</td>
<td>None</td>
<td>386</td>
<td>Yes</td>
<td>(Kimball et al., 2007)</td>
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<tr>
<td>VitD&lt;sub&gt;8&lt;/sub&gt;</td>
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<td>IFNβ</td>
<td>52</td>
<td>66</td>
<td>32</td>
<td>Yes</td>
<td>Yes</td>
<td>163</td>
<td>Yes</td>
<td>Soilu-Hänninen, Åivo et al. 2012</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;9&lt;/sub&gt;</td>
<td>50,000 IU five days</td>
<td>IFNβ</td>
<td>13</td>
<td>94</td>
<td>N/A</td>
<td>Yes</td>
<td>None</td>
<td>N/A</td>
<td>Yes</td>
<td>Ashtari, Toghianifar et al. 2016</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>10,400-800 IU</td>
<td>Various</td>
<td>26</td>
<td>40</td>
<td>No Control</td>
<td>Yes</td>
<td>Yes</td>
<td>101</td>
<td>Yes</td>
<td>Sotirchos, Bhargava et al. 2016</td>
</tr>
<tr>
<td>VitD</td>
<td>Type of Treatment</td>
<td>Number of MS patients</td>
<td>Number of Controls</td>
<td>Blinded Trial</td>
<td>Adverse Events</td>
<td>Max Levels Mean Serum 25(OH)D</td>
<td>Therapeutic Effect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
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<td>-------------------</td>
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<td>-----------------------------</td>
<td>------------------</td>
<td></td>
<td></td>
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<tr>
<td>4000 IU-8000 IU</td>
<td>Various</td>
<td>52</td>
<td>25</td>
<td>25</td>
<td>Yes</td>
<td>Yes</td>
<td>N/A</td>
<td>No</td>
<td>(Shaygannejad et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>20,000 IU</td>
<td>Various</td>
<td>96</td>
<td>35</td>
<td>33</td>
<td>Yes</td>
<td>Yes</td>
<td>121</td>
<td>No</td>
<td>(Kampman et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>6000 IU-10,000 IU</td>
<td>IFNβ</td>
<td>26</td>
<td>23</td>
<td>None</td>
<td>Yes</td>
<td>None</td>
<td>120</td>
<td>No</td>
<td>(Stein et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>100,000 IU</td>
<td>IFNβ</td>
<td>104</td>
<td>63</td>
<td>66</td>
<td>Yes</td>
<td>None</td>
<td>N/A</td>
<td>Yes</td>
<td>(Camu et al., 2017)</td>
<td></td>
</tr>
</tbody>
</table>

1 Type vitamin supplemented

2 Other treatment, supplements or restrictions (Ca= calcium supplementation, diet= dietary calcium restriction)

3 Number of MS patients treated

4 Number of controls (placebo or unrelated) patients

5 Blinded trial yes/no

6 Adverse events

7 Maximum levels of the mean concentration of serum 25(OH)D

8 Therapeutic effect of vitamin D supplementation (trend= non-significant; NR= not report)
1.5 Psoriasis

Psoriasis is a chronic inflammatory skin disease, where erythematous plaques or lesions can be found on the knees, elbows, scalp or intergluteal cleft (Green et al., 1996). The most common type of psoriasis is psoriasis vulgaris which also known as plaque psoriasis, which affects more than 80% of patients (Lebwohl, 2003). It was previously thought that psoriasis was an epidermal disease, however the immune system has a prominent role in the disease pathogenesis (Bjerke and Krogh, 1978, Bjerke et al., 1978).

1.5.1 Pathogenesis of psoriasis

Psoriasis has been previously thought to be mainly an epidermal disease, however in recent years it has been shown that the epidermal change observed in psoriasis is due to the infiltration of immune cells into the skin (Lebwohl, 2003, Greb et al., 2016). T cells have been strongly implicated in psoriasis, and early studies showed that early psoriatic lesions contain T cells and macrophages (Krogh and Bjerke, 1979, Bjerke et al., 1978). It was demonstrated that cyclosporine, a general T cell immunosuppressant, was capable of clearing psoriasis, suggesting that T cells were involved in the disease. It was later found that the drug DAB389IL-2 which specifically targets T cells but not keratinocytes, was capable of clearing psoriasis, providing further evidence that T cells were implicated in psoriasis (Gottlieb et al., 1995). Psoriasis is triggered by an unknown antigen which matures epidermal antigen specific tissue resident Langerhans cells. Langerhans cells then migrate to the lymph nodes where they interact with naïve T cells, which in turn activates T cells. These activated T cells proliferate and enter the bloodstream and traffic towards the skin. In order to migrate to the skin, a combination of chemokine receptors and glycoproteins on T cells must interact with adhesion molecules on the endothelium (Lebwohl, 2003).

1.5.2 Inflammatory T cells in psoriasis

The inflammation, infiltration, epidermal hyper proliferation and keratinocyte differentiation in psoriasis is driven mainly by cytokines and chemokines from activated pathogenic T cells. These cytokines from T cells are capable of stimulating cells such as DC, macrophages and keratinocytes, which in turn secrete cytokines that play a role in maintaining the chronic inflammatory disease (Robert and Kupper, 1999, Bonifati and Ameglio, 1999). It has been demonstrated that psoriatic lesions contain a high number of T cells (Bos et al., 1983), and that T cells from psoriatic skin lesions activated epidermal keratinocyte proliferation (Bata-Csorgo et al., 1995b, Bata-Csorgo et al., 1995a). For many years psoriasis was known as a Th1 mediated disease in which IFNγ and TNFα were the main proinflammatory cytokines.
This was later revised with the discovery of Th17 cells (Di Cesare et al., 2009). Th17 cells and their effector cytokines IL-17A, IL-17F, IL-21, GM-CSF, IL-22 and TNFα have been identified as playing a key role in psoriatic lesions, and elevated levels of these cytokines were also observed in the peripheral blood of psoriasis patients (Kagami et al., 2010). The Th17 signature cytokine IL-17A has been shown to induce epidermal hyperplasia in an IL-23-driven murine model of psoriasis (Chan et al., 2006). IL-17 has also been shown to activate the immortalised human keratinocyte cell line HaCat in vitro, via inhibition of CCATT/enhancer binding protein alpha (Ma et al., 2016). IL-17 can also synergise with TNFα to induce a higher expression of proinflammatory messenger ribonucleic acid (mRNA) of IL-8 and S100A7 from keratinocytes compared with IL-17 or TNFα alone (Chiricozzi et al., 2011). Another cytokine produced by Th17 cells is IL-22, which has been shown to increase keratinocyte proliferation and inhibit keratinocyte differentiation in psoriasis (Boniface et al., 2005). The IL-23/Th17 signalling pathway plays a crucial role in the pathogenesis of psoriasis (Kim and Krueger, 2017). IL-23 plays a critical role in maintaining the effector functions of Th17 cells (McGeachy et al., 2009) and it has been suggested that IL-23/IL-23R plays a role in the differentiation maintenance and pathogenicity of Th17 cells (Hirota et al., 2011). Further evidence for a key role of IL-23 in psoriasis come from the success of IL-23p19 inhibitors which have recently been approved for the treatment of psoriasis (Chan et al., 2018).

### 1.5.3 Treg cells in psoriasis

Treg cells play a crucial role in maintaining antigen specific self-tolerance and help to prevent tissue damage caused by inflammation. Treg cells are found in abundance in the skin compared to the circulation or other non-lymphoid tissue, which makes them a likely candidate for controlling psoriatic skin inflammation (Ali and Rosenblum, 2017). It was also demonstrated in some studies that there is an increased frequency of Treg cells in psoriatic lesions compared with controls or uninvolved skin biopsies (Yan et al., 2010a, Bovenschen et al., 2006, Zhang et al., 2010). However, in the study by Zhang et al, they found that even though there was an increase in Treg cells in psoriatic lesions they were unable to suppress the inflammation, suggesting that they were functionally defective (Zhang et al., 2010). It was also shown by another study that Treg cells isolated from psoriatic lesions or blood from psoriasis patients were reduced or unable to suppress effector cells in vitro in an alloantigen-specific response or polyclonal TCR response (Sugiyama et al., 2005). It has also been shown that the dysfunction of Treg cells observed in psoriatic lesions was due to the proinflammatory cytokine milieu, specifically IL-6 secreted by endothelial cells, DC and...
Th17 cells, which has been shown to inhibit the activity of Treg cells; this allows effector cells to evade suppression by Treg cells (Goodman et al., 2009, Clark, 2010). It was also demonstrated in vitro that an IL-6-specific antibody reversed the impairment in Treg cells from psoriasis patients (Goodman et al., 2009).

1.5.4 Risk factors for psoriasis

The risk factors associated with psoriasis include both genetic and environmental factors. The evidence for genetic predisposition to psoriasis was proven by twin studies in the 1970s, which showed increased concordance in monozygotic twins compared with dizygotic twins (Lomholt, 1963). Since then, many advances have occurred in understanding the genetics of psoriasis. One example of this is psoriasis susceptibility 1 (PSORS1) which is a major determinant of psoriasis that was identified in the mid-1970s (Lomholt, 1963, Trembath et al., 1997). The most likely implicated PSORS1 gene is HLA-C and its HLA-Cw*060 allele, and 60% of psoriasis patients carry this allele (Russell et al., 1972, Tiilikainen et al., 1980). Several psoriasis associated genes from signalling pathways have been identified, and it has been suggested that these pathways are involved in psoriasis pathogenesis, such as the IL-23/Th17 pathway (Di Cesare et al., 2009), the nuclear factor kappa B (NF-κB) signalling pathway (Nair et al., 2009) and the epidermal differentiation complex (Zhang et al., 2009, Capon et al., 1999a, Capon et al., 1999b). Several environmental triggers have been implicated in psoriasis such as streptococcal infection (Valdimarsson et al., 2009), physical trauma (Raychaudhuri et al., 2008), medication such as lithium, β-blockers and IFNα used for the treatment of malignant melanoma and hepatitis C (Abel et al., 1986), and smoking (Jin et al., 2009).

1.5.5 Diagnosis of psoriasis

The most common form of psoriasis is psoriasis vulgaris, affecting more than 80% of patients. Other types of psoriasis include guttate, inverse, pustular, erythrodermic, palmoplantar, and drug-associated psoriasis (Lebwohl, 2003, Nestle et al., 2009). Diagnosis of psoriasis is solely dependent on clinical features. It is usually identified by the salmon-coloured erythematous macules with silvery white dry scale located on the knees, elbows and scalp, however it can be on any part of the body (Lebwohl, 2003). Psoriasis can be measured and assessed using the psoriasis activity and severity index (PASI) score. This assessment tool is used to measure the severity and area of erythema, induration, desquamation of the plaques in different areas, with ranges from 0 to 72 (Fredriksson and Pettersson, 1978).
1.5.6 Immunomodulatory therapies for psoriasis

The treatment of psoriasis is divided into four categories depending on the location and severity of the disease. Topical treatment is usually used when psoriasis is mild to moderate, with up to 80% of psoriasis patients using topical treatments as their main therapy (Menter et al., 2010, Koo et al., 2017). Corticosteroids are the most commonly prescribed treatment for psoriasis, they are very effective and have many strengths however, side effects include cutaneous atrophy formation of telangiectasia and striae (Lebwohl, 2003). Topical vitamin D analogues have been shown to be moderately effective are not associated with the cutaneous atrophy that is associated with corticosteroid use (Nagpal et al., 2001). When psoriasis is resistant to topical treatments, or body surface area (BSA) involvement is too widespread, phototherapy or systemic therapy is used. Broadband UVB phototherapy has been used for psoriasis since 1920 (Larko, 1989). However, narrowband UVB is the primary choice and far more effective than broadband UVB (Coven et al., 1997). Psoralen-Ultraviolet A (PUVA) therapy is highly effective but only used if narrowband UVB fails, or in specific types of psoriasis such as pustular or erythrodermic psoriasis. PUVA, which was developed in the 1970s, requires the ingestion of psoralen followed by UVA photo treatment (Melski et al., 1989), however PUVA has been associated with cancer (Stern et al., 1998, Stern et al., 1997). If phototherapy is unsuccessful, then a range of systemic drugs can be administered, such as methotrexate, which has been used since 1958 for the treatment of psoriasis (Edmundson and Guy, 1958). The main mechanism of action of methotrexate is thought to be the inhibition of nucleic acid synthesis in T cells and in keratinocytes, which has been suggested to have anti-proliferative and immunomodulatory effects (Cronstein et al., 1994, Elango et al., 2014). Cyclosporine is one of the most effective and rapid acting drugs which is often used as a first line therapy for psoriasis. Its mechanism of action is to inhibit T cell activation by binding to the intracellular receptor protein cyclophilin-1, this in turn inhibits calcineurin, which inhibits the dephosphorylation of transcription nuclear factor of activated T cells (NF-AT) (Guaguere et al., 2004, Mueller and Herrmann, 1979). Retinoids such as etretinate, acitretin and isotretinoin have been used for the treatment of psoriasis. The exact mechanism of action of retinoids is not fully clear; however, retinoids have anti-proliferative and immunomodulatory properties and can inhibit production of vascular endothelial growth factor by keratinocytes (Young et al., 2006). Fumaric acid esters have been approved in Germany since 1994 for the treatment of psoriasis (Altmeyer et al., 1994). Fumaric acid has many immunomodulatory effects however the exact mechanism of action has yet to be uncovered and will be explored in greater detail further on. If systemic treatments have not been successful for the psoriatic patients, then the next line of treatments
are biologics such anti-TNFα drugs including etanercept 145 (Nestorov et al., 2006), infliximab (Gottlieb et al., 2004), adalimumab 252 (Gordon et al., 2005) and certolizumab (Lebwohl et al., 2018). Biologics that target the Th17 cytokine IL-17 including secukinumab and ixekizumab and brodalumab have proven highly effective in treating psoriasis (Tobin and Kirby, 2005, van de Kerkhof et al., 2016, Krueger et al., 2012, Blauvelt et al., 2017). Finally, ustekinumab, which target the IL-12/23p40 molecule and thereby inhibit both Th1 and Th17 cells have been used to successfully treat psoriasis (Gordon et al., 2012). Finally, monoclonal antibodies that inhibit the IL-23p19 subunit, gueselkumab (Gordon et al., 2015), tildrakizumab (Papp et al., 2015) and risankizumab (Krueger et al., 2015) are in clinical trials for the treatment of moderate to severe psoriasis.

1.6 DMF
DMF is derived from fumaric acid and has significant immunomodulatory effects. DMF is a fumaric acid ester derivative of the Krebs cycle intermediate fumarate, and in 1959 the German chemist Schweckendieck, who himself suffered from psoriasis, hypothesised that the disease might be caused by a disturbance in the citric acid cycle. He suggested that the oral supplementation of fumaric acid might reverse the disease. Schweckendieck used esters of fumaric acid since fumaric acid itself was a gastrointestinal irritant (Schweckendieck, 1959). Although Schweckendieck never proved that there was a deficiency in the citric acid cycle in psoriasis, patients nevertheless seemed to have benefited from this oral supplementation (Schweckendieck, 1966). In Germany, the combination of DMF and monomethyl fumarate (MMF) is marketed as Fumaderm™ which is a registered drug for the treatment of psoriasis (Altmeyer et al., 1994). BG-12 (Tecfidera®), in which DMF is the active ingredient, is approved by the Food and Drug Administration (FDA) and European Medicine Agency (EMA) as an oral treatment for relapsing forms of MS (Bomprezzi, 2015). DMF can mediate a broad range of anti-inflammatory and cytoprotective effects. These beneficial effects were demonstrated in preclinical models of neuro-inflammation, neurodegeneration, and toxic oxidative stress (Linker et al., 2011, Scannevin et al., 2012). However, its exact mechanism of action still remains unclear and it is evident that DMF exerts profound effects on the redox status of cells via its oxidative effects. DMF has the capacity to modify cysteines within various proteins in the cell, with the potential to modify their function. Paradoxically however, the oxidative capacity of DMF may trigger anti-oxidant responses within the cell, resulting in overall anti-oxidant and anti-inflammatory effects.
1.6.1 Oxidative stress

Oxygen is crucial for multicellular life; however, it is the most reactive and life-threatening agent recognised. To prevent the possible harmful effects of oxygen, intracellular homeostasis is maintained by a balance between oxidation and reduction reactions, known as the intracellular redox equilibrium (Winterbourn, 2008). When this balance is disturbed, and the pro-oxidants outbalance the anti-oxidants, this causes oxidative stress. Oxidative stress results in oxidation induced damage to proteins, lipids, carbohydrates, and nucleic acid, which ultimately leads to cell death. The agents that induce oxidative stress are chemical compounds called ROS, or reactive nitrogen species (RNS) (Miller, 2012, Devasagayam et al., 2004, Kowaltowski et al., 2009). Both are unstable, and have been suggested to play a role in the pathogenesis of autoimmune diseases such as MS and psoriasis. One of the major sources of ROS in the cell are from the electron transport chain (ETC) within the mitochondria (Kowaltowski et al., 2009). There are also enzymes that are found in the mitochondria, endoplasmic reticulum, peroxisomes and cytosol, that produce ROS as a by-product of their reactions (Hanschmann et al., 2013). It is important to note that ROS is not only produced endogenously via mitochondria but can also be exogenously induced via ultra violet light, gamma radiation, smoke, and other air pollutants, and several drugs which include fumarate esters; DMF and MMF (Hanschmann et al., 2013).

1.6.2 Mechanisms of action of DMF

DMF is not detectable in the plasma of patients treated with DMF. It was previously assumed that DMF was converted to MMF in the small intestine mucosa (Litjens et al., 2004b). Glutathione and DMF form an adduct S(1,2-dimethoxycarbonylethyl) glutathione (GS-DMS). It has been found that mercapturic acid can be found in the urine of Fumaderm™ treated psoriasis patients, mercapturic acid is the breakdown of GS-DMS (Rostami and Ciric, 2013). It was also suggested that DMF is not completely hydrolysed into MMF in the small intestine as was once thought (Rostami-Yazdi et al., 2010). Until now the precise mechanism of action of DMF in psoriasis has not yet been established, although several mechanisms of action in various cell types have been described in the literature. A summary of these mechanisms in T cells are described in Fig 1.4 and in innate cells in Fig 1.5.

1.6.2.1 Induction of the antioxidant pathway by DMF

Stabilisation of Nrf2 by DMF

All cells have an innate mechanism that neutralises excess ROS, and this oxidative stress response is mainly achieved by the Nrf2. Nrf2 is important in maintaining cellular homeostasis, and its role is more prominent when cells are exposed to chemical or oxidative
stress. Nrf2 has the ability to induce the expression of antioxidant proteins, detoxifying enzymes and xenobiotic transporters, if the cell is under oxidative stress, this is called the anti-oxidative stress response. These Nrf2-induced molecules include glutathione, thioredoxin and detoxifying enzymes like heme oxygenase-1 (HO-1) amongst others.

It was first described that DMF can reduce oxidative stress by activating Nrf2 and inducing the expression of the stress response protein HO-1 (Kwak et al., 2004, Wilms et al., 2010, Linker et al., 2011, Gold et al., 2012, Scannevin et al., 2012). Under normal conditions, Nrf2 is constantly degraded through the ubiquitin-proteasome pathway in a Keap1 dependent manner (Sekhar et al., 2002). However, in the presence of electrophiles such as DMF or ROS, which can modify reactive cysteine residues within Keap1, Nrf2 is stabilized and no longer degraded. This modification of reactive cysteine residues within Keap1 impairs the structural integrity of the Keap-Cul3 E3 ligase complex, which declines ubiquitination activity (Itoh et al., 2004). This allows Nrf2 to accumulate in the nucleus, heterodimerise with small Maf proteins, and activate target genes for cytoprotection through antioxidant response elements (ARE) and electrophile response elements (EpRE) (Itoh et al., 2004). Thus, by acting in a similar manner to ROS, DMF mimics conditions of oxidative stress which turns on antioxidant pathways with the cell.

**Inhibition of NF-κB by DMF**

NF-κB plays a crucial role in regulating a range of immune functions such activation and differentiation of lymphocytes and the maturation and inflammatory functions of innate cells. DMF was shown to be capable of directly inhibiting NF-κB in the breast cancer cell line MCF-7 by directly binding to p65 via its numerous reactive cysteines (Kastrati et al., 2016). In stimulated human T cells, DMF inhibited NF-κB in a dose dependant manner (Gerdes et al., 2007). Similarly, a study also showed that MMF inhibited NF-κB in human monocyte derived DC (Litjens et al., 2004a). Interestingly, in NIH 3T3 fibroblast cells, DMF induced HO-1, which interacted with NF-κB sites of the IL-23p19 promoter (Ghoreschi et al., 2011). This prevented the transcription of IL-23p19 without affecting IL-12p35 (Ghoreschi et al., 2011). In endothelial cells DMF inhibited nuclear entry of NF-κB (Loewe et al., 2002). In LPS stimulated bone marrow derived dendritic cell (BMDC) maturation of DC was inhibited by DMF via inhibition of the NF-κB pathway as a result of suppression of p65 phosphorylation and nuclear localization (Peng et al., 2012). These studies suggest that via the inhibition of NF-κB activation, DMF treatment results in downstream reduction of
proinflammatory cytokines, inhibition of maturation of APC and the modulation of T cell function.

**Nrf2 independent effects of DMF**

Although as described above, DMF has been shown to exert anti-inflammatory activity via Nrf2, there is also evidence to suggest that the mechanism of action of DMF *in vivo* may be independent of Nrf2. A study by Schulz-Topphoff et al., 2016 demonstrated that oral DMF provided equal anti-inflammatory benefit in acute EAE in Nrf2<sup>-/-</sup> vs wild type mice, and suggested that the anti-inflammatory effect of DMF in MS patients may occur independently of Nrf2 (Schulze-Topphoff et al., 2016). Another study showed that NF-κB mediated cytokine production was inhibited in Nrf2<sup>-/-</sup> primary splenocytes, confirming that DMF inhibits proinflammatory cytokines independent of Nrf2 (Gillard et al., 2015). Gillard et al., 2015 also demonstrated that DMF inhibited proinflammatory cytokine production by primary splenocytes in both wild type mice and in Nrf2<sup>-/-</sup> deficient mice equally demonstrating that DMF can suppress NF-κB dependent responses independently of Nrf2 (Gillard et al., 2015) Studies have also shown that *in vitro*, DMF inhibited the expression of inflammatory cytokines via Nrf2-independent pathways in human umbilical vein endothelial cells (Bista et al., 2012, de Martin et al., 2001), and in human spinal cord astrocytes (Bista, Zeng et al. 2012).

**DMF induces the antioxidant pathway through reaction with glutathione**

DMF which is a fumaric acid ester, is also known as an electrophile as it reacts with nucleophiles such as sulfhydryl groups. It been established that DMF represents an alpha beta unsaturated carbonyl system and can react with glutathione by the addition of a nucleophile to an alpha beta unsaturated carbonyl compound in near physiological conditions *in vitro* (Schmidt et al., 2007b). It was also demonstrated that DMF reacted with glutathione in the portal vein blood *in vivo* in rats (Dibbert et al., 2013). Interestingly, only MMF and GS-DMS could be detected in plasma after intestinal administration of DMF in rats (Dibbert et al., 2013). This demonstrated that DMF forms adducts with glutathione and is rapidly metabolised. DMF has been shown to reduce cytokine production via depletion of glutathione; the depletion of glutathione induces oxidative stress, which ultimately induces HO-1 through induction of the anti-oxidant pathway (Lehmann et al., 2007). Another study suggested that DMF could enhance glutathione, most likely via induction of the Nrf2 antioxidant pathway, which helped cells to deal with oxidative stress (Scannevin et al., 2012). Additionally, DMF has been shown to inhibit PMA induced neutrophil extracellular traps (NET) and this is directly associated with DMF ability to interact or deplete
glutathione, the inhibition of PMA induced NET was reversed when supplemented with glutathione (Hoffmann et al., 2018).

**Modification of protein kinase c theta (PKCθ) by DMF**

PKCθ plays a crucial role in the early activation of T cells. It is an important kinase in T cell activation through ligation of the TCR and co-stimulatory marker CD28. When T cells are activated via TCR and costimulatory molecules, PKCθ is recruited to interact with CD28 (Kong et al., 2011, Altman and Villalba, 2003). A study detected two DMF sensitive cysteine residues located on PKCθ, namely Cys14 and Cys17 in both human and murine T cells. Interestingly Cys14 and Cys17 form a CXXC motif that is only found in the C2 domain of PKCθ and not any other PKC isoforms. Furthermore, this study showed that DMF was capable of blocking the interaction between T cell PKCθ and CD28 in mice, which in turn impaired T cell activation (Blewett et al., 2016). This study suggested that this particular C domain that is only found in PKC could be a good potential for suppressive therapies. Taken together, these studies suggest that DMF can inhibit T cell activation via modification of PKCθ.

**1.6.2.2 Modulation of metabolism by DMF**

Both Th1 and Th17 cells contribute to the pathogenesis of psoriasis, and it has been shown that Th1 and Th17 cells require glycolysis, whereas oxidative phosphorylation favours the differentiation of Treg cells (Chang et al., 2013, Gerriets et al., 2015). Similarly, the activation of macrophages and DC allows them to favour glycolysis over oxidative phosphorylation (Kelly and O'Neill, 2015). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) is an important enzyme that breaks down glucose in the glycolytic pathway (Sirover, 1999). Recently, a study by Kornberg et al. 2018, showed that DMF and its metabolite MMF can target the active cysteine site of the enzyme GAPDH in vitro and in vivo in humans PBMC and mice peritoneal macrophages (mPM). It was also demonstrated that this inhibition of GAPDH downregulated glycolysis in myeloid and lymphoid cells. Interestingly glycolysis was only inhibited when mouse mPM were stimulated with LPS or when mouse CD4 were stimulated with anti-CD3/CD28, this agrees with studies that showed that GAPDH becomes a rate limiting enzyme only when glycolysis is upregulated (Shestov et al., 2014). The study also demonstrated that the inhibition of glycolysis by DMF inhibited macrophage maturation, and survival and differentiation of T cell subsets. Furthermore, use of the GAPDH inhibitor heptelidic acid could replicate the inhibition of GAPDH that was seen with DMF (Kornberg et al., 2018). It has been demonstrated that hypoxia inducible factor 1 alpha (HIF-1α) induces the glycolytic pathway (Cheng et al., 2014). However, the
evidence of the effect of DMF on HIF-1α is conflicting. One study showed that DMF could inhibit HIF-1α in MC3T3 E1 cells (Zhao et al., 2014). However, contrasting results suggest that DMF can induce HIF-1α in astrocytes (Wiesner et al., 2013). More studies are needed to fully understand the effects of DMF on metabolic pathways, and strategies to target metabolism could be a viable therapeutic option for autoimmunity.

1.6.2.3 Modulation of innate cell function by DMF

Modulation of APC function by DMF

APC can influence the differentiation of pathogenic T cells such as Th1 and Th17 cells that play a key role in autoimmune diseases. The importance of DC in directing CD4+ T cell differentiation have prompted studies to investigate the effect of DMF on APC function, including their expression of co-stimulatory molecules/maturation markers and polarising cytokines in response to PRR activation. A study showed that DMF can block the formation of M1 and K63 ubiquitin chains downstream of TLR signalling in murine BMDC, and also inhibit E2 enzymes Ubc13 and UbcH7 that are involved in the formation of both K63 and M1 chains (McGuire et al., 2016). This study also demonstrated that DMF can inhibit the transcription of cytokines by BMDC independently of Nrf2 (McGuire et al., 2016). Interestingly, a study by Peng et al., 2012 showed that DMF inhibited the expression of MHC class II, CD80, and CD86 on BMDC from mice (Peng et al., 2012). They also showed that p65 activation was fully reduced by DMF-mediated MSK1 reduction, resulting in a decrease in phosphorylation of p65 at serine 276 and histone-3 at serine 10, which played a vital role in suppressing maturation markers on DC (Peng et al., 2012). Another study also demonstrated that MMF treatment impaired the maturation of human myeloid dendritic cells (mDC), as shown by reduced expression of CD86, CD40, CD83, MHC class II and NF-κB (Mazzola et al., 2017). This immature phenotype of mDC is associated with an impaired ability of the mDC to activate T cells, which results in a decrease in proliferation and inflammatory cytokine production by T cells (Mazzola et al., 2017). It was also shown that MMF reduced the expression of CCR7, suggesting that MMF might reduce the ability of mDC migrate to lymphoid organs and induce inflammation (Mazzola et al., 2017).

Cytokine production is also initiated when APC are activated and mature. This unique microenvironment of cytokines influences naïve T cell differentiation. Interestingly, a study showed that DMF affected maturation of 6-sulfo LacNAc DC (slanDC) by decreasing inflammatory cytokines IL-12 and IL-23 (Oehrl et al., 2017). It was also demonstrated that DMF can impair the capacity of DC to produce IL-12 or IL-23, with a significant increase in IL-10, which makes them phenotypically type II DC (Ghoreschi et al., 2011). However,
another study showed that MMF inhibited IL-12p70 and IL-10 in DC, which were less effective in polarising T cells into Th1 and Treg cells (Litjens et al., 2006). The same group showed that, in comparison to the control, MMF-DC that were LPS stimulated dramatically reduced IL-12p70 and IL-10 but not IL-8 or TNFα (Litjens et al., 2004a). Another interesting study showed that DMF induced type II DC in both human and mice (Ghoreschi et al., 2011). These induced type II DC shared the major features of classical type II DC, which suppress Th1 cell development and prevent diseases such as EAE (Racke et al., 1994, Chora et al., 2007). Taken together these studies suggest that DMF exerts anti-inflammatory effects on various types of DC, which would be expected to reduce the activation and differentiation of inflammatory T cell subsets.

**Activation of the hydroxycarboxylic acid receptor (HCA2) by DMF**

HCA were formally known as nicotinic acid receptors and are G-protein coupled receptors and HCA2 is one of the family subtypes (Wise et al., 2003). Niacin is a ligand for HCA2, and has been shown to inhibit the expression of proinflammatory molecules by LPS stimulated adipocytes and THP-1 macrophages (Mandrika et al., 2018). The same study also demonstrated that LPS stimulation upregulated the expression of HCA2 in both adipocytes and macrophages, and its ligand was capable of counteracting the LPS mediated proinflammatory cytokine production.

It was also demonstrated that DMF acts as an agonist for HCA2 and thereby inhibited neuroinflammation (Chen et al., 2014, Offermanns and Schwaninger, 2015). DMF reduced neutrophil adhesion, migration and infiltration into the CNS in the EAE mode (Chen et al., 2014). The same study also showed that DMF inhibited the severity of EAE in wild type mice but not in HCA2−/− deficient mice. DMF treatment also lowered the number of CD11c+ DC and this trend wasn’t seen in HCA2−/− deficient mice (Chen et al., 2014). They also demonstrated that DMF reduced the infiltration of CD4+ T cells into the CNS in wild type mice but not in HCA2−/− deficient mice (Chen et al., 2014). This showed that DMF exerted its effects in a HCA2-dependant manner (Chen et al., 2014). Another study showed that activation of HCA2 by MMF induced Ca2+ which inhibited NF-κB dependent gene expression, and it was also suggested by the study that MMF initiated a downstream signalling pathway that inhibited NF-κB in microglial cells (Parodi et al., 2015). These data suggest a role for the HCA2 in mediating the effects of DMF, at least in the EAE model. However, the role of the HCA2 in other settings still needs to be evaluated.
1.6.2.4 Induction of apoptosis by DMF

As discussed above, many different mechanisms of action have been described for DMF. However, DMF can also induce apoptosis in CD4\(^+\) and CD8\(^+\) T cells \textit{in vitro} (Treumer et al., 2003a) and such an attrition of lymphocytes by DMF may be another way in which it exerts therapeutic effects in patients. Indeed, both psoriasis and MS patients treated with DMF are known to experience lymphopenia to different degrees. Interestingly, the study by Ghadiri et al., 2017, showed that compared with naïve subsets, memory CD4\(^+\) and CD8\(^+\) T cells were more susceptible to DMF-induced apoptosis in RRMS patients \textit{in vivo} and HC \textit{in vitro}. They also indicated that there was a greater loss of CD8\(^+\) T cells in comparison to CD4\(^+\) T cells.

This study revealed that DMF induced apoptosis in CD4\(^+\) and CD8\(^+\) T cells within PBMC and purified T cells from HC, and to a greater extent in CD8\(^+\) T cells than CD4\(^+\) T cells in PBMC and purified T cells from HC. (Ghadiri et al., 2017).

In contradiction to the \textit{in vitro} data, the \textit{in vivo} treatment of RRMS patients with DMF did not make circulating T cells more susceptible to apoptosis, and it was suggested that these circulating cells within RRMS patients were resistant to treatment (Ghadiri et al., 2017). However other studies revealed that there were reductions in memory T cell subsets were when RRMS patients were treated with DMF (Longbrake et al., 2016, Gross et al., 2016). This indicated that naïve cells are relatively more protected from the effects of DMF treatment. A study by Diebold, 2017 indicated that DMF \textit{in vivo} increased ROS significantly after 3 month of treatment in RRMS patients, and all lymphocyte subpopulation counts dropped significantly over the course of 12 months (Diebold et al., 2017). CD8\(^+\) T cells and EM CD4\(^+\) T cells were most significantly affected. They also demonstrated that \textit{in vitro}, DMF induced cell death and inhibited proliferation of T cells (Diebold et al., 2017). In agreement with the Diebold, 2017 study, an \textit{in vitro} study showed that DMF upregulated the expression of Apo2.7, which is a mitochondrial protein that can indicate early apoptosis (Diebold et al., 2017). Another study showed a dose dependent decrease in Bcl-2 expression in IL-2 stimulated human T cells that had been treated with DMF \textit{in vitro} (Treumer et al., 2003b). Another study also demonstrated that \textit{in vitro} DMF increased apoptosis and decreased proliferation of CD4\(^+\) T cells (Wu et al., 2017). They also showed that DMF reduced ROS, which is conflicting with the Diabold et al., 2017 study, suggesting that the ability of DMF to induce or reduce ROS is still unclear (Diebold et al., 2017, Wu et al., 2017).
Fig 1.4 Summary of the mechanisms of DMF on adaptive immune cells
DMF has various mechanisms of action in which it can exert its effects on the adaptive immune cells including: 1. DMF induces the antioxidant pathway via Nrf2 (Kwak, Wakabayashi et al. 2004, Wilms, Sievers et al. 2010, Linker, Lee et al. 2011, Gold, Linker et al. 2012, Scannevin, Chollate et al. 2012) 2. DMF can deplete glutathione and increase ROS, which can induce the antioxidant pathway via Nrf2 (Lehmann, Listopad et al. 2007) 3. DMF can inhibit PKCθ at the cysteine level, which inhibits the TCR signaling pathway (Blewett et al., 2016). 4. DMF inhibits NF-κB directly (Gerdes, Shakery et al. 2007) 5. DMF inhibits the glycolytic enzyme GAPDH (Kornberg et al., 2018).
Fig 1.5 Summary of the mechanisms of DMF on innate immune cells

DMF has various mechanisms in which it can exert its effects on the innate immune cells including:


2. DMF can deplete glutathione and form an adduct with glutathione and increase ROS, which can induce the antioxidant pathway via Nrf2 (Schmidt, Ak et al. 2007, Dibbert, Clement et al. 2013, Lehmann, Listopad et al. 2007)

3. DMF is an agonist to the HCA2 receptor, HCA2 activation leads to inhibition of NF-κB (Offermanns and Schwaninger 2015)

4. DMF inhibits the recruitment of K63 and M1 downstream of TLR activation, which inhibits NF-κB activation and translocation (McGuire et al., 2016)

5. DMF inhibits the glycolytic enzyme GAPDH (Kornberg et al., 2018)
1.6.3 Effect of DMF therapy in patients

It has been described that Fumaric Acid Esters (FAE) such as DMF had adverse effects in phase 3 clinical trials in RRMS on lymphocyte counts (Fox et al., 2012, Gold et al., 2012). Recently there have been many reports that DMF induces lymphopenia in both psoriasis and RRMS patients. This lymphopenia has been associated in rare cases with the development of PML in patients receiving DMF therapy for either psoriasis or RRMS (Ermis et al., 2013b, Ermis et al., 2013a, van Oosten et al., 2013, Rosenkranz et al., 2015).

The DMF associated lymphopenia is most likely due to the apoptosis of T cells that was observed in the studies mentioned above. It was also reported that DMF induced more apoptosis in CD8\(^+\) T cells compared with CD4\(^+\) T cells (Ghadiri et al., 2017, Spencer et al., 2015b), and the reduction of absolute numbers of CD8\(^+\) T cells was more prominent than that of CD4\(^+\) T cells in RRMS patients treated with DMF (Spencer et al., 2015a). RRMS patients treated with DMF for 6 months exhibited a reduction in the frequency of both CD4 and CD8 memory T cells as well as inhibition of Th1, Th17 cells and a relative increase in the frequency of peripheral Treg cells (Gross et al., 2016).

There was also a pilot study comprised of 10 RRMS patients, of which six completed the study. In this in vivo study, they showed that DMF enhanced the production of IL-10 from CD4\(^+\) T cells, whereas no change was seen in IFN\(\gamma\) (Schimrigk et al., 2006). Interestingly a similar in vivo study showed that RRMS patients (n=15) that were treated with DMF for 6 months decreased IFN\(\gamma\), GM-CSF, and TNF\(\alpha\) expressing CD4\(^+\) T cells (Gross et al., 2016). They also showed that CD4\(^+\) memory T cells were skewed from a Th1 phenotype toward a Th2 phenotype when patients were treated with DMF for 6 months (Gross et al., 2016). Furthermore, they also demonstrated that memory CD4\(^+\) and CD8\(^+\) T cells in RRMS patients were decreased following 6 months of DMF treatment (Gross et al., 2016). However, it is not yet clear as to how DMF preferentially targets memory T cell subsets over naïve T cells. Further analysis is needed as memory T cell subsets are important for the maintenance of antiviral immunity. Another group also showed that in RRMS patients (n=13) DMF decreased the frequency of memory T cell subsets and also decreased CD4\(^+\)IFN\(\gamma^+\) T cells while Treg cells remained stable. They also showed a reduction in CD8\(^+\) and CD4\(^+\) T cell counts when patients were treated with DMF (Ghadiri et al., 2017). Interestingly, an in vivo study in RRMS patients (n=20) that were treated with DMF showed significant decreases in subpopulations of CD4\(^+\) and CD8\(^+\) T cells and an increase in Treg cells after 3 months of treatment (Diebold et al., 2017). They also showed that patients that were treated with DMF...
had higher cytosolic ROS after 3 months of treatment in comparison to HC (Diebold et al., 2017). Another interesting study recruited DMF treated RRMS patients (n=17 lymphopenic, n=24 non-lymphopenic), untreated RRMS patients (n=17) and HC (n=23). In this study they showed that lymphopenic DMF treated patients had significantly lower absolute counts of CD8 and CD4 T cells compared with HC and RRMS untreated controls, however Treg cells remained unchanged. They also showed that DMF treated patients (lymphopenic and non-lymphopenic) had lower frequencies of circulating CM and EM CD4 and CD8 T cells, with a relative expansion of naïve T cells (Longbrake et al., 2016). Overall, DMF treatment in RRMS patients appears to result in a decrease in CD4$^+$ and CD8$^+$ T cells affecting predominantly the memory T cells, and a decrease in proinflammatory cytokines. However, to date there are no studies on the effects of DMF on memory CD4$^+$ and CD8$^+$ T cells and their associated cytokines in psoriasis patients. Further studies are necessary to determine the effects of DMF in psoriasis patients.
1.7 Aims

- **Determine the effects of vitamin D supplementation on T cell responses in HC and CIS patients**
  The effects of vitamin D₃ supplementation on CD4⁺ T cell proliferation and cytokine production using various stimulation methods will be investigated in HC and CIS patients. There are a number of ways to stimulate T cells, which can determine the effects of vitamin D supplementation directly and indirectly. The effects of vitamin D supplementation on CD4⁺ T cell proliferation and cytokine production was examined using polyclonal anti-CD3 stimuli, myelin specific antigens, recall antigens, and allogeneic stimuli.

- **Investigate the immunomodulatory effects of DMF in vitro and in vivo in psoriasis patients.**
  Exploring the effects of DMF in vitro might give us an insight into its effects in vivo. The effects of DMF on LPS induced DC maturation and its cytokines were investigated, also the effects of DMF to alter T cell survival, proliferation, and cytokine production were examined within PBMC and sorted CD3 T cells. Additionally, investigating the effects of DMF on the frequency of Treg cells, and examining the ability of Treg cells to counteract DMF induced oxidative stress compared with T conventional (Tconv) cells within PBMC and sorted CD3 T cells. Finally, examining the frequency of T cell subsets and their associated cytokines in Fumaderm™ treated patients.
Chapter 2:
Materials and methods


2.1 Materials

Cell Culture Medium

Roswell Park Memorial Institute (RPMI) - 1640 (BioSera) was supplemented with heat-inactivated (56°C for 30 minutes (min)) 10% foetal calf serum (FCS, BioSera), 100 mM L-glutamine (Sigma Aldrich) and 100 µg/ml penicillin/streptomycin (Sigma Aldrich) to generate complete RPMI (cRPMI).

Fluorescence activated cell sorting (FACS) Buffer

Dulbecco’s phosphate buffered saline (PBS; Sigma Aldrich) supplemented with FCS (2%) and sodium azide (0.02%).

MACS Buffer

PBS supplemented with FCS (2%) and EDTA (2 mM) and maintained at 4°C.

ELISA Wash Buffer

20x PBS: NaCl, 2.73 M, Na₂HPO₄, 0.197 M, KH₂PO₄, 0.0293 M, KCL 0.0536 M dissolved in dH₂O, Tween 20 (0.1%, Sigma Aldrich).

ELISA Substrate solution TMB (eBioscience, BioLegend, Biosciences)

ELISA Stopping solution 1M H₂SO₄ (Sigma Aldrich)

ELISA KITS

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ELISA software

ELISA spectrophotometric analysis was carried out at a wavelength of 450 nm using the Epoch microplate spectrophotometer (BioTek) and Gen5 software (BioTek).

Flow cytometry software

Cells were acquired on LSR Fortessa™ (BD Biosciences) using FACS Diva software (BD) analysis was carried out with FlowJo 9.4.10.

Fluorochrome-labelled Antibodies for Flow Cytometry

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**General Reagents**

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<td>Serum Staten Institute</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Recombinant human GM-CSF</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>Recombinant human IL-4</td>
<td>Miltenyi Biotech</td>
</tr>
</tbody>
</table>

### 2.2 Source of blood samples

Leukocyte enriched buffy coats from anonymous healthy donors were obtained from the Irish Blood Transfusion Board, St James’s Hospital, Dublin and ethical approval was granted by the School of Biochemistry and Immunology, Trinity College Research Ethics Committee. MS patient samples were obtained from Dr Karen O’Connell, Department of Neurology, St. Vincent’s University Hospital, and psoriasis patient samples were obtained from Prof. Kirby, Department of Dermatology, St Vincent’s University Hospital.
2.3 Isolation of PBMC from buffy coats
Leukocyte enriched healthy donor blood was removed from buffy coats and diluted 1 in 2 with sterile PBS and centrifuged at 1250 g for 10 min at room temperature with the brake off. The buffy coat layer containing leukocytes was removed using a Pasteur pipette, diluted in 200 ml of sterile PBS and layered over Lymphoprep (Biosciences) (25 ml of diluted blood and 20 ml of Lymphoprep per tube). PBMC were isolated by density-gradient centrifugation at 800 g for 20 min at room temperature, with the brake off. The PBMC layer was removed using a Pasteur pipette, washed in sterile PBS and centrifuged at 650 g for 10 min. The supernatant was removed, the pelleted PBMC was washed again in sterile PBS and centrifuged at 290 g for 10 min in order to remove platelets. PBMC were resuspended in cRPMI and counted.

2.4 Isolation of PBMC from human venous blood
Blood samples were collected in 9 ml lithium heparin-coated tubes, diluted 1 in 2 with sterile PBS and layered over Lymphoprep. PBMC were isolated by density-gradient centrifugation (900 g, 20 min, at room temperature, with the brake off). The PBMC layer was removed using a Pasteur pipette, diluted in sterile PBS and centrifuged at 650 g for 10 min. The supernatant was discarded and the pellet resuspended in sterile PBS and centrifuged at 290 g for 10 min. The pellet was washed again in sterile PBS (290 g, 7 min) to remove platelets. Cells were cryopreserved using CoolCell™ freezing containers in FCS containing 10% DMSO at -80°C overnight then transferred to liquid nitrogen for long-term storage. Cells were thawed rapidly into pre-warmed cRPMI, washed, resuspended in cRPMI and counted.

2.5 Cell counting
Cells in cRPMI were diluted in trypan blue (Sigma Aldrich) and viable mononuclear cells were counted on a light microscope (Leica DM500) using a haemocytometer (Improved Neubauer) or Hycor Glasstic slide (Kova). The total number of cells was calculated from the formula:

\[
\text{Cell count (in one quadrant)} \times 10^4 \times \text{dilution factor} = \text{Number of cells per ml}
\]

2.6 Vitamin D and PTH measurement
Serum 25(OH)D and PTH concentrations were measured at Mater Misericordiae University Hospital, Dublin. Serum 25(OH)D levels were measured by liquid chromatography-mass spectrometry using a Waters Acquity UPLC coupled to a triple quadruple mass (TQD) (Waters Corp., Milford, USA) with a semi-automated solid phase extraction preparation of
patient’s serum samples (Tecan Freedom Evo 100, Switzerland). Serum samples, calibrators (traceable to NIST 972a, Chromsystems, Germany) & quality control samples (150 µl) were placed on a Tecan Freedom Evo 100, internal standards (IS) were added (25(OH) vitamin D2 (D3) and 26,27-[d₆]-25(OH) vitamin D₃, Isoscience, PA, USA and Synthetica AS, Norway respectively) to the dispensed samples prior to protein precipitation with methanol and ZnSO₄ (0.2 M). Following centrifugation (off-line), the supernatant was transferred to a conditioned Oasis uElution (Waters) Hydrophilic Lipophilic Balance (HLB) plate, washed & the retained analytes eluted in a two-step procedure. Preparation time for 96 samples was 1.75 hr. 20 µl of eluent was injected onto an Acquity UPLC BEH Phenyl column (Waters) maintained at 35°C and eluted with a step gradient of 65-85% Methanol, 2 mmol/L ammonium acetate & 0.1 (v/v) formic acid over 3 min. Injection-injection time was 4.9 min. 25(OH)D₂, 25(OH)D₃ and IS were detected using multiple monitoring (MRM) with the following mass-to-charge (m/z) transitions: 413.4>355.3, 416.4>358.3 for 25(OH)D₂ and D₁-25(OH)D₂, 401.4>159.2, 407.3>159.2 for 25(OH)D₃ and d₆-25(OH)D₃ respectively. The instrument was operated in positive electrospray ionisation (ESI) mode using MassLynx 4.1 software with auto data processing by the Target Aplication Manager. PTH was measured using the Roche intact PTH assay on the cobas® e411 chemiluminescent immunoassay analyser (Roche Diagnostics GmbH, Mannheim, Germany).

2.7 Power calculations
Power calculations for the vitamin D₃ supplementation trial were performed based on a previously performed pilot study and were conducted by Prof. Jean Fletcher (Allen et al., 2012, O'Connell et al., 2017). These were based on an alpha of 5% and a beta of 80%. The change in IL-17⁺CD4⁺ T cells (Th17) using flow cytometric analysis had an effect of 1.9 (SD 1.8) indicating a need for five study subjects per group of 80% power. For IFNγ producing CD4⁺ T cells (Th1 cells) the effect size was 4.3 (SD 6.3) indicating a need for 19 subjects per group for 80% power. The changes noted in cytokines in response to anti-CD3 stimulation of PBMC were a) for IL-17 (delta=44.5, SD 44.3); thus, ten participants needed in each group, b) for IFNγ (delta=32.8, SD1.2); thus, six needed in each group, c) For IL-10 (delta-455, SD127); number needed < 5. On the basis of these results at least ten subjects should be included in each group (the only immunological end-point not powered would be the change in IFNγ⁺CD4⁺ T cells). 39 HC and 45 CIS patients were recruited; this allowed a dropout rate of 20% and 30%
2.8 Isolation of CD14$^+$ monocytes and CD3$^+$ T cells using magnetic beads

CD14$^+$ monocytes and CD3$^+$ T cells were isolated from PBMC by magnetic-activated cell sorting (MACS), using CD14 and CD3 positive magnetic beads (Miltenyi Biotec). PBMC were isolated as previously described in section 2.3. Cells were washed in MACS buffer (PBS supplemented with 2% FBS and 2 mM EDTA) and pelleted by centrifugation at 650 g for 10 min. The supernatant was removed, and resuspended in 80 µl of MACS buffer per 10 million cells and CD14 or CD3 magnetic beads were added to the cells at 10 µl per 10 million cells for 15 min at 4°C. The cell/bead solution was washed in MACS buffer and pelleted by centrifugation at 650 g for 10 min. The pellet was resuspended in 500 µl (for up to 100 million cells) of MACS buffer. A LS magnetic column (Miltenyi Biotec) was placed in the magnet and washed with 3 ml of MACS buffer. The cell sample was loaded onto the column and washed 3 times with 3 ml of MACS buffer. The eluent of CD3-depleted or CD14-depleted was collected and discarded. The column was removed from the magnet and 5 ml of MACS buffer was placed in the column. CD3$^+$ T cells or CD14$^+$ monocytes were expelled using a plunger. Purified CD3$^+$ T cells or CD14$^+$ monocytes were washed in cRPMI, pelleted by centrifugation at 340 g for 10 min and counted. The purity of CD14$^+$ monocytes as described in Fig 2.1 was determined by the expression of CD14 and CD209 (DC-SIGN) by flow cytometry; CD14$^-$$CD209^{hi}$ cells were defined as immature DC and were routinely greater than 90%.

2.9 Culture of monocyte derived DC

Primary human DC were cultured from CD14$^+$ monocytes isolated from healthy human PBMC derived from buffy coats. Purified CD14$^+$ monocytes as described in Fig 2.1 were cultured at 1 x 10$^6$ cells/ml in 6 well plates in RPMI supplemented with human recombinant GM-CSF (70 ng/ml) and IL-4 (50 ng/ml). After 3 days of culture half the cRPMI was carefully removed and replaced with fresh cRPMI containing GM-CSF and IL-4. After 6 days of culture cells were gently removed from the wells and pelleted by centrifugation at 300 g. Immature DC were resuspended in RPMI and counted. The purity of DC as described in Fig 2.2 was determined by the expression of CD14 and CD209 (DC-SIGN) by flow cytometry; CD14$^-$$CD209^{hi}$ cells were defined as immature DC and were routinely greater than 90%.

2.10 In vitro stimulation

2.10.1 In vitro stimulation for vitamin D trial

PBMC were isolated and counted as described above, and labelled with CTV. Cells (2 x 10$^6$/ml) were seeded in 48 well plates in a volume of 250 µl per well (0.5 x 10$^6$ cells per well) and left unstimulated or stimulated with polyclonal or antigen-specific stimuli. For
polyclonal stimulation with anti-CD3, PBMC were transferred to a 48 well plate that was previously coated with 1 µg/ml anti-CD3 in PBS. In addition, PBMC were stimulated with the myelin antigens PepTivator MPB and PepTivator MOG in combination (0.625 µg/ml/0.375 nM; Miltenyi Biotech). MBP and MOG are a pool of lyophilized peptides that can activate antigen specific T cells; recall antigens PPD (purified protein derivative; 1 µg/ml; Serum Staten Institute) and TT (tetanus toxoid; 1 µg/ml; a kind gift from Prof. Kingston Mills), and a single batch of irradiated allogeneic PBMC from a HC buffy coat. PBMC were cultured for 3 days (anti-CD3 stimulation) or 7 days (antigen specific stimuli) after which supernatants and cells were harvested for analysis. Cell culture plates were centrifuged for 1 min at 300 g and the supernatants were removed for analysis by ELISA assay. The cRPMI was replaced on the cells, which were then harvested and T cell proliferation and intracellular cytokine production analysed by flow cytometry.

2.10.2 In vitro stimulation of DC in the presence of DMF
Immature DC (2 x 10^6/ml) were seeded in 96 well plates in a volume of 100 µl per well (0.2 x 10^6 cells per well). Immature DC were pre-treated with DMF (0, 25, 50, 100 µM) or curcumin (10 µM) for 6 hr and then left unstimulated or stimulated with TLR4 agonist LPS. After 24 hr supernatants were removed and collected for analysis by ELISA assay. The cRPMI was replaced on the cells, which were then harvested and DC co-stimulatory and maturation markers were analysed by flow cytometry.

2.10.3 In vitro stimulation of PBMC in the presence of DMF
PBMC were isolated and counted as described above, and labelled with CTV. Cells (2 x 10^6/ml) were seeded at 100 µl per well (0.2 x 10^6 cells per well) in 96 well plate and left unstimulated or stimulated with anti-CD3 polyclonal antibody in the absence or presence of different concentrations of DMF (5, 10, 25, 50 µM). For polyclonal stimulation with anti-CD3, PBMC were transferred to a 96 well plate that was previously coated with 1 µg/ml anti-CD3 in PBS. PBMC were cultured for 24 hr or 5 days (anti-CD3 stimulation) after which supernatants and cells were harvested for analysis. Cell culture plates were centrifuged for 1 min at 300 g and the supernatants were removed. The cRPMI was replaced on the cells, which were then harvested and T cell proliferation, intracellular cytokine, intracellular and intranuclear proteins were analysed by flow cytometry.

2.10.4 In vitro stimulation of sorted CD3^+ T cells in the presence of DMF
CD3^+ T cells were isolated from HC PBMC using magnetic beads and labelled with CTV and left unstimulated or stimulated with anti-CD3 (1 µg/ml) and anti-CD28 (0.5 µg/ml) in
the presence of different concentrations of DMF (0, 5, 10, 25, 50 µM) or glutathione (0, 1 mM) or DTT (5 µM). Cells (2 x 10^6/ml) were seeded at 100 µl per well (0.2 x 10^6 cells per well) in 96 well plate and left unstimulated or stimulated with anti-CD3 and anti-CD28 antibody in the absence or presence of different concentrations of DMF (5, 10, 25, 50 µM). PBMC were cultured for 24 hr or 5 days after which supernatants and cells were harvested for analysis. Cell culture plates were centrifuged for 1 min at 300 g and the supernatants were removed. The cRPMI was replaced on the cells, which were then harvested and T cell proliferation, intracellular cytokine, intracellular and intranuclear proteins were analysed by flow cytometry.

2.11 Flow cytometric staining and analysis

2.11.1 Surface and intracellular staining

Cytokine analysis required stimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (5 µg/ml) (all Sigma Aldrich) for 5 hr. PMA/ionomycin stimulation down regulates the expression of CD4 on CD4^+ T cells; therefore, stimulated cells and controls were stained for CD3 and CD8 instead, gating on the CD3^+CD8^- population to identify the CD8^- T cells, however this population will be referred to as CD4^+ T cells for the remainder of this thesis. Cells were washed in PBS, pelleted by centrifugation at 300 g for 5 min and resuspended in 50 µl of PBS containing conjugated antibodies specific for extracellular markers of interest. The samples were incubated for 15 min in the dark at room temperature, washed in PBS and pelleted by centrifugation. Supernatants were removed and pellet was resuspended in 50 µl of fixative solution part A (Caltag Fix and Perm kit, Biosciences) for 15 min in the dark at room temperature. Cells were then washed, pelleted, and resuspended in 50 µl of permeabilisation buffer (part B) containing the conjugated antibodies specific for the intracellular makers of interest. Intracellular stains were incubated for 15 min in the dark at room temperature, washed in PBS and acquired on the LSR Fortessa™. If not analysed immediately, cells were placed in the dark at 4°C and acquired within 24 hr.

2.11.2 Intranuclear staining

In order to stain for transcription factor expression, the nucleus of the cell must be permeabilised. Cells were washed twice with PBS and centrifuged at 340 g for 5 min. Supernatants were removed and cells were resuspended in 50 µl of PBS containing antibody-conjugated-fluorochromes against extracellular makers of interest. The samples were incubated for 15 min in the dark at room temperature, washed in PBS and pelleted by centrifugation. Supernatants were removed and after extracellular staining the Foxp3
fixation and permeabilisation buffer (eBiosciences) solution was diluted 1 in 4 with diluent and 250 µl was applied to cells for 30 min in the dark at 4°C. Cells were then washed and pelleted twice in 1 ml of permeabilisation buffer (diluted 1 in 10 with dH2O) and then resuspended in 50 µl of permeabilisation buffer containing conjugated antibodies against intracellular and intranuclear proteins including Foxp3, Ki67, pS6 and incubated for 30 min in the dark at 4°C. Cells were then washed in PBS and acquired on the LSR Fortessa™. If not analysed immediately, cells were placed in the dark at 4°C and acquired within 24 hr.

2.11.3 Cell tracer labelling

In order to track cell proliferation, PBMC were labelled with a cell tracer dye, CTV (Biosciences). PBMC were washed in sterile PBS to remove FCS or glutamine as the presence of amine groups quenches tracer dyes. CTV was diluted in sterile, warm PBS to avoid precipitation, and an equal volume added to cells suspended in warm PBS (2 x 10^6 cells/ml) to yield a final concentration of 0.25-1 µM. Cells were incubated at 37°C for 10 min. Tracer dye labelling was stopped by the addition of at least 4 times the labelling volume of cRPMI. Cells were cooled on ice for 5 min, pelleted by centrifugation (360 g for 5 min) and resuspended in cRPMI. A sample of unlabelled cells and CTV labelled cells were fixed with 4% paraformaldehyde (PFA; Santa Cruz Biotech) immediately after staining for use as compensation controls.

2.11.4 Annexin V & 7AAD staining

For the measurement of apoptosis, cells were stained with fluorochrome-conjugated Annexin V and 7AAD. Cells were washed in Annexin V binding buffer and centrifuged at 300 g. Supernatant was removed and cells were incubated in 30 µl binding buffer containing 1.25 µl fluorochrome-conjugated anti-Annexin V for 15 min at room temperature in the dark. Cells were then washed in binding buffer and centrifuged at 300 g. Supernatant was discarded and cells were resuspended in 200 µl 1x binding buffer containing (25 µg) of 7AAD per test per million cell and acquired immediately.

2.11.5 ALM633 maleimide staining

In order to detect surface thiols, cells were stained with ALM633 maleimide. Cells were washed in cold PBS and centrifuged at 300 g for 5 min at 4°C. Supernatants were removed and cells were stained with ALM633 maleimide at a final concentration of 4 µM in PBS. Cells were incubated for 15 min on ice. Cells were washed twice in cold PBS and centrifuged at 300 g for 5 min at 4°C. Surface staining was conducted at 4°C after ALM633 maleimide staining as shown in section 2.11.1. Intracellular and intranuclear staining was not
compatible with ALM633 maleimide. Cells were then washed in PBS and centrifuged at 300g for 5 min at 4°C. Supernatants were removed and cells were acquired immediately. To validate the use of ALM633 the antioxidant DTT was used to induce reduced surface thiols as shown in Fig 2.8.

2.11.6 Flow cytometry acquisition, compensation and analysis

Flow cytometry experiments were acquired on the LSR Fortessa™. A single-stained control was prepared for each fluorochrome using BD compensation beads in order to compensate for spectral overlap, in flow cytometry experiments that contained more than one fluorochrome. Fluorescence minus one (FMO) controls were acquired and, where appropriate, to set negative gates during analysis. An unstimulated stained control for experiments such as CTV, Ki67, cytokine analysis, activation markers, ALM maleimide were used to appropriately set positive gates during analysis.

2.12 Measurement of cytokine production by ELISA

ELISA assays were utilised to quantify the concentration of human IL-17, IL-10, IFNγ, IL-12p70 and IL-23 within cell culture supernatants, using the manufacturer’s protocol. Purified mouse anti-human capture antibody in coating buffer was applied to high binding 96 well plates. Plates were incubated overnight at 4°C, capture antibody was then flicked off and non-specific binding sites were blocked with the recommended blocking solution for 1 hr at room temperature. After blocking, plates were washed in PBS-tween solution (general reagents), and duplicate supernatants were loaded into wells either neat or diluted 1 in 10 with assay diluent. A standard curve of serially diluted recombinant cytokine standard was also loaded onto the plates. Triplicate blank wells, containing assay diluents only were loaded on every plate to allow subtraction of background from each sample. Samples were incubated overnight at 4°C. After washing, biotinylated detection antibody was added to each well and incubated for 2 hr at room temperature. Plates were washed and horseradish-peroxide (HRP) conjugated to streptavidin was applied to wells for 30 min in the dark. Wells were thoroughly washed and the substrate, TMB, was added. The enzyme-mediated colour reaction was protected from light while developing and stopped with the addition of 1 M H2SO4 (1 in 3 dilution). The optical density of the colour was determined by measuring the absorbance at 450 nm using a microtiter plate reader (Biotek, Epoch). Cytokine concentrations were calculated from the standard curve using Gen5© software.
2.13 Statistical analysis
Statistical analyses were performed using Graphpad Prism version 6. Paired or unpaired t-test as appropriate was used to compare variance between 2 groups, One-way or two-way ANOVA was applied with Tukey’s multiple comparison and Bonferroni post-test to compare three or more groups, respectively. P values of <0.05 were considered significant and denoted with an asterisk or hash symbol.
Figure 2.1 Analysis of monocyte purity by flow cytometry
Monocytes were purified by MACS as described in 2.8 and their purity assessed on the basis of CD14 expression by flow cytometry. Dead cells and cellular debris were excluded on the basis of size (FSC) and granularity (SSC) (A). Monocytes were then gated on the basis of CD14 expression (B).
Figure 2.2 Analysis of DC purity by flow cytometry

Human immature monocyte-derived DC were generated as described in 2.9 and purity was assessed on the basis of CD14<sup>-</sup>CD209<sup>+</sup>CD11c<sup>+</sup> by flow cytometry. Dead cells and cellular debris were excluded on the basis of size (FSC) and granularity (SSC) (A). Viable cells were then gated as CD209<sup>+</sup>CD14<sup>-</sup> (B). Within the CD209<sup>+</sup> gate CD209<sup>+</sup>CD11c<sup>+</sup> was gated to determine the purity of the immature DC population (C).
Figure 2.3 Analysis of CD3+ T cell purity by flow cytometry
CD3+ T cells were purified by MACS as described in 2.8. Purified CD3+ T cells were identified as CD3+ by flow cytometry. Dead cells and cellular debris were excluded on the basis of size (FSC) and granularity (SSC) (A). Viable cells were then gated for the expression of CD3 (B).
Figure 2.4 Validation of phospho-S6 ribosomal protein (pS6) staining
PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the presence of PMA for 20 min or in the presence of rapamycin 20nM for 24 hr. Cells were analysed by flow cytometry and gated on CD4 and CD8 as described above. PMA upregulates pS6 in CD4 and CD8 T cells. PS6 were identified on the basis of CD3 and pS6 in PMA stimulated CD4$^+$ T cells (A). PS6 were identified on the basis of CD3 and pS6 in PMA stimulated CD8$^+$ T cells (B). Rapamycin is an inhibitor of mTOR and results in the downregulation of pS6. PS6 within CD4$^+$ T cells were gated on the basis of CD3 and pS6 (C). PS6 within CD8$^+$ T cells were gated on the basis of CD8 and pS6.
Figure 2.5 Flow cytometry gating strategy to identify Treg cells in vitamin D₃ trial
Cryopreserved PBMC were stained with fluorochrome-conjugated antibodies specific for CD4 and Treg markers CD4, CD25, CD127 and Foxp3. Cells were analysed by flow cytometry and gated on CD4 as shown above (A). Treg cells were initially identified on the basis of the expression of Foxp3 and CD127 within the CD4 population and Foxp3⁺CD127lo cells were gated (B) and were subsequently gated further on the basis of CD25⁺. Treg cells were identified as CD4⁺CD25⁺CD127loFoxp3⁺ and Tconv cells were identified as CD4⁺CD25⁻CD127hi.
Fig 2.6 Flow cytometry gating strategy to identify CD4$^+$ T cells after stimulation with PMA and Ionomycin in vitamin D$_3$ trial

PBMC were isolated, stimulated anti-CD3 or antigen specific stimuli and cultured for 3 or 7 days respectively. Cells were re-stimulated with PMA and ionomycin, and stained with fluorochrome-conjugated antibodies specific for CD3, and CD8, and analysed by flow cytometry. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC) (A). Since PMA and Ionomycin stimulation results in the downregulation of CD4 expression, CD4$^+$ T cells were identified indirectly as the CD3$^+$ CD8$^-$ population (B).
Fig 2.7 Gating strategy used to analyse T cell proliferation using CTV in vitamin D₃ trial

PBMC were labelled with CTV or left unlabelled. Cells were stimulated with anti-CD3 or antigen-specific stimuli for 3 or 7 days respectively. Cells were analysed by flow cytometry and the proliferation of cells was determined by the dilution of the CTV label. (A) Shows the proliferation of cells by CTV dilution as a histogram. (B) Shows the proliferation of cells vs the cytokine IFN-γ being secreted.
Figure 2.8 Validation of ALM633 staining to detect cell surface thiols using antioxidant DTT as positive control

CD3⁺ T cells were isolated from HC PBMC using magnetic beads and left unstimulated or stimulated with anti-CD3 and anti-CD28. After 24 hr cells were left untreated or treated with 5 mM DTT for 15 min. Cells were then stained with fluorochrome-conjugated antibodies specific for ALM633 and viability dye, and analysed by flow cytometry. Surface thiols were detected with ALM633 staining as shown in the representative histograms for unstimulated, stimulated and stimulated/DTT treated cells (A). The graph shows the mean (+/- SEM) of ALM633 median fluorescence intensity (MFI), expressed as a percentage of unstimulated control (n=3) (B). Statistical differences between unstimulated cells that were untreated vs those stimulated or treated with DTT were determined by one-way ANOVA with Tukey’s multip
Chapter 3:

The effects of vitamin D$_3$ supplementation on T cell responses in HC and CIS patients
3.1 Introduction

Although there are a number of therapies for MS, some patients do not respond to therapy and adverse events can be problematic. Therefore, there is still an unmet need for safer therapies for MS patients. As described previously, MS is thought to be triggered in genetically susceptible individuals in response to environmental factors, including vitamin D deficiency. There is substantial epidemiological evidence that low vitamin D levels in utero/early life increases the prevalence of MS. It has been demonstrated that the prevalence of MS is correlated with increasing latitude in both hemispheres, and this corresponds to the amount and duration of UVB that individuals are exposed to (Acheson et al., 1960, Hayes et al., 1997). An increased odds ratio for MS was also correlated with births that occurred in spring, as this was related to low maternal vitamin D levels during the winter months (Dobson et al., 2013). It is thought that such vitamin D deficiency in early life may adversely influence thymic selection of T cells, thereby predisposing genetically susceptible individuals to developing MS in later life.

In addition to this, there is also evidence to suggest that once individuals have already developed MS as a result of combined genetic and environmental factors, that vitamin D might have a therapeutic effect in MS. Firstly, there is a large body of evidence to show that 1,25(OH)₂D₃ exerts immunomodulatory effects. It has been shown that 1,25(OH)₂D₃ can inhibit the maturation and differentiation of APC, and can inhibit their production of proinflammatory cytokines (D'Ambrosio et al., 1998). However conflicting evidence suggests that 1,25(OH)₂D₃ can also enhance the differentiation of monocytes and mononuclear phagocytes (McCarthy et al., 1983) (Provvedini et al., 1986, Miyaura et al., 1981).

There is evidence that suggests that vitamin D can exert anti-inflammatory effects on T cells in vitro via activation of the VDR (da Costa et al., 2016, Boonstra et al., 2001, Correale et al., 2009). 1,25(OH)₂D₃ mediates its effects through VDR activation, once activated the VDR heterodimerises, translocates the nucleus and interacts with VDRE to suppress cytokine transcription or exert multiple other effects (Nagpal et al., 2005). 1,25(OH)₂D₃ was shown to inhibit cytokines from Th1 cells such as IFNγ, IL-2 and TNFα (Palmer et al., 2011), but on the other hand promoted Th2 cytokines IL-4, IL-5 and IL-10 (Boonstra et al., 2001). Another study showed that 1,25(OH)₂D₃ was also capable of decreasing IL-17, IFNγ and IL-21 from activated T cells (Jeffery et al., 2009). 1,25(OH)₂D₃ was shown to also directly suppress the Th17 cytokine IL-17 transcription via blocking of NF-AT, recruitment of histone deacetylase and sequestration of Runt related transcription factor 1 via a VDR
 dependant pathway (Joshi et al., 2011). Treg cells play a role in suppressing proinflammatory responses and help to prevent autoimmunity and are therefore promoting Treg cells in autoimmunity is desirable. An in vitro study showed that 1,25(OH)₂D₃ could induce IL-10 producing Treg cells (Barrat et al., 2002). Thus, taken together, the in vitro data suggests that vitamin D could favourably alter the ratio between inflammatory Th1/Th17 cells and Treg cells.

Secondly, low serum vitamin D levels have been correlated with MS, and also with increased disease activity. Studies that have found that MS patients have low serum vitamin D compared with HC (Munger et al., 2004, Munger et al., 2006). Furthermore, it has also been demonstrated that higher serum vitamin D levels in MS patients was associated with a lower relapse rate (Smolders et al., 2008, Simpson et al., 2010). Thirdly, studies in the EAE model for MS have shown that vitamin D can have a therapeutic or preventative role. A study showed that UV treatment prevented the development of EAE when UV administration was prior to the induction of EAE (Hauser et al., 1984). Given that skin exposure to UV results in the synthesis of vitamin D₃, it was suggested that the therapeutic effect of UV treatment was mediated via vitamin D. In addition, therapeutic administration of 1,25(OH)₂D₃ was shown to inhibit EAE in wild type mice but not in IL-10 deficient mice, indicating a protective effect of vitamin D that required IL-10 (Spach et al., 2006). Interestingly, when administered during the immunisation phase of EAE, 1,25(OH)₂D₃ prevented the development of disease (Cantorna et al., 1996).

Taken together, these lines of evidence suggest that 1,25(OH)₂D₃ might modulate immune responses and exert anti-inflammatory effects in vitro and in vivo, prompting the idea of testing vitamin D₃ as a potential therapy for MS. The obvious advantage would be that as a vitamin, vitamin D₃ is considered safe even in relatively high doses. At the point when recruitment for the trial presented in this thesis began (2012), there were a limited number of trials of vitamin D₃ supplementation in RRMS patients. The majority of these trials had one or more defects in trial design, such as a lack of placebo control group, double blinding or randomisation. The majority of the trials were conducted in RRMS patients that were already on disease modifying therapies (DMT) and vitamin D₃ supplementation was an addition to their treatment. The dose ranges in these trials were not similar, this indicated that there is not a unified dose for vitamin D₃ supplementation. Finally, the duration of these trials varied from as short as 12 wk to as long as 52 wk. The outcomes of these studies were also conflicting and not all the trials met their primary endpoints. Moreover, these trials did not investigate the effects of vitamin D₃ supplementation on T cell responses.
Thus, the trial conducted as part of this study was designed to overcome the deficits of previous trials and in addition recruited CIS patients and HC. CIS patients are those who had experienced their first neurological episode, but had not yet been diagnosed as clinically definite MS. Thus, the rationale for using CIS patients is that they are not administered any DMT, thus making CIS patients ideal candidates for the trial since the effect of vitamin D₃ alone could be examined. Although the vast majority of therapies target inflammation in clinically definite MS, whereas this study is examining the immunomodulatory effects of vitamin D in early MS patients and its effects on the prevention of clinically definite MS. In addition, since protocol does not allow for DMT use in CIS patients, the possibility that vitamin D₃ could be used in these patients to delay or prevent progression to RRMS is an attractive one. HC were also recruited for this trial to compare with CIS patients. The design of the trial conducted in this study was a randomised double blinded placebo controlled clinical trial for 24 wk, and serum and blood samples were collected at baseline, 16 and 24 wk (O’Connell et al., 2013).

Prior to the clinical trial, a pilot study was carried out to examine the effects of vitamin D₃ supplementation on immunological responses in HC in order to optimise the immunological analyses prior to the clinical trial. The pilot study examined the immunomodulatory effects of vitamin D₃ in four HC that were supplemented with 5,000 IU or 10,000 IU of vitamin D₃ daily for 15 wk. 2 participants were administered 5,000 IU per day for 10 wk and the other 2 participants received 5,000 IU and this was increased to 10,000 IU in the 5-remaining wk. After 15 wk serum 25(OH)D was significantly increased in all participants compared to baseline. The pilot study demonstrated that vitamin D₃ increased production of IL-10 by a non-T cell population and reduced the frequency of Th17 cells (Allen et al., 2012).

The randomised, double blinded, placebo-controlled trial of vitamin D₃ supplementation in CIS patients that was conducted at St. Vincent’s University Hospital was the first properly controlled trial at the time, and the first to recruit CIS patients (O’Connell et al., 2017). The primary endpoint was the effect of 5,000 IU and 10,000 IU of vitamin D₃ supplementation daily compared to placebo on the frequency of CD4⁺ T cell subsets (IL-17⁺CD4⁺ T cells and IFNγ⁺CD4⁺ T cells) and cytokines IL-17, IFNγ and IL-10 in PBMC in CIS patients and HC at 16 and 24 wk compared to baseline. The secondary endpoints were the effects of 5,000 IU and 10,000 IU of vitamin D₃ supplementation daily compared to placebo on radiological activity as measured by the number of new or enlarging T2 lesions and gadolinium-enhancing lesions on brain MRI at 24 wk compared to baseline. Clinical activity was measured by the annualised relapse rate, percentage of patients relapse-free and time to first
relapse. Disease freedom was measured by the percentage of patients with no evidence of either radiological or clinical activity (O'Connell et al., 2013). Overall the aim of the trial was to determine whether vitamin D3 supplementation could prevent or delay progression in CIS patients, and whether vitamin D3 exerted anti-inflammatory effects on T cell function.

3.1.1 Aims and Hypothesis

Hypothesis:

Th1 and Th17 cells play a central role in mediating the pathogenesis of MS. On the other hand, Treg cells are crucial in maintaining tolerance and preventing autoimmunity by suppressing effector T cell proliferation and secretion of pro-inflammatory cytokines, including IFNγ and IL-17.

The hypothesis for this study was that vitamin D3 supplementation shifts the balance from a pro-inflammatory to an anti-inflammatory T cell response by suppressing pathogenic T cell cytokines and/or inducing Treg cells. The specific aims were as follows:

- Examine the effects of vitamin D3 supplementation on the frequency of Treg cells and memory T cells in HC.
- Examine the effects of vitamin D3 supplementation on CD4+ T cell proliferation and cytokine production indirectly via APC using polyclonal anti-CD3 stimuli on PBMC from HC and CIS patients.
- Examine the effects of vitamin D3 supplementation on CD4+ proliferation and cytokine production indirectly via APC using myelin specific antigens MOG and MBP stimuli on PBMC from HC and CIS patients.
- Examine the effects of vitamin D3 supplementation on CD4+ proliferation and cytokine production indirectly via APC using recall antigens specific antigens PPD and TT stimuli on PBMC from HC and CIS patients.
- Examine the effects of vitamin D3 supplementation on CD4+ proliferation and cytokine production indirectly via APC using allogeneic stimuli on PBMC from CIS patients.
3.1.2 Recruitment of HC and CIS patients

**Inclusion criteria:**

A. CIS patients aged between 18 and 55 years with symptom onset within 3 months of screening having two or more asymptomatic T2 lesions on MRI brain consistent with demyelination, not treated with steroids within 30 days of screening and not on any other DMT

B. HC were aged between 20 and 40 years with a female: male ration of 2:1, broadly in line with the gender ration of CIS

**Exclusion criteria:**

A. Exclusion criteria in both CIS patients and HC were: no history or evidence of hypercalcemia, renal impairments, vitamin D₃ intolerance, parathyroid dysfunction, sarcoidosis, pregnancy or refusal to use contraception, prior or current treatment with thiazide diuretics or vitamin D₃ supplementation of greater than 1000 IU/day

B. Exclusion criteria in patients with CIS were:
   a. Patients whose symptoms might be explained by a diagnosis other than MS
   b. Patients with occurrence of an exacerbation less than six wk prior to entry to the study
   c. Previous treatment with any immunomodulating therapy in the last three months, steroids in the last four wk or any previous treatment with mitoxantrone or other immunosuppressant

A schematic diagram outlining the recruitment to the study, randomisation and completion and withdrawal shown in Fig 3.1, and a baseline characteristics of HC and CIS patients shown in Table 3.1.
The study was a double-blinded placebo controlled randomised trial. Both HC and CIS patients were recruited over the same time period. Both HC and CIS patients were randomised to one of three groups: placebo, 5,000 IU, and 10,000 IU vitamin D_{3} daily. The vitamin D_{3} was in the form of Vigantol Oil®, supplied by Merk KgaA (Darmstsdt, Germany). Both placebo and the active product were identical in appearance.
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Table 3.1 Baseline characteristics of HC and CIS patients
3.2 Results

3.2.1 The effects of vitamin D₃ supplementation in HC subjects on 25(OH)D and PTH

HC were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period. Serum samples were collected at baseline, 16, and 24 wk. In order to determine whether vitamin D₃ supplementation at either 5,000 or 10,000 IU vitamin D₃ resulted in increased serum levels of 25(OH)D; serum 25(OH)D was measured by (LC-MS/MS) as described in the methods section. In addition, because PTH regulates vitamin D metabolism, serum PTH was measured. Serum samples were prepared by Dr Karen O’Connell, Department of Neurology, St. Vincent’s University Hospital and collected, frozen and analysed by the Department of Neurology, Mater Misericordiae University Hospital, Dublin.

As shown in Fig 3.2A (left) serum 25(OH)D did not alter when HC were supplemented with placebo across the three time points. Similarly, there was no change in serum PTH across the time points, when HC were administered placebo (Fig 3.2A, right). In Fig 3.2B (left) serum 25(OH)D significantly increased from baseline to 16 wk (p<0.0001), and from baseline to 24 wk (p<0.0001), when HC were supplemented with 5,000 IU vitamin D₃. However, the PTH levels did not significantly change throughout the time points, when HC were administered 5,000 IU of vitamin D₃ (Fig 3.2B, right).

In Fig 3.2C (left) serum 25(OH)D significantly increased in HC from baseline to 16 wk (p<0.0001), and from baseline to 24 wk (p<0.0001), when supplemented with 10,000 IU of vitamin D₃. Although, the PTH levels did not significantly change at baseline vs 16 wk, there was however a significant reduction from baseline vs 24 wk (p<0.05) (Fig 3.2C, right). In summary, as would be expected, there was a significant increase in serum 25(OH)D in HC after supplementation with either 5,000 or 10,000 IU of vitamin D₃ and no significant change in the placebo group.
Figure 3.2 The effects of vitamin D₃ supplementation in HC subjects on serum 25(OH)D and PTH

HC were randomised into three groups and administered placebo, 5,000 or 10,000 IU vitamin D₃. Serum 25(OH)D and PTH concentrations were measured at baseline, 16 wk and 24 wk. Graphs show serum 25(OH)D (left) and PTH (right) concentrations in HC controls that were administered placebo (A) \( (n=10-12) \), 5,000 IU vitamin D₃ (B) \( (n=13) \) or 10,000 vitamin D₃ (C) \( (n=13) \). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. *\( p \leq 0.05 \), **\( p \leq 0.01 \), ***\( p \leq 0.001 \), versus baseline. Data expressed as mean (+/- SEM). Samples were collected by Dr Karen O’Connell and analysed by the Department of Neurology, Mater Misericordiae University Hospital, Dublin.
3.2.2 The effects of vitamin D₃ supplementation in HC subjects on the frequency of memory T cells

Vitamin D has previously been shown to preferentially inhibit the memory T cell compartment rather than naïve T cells, therefore the effects of vitamin D₃ supplementation on memory CD4 T cells was examined. HC were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual and analysed directly for memory T cells. PBMC were stained for memory T cells using fluorochrome-conjugated antibodies specific for CD4 and the memory marker CD45RO, and analysed by flow cytometry.

For flow cytometric analysis, CD4⁺ T cells were gated as described in materials and methods. The representative dot plot in Fig 3.3A shows CD4 vs CD45RO staining in CD4⁺ T cells, where the gate on the top right indicates the frequency of CD4⁺CD45RO⁺ memory T cells, while the gate on the top left indicated the frequency of naïve CD45RO⁻ T cells.

The frequency of memory CD4⁺ T cells at 0, 16 and 24 wk in HC that were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ are shown in Fig 3.3B. As expected, no significant differences between time points were observed after placebo treatment in HC. There were also no significant differences observed in the frequency of memory T cells between time points for HC that were supplemented.

In summary, the frequencies of memory T cells in HC subjects were not significantly altered over the course of 24 wk when administered either placebo or vitamin D₃ at 5,000 IU or 10,000 IU.
Figure 3.3 The effects of vitamin D₃ supplementation in HC subjects on the frequency of memory CD4⁺ T cells

HC were administered placebo over 24 wk and PBMC were isolated at baseline, 16 wk, and 24 wk. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4 and CD45RO and analysed by flow cytometry. The lymphocyte population was identified on the basis of FSC and SSC (shown in methods) and CD4 T cells within the lymphocyte gate were gated on the basis of CD4⁺CD3⁺. A representative dot plot shows the gating strategy for identifying the CD4⁺CD45RO⁺ memory T cell population (A). The effect of placebo (n=9-11), 5,000 IU (n=12-13) or 10,000 IU (n=12) of vitamin D₃ supplementation on the frequency of memory T cells is shown in (B). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test.
3.2.3 The effects of vitamin D₃ supplementation in HC subjects on the frequency of Treg cells

Vitamin D has been shown to induce Treg cells *in vitro*, and Treg cells play an important role in preventing and controlling autoimmune disease. Therefore, it was of great interest to determine whether vitamin D₃ supplementation *in vivo* affected the frequency of Treg cells. HC were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual and analysed directly for Treg cells. PBMC were stained for Treg cells using fluorochrome-conjugated antibodies specific for CD4, CD25, CD127 and Foxp3, and analysed by flow cytometry.

For flow cytometric analysis, Treg cells were gated as described in Fig 2.5. Fig 3.4A, (left) shows a representative dot plot of CD25 vs Foxp3 staining in CD4⁺ T cells, where the gate indicates the frequency of CD4⁺CD25⁺Foxp3⁺ T cells. These cells were then gated and further assessed for expression for CD127 (vs Foxp3) as indicated in the dot plot Fig 3.4A, right. Foxp3⁺CD127lo were gated as indicated in Fig 3.4A, right. Treg cells were identified as being CD4⁺CD25⁺CD127loFoxp3⁺.

The frequency of Treg cells at 0, 16 and 24 wk in HC that were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ are shown in (Fig 3.4B). As expected, no significant differences in Treg frequency was observed between time points after placebo treatment. In addition, however, there were no significant differences observed between time points for HC that were supplemented with either 5,000 IU or 10,000 IU of vitamin D₃.

In summary, the frequencies of Treg cells in HC subjects were not significantly altered over the course of 24 wk when administered either placebo or vitamin D₃ at 5,000 IU or 10,000 IU.
Figure 3.4 The effects of vitamin D₃ supplementation in HC subjects on the frequency of Treg cells

HC were administered placebo over 24 wk, and PBMC were isolated at 0, 16 and 24 wk. PBMC were stained for Treg cell markers using fluorochrome-conjugated antibodies specific for CD4, Foxp3, CD25 and CD127, and analysed by flow cytometry. Representative dot plots show the gating strategy for identifying the Treg cell population. The lymphocyte population was identified on the basis of FSC and SSC (shown in methods). CD4⁻ T cells within the lymphocyte gate were gated on the basis of CD4⁺CD3⁻. Expression of Foxp3 and CD25 were examined in the CD4⁻ population, and CD4⁺Foxp3⁻CD25⁻ cells were gated (A, Left) and then assessed further for the expression of CD127lo and Foxp3 (A, right). Treg cells are identified as being CD4⁺Foxp3⁺CD25⁺CD127lo. The effect of placebo (n=8-10), 5,000 IU (n=12-13) or 10,000 IU (n=9) of vitamin D₃ supplementation on the frequency of Treg cells is shown in (B) Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test.
3.2.4 The effect of vitamin D₃ supplementation in HC subjects on their anti-CD3 induced T cell proliferation and cytokine production

Vitamin D has been shown to exert anti-proliferative and anti-inflammatory effects *in vitro*, therefore the effects of vitamin D₃ supplementation on T cell proliferation and cytokine responses was examined. HC were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual, and analysed for proliferation and cytokine production in response to polyclonal anti-CD3 stimulation. A standardised single HC sample was run in parallel with each batch of samples in order to control for inter-experimental variation. PBMC were labelled with cell tracker dye CTV, stimulated for 3 days with anti-CD3 and then harvested and re-stimulated with PMA and ionomycin for 5 hr in order to measure cytokine production. The proliferation of CD4+ T cells was measured by the dilution of CTV using flow cytometry. In addition, an unstimulated control was used to determine the proliferation of CD4+ T cells using CTV dilution. Cells were also stained for expression of CD3, CD8 and intracellular IFNγ and IL-17 and analysed by flow cytometry. In addition, the concentrations of IL-17F, IFNγ and IL-10 were determined by ELISA in cell culture supernatants harvested on day 3.

**Placebo:**

For flow cytometric analysis, CD4+ T cells were gated as described in Fig 2.6 and proliferated cell cytokine production by CTV dilution was gated as described in Fig 2.7. Fig 3.5A, left shows a representative histogram of CTV dilution, where the bar indicates the frequency of proliferated CD4+ T cells in response to anti-CD3 stimulation. The frequency of proliferated CD4 T cells at 0, 16 and 24 wk for HC that were administered placebo is shown in Fig 3.5A, right, and no significant differences were observed between time points. A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the proliferated (CTVlo) cells (Fig 3.5B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for HC that were administered placebo (Fig 3.5B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.5C left, where proliferating cells produced the majority of cytokines. The graph shows the frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for HC that were administered placebo (Fig 3.5C right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.5D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the
supernatants from anti-CD3 stimulated PBMC, however no significant differences were observed between time points. In summary, CD4⁺ T cells from HC administered placebo for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to anti-CD3 stimulation.

5,000 IU vitamin D₃:
Figure 3.6A, left, shows a representative histogram of CTV dilution, where the bar indicates the frequency of proliferated CD4⁺ T cells in response to anti-CD3 stimulation. The graph shows the frequency of proliferated CD4 T cell at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D₃, and no significant differences were observed between time points (Fig 3.6A, right). A representative dot plot shows IFNγ vs CTV staining in CD4⁺ T cells, where the majority of IFNγ was produced by the proliferated (CTVlo) cells (Fig 3.6B, left). The graph shows the frequency of CD4⁺ T cells producing IFNγ at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D₃ (Fig 3.6B, right). There were no significant differences observed between time points. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.6C left; the graph shows the frequency of CD4⁺ T cells producing IL-17 at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D₃ (Fig 3.6C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.6D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from anti-CD3 stimulated PBMC, however no significant differences were observed between time points. In summary, CD4⁺ T cells from HC administered 5,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to anti-CD3 stimulation.

10,000 IU vitamin D₃:
Figure 3.7A, left, shows a representative histogram of CTV dilution, where the bar indicates the frequency of proliferated CD4⁺ T cells in response to anti-CD3 stimulation. The graph shows the frequency of proliferated CD4 T cell at 0, 16 and 24 wk for HC that were administered 10,000 IU of vitamin D₃, and no significant differences were observed between time points (Fig 3.7A, right). A representative dot plot shows IFNγ vs CTV staining in CD4⁺ T cells, where the majority of IFNγ was produced by the proliferated (CTVlo) cells (Fig 3.7B, left). The graph shows the frequency of CD4⁺ T cells producing IFNγ at 0, 16 and 24 wk for HC that were administered 10,000 IU of vitamin D₃ (Fig 3.7B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.7C, left the graph shows the frequency of CD4⁺ T cells
producing IL-17 at 0, 16 and 24 wk for HC that were administered 10,000 IU of vitamin D₃ (Fig 3.7C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.7D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from anti-CD3 stimulated PBMC, however no significant differences were observed between time points. In summary, CD4⁺ T cells from HC administered 10,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to anti-CD3 stimulation.
Figure 3.5 The effect of placebo supplementation in HC subjects on their anti-CD3 induced T cell proliferation and cytokine production

HC were administered placebo over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with anti-CD3 for 3 days. After 3 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4+ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=9-11). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4+ T cells and production of total IFNγ (B) (n=9-10) and IL-17 (C) (n=9-11) from CD4+ T cells, and analysed by flow cytometry. After 3 days, the supernatants were harvested and the concentrations of IL-10 (n=9-11), IFNγ (n=9-11) and IL-17F (n=9-11) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.6 The effect of 5,000 IU of vitamin D₃ supplementation in HC subjects on their anti-CD3 induced T cell proliferation and cytokine production

HC were administered 5,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with anti-CD3 for 3 days. After 3 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=11-13). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=11-13) and IL-17 (C) (n=11-13) from CD4⁺ T cells, and analysed by flow cytometry. After 3 days, the supernatants were harvested and the concentrations of IL-10 (n=11-13), IFNγ (n=11-13) and IL-17F (n=11-13) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/− SEM).
Figure 3.7 The effect of 10,000 IU of vitamin D₃ supplementation in HC subjects on their anti-CD3 induced T cell proliferation and cytokine production

HC were administered 10,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with anti-CD3 for 3 days. After 3 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=12). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=12) and IL-17 (C) (n=12) from CD4⁺ T cells, and analysed by flow cytometry. After 3 days, the supernatants were harvested and the concentrations of IL-10 (n=12), IFNγ (n=12) and IL-17F (n=12) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
3.2.5 The effect of vitamin D$_3$ supplementation in HC subjects on their MOG and MBP induced T cell proliferation and cytokine production

The auto-antigens that are targeted by T cells in MS are thought to include the myelin antigens MOG and MBP, therefore it was of interest to determine whether vitamin D$_3$ supplementation could affect recall responses to these antigens. HC were administered placebo, 5,000 IU or 10,000 IU of vitamin D$_3$ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual, and analysed for proliferation and cytokine production in response to myelin antigens MOG and MBP. A standardised single HC sample was run in parallel with each batch of samples in order to control for inter-experimental variation. PBMC were labelled with cell tracker dye CTV, stimulated for 7 days with MOG and MBP, and then harvested and re-stimulated with PMA and ionomycin for 5 hr in order to measure cytokine production. The proliferation of CD4$^+$ T cells was measured by the dilution of CTV using flow cytometry. In addition, an unstimulated control was used to determine the proliferation of CD4$^+$ T cells using CTV dilution. CD4$^+$ T cell proliferation and cytokine production were analysed by flow cytometry. In addition, the concentrations of IL-17F, IFN$\gamma$ and IL-10 were determined by ELISA in cell culture supernatants harvested on day 7.

Placebo:

Fig 3.8A, left shows a representative histogram of CTV dilution, where the bar indicates the frequency of proliferated CD4$^+$ T cells in response to MOG and MBP stimulation. The frequency of proliferated CD4$^+$ T cell at 0, 16 and 24 wk for HC that were administered placebo is shown in Figure 3.8A, right where no significant differences were observed between time points. A representative dot plot shows IFN$\gamma$ vs CTV staining in CD4$^+$ T cells, where the majority of IFN$\gamma$ was produced by the unproliferated (CTV$^{hi}$) cells (Fig 3.8B, left). The graph shows the frequency of CD4$^+$ T cells producing IFN$\gamma$ at 0, 16 and 24 wk for HC that were administered placebo (Fig 3.8B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.8C, left, where proliferating cells produced the majority of cytokines. The graph shows the frequency of CD4$^+$ T cells producing IL-17 at 0, 16 and 24 wk for HC that were administered placebo (Fig 3.8C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.8D shows the concentrations of IL-10, IFN$\gamma$ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from MOG and MBP stimulated PBMC, however no significant differences were observed between time points. In summary,
CD4+ T cells from HC administered placebo for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to MOG and MBP stimulation.

5,000 IU vitamin D₃:
The graph shows the frequency of proliferated CD4 T cells at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D₃ (Fig 3.9A, left), and no significant differences were observed between time points (Fig 3.9A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the unproliferated (CTV\textsuperscript{hi}) cells (Fig 3.9B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D₃ (Fig 3.9A, right), however no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.9C, left, where unproliferated cells produced the majority of cytokine. The graph shows the frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D₃ (Fig 3.9C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.9D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from MOG and MBP stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from HC administered 5,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to MOG and MBP stimulation.

10,000 IU vitamin D₃:
The graph shows the frequency of proliferated CD4 T cells at 0, 16 and 24 wk for HC that were administered 10,000 IU of vitamin D₃ (Fig 3.10A, left), and no significant differences were observed between time points (Fig 3.10A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the unproliferated (CTV\textsuperscript{hi}) cells (Fig 3.10B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for HC that were administered placebo (Fig 3.10B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.10C, left, where the majority of cytokine was produced by unproliferated cells. The graph shows the frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for HC that were administered 10,000 IU of vitamin D₃ (Fig 3.10C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.10D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines
were detectable in the supernatants from MOG and MBP stimulated PBMC, however no significant differences were observed between time points, except for a significant decrease in IL-10 from baseline to 16 wk ($p \leq 0.05$), and from baseline to 24 wk ($p \leq 0.05$). In summary, CD4$^+$ T cells from HC administered 10,000 IU of vitamin D$_3$ for 24 wk did not exhibit any changes in their proliferation or pro-inflammatory cytokine production in response to MOG and MBP stimulation.
Figure 3.8 The effect of placebo supplementation in HC subjects on their MOG and MBP induced T cell proliferation and cytokine production

HC were administered placebo over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with MOG and MBP for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4\(^+\) T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=7-11). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4\(^+\) T cells and production of total IFN\(\gamma\) (B) (n=7-11) and IL-17 (C) (n=4-8) from CD4\(^+\) T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=4-11), IFN\(\gamma\) (n=4-11) and IL-17F (n=4-11) were determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.9 The effect of 5,000 IU of vitamin D₃ supplementation in HC subjects on their MOG and MBP induced T cell proliferation and cytokine production

HC were administered 5,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with MOG and MBP for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=11-13). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=11-13) and IL-17 (C) (n=8-10) from CD4⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=8-13), IFNγ (n=8-13) and IL-17F (n=8-13) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.10 The effect of 10,000 IU of vitamin D₃ supplementation in HC subjects on their MOG and MBP induced T cell proliferation and cytokine production

HC were administered 10,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with MOG and MBP for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=11). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=10) and IL-17 (C) (n=8) from CD4⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=8-11) IFNγ (n=8-11) and IL-17F (n=8-11) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. *p≤0.05, versus baseline. Data expressed as mean (+/- SEM).
3.2.6 The effect of vitamin D₃ supplementation in HC subjects on their PPD- and TT-induced T cell proliferation and cytokine production

PPD and TT are recall antigens that stimulate memory antigen-specific CD4⁺ T cells, which have been previously generated after exposure via vaccination for or infection with TB and tetanus respectively. These antigens need to be presented to T cells via APC, thus recall antigen stimulation also takes into account the indirect effects of vitamin D on T cells via APC. Therefore, it was of interest to determine whether vitamin D₃ supplementation could affect recall responses to these antigens. HC were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual, and analysed for proliferation and cytokine production in response to recall antigens PPD and TT. A standardised single HC sample was run in parallel with each batch of samples in order to control for inter-experimental variation. PBMC were labelled with cell tracker dye CTV, stimulated for 7 days with PPD and TT, and then harvested and re-stimulated with PMA and ionomycin for 5 hr in order to measure cytokine production. CD4 T cell proliferation and cytokine production were analysed by flow cytometry. The proliferation of CD4⁺ T cells was measured by the dilution of CTV using flow cytometry. In addition, an unstimulated control was used to determine the proliferation of CD4⁺ T cells using CTV dilution. In addition, the concentrations of IL-17F, IFNγ and IL-10 were determined by ELISA in cell culture supernatants harvested on day 7.

Placebo:

Fig 3.11A left shows a representative histogram of CTV dilution, where the bar indicates the frequency of proliferated CD4⁺ T cells in response to PPD and TT stimulation. The frequency of proliferated CD4 T cell at 0, 16 and 24 wk for HC that were administered placebo is shown in Fig 3.11A, right, where no significant differences were observed between time points. A representative dot plot shows IFNγ vs CTV staining in CD4⁺ T cells, where the majority of IFNγ was produced by the unproliferated (CTV<sub>hi</sub>) cells (Fig 3.11B, left). The graph shows the frequency of CD4⁺ T cells producing IFNγ at 0, 16 and 24 wk (Fig 3.11, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.11C, left, where unproliferated cells produced the majority of cytokines. The graph shows the frequency of CD4⁺ T cells producing IL-17 at 0, 16 and 24 wk for HC that were administered placebo (Fig 3.11C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.11D shows the concentrations of IL-10, IFNγ and IL-
measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from PPD and TT stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from HC administered placebo for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to PPD and TT stimulation.

5,000 IU vitamin D3:
Figure 3.12A, left, shows a representative histogram of CTV dilution, where the bar indicates the frequency of proliferated CD4+ T cells in response to PPD and TT stimulation. The graph shows the frequency of proliferated CD4+ T cell at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D3, and no significant differences were observed between time points (Fig 3.12A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the unproliferated (CTV^{hi}) cells (Fig 3.12B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D3 (Fig 3.12B, right). There were no significant differences observed between time points. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.12C, left where unproliferated cells produced the majority of cytokines. The graph shows the frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for HC that were administered 5,000 IU of vitamin D3 (Fig 3.12C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.12D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from PPD and TT stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from HC administered 5,000 IU of vitamin D3 for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to PPD and TT stimulation.

10,000 IU vitamin D3:
Figure 3.13A, left, shows a representative histogram of CTV dilution, where the bar indicates the frequency of proliferated CD4+ T cells in response to PPD and TT stimulation. The graph shows the frequency of proliferated CD4+ T cell at 0, 16 and 24 wk for HC that were administered 10,000 IU of vitamin D3, and no significant differences were observed between time points (Fig 3.13A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the unproliferated (CTV^{hi}) cells (Fig 3.13B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for HC that were administered placebo Fig 3.13B, right. As expected, no
significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.13C, left, where the majority of cytokine was produced by unproliferated cells. The graph shows the frequency of CD4$^+$ T cells producing IL-17 at 0, 16 and 24 wk for HC that were administered 10,000 IU of vitamin D$_3$ (Fig 3.13C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.13D shows the concentrations of IL-10, IFN$\gamma$ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from PPD and TT stimulated PBMC, however no significant differences were observed between time points. In summary, CD4$^+$ T cells from HC administered 10,000 IU of vitamin D$_3$ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to PPD and TT stimulation.
Figure 3.11 The effect of placebo supplementation in HC subjects on their PPD and TT induced T cell proliferation and cytokine production
HC were administered placebo over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with PPD and TT for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4+ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=7-12). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4+ T cells and production of total IFNγ (B) (n=11-13) and IL-17 (C) (n=4-7) from CD4+ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=4-13), IFNγ (n=4-13) and IL-17F (n=4-13) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.1 The effect of 5,000 IU of vitamin D₃ supplementation in HC subjects on their PPD and TT induced T cell proliferation and cytokine production

HC were administered 5,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with PPD and TT for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD₄⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=9-12). Cells were stained with fluorochrome-conjugated antibodies to identify the CD₄⁺ T cells and production of total IFNγ (B) (n=9-12) and IL-17 (C) (n=8-11) from CD₄⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10, IFNγ and IL-17F determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.13 The effect of 10,000 IU of vitamin D₃ supplementation in HC subjects on their PPD and TT induced T cell proliferation and cytokine production

HC were administered 10,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with PPD and TT for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=11). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=11) and IL-17 (C) (n=8) from CD4⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=8-11), IFNγ (n=8-11) and IL-17F (n=8-11) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
3.2.7 The effects of vitamin D₃ supplementation in CIS patients on serum vitamin D₃ and PTH

CIS patients were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period, and serum samples were collected at baseline, 16, and 24 wk. Serum 25(OH)D and PTH were measured as described in the methods. Serum samples were collected by Dr Karen O’ Connell, Department of Neurology, St. Vincent’s University Hospital and sent to the Department of Neurology, Mater Misericordiae University Hospital, Dublin for analysis of 25(OH)D and PTH.

As shown in Fig 3.1A (left) there was no change in serum 25(OH)D concentrations in CIS patients were supplemented with placebo across three time points. Similarly, the PTH concentrations did not significantly alter in response to supplementation with placebo (Fig 3.1A, right).

When CIS patients were supplemented with 5,000 IU of vitamin D₃, serum 25(OH)D concentrations significantly increased from baseline to 16 wk (p≤0.001), and from baseline to 24wk (p≤0.01) (Fig 3.1B, left). However, the serum PTH levels did not change significantly over the time points (Fig 3.1B, right).

In Fig 3.1C (left) serum 25(OH)D significantly increased from baseline to 16 wk (p≤0.0001), and from baseline to 24wk (p≤0.0001), when supplemented with 10,000 IU of vitamin D₃. In Fig 3.1C (right) the PTH levels did not significantly change at baseline vs 16 wk, or vs 24 wk.

In summary, CIS patients exhibited a significant increase in serum 25(OH)D levels after 16 or 24 wk of vitamin D₃ supplementation at either 5,000 or 10,000 IU. This was accompanied by no significant decrease in PTH levels in both 5,000 and 10,000 IU vitamin D₃ group, suggesting that the increased 25(OH)D levels were having no effect on PTH release.
Figure 3.14 The effects of vitamin D₃ supplementation in CIS patients on serum 25(OH)D and PTH concentrations

CIS patients were randomised into three groups and administered placebo, 5,000 or 10,000 IU vitamin D₃. Serum 25(OH)D and PTH concentrations were measured at baseline, 16 wk and 24 wk. Graphs show the serum 25(OH)D (left) and PTH (right) concentrations in HC that were administered placebo (A) (n=7), 5,000 IU vitamin D₃ (B) (n=10) or 10,000 vitamin D₃ (C) (n=12). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. **p≤0.01, ***p≤0.001, versus baseline. Data expressed as mean (+/- SEM). Samples were collected by Dr Karen O’Connell and analysed by the Department of Neurology, Mater Misericordiae University Hospital, Dublin.
3.2.8 The effect of vitamin D₃ supplementation on serum 25(OH)D and PTH concentrations-comparison between HC and CIS patient groups

HC and CIS patients were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period, and serum samples were collected at baseline, 16, and 24 wk. Serum vitamin D 25(OH)D and PTH were measured as described in the methods. Serum samples were collected by Dr Karen O’Connell, Department of Neurology, St. Vincent’s University Hospital and sent to the Department of Neurology, Mater Misericordiae University Hospital, Dublin for analysis of 25(OH)D and PTH.

As shown in Fig 3.15A there was no change in serum 25(OH)D concentrations in HC vs CIS patients when supplemented with placebo, 5,000 IU, and 10,000 IU vitamin D₃ across three time points. Similarly, the PTH concentrations did not significantly alter in response to supplementation with placebo, 5,000 IU, and 10,000 IU (Fig 3.15B) (table 3.2)(i-iii).

In HC, comparing placebo group at baseline against 5,000 IU group baseline showed no significance which is demonstrated in table 3.2(iv), however comparing placebo group at 16 wk vs 5,000 IU vitamin D₃ group at 16 wk showed a significant difference (p≤0.001), and similarly when comparing placebo group at 16 wk vs 10,000 IU vitamin D₃ group at 16 wk showed a significant increase in the treatment groups. This was also significant when comparing placebo group at 24 wk vs 5,000 IU and 10,000 IU vitamin D₃ at 24 wk (p≤0.0001). This was not seen in CIS patients when comparing placebo group at 16 or 24 wk when compared to 5,000 IU vitamin D₃ group. In CIS patients, comparing placebo group at baseline against 5,000 IU group baseline showed no significance which is demonstrated in table 3.2(v). Similarly comparing Placebo group at 16 wk vs 5,000 IU showed no significance. However, when comparing placebo group at 16 wk vs 10,000 IU vitamin D₃ group at 16 wk showed a significant (p≤0.0001) increase in the treatment groups table 3.1(v). This was also significant when comparing placebo group at 24 wk vs 10,000 IU vitamin D₃ at 24 wk (p≤0.0001), but not at 5,000 IU.

In summary, there was no significant difference between HC and CIS in serum 25(OH)D and PTH concentrations. However, in both HC and CIS patients both had a significant increase from placebo at 16 and 24 wk against 16 and 24 wk 10,000 IU vitamin D₃ group. HC also demonstrated a significant increase at 16 and 24 wk placebo against 16 and 24 wk 10,000 IU vitamin D₃ group. This was not seen in the CIS group which suggests vitamin D dysfunctional metabolism in CIS patients.
Figure 3.15 The effect of vitamin D₃ supplementation on serum 25(OH)D and PTH concentrations-comparison between HC and CIS patient groups

(A) 25(OH)D concentrations in HC (n=10-13) and CIS patients (n=7-12) at 0, 16 and 24 wk after supplementation with placebo, 5,000 or 10,000 IU vitamin D₃. (B) PTH concentrations in HC (n=10-13) and CIS patients (n=7-12) at 0, 16 and 24 wk after supplementation with placebo, 5,000 or 10,000 IU vitamin D₃. Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 versus baseline. Data expressed as mean (+/- SEM). Refer to table 3.1 for statistical analysis.
Table 3.2 The effects of vitamin D₃ supplementation in HC and CIS patients on serum 25(OH)D and PTH concentrations – comparisons between groups

PTH concentrations in HC (i) and CIS patients (ii) at each time point were compared between placebo and 5,000 IU or 10,000 IU vitamin D₃ groups. PTH concentrations at each time point were compared between HC and CIS for each treatment arm (iii). 25(OH)D concentrations in HC (iv) and CIS patients (v) at each time point were compared between placebo and 5,000 IU or 10,000 IU vitamin D₃ groups. PTH concentrations at each time point were compared between HC and CIS for each treatment arm (vi). Statistical differences between groups were determined by one-way ANOVA with Bonferroni post-test. ***p<0.0001 versus baseline.
3.2.9 The effect of vitamin D₃ supplementation in CIS patients on their anti-CD3 induced T cell proliferation and cytokine production

Vitamin D has been shown to exert anti-proliferative and anti-inflammatory effects in vitro, therefore the effect of vitamin D₃ supplementation on T cell proliferation and cytokine responses was examined. CIS patients were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual, and analysed for proliferation and cytokine production in response to polyclonal anti-CD3 stimulation. A standardised single HC sample was run in parallel with each batch of samples in order to control for inter-experimental variation. PBMC were labelled with cell tracker dye CTV, stimulated for 3 days with anti-CD3 and then harvested and re-stimulated with PMA and ionomycin for 5 hr in order to measure cytokine production. The proliferation of CD4⁺ T cells was measured by the dilution of CTV using flow cytometry. In addition, an unstimulated control was used to determine the proliferation of CD4⁺ T cells using CTV dilution. Cells were stained for expression of CD3, CD8 and intracellular IL-17 and analysed by flow cytometry. In addition, the concentrations of IL-17F, IFNγ and IL-10 were determined by ELISA in cell culture supernatants harvested on day 3.

Placebo:

For flow cytometric analysis, CD4⁺ T cells were gated as described in Fig 2.6 and proliferated cell cytokine production by CTV dilution was gated as described in Fig 2.7. The graph shows the frequency proliferation at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.16A, left), where no significant differences were observed between time points (Fig 3.16A, right). A representative dot plot shows IFNγ vs CTV staining in CD4⁺ T cells, where the majority of IFNγ was produced by the proliferated (CTVlo) cells (Fig 3.16B, left). The graph shows the frequency of CD4⁺ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.15B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.16C, left, where proliferating cells produced the majority of cytokines. The graph shows the frequency of CD4⁺ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.16C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.16D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in
the supernatants from anti-CD3 stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from CIS patients administered placebo for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to anti-CD3 stimulation.

5,000 IU vitamin D₃:

The graph shows the frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.17A, left), and no significant differences were observed between time points (Fig 3.17A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the proliferated (CTVlo) cells (Fig 3.17B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.17B, right). There were no significant differences observed between time points. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.17C, left, where unproliferated cells produced the majority of cytokine. The graph shows the frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.17C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.17D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from anti-CD3 stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from CIS patients administered 5,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to anti-CD3 stimulation.

10,000 IU vitamin D₃:

The graph shows the frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered 10,000 IU of vitamin D₃ (Fig 3.18A left), and no significant differences were observed between time points (Fig 3.18A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the proliferated (CTVlo) cells (Fig 3.18B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.18B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.18C, left, where the majority of cytokine was produced by unproliferated cells. The graph shows the frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered 10,000 IU of vitamin D₃ (Fig 3.18C, right). No significant differences in the
frequency of IL-17 producing cells were observed between time points. Fig 3.18D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from anti-CD3 stimulated PBMC, however no significant differences were observed between time points. In summary, CD4⁺ T cells from CIS patients administered 10,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to anti-CD3 stimulation.
The effect of placebo supplementation in CIS patients on their anti-CD3 induced T cell proliferation and cytokine production

CIS patients were administered placebo over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with anti-CD3 for 3 days. After 3 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4+ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=6-7). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4+ T cells and production of total IFNγ (B) (n=6-7) and IL-17 (C) (n=6-7) from CD4+ T cells, and analysed by flow cytometry. After 3 days, the supernatants were harvested and the concentrations of IL-10 (n=6-7), IFNγ (n=6-7) and IL-17F (n=6-7) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.17 The effect of 5,000 IU vitamin D₃ supplementation in CIS patients on their anti-CD3 induced T cell proliferation and cytokine production

CIS patients were administered 5,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with anti-CD3 for 3 days. After 3 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=10). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=9-10) and IL-17 (C) (n=9-10) from CD4⁺ T cells, and analysed by flow cytometry. After 3 days, the supernatants were harvested and the concentrations of IL-10 (n=9-10), IFNγ (n=9-10) and IL-17F (n=9-10) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.18 The effect of 10,000 IU of vitamin D₃ supplementation in CIS patients on their anti-CD3 induced T cell proliferation and cytokine production

CIS patients were administered 10,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with anti-CD3 for 3 days. After 3 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=10-12). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=10-12) and IL-17 (C) (n=10-12) from CD4⁺ T cells, and analysed by flow cytometry. After 3 days, the supernatants were harvested and the concentrations of IL-10 (n=10-12), IFNγ (n=10-12) and IL-17F (n=10-12) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM)
3.2.10 The effect of vitamin D₃ supplementation in CIS patients on their MOG and MBP induced T cell proliferation and cytokine production

The auto-antigens that are targeted by T cells in MS are thought to include the myelin antigens MOG and MBP, therefore it was of interest to determine whether vitamin D₃ supplementation could affect recall responses to these antigens. CIS patients were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual, and analysed for proliferation and cytokine production in response to myelin antigens MOG and MBP. A standardised single HC sample was run in parallel with each batch of samples in order to control for inter-experimental variation. PBMC were labelled with cell tracker dye CTV, stimulated for 7 days with MOG and MBP and then harvested and re-stimulated with PMA and ionomycin for 5 hr in order to measure cytokine production. The proliferation of CD4⁺ T cells was measured by the dilution of CTV using flow cytometry. In addition, an unstimulated control was used to determine the proliferation of CD4⁺ T cells using CTV dilution. The production of cytokines IFNγ, and IL-17 by CD4⁺ T cells were analysed by flow cytometry. In addition, the concentrations of IL-17F, IFNγ and IL-10 were determined by ELISA in cell culture supernatants harvested on day 7.

**Placebo:**

For flow cytometric analysis, CD4⁺ T cells were gated as described in materials and methods. The frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.19A left), and no significant differences were observed between time points (Fig 3.19A, right). A representative dot plot shows IFNγ vs CTV staining in CD4⁺ T cells, where the majority of IFNγ was produced by the unproliferated (CTV<hi>) cells (Fig 3.19B, left). The graph shows the frequency of CD4⁺ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.19B right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.18C, left, where unproliferated cells produced the majority of cytokines. The graph shows the frequency of CD4⁺ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.19C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.19D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from MOG and MBP stimulated PBMC, however no significant differences were observed between time points. In summary, CD4⁺ T cells from CIS patients administered placebo for
24 wk did not exhibit any changes in their proliferation or cytokine production in response to MOG and MBP stimulation.

5,000 IU vitamin D₃:

For flow cytometric analysis, CD4⁺ T cells were gated as described in materials and methods. Figure 3.20A, left, shows a representative histogram of CTV dilution, where the bar indicates the frequency of proliferated CD4⁺ T cells in response to MOG and MBP stimulation. The graph shows the frequency proliferation at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃, and no significant differences were observed between time points (Fig 3.20A, right). A representative dot plot shows IFNγ vs CTV staining in CD4⁺ T cells, where the majority of IFNγ was produced by the unproliferated (CTVₕi) cells (Fig 3.20B, left). The graph shows the frequency of CD4⁺ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.20B, right). There were no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.20C, left, where unproliferated produced the majority of cytokine cells. The graph shows the frequency of CD4⁺ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.20C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.20D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from MOG and MBP stimulated PBMC, however no significant differences were observed between time points. In summary, CD4⁺ T cells from CIS patients administered 5,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to MOG and MBP stimulation.

10,000 IU vitamin D₃:

For flow cytometric analysis, CD4⁺ T cells were gated as described in materials and methods. The graph shows the frequency proliferation at 0, 16 and 24 wk for CIS patients that were administered 10,000 IU of vitamin D₃ (Fig 3.21A left), and no significant differences were observed between time points (Fig 3.21A, right). A representative dot plot shows IFNγ vs CTV staining in CD4⁺ T cells, where the majority of IFNγ was produced by the unproliferated (CTVₕi) cells (Fig 3.21B, left). The graph shows the frequency of CD4⁺ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.21B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.21C, left, where the majority of cytokine was produced by proliferating cells. The graph shows the
frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered 10,000 IU of vitamin D3 (Fig 3.21C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.21D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from MOG and MBP stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from CIS patients administered 10,000 IU of vitamin D3 for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to MOG and MBP stimulation.
Figure 3.19 The effect of placebo supplementation in CIS patients on their MOG and MBP induced T cell proliferation and cytokine production

CIS patients were administered placebo over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with MOG and MBP for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4+ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=6-7). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4+ T cells and production of total IFNγ (B) (n=6-7) and IL-17 (C) (n=6-7) from CD4+ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=6-7), IFNγ (n=6-7) and IL-17F (n=6-7) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.20 The effect of 5,000 IU of vitamin D$_3$ supplementation in CIS patients on their MOG and MBP induced T cell proliferation and cytokine production

CIS patients were administered 5,000 IU of vitamin D$_3$ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with MOG and MBP for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD$^+$ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) ($n=10$). Cells were stained with fluorochrome-conjugated antibodies to identify the CD$^+$ T cells and production of total IFN$\gamma$ (B) ($n=10$) and IL-17 (C) ($n=10$) from CD$^+$ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 ($n=10$), IFN$\gamma$ ($n=10$) and IL-17F ($n=10$) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.21 The effect of 10,000 IU of vitamin D₃ supplementation in CIS patients on their MOG and MBP induced T cell proliferation and cytokine production

CIS patients were administered 5,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with MOG and MBP for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=10-12). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=10-12) and IL-17 (C) (n=10-12) from CD4⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=10-12), IFNγ (n=10-12) and IL-17F (n=10-12) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
3.2.11 The effect of vitamin D₃ supplementation in CIS patients on their PPD and TT induced T cell proliferation and cytokine production

PPD and TT are recall antigens that stimulate memory antigen-specific CD4⁺ T cells, which have been previously generated after exposure via vaccination for or infection with TB and tetanus respectively. These antigens need to be presented to T cells via APC, thus recall antigen stimulation also accounts for the indirect effects of vitamin D on T cells via APC. Therefore, it was of interest to determine whether vitamin D₃ supplementation could affect recall responses to these antigens. CIS patients were administered placebo, 5,000 IU or 10,000 IU of vitamin D₃ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual, and analysed for proliferation and cytokine production in response to recall antigens PPD and TT. A standardised single HC sample was run in parallel with each batch of samples in order to control for inter-experimental variation. PBMC were labelled with cell tracker dye CTV, stimulated for 7 days with PPD and TT, then harvested and re-stimulated with PMA and ionomycin for 5 hr in order to measure cytokine production. The proliferation of CD4⁺ T cells was measured by the dilution of CTV using flow cytometry. In addition, an unstimulated control was used to determine the proliferation of CD4⁺ T cells using CTV dilution. The production of cytokines IFNγ, and IL-17 by CD4⁺ T cells were analysed by flow cytometry. In addition, the concentrations of IL-17F, IFNγ and IL-10 were determined by ELISA in cell culture supernatants harvested on day 7.

Placebo:
For flow cytometric analysis, CD4⁺ T cells were gated as described in materials and methods. The frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.22, left), and no significant differences were observed between time points (Fig 3.22A, right). A representative dot plot shows IFNγ vs CTV staining in CD4⁺ T cells, where the majority of IFNγ was produced by the unproliferated (CTV<sup>hi</sup>) cells (Fig 3.22B, left). The graph shows the frequency of CD4⁺ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.22B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.22C, left where unproliferated cells produced the majority of cytokines. The graph shows the frequency of CD4⁺ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.22C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.22D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell
culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from PPD and TT stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from CIS patients administered placebo for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to MOG and MBP stimulation.

5,000 IU vitamin D₃:
For flow cytometric analysis, CD4+ T cells were gated as described in materials and methods. The graph shows the frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.23A, left), and no significant differences were observed between time points (Fig 3.23A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the unproliferated (CTV hi) cells (Fig 3.23B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.23B, right). There were no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.23C, left, where unproliferated cells produced the majority of cytokine cells. The graph shows the frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.23C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.23D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from PPD and TT stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from CIS patients administered 5,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to PPD and TT stimulation.

10,000 IU vitamin D₃:
For flow cytometric analysis, CD4+ T cells were gated as described in materials and methods. The graph shows the frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered 10,000 IU of vitamin D₃ (Fig 3.24A, left) and no significant differences were observed between time points (Fig 3.24A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the unproliferated (CTV hi) cells (Fig 3.24B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.24B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.24C, left,
where the majority of cytokine was produced by unproliferated cells. The graph shows the frequency of CD4⁺ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered 10,000 IU of vitamin D₃ (Fig 3.24C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.24D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from PPD and TT stimulated PBMC, however no significant differences were observed between time points. In summary, CD4⁺ T cells from CIS patients administered 10,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to PPD and TT stimulation.
Figure 3.2 The effect of placebo supplementation in CIS patients on their PPD and TT induced T cell proliferation and cytokine production
CIS patients were administered placebo over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with PPD and TT for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=6-7). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=6-7) and IL-17 (C) (n=6-7) from CD4⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=6-7), IFNγ (n=6-7) and IL-17F (n=6-7) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM)
Figure 3.23 The effect of 5,000 IU of vitamin D₃ supplementation in CIS patients on their PPD and TT induced T cell proliferation and cytokine production

CIS patients were administered 5,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with PPD and TT for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD⁴⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=10). Cells were stained with fluorochrome-conjugated antibodies to identify the CD⁴⁺ T cells and production of total IFNγ (B) (n=10) and IL-17 (C) (n=10) from CD⁴⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=10), IFNγ (n=10) and IL-17F (n=10) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
The effect of 10,000 IU of vitamin D₃ supplementation in CIS patients on their PPD and TT induced T cell proliferation and cytokine production

CIS patients were administered 10,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with PPD and TT for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) \((n=10-11)\). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) \((n=10-11)\) and IL-17 (C) \((n=10-11)\) from CD4⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 \((n=10-11)\), IFNγ \((n=10-11)\) and IL-17F \((n=10-11)\) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
3.2.12 The effect of vitamin D$_3$ supplementation in CIS patients on their allogeneic induced T cell proliferation and cytokine production

As described above, cells from HC or CIS patients in different treatment arms were stimulated with anti-CD3, which stimulates all T cells, or antigen specific stimuli which stimulated memory responses to previously encountered antigens. It is possible however that vitamin D$_3$ supplementation may have skewed the differentiation of naïve T cells. In order to address this, PBMC from CIS patients were stimulated with irradiated allogeneic APC which would stimulate allo-specific naïve T cells within the PBMC. CIS patients were administered placebo, 5,000 IU or 10,000 IU of vitamin D$_3$ over a 24 wk period. Blood samples were taken at 0, 16, and 24 wk and PBMC were isolated and cryopreserved. On conclusion of the study, PBMC were thawed in batches that included all 3 time points for a given individual, and analysed for proliferation and cytokine production in response to allogeneic stimulation. A standardised single HC sample was run in parallel with each batch of samples in order to control for inter-experimental variation. PBMC were labelled with cell tracker dye CTV, stimulated for 7 days with irradiated cells and then harvested and re-stimulated with PMA and ionomycin for 5 hr in order to measure cytokine production. The proliferation of CD4$^+$ T cells was measured by the dilution of CTV using flow cytometry. In addition, an unstimulated control was used to determine the proliferation of CD4$^+$ T cells using CTV dilution. The production of cytokines IFN$\gamma$, and IL-17 by CD4$^+$ T cells were analysed by flow cytometry. In addition, the concentrations of IL-17F, IFN$\gamma$ and IL-10 were determined by ELISA in cell culture supernatants harvested on day 7.

**Placebo:**

The frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.25A, left), and no significant differences were observed between time points (Fig 3.25A, right). A representative dot plot shows IFN$\gamma$ vs CTV staining in CD4$^+$ T cells, where the majority of IFN$\gamma$ was produced by the proliferated (CTV$^{lo}$) cells (Fig 3.25B, left). The graph shows the frequency of CD4$^+$ T cells producing IFN$\gamma$ at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.25B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.25C, left, where unproliferated cells produced the majority of cytokines. The graph shows the frequency of CD4$^+$ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.25C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.25D shows the concentrations of IL-10, IFN$\gamma$ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in
the supernatants from allogeneic stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from CIS patients administered placebo for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to allogeneic stimulation.

5,000 IU vitamin D₃:

For flow cytometric analysis, CD4+ T cells were gated as described in materials and methods. The graph shows the frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.26A, left), and no significant differences were observed between time points (Fig 3.26A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the proliferated (CTVlo) cells (Fig 3.26B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.26B, right). There were no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.26C, left, where unproliferated cells produced the majority of cytokine cells. The graph shows the frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered 5,000 IU of vitamin D₃ (Fig 3.26C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.25D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from allogeneic stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from CIS patients administered 5,000 IU of vitamin D₃ for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to allogeneic stimulation.

10,000 IU vitamin D₃:

For flow cytometric analysis, CD4+ T cells were gated as described in materials and methods. The graph shows the frequency of proliferated CD4 T cells at 0, 16 and 24 wk for CIS patients that were administered 10,000 IU of vitamin D₃ (Fig 3.27A, left), and no significant differences were observed between time points (Fig 3.27A, right). A representative dot plot shows IFNγ vs CTV staining in CD4+ T cells, where the majority of IFNγ was produced by the proliferated (CTVlo) cells (Fig 3.27B, left). The graph shows the frequency of CD4+ T cells producing IFNγ at 0, 16 and 24 wk for CIS patients that were administered placebo (Fig 3.27B, right). As expected, no significant differences between time points were observed. A representative dot plot of IL-17 vs CTV staining is shown in Fig 3.27C, left, where the majority of cytokine was produced by unproliferated cells. The graph shows the
frequency of CD4+ T cells producing IL-17 at 0, 16 and 24 wk for CIS patients that were administered 10,000 IU of vitamin D3 (Fig 3.2C, right). No significant differences in the frequency of IL-17 producing cells were observed between time points. Fig 3.2D shows the concentrations of IL-10, IFNγ and IL-17 measured in cell culture supernatants by ELISA. All of the cytokines were detectable in the supernatants from allogeneic stimulated PBMC, however no significant differences were observed between time points. In summary, CD4+ T cells from CIS patients administered 10,000 IU of vitamin D3 for 24 wk did not exhibit any changes in their proliferation or cytokine production in response to allogeneic stimulation.
The effect of placebo supplementation in CIS patients on their allogeneic induced T cell proliferation and cytokine production

CIS patients were administered placebo over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with irradiated cells from an unrelated donor to stimulate allo-reactive T cells for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4\(^+\) T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=6-7). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4\(^+\) T cells and production of total IFN\(\gamma\) (B) (n=6-7) and IL-17 (C) (n=6-7) from CD4\(^+\) T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=6-7), IFN\(\gamma\) (n=6-7) and IL-17F (n=6-7) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
The effect of 5,000 IU of vitamin D₃ supplementation in CIS patients on their allogeneic induced T cell proliferation and cytokine production

CIS patients were administered 5,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with irradiated cells from an unrelated donor to stimulate allo-reactive T cells for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=9-10). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=9-10) and IL-17 (C) (n=9-10) from CD4⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=9-10), IFNγ (n=9-10) and IL-17F (n=9-10) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
Figure 3.27 The effect of 10,000 IU of vitamin D₃ supplementation in CIS patients on their allogeneic induced T cell proliferation and cytokine production
CIS patients were administered 10,000 IU of vitamin D₃ over 24 wk, and PBMC were isolated at 0, 16, and 24 wk. PBMC were labelled with CTV, and then stimulated with irradiated cells from an unrelated donor to stimulate allo-reactive T cells for 7 days. After 7 days, the cells were harvested and stimulated with PMA/ionomycin for 5 hr. The proliferation of CD4⁺ T cells was measured by the dilution of CTV, and analysed by flow cytometry (A) (n=10-12). Cells were stained with fluorochrome-conjugated antibodies to identify the CD4⁺ T cells and production of total IFNγ (B) (n=10-12) and IL-17 (C) (n=10-12) from CD4⁺ T cells, and analysed by flow cytometry. After 7 days, the supernatants were harvested and the concentrations of IL-10 (n=10-12), IFNγ (n=10-12) and IL-17F (n=10-12) determined by ELISA (D). Statistical differences between baseline and time points were determined by one-way ANOVA with Bonferroni post-test. Data expressed as mean (+/- SEM).
3.3 Discussion

In this study, we conducted a dose ranging, randomised double-blind placebo-controlled trial, which examined the immunomodulatory effects of high dose vitamin D₃ supplementation in HC subjects and CIS patients. The primary endpoint of the study was the effect of oral vitamin D₃ compared with placebo on the frequency of CD4⁺ T cell subsets (IL-17⁺CD4⁺ and IFNγ⁺CD4⁺ T cells) and cytokine (IL-17, IFNγ, and IL-10) responses in PBMC. The secondary endpoints were radiological activity, clinical activity, and disease freedom. The safety and tolerability of high dose vitamin D₃ in HC and in CIS patients were also determined. The immunological assessments carried out in this clinical trial were based on a pilot study conducted in 2012, which studied the immunological effect of vitamin D₃ in HC (Allen et al., 2012). In the pilot study, 4 healthy individuals were administered 5,000 IU or 10,000 IU of vitamin D₃ over a course of 15 wk. The pilot study demonstrated that vitamin D₃ supplementation increased serum 25(OH)D levels significantly from baseline (Allen et al., 2012). It also showed that IL-10 production increased in PBMC and the frequency of Th17 cells was reduced (Allen et al., 2012). The findings of the pilot study were broadly in agreement with studies in the EAE model (Joshi et al., 2011, Chang et al., 2010). Power calculations were conducted to inform the design of this clinical trial, on the basis of the findings from the pilot study (Allen et al., 2012).

Safety and tolerability of vitamin D₃

This trial showed that vitamin D₃ at 5,000 IU and at 10,000 IU was tolerable and safe in HC subjects and in CIS patients, since there were no serious adverse events related to vitamin D₃ supplementation during the study. A recent study by Sotirchos et al. in 2016 administered a similar high dose of vitamin D₃ (10,400 IU) daily for 6 months and concluded that this dosage of vitamin D₃ was safe and tolerable. However, 3 patients withdrew from the study due to hypercalcemia or gastrointestinal disturbances (Sotirchos et al., 2016). In another trial conducted by Burton et al., in 2010 patients were supplemented with up to 40,000 IU vitamin D₃ daily for 28 wk, and no adverse events were reported. However, four patients experienced mild constipation due to the calcium supplementation in powdered form, and symptoms were resolved by change in calcium formulation or discontinuation (Burton et al., 2010). In support of the current study, several other trials showed that supplementation of vitamin D₃ was safe and tolerable with no adverse events reported (Kimball et al., 2007, Kimball et al., 2011, Smolders et al., 2010, Wingerchuk et al., 2005, Ashtari et al., 2016, Mahon et al., 2003a).
The majority of patients and HC recruited during this trial were borderline sufficient rather than insufficient or deficient in 25(OH)D levels at baseline. After vitamin D₃ supplementation, serum 25(OH)D concentrations increased to a similar extent in CIS patients and in HC. In HC serum 25(OH)D levels increased from 63 nmol/L to 155 nmol/L and from 40 nmol/L to 176 nmol/L after supplementation with 5,000 or 10,000 IU of vitamin D₃ respectively. In CIS patients serum 25(OH)D levels increased from 56 nmol/L to 132 nmol/L and from 52.2 nmol/L to 183 nmol/L after supplementation with 5,000 or 10,000 IU of vitamin D₃ respectively. Thus, there did appear to be a dose effect, with higher serum levels in response to 10,000 vs 5,000 IU of vitamin D₃ supplementation. Although there is no general agreement on the serum level of 25(OH)D that is required to exert immunomodulatory effects, it has been suggested that serum 25(OH)D levels above 100 nmol/L could be required to produce the immunological effects of vitamin D₃ supplementation (Munger et al., 2006) This suggests that the serum 25(OH)D levels achieved in this study are likely to have been in the range required for immune modulation. Direct comparisons between this study and other vitamin D₃ supplementation studies in MS are not always possible due to differences in dosing and duration. However, in a study that used a similar dose of 10,400 IU of vitamin D₃ daily for 6 months in RRMS patients, serum 25(OH)D only increased from 49.9 nmol/L to 67.3 nmol/L (Sotirchos et al., 2016). Thus, although the dose, duration and baseline 25(OH)D level was similar to the current study, the increase in 25(OH)D was much lower than that observed here. The reasons for this are not clear, however, a study which investigated the 25(OH)D serum levels in RRMS patients and HC in response to vitamin D₃ supplementation found that MS patients had a lesser average increase in serum 25(OH)D than HC after oral vitamin D₃ supplementation, despite having similar concentrations at baseline (Bhargava et al., 2016). One possible reason for this low serum 25(OH)D level in MS patients could be the presence of a systematic inflammatory response, which is associated with an increase in C reactive protein (CRP). Low serum 25(OH)D has been independently associated with an increase in CRP and a decrease in albumin (Ghashut et al., 2014). It is possible that the CIS patients in our study had less systemic inflammation that RRMS patients, thereby allowing higher serum 25(OH)D levels to be achieved after supplementation.

Another trial showed that supplementation of 4,000 IU of vitamin D₃ daily for 52 wk in RRMS and SPMS patients increased mean serum 25(OH)D from 78 nmol/L to 413 nmol/L.
(Burton et al., 2010); which is notably higher than the levels achieved in this study using higher doses, albeit for a shorter duration. It is not clear whether the serum 25(OH)D levels achieved in our study had plateaued for each dose, or whether the levels would have increased further after 24 wk. Another study showed similar baseline serum 25(OH)D to the current trial with a similar serum 25(OH)D increase, however they administered 20,000 IU weekly to RRMS patients for one year (Soilu-Hänninen et al., 2012).

When considering serum 25(OH)D levels in response to supplementation, it is important to note that the immunomodulatory function of vitamin D only occurs when the kidneys convert the serum 25(OH)D to 1,25(OH)\textsubscript{2}D\textsubscript{3}, and thus the measurement of 25(OH)D is not necessarily an indicator of the level of active 1,25(OH)\textsubscript{2}D\textsubscript{3} in the body. PTH plays a key role in regulating both this conversion of vitamin 25(OH)D to its active form and calcium uptake in the body. PTH enhances the uptake of calcium by increasing 1,25(OH)\textsubscript{2}D\textsubscript{3}. If calcium levels are low, the parathyroid gland secretes PTH, which increases the amount of 25(OH)D that is converted to active 1,25(OH)\textsubscript{2}D\textsubscript{3} in the kidneys to help absorb more calcium. If calcium levels are high, this downregulates PTH secretion which in turn prevents the conversion of 25(OH)D into 1,25(OH)\textsubscript{2}D\textsubscript{3}. Thus PTH levels decrease when serum 25(OH)D is converted to its active form, acting as a negative feedback mechanism (Holick, 2010, Holick et al., 2012). In this study, the increase in serum 25(OH)D levels after supplementation of HC with 10,000 IU vitamin D\textsubscript{3} was associated with a significant decrease in serum PTH. This suggests that high dose supplementation in HC resulted in increased active vitamin D and increased calcium levels, which had a negative feedback effect on PTH release. In contrast however, this effect was not seen in CIS patients, where there was no significant decrease in PTH after supplementation. This raises the possibility that vitamin D metabolism may be dysfunctional in CIS and MS patients. In support of this concept a study in RRMS patients identified abnormalities in two genes involved in vitamin D metabolism, CYP27B1, which encodes the rate limiting enzyme 1α-hydroxylase for vitamin D, and CYP24A1, which encodes an enzyme that degrades 1,25(OH)\textsubscript{2}D\textsubscript{3}, that were associated with MS (Sawcer et al., 2011). Furthermore, another study showed that the known risk single nucleotide polymorphisms (SNP) rs2248359 regulates CYP24A1 expression in the human brain (Ramasamy et al., 2014). Taken together these studies suggest that there may be a higher expression of CYP24A1 in MS patients, leading to increased degradation of 1,25(OH)\textsubscript{2}D\textsubscript{3}, which could in part explain the dysfunction of vitamin D metabolism in MS patients. In addition, a number of studies have shown an association between polymorphisms in the VDR and MS (Smolders et al., 2009a). These or other possible alterations in vitamin
D metabolism in MS patients could account for our observation that PTH levels did not decrease in CIS patients in response to high dose vitamin D₃.

Memory T cells
In this study, the effects of vitamin D₃ supplementation on memory T cells in HC were investigated, since it is assumed that EM T cells play an important role in the pathogenesis of MS, due to their ability to migrate to peripheral tissues, consistent with data that T cells in the brain in MS patients are predominantly CCR7⁻ EM T cells (Bhargava et al., 2015). However, the data from this study showed that supplementation of HC with 5,000 IU or 10,000 IU of vitamin D₃ had no effect on the frequency of memory T cells over the course of 24 wk. In contrast to the present trial, two studies showed that vitamin D₃ supplementation reduced the frequency of EM T cells within blood (Bhargava et al., 2015, Sotirchos et al., 2016). In the study by Sotirchos et al., 2016 they showed that patients supplemented with 10,400 IU of vitamin D₃ had a reduction in the frequency of EM T cells, with a corresponding increase in CM T cells; however, they did not measure the frequency of total memory CD4 T cells (Sotirchos et al., 2016). Thus, our study was not directly comparable, and it is possible that had we analysed the effector and central memory compartments we may have seen similar results. However the Bhargava study also showed an increase in the frequency of naïve CD4 T cells after vitamin D₃ supplementation, which would correspond with a decrease in total memory CD4 T cells, although this was not directly shown (Bhargava et al., 2015).

Treg cells
The rationale for investigating the effect of vitamin D₃ supplementation on Treg cells was that it is has been shown that vitamin D increases the proportion of Treg cells in vitro and in vivo. However, this study found no significant difference between the frequencies of CD4⁺CD25⁺CD127loFoxp3⁺ Treg cells when HC were administered placebo, 5,000 IU, or 10,000 IU of vitamin D₃. Data elsewhere showed that the frequency of CD4⁺CD25⁺CD127loFoxp3⁺ Treg cells significantly increased in HC that were administered 140,000 IU vitamin D₃ monthly for 3 months (Bock et al., 2011). Another study where HC were administered 140,000 IU of vitamin D₃ at baseline and 1 month later, showed a significant increase in Treg cells from baseline (Prietl et al., 2010). The same group conducted a double blinded placebo controlled trial in 60 healthy individuals and were supplemented with 140,000 IU of vitamin D₃ monthly for 12 months, and it demonstrated that vitamin D₃ supplementation increased Treg cells significantly from baseline (Prietl et al., 2014). Since these studies used higher single doses of vitamin D₃ it is possible that higher
vitamin D₃ doses than were used in our study might be required to have an effect on Treg cells. In agreement with our findings, a study similar to the current trial supplemented RRMS patients with 20,000 IU of vitamin D₃ daily for 12 wk and found that the frequency of Treg cells was unaffected (Smolders et al., 2010). However, an earlier study by Smolders et al., 2009 showed that high serum level status in RRMS patients showed improved Treg cell function in RRMS patients, these patients were on immunomodulatory therapy but were not supplemented with vitamin D₃ (Smolders et al., 2009b).

**In vitro stimulation**

In the trial, PBMC from both HC and CIS patients were stimulated *in vitro* with either polyclonal or antigen-specific stimuli in order to determine whether vitamin D₃ supplementation had an effect on T cell proliferation or cytokine production. Anti-CD3 is a polyclonal stimulation that mimics the activation of T cells that usually takes place via antigen binding to the TCR, and stimulates all T cells regardless of their antigen-specificity. PBMC contain both naïve and memory T cells, and both would be stimulated by anti-CD3, with the co-stimulatory signal required for full activation of T cells being provided by the APC present within PBMC.

PPD and TT are recall antigens that stimulate memory antigen-specific CD4⁺ T cells, which have been previously generated after exposure via vaccination for or infection with TB and tetanus, respectively. These antigens need to be presented to T cells via APC, thus recall antigen stimulation also considers the indirect effects of vitamin D on T cells via APC.

MOG and MBP are myelin antigens assumed to be among the auto antigens involved in MS, however MOG/MBP responses can also be detected in HC. Studies suggest that they stimulate antigen specific memory cells, but they can also stimulate antigen specific naïve T cells (Ota et al., 1990, Chou et al., 1991). Therefore, MOG and MBP can act as recall antigens in MS patients, however, they can also activate naïve T cells in lower frequencies.

PBMC were also stimulated with irradiated PBMC from an unrelated donor, in order to stimulate allo-reactive T cells. These T cells are specific for non-self MHC molecules, and should be naïve unless there was a history of transplantation (Murphy and Weaver, 2016). This stimulation was included in order to demonstrate possible effects of vitamin D₃ supplementation on the differentiation of naïve T cells.

The primary end points of the trial were to determine the effects of vitamin D₃ supplementation on the frequency of IL-17⁺CD4⁺ T cells and IFNγ⁺CD4⁺ T cells and the proliferation of CD4⁺ T cells.
When PBMC were stimulated with anti-CD3, the proliferation of CD4⁺ T cells from HCl subjects or CIS patients did not alter from baseline over the course of 24 wk when administered placebo. However, similarly, no difference was seen in HC or CIS patients that were administered with 5,000 IU or 10,000 IU of vitamin D₃. In agreement with our study, a Dutch study examined the effects of vitamin D₃ where participants were supplemented with 20,000 IU of vitamin D₃ daily for 12 wk and the anti-CD3 induced proliferation of responder T cells was not altered (Smolders et al., 2010). However, a study showed that the active form of vitamin D inhibited proliferation of CD4⁺ T cells and MBP-specific T cells in vitro (Correale et al., 2011, Rigby et al., 1984, Mahon et al., 2003b). Similarly another study showed that 1,25(OH)₂D₃ decreased the proliferation of all T helper cells (Mahon et al., 2003b). However, as there is no evidence to date that vitamin D₃ supplementation in vivo affects T cell proliferation, it is possible that these in vitro findings do not translate to the in vivo situation. It is also possible that the doses of 1,25(OH)₂D₃ used in vitro may not be comparable to those achieved in the body after supplementation since it is unclear what 1,25(OH)₂D₃ levels are achieved as this is dependent on the metabolism (Correale et al., 2009).

Similarly, when PBMC from HC or CIS patients were stimulated with MOG and MBP no difference in proliferation was seen in response to placebo, 5,000 IU, or 10,000 IU. The autoreactive T cells specific for myelin antigens are said to be involved in the pathogenesis of MS (Bielekova et al., 2004, Ota et al., 1990, Chou et al., 1991). These autoreactive T cells are thought to be high avidity cells that escaped thymic selection. However, even though these myelin specific T cells are found in healthy subjects, studies have shown that they are increased or altered in MS patients (Bielekova et al., 2004, Ota et al., 1990). Although there was no difference in this study between MOG/MBP induced proliferation in HC and CIS patients, this could be an indicator that CIS patients might not have as many myelin specific T cells as MS patients (Chou et al., 1991, Ota et al., 1990, Bielekova et al., 2004). In addition, epitope spreading is thought to occur as the disease progresses. Indeed a study showed that T cells from patients with long term MS recognised more myelin epitopes than patients with early onset such as CIS patients (Davies et al., 2005). Thus, it is possible that the MOG/MBP responses are more dominant in MS than CIS and that this is why we did not observe any differences between HC and CIS patients.

In addition, the investigation of the effect of vitamin D₃ supplementation on antigen specific CD4⁺ T cells was analysed by stimulating PBMC with PPD and TT. However, similar to
MOG and MBP stimulation, there was no change in proliferation in HC or in CIS patients when administered placebo, 5,000 IU, or 10,000 IU of vitamin D$_3$ across time points. The study also demonstrated the effects of vitamin D$_3$ on allo-reactive T cells. Similar to the results described above, PPD/TT induced proliferation was not altered when in CIS patients administered placebo, 5,000 IU or 10,000 IU of vitamin D$_3$. To date, no other supplementation studies have investigated the effect of vitamin D$_3$ supplementation on antigen-specific T cell proliferation.

*IFN$_\gamma$*

When PBMC were stimulated with anti-CD3, the frequency of IFN$_\gamma$+CD4+ T cells from HC or CIS patients did not change when administered placebo over the course of 24 wk. Similarly, no difference was seen in HC or CIS patients that were administered with 5,000 IU or 10,000 IU of vitamin D$_3$. In agreement with our findings, a clinical trial examined the effects of 10,400 IU of vitamin D$_3$ supplementation on IFN$_\gamma$+CD4+ T cells and there was no significant decrease compared to baseline (Sotirchos et al., 2016). In a study by Smolders et al., 2009 the serum 25(OH)D status of RRMS patients was associated with skewing from a IFN$_\gamma$+ producing Th1 cell phenotype to an IL-4 producing Th2 phenotype, suggesting that vitamin D is important in T cell regulation in MS patients, however this was not an intervention study (Smolders et al., 2009b). A study using the active form of vitamin D showed a decrease in IFN$_\gamma$+CD4+ T cells in mice that were treated with 1,25(OH)$_2$D$_3$ (Mahon et al., 2003b). However, as discussed above, administration of the active form of vitamin D versus vitamin D$_3$ may exert different effects *in vivo*.

Similarly, when PBMC from HC or CIS patients were stimulated with MOG and MBP no difference was seen in the frequency of IFN$_\gamma$+CD4+ T cells when administered placebo, 5,000 IU, or 10,000 IU of vitamin D$_3$. A study elsewhere indicated the effects of MOG stimulation of myelin specific T cells showed that T cells in the CSF and blood of MS patients have the ability to produce IFN$_\gamma$ in response to MOG stimulation *in vitro* (Sun et al., 1991), and in agreement with this we did detect measurable IFN$_\gamma$ responses to MOG/MBP. They also showed that MS patients have more MOG reactive T cells compared to controls (Sun et al., 1991). Both studies by Sun et al., 1991 and Olsson et al., showed that there are more MBP reactive T cells in MS patients compared to HC (Olsson et al., 1990, Sun et al., 1991). Interestingly, Sun et al. suggested that if T cells can produce IFN$_\gamma$+ after recognition of MOG and MBP *in vitro*, and can do so *in vivo*, this would be important in the
pathogenesis of MS. However, in this case, vitamin D₃ supplementation did not have an effect on the production of IFNγ⁺CD4⁺ T cells from myelin specific T cells (Sun et al., 1991).

The effect of vitamin D₃ supplementation on PPD/TT specific IFNγ responses was examined, and, similar to MOG and MBP stimulation, there was no change in the frequency of PPD/TT specific IFNγ⁺CD4⁺ T cells in HC or in CIS patients when administered placebo, 5,000 IU, or 10,000 IU of vitamin D₃ across time points. There was no difference in the frequency of IFNγ⁺CD4⁺ T cells in CIS compared to HC subjects when stimulated with recall antigens. This suggests that vitamin D₃ might not have an effect on these antigen specific T cells.

The study also demonstrated the effects of vitamin D₃ on allo-reactive T cell responses. Similarly, to MOG and MBP stimulation, and PPD and TT stimulation, IFNγ from allo-specific T cells was not altered when administered placebo 5,000 IU and 10,000 IU of vitamin D₃. This showed that vitamin D₃ has no effect on IFNγ⁺ production by allo-reactive T cells in CIS patients. However, the effects of vitamin D₃ on allo-reactive T cells were not performed on HC.

In addition, the concentration of IFNγ secreted from PBMC cultures was measured for all stimulations, and there was no significant change when supplemented with 5,000 IU or 10,000 IU of vitamin D₃. This shows that vitamin D₃ did not have an overall effect on IFNγ from PBMC when stimulated with anti-CD3, or PPD and TT, or MOG and MBP, or irradiated APC.

**IL-17**

When PBMC were stimulated with anti-CD3, the frequency of IL-17⁺CD4⁺ T cells from HC or CIS patients did not change when administered placebo, 5,000 IU or 10,000 IU of vitamin D₃. In support of these findings, another study showed no effect of 20,000 IU vitamin D₃ supplementation per day on Th17 cells in MS patients (Smolders et al., 2010). Furthermore, a study that examined the effects of 14,000 IU of vitamin D₃ supplementation on cytokine production by anti-CD3 stimulated PBMC in IFNγ treated RRMS patients found that it had no effect on the production of IL-17 after 48 wk (Muris et al., 2016). They also demonstrated that after 48 wk, the effects of in vitro 1,25(OH)₂D₃ significantly reduced cytokine production by anti-CD3 stimulated PBMC (Muris et al., 2016). This shows that supplementation effects in vivo may not have similar effects to 1,25(OH)₂D₃ in vitro. In contrast however, a trial study by Sotirchos et al., 2016 which examined the effects of 10,400 IU of vitamin D₃ supplementation in RRMS patients, found a reduction in IL-17⁺CD4⁺ T
cells which correlated with the increase of serum 25(OH)D (Sotirchos et al., 2016). However, the patients recruited for this trial were RRMS patients that were on different immunomodulating treatments (Sotirchos et al., 2016). In addition, the PBMC from RRMS patients in the Sotirchos trial stimulated their PBMCs with both anti-CD3 and anti-CD28 for 5 days, this would have given a stronger response in IL-17 production. A study by Correale et al., 2009 showed a reduction in the frequency of Th17 cells when 1,25(OH)$_2$D$_3$ was added directly to activated CD4$^+$ T cells in vitro (Correale et al., 2009). This showed that 1,25(OH)$_2$D$_3$ has a direct effect on Th17 cells in vitro, however, it is not clear whether these in vitro findings will necessarily translate to in vivo supplementation. Similar studies showed that the direct effects of 1,25(OH)$_2$D$_3$ reduced Th17 cells in the CNS in an EAE prevention study by Spanier et al., 2012 and in two EAE treatment studies by Joshi et al., 2011 and Nashold et al., 2013 (Joshi et al., 2011, Nashold et al., 2013, Spanier et al., 2012).

Similarly, when PBMC from HC or CIS patients, were stimulated with MOG and MBP no difference was seen in the frequency of IL-17$^+$CD4$^+$ T cells when administered placebo, 5,000 IU, and 10,000 IU of vitamin D$_3$. A study by Venken et al., 2010 suggested that there is an increased proportion of myelin reactive T cells that have a Th17 phenotype in MS patients (Venken et al., 2010). Another study showed that the amount of IL-17 producing cells in MBP stimulated PBMC, was very low in HC in comparison to MS patients (Durelli et al., 2009). However, this was detected by ELISPOT, whereas the study by Venken et al., 2010 was measuring intracellular FACS analysis and our trial measured intracellular FACS analysis and ELISA, but not ELISPOT (Venken et al., 2010).

The investigation of the effect of vitamin D$_3$ supplementation on IL-17$^+$CD4$^+$ antigen specific T cells was analysed by stimulating PBMC with PPD and TT. However, similar to MOG and MBP stimulation, there was no change in the frequency of IL-17$^+$CD4$^+$ in HC or in CIS patients when administered placebo, 5,000 IU, or 10,000 IU of vitamin D$_3$ across time points. This suggests that vitamin D might not have an effect on antigen specific T cells, as there was no difference in the frequency of IL-17$^+$CD4$^+$ in CIS patients compared to HC subjects when stimulated with recall antigens. A study compared the cytokine production in HC and in RRMS and SPMS, when stimulated with MBP, MOG, or TT (Venken et al., 2010). Interestingly they found that TT reactive memory T cells were IL-17 positive but no difference was seen between control groups and MS patients. However, MOG and MBP reactive T cells were significantly increased in IL-17 production in MS patients in comparison to HC (Venken et al., 2010). Similar to MOG and MBP stimulation and PPD
and TT stimulation, 5,000 or 10,000 IU vitamin D₃ had no effect on proliferation or cytokine production by allo-stimulated T cells.

In addition, the concentration of IL-17 secreted from PBMC cultures was measured for all stimulations, and there was no significant change when supplemented with 5,000 IU or 10,000 IU of vitamin D₃. This shows that vitamin D did not have an overall effect on IL-17 secretion from PBMC cultures when stimulated with anti-CD3, or PPD and TT, or MOG and MBP, or irradiated APC. Vitamin D₃ supplementation had no effect on these stimulations, nor did it have an effect on its production of cytokines from CD4⁺ T cells or from PBMC. One possible explanation for this result is the pilot study used phytohaemagglutinin (PHA) to stimulate T cells for 5 days and vitamin D₃ supplementation reduced the frequency of IL-17⁺CD4⁺ T cells, whereas the trial stimulated T cells with anti-CD3 for only 3 days.

**IL-10**

Similarly, the concentration of IL-10 secreted from PBMC cultures was measured for all stimulations, and there was no significant change when supplemented with 5,000 IU or 10,000 IU of vitamin D₃. This shows that vitamin D did not have an overall effect on IL-10 from PBMC when stimulated with anti-CD3, or PPD and TT, or MOG and MBP, or irradiated APC. Vitamin D₃ supplementation had no effect on these stimulations, nor did it have an effect on its production of cytokines from PBMC. However, a study showed that the treatment of control C57BL/6 mice with 1,25(OH)₂D₃ suppresses the inflammatory and T cell stimulatory capacity of macrophages, but this effect was abrogated in IL-10 deficient mice. Hence the suppressive effects of 1,25(OH)₂D₃ on macrophages appears to be mediated via IL-10 (Korf et al., 2012). Another study showed that 1,25(OH)₂D₃ inhibited EAE in wild type mice, however did not inhibit EAE in mice that had a disrupted IL-10 or IL-10 gene. This showed that the IL-10-IL-10R pathway is essential for the inhibition of EAE in mice (Spach et al., 2006). In addition, a study showed that addition of 1,25(OH)₂D₃ in vitro enhanced the development of IL-10 producing CD4⁺ T cells, whilst reducing IL-17 and IL-6 in PBMC From patients with MS (Correale et al., 2009). Another study demonstrated that MS patients PBMC had secreted less IL-10 compared to those from control groups. However, in patients undergoing IFNβ treatment, IL-10 producing cells were the same as controls (Özenci et al., 2000). Taken together, these studies suggest that the induction of anti-inflammatory IL-10 by vitamin D₃ supplementation could be a mechanism whereby supplementation exerts anti-inflammatory effects in vivo. Indeed, the pilot study prior to this trial suggested that vitamin D₃ supplementation in HC resulted in increased IL-10 production.
via PHA, PPD and DT/TT stimulation by non-T cells (Allen et al., 2012). However, these findings were not replicated here, and the reasons for this are unclear. One possible explanation is that anti-CD3 was used for T cell stimulation whereas PHA was used for the pilot study, this could explain the difference seen with PHA and anti-CD3 stimulation. However, an increase was observed in IL-10 with PPD and TT, and this was not seen in the trial with the same stimulation.

Summary
In summary, this study was a placebo controlled, randomised, double blinded trial; thus, the design was preferable to some other studies that were not adequately controlled. Overall this study did not meet its primary or secondary endpoints, and clinical results for secondary endpoints were not given as it would have been of interest to correlate the immunological and clinical outcomes. Since there was no effect observed on both primary and secondary endpoints, the evidence from several studies suggests that the impact of vitamin D3 supplementation in vivo is not yet clear. Our study indicated that vitamin D3 supplementation did not exert anti-inflammatory effects on T cells in either HC or CIS patients. One possible reason is the vitamin D3 supplementation in the pilot study used Vigantol Tablets®, whereas the trial used vitamin D3 Vigantol Oil®, this could have impacted the conversion of vitamin D3 to its active form, however both were from the same supplier. Another confounding factor was that the pilot study used PHA to stimulate T cells and showed that vitamin D supplementation inhibited the frequency of CD4+IL-17+ T cells while the trial used anti-CD3 and no effect was seen, this difference in stimulation might have impacted the effects on inflammatory cytokines with vitamin D3 supplementation. It is possible that vitamin D3 supplementation does not in fact exert effects on the T cell responses that were measured in this study. Alternatively, the readouts used in this trial may not have been the most appropriate, although these were selected on the basis of the pilot study (Allen et al., 2012). It is possible that a longer trial duration or higher supplementation in larger groups including MS patients and CIS patients may have been needed to demonstrate an immunological effect of vitamin D3 supplementation. Also, Correale et al., 2009 suggests that 25(OH)D levels are lower in MS patients than HC during relapses of the disease. The vitamin D serum level could be a predictor for a relapse in MS patients, and monitoring serum 25(OH)D levels could be used to prevent future relapses (Correale et al., 2009). In addition, the treatment of naïve CIS patients may have had different outcomes compared with other trials where MS patients were being treated with disease modifying therapies, since the effects of modifying treatments could have a positive effect on the endpoints of the trial.
Vitamin D in its active form potentially has many benefits in autoimmune disease. However, the potential role of vitamin D still needs to be clarified, as its metabolism, particularly in patients with inflammatory disease is not fully understood. It is still not fully clear; how much vitamin D$_3$ supplementation is required to exert immunomodulatory effects. It is possible that vitamin D$_3$ supplementation over a much longer period could be of value in preventing the conversion of CIS to clinically definite MS, however the duration of our study was insufficient to determine this and additional studies would be required in future.
Chapter 4:

The immunomodulatory effects of DMF \textit{in vitro}
and \textit{in vivo}
4.1 Introduction

DMF is a derivative of fumaric acid which has many immunomodulatory effects. DMF is the active ingredient in the drug Fumaderm™ and it has been used in Germany since 1994 for the treatment of psoriasis (Altmeyer et al., 1994). It was hypothesised by the German chemist Schweckendiek, who was himself affected by psoriasis, that the disease was caused by a deficiency in the citric acid cycle. He used fumaric acid esters to replenish fumaric acid deficiency (Schweckendiek, 1959), however this theory of a deficiency in the citric acid cycle was never proven. Nonetheless, DMF was found to be clinically effective for the treatment of psoriasis and more recently also for RRMS. DMF is an electrophile and cysteine modifier that has been shown to have a range of anti-inflammatory and immunomodulatory effects in vitro (Scannevin et al., 2012, Blewett et al., 2016). However, the precise mechanism of action of Fumaderm™ in psoriasis patients is unknown. Six mechanisms of action have been described thus far, first, it was widely thought that the mechanism of action of DMF was via activation of the Nrf2 pathway (Gold et al., 2012, Linker et al., 2011, Scannevin et al., 2012). Second, DMF has been shown to inhibit NF-κB in an Nrf2 independent manner (Gillard et al., 2015, Schulze-Topphoff et al., 2016, Bista et al., 2012). Third, due to the nature of DMF as an alpha beta unsaturated carboxylic acid ester it can react with thiol groups and also modifies glutathione (Dibbert et al., 2013, Mrowietz and Asadullah, 2005, Lehmann et al., 2007, Scannevin et al., 2012). Fourth, DMF is an agonist for the HCA2, thereby inhibiting NF-κB (Chen et al., 2014, Offermanns and Schwaninger, 2015). Fifth, it was demonstrated that DMF succinates and inactivates the enzyme GAPDH and thereby inhibits glycolysis (Kornberg et al., 2018). Finally, DMF can block the formation of M1 and K63 poly-ubiquitin chains downstream of TLR signalling, and also inhibit E2 enzymes UBC13 and UbcH7 that are involved in the formation of both K63 and M1 chains (McGuire et al., 2016). The same study also demonstrated that DMF can inhibit the transcription of cytokines independently of Nrf2. Although there are many published studies of the mechanism of action of DMF, the majority are not specific to T cells.

It’s widely known that T cells play a key role in the pathogenesis of psoriasis, however, IL-23, IL-12 and TNFα producing mDC play a major role in instructing the differentiation of Th1 and Th17 cells that are involved in the disease (Lowes et al., 2014). DMF was capable of inhibiting maturation markers CD80, CD86, and CD83 in slanDC and inhibited the expression of MHC II, CD86 and CD80 in BMDC, this immature phenotype led to less activation of T cells (Peng et al., 2012, Oehrl et al., 2017). DMF not only could inhibit the expression of maturation markers on DC, but was capable of inhibiting the inflammatory
cytokines IL-12 and IL-23 that promoted in the differentiation of pathogenic Th1 and Th17 cells respectively. In both mice and humans, DMF induced IL-4 producing Th2 cells and induced type II DC, DMF also inhibited the capacity of DC to produce IL-12 or IL-23 with a slight increase in IL-10 (Ghoreschi et al., 2011). These studies suggest that DMF can inhibit the differentiation of Th1 and Th17 cells indirectly by preventing the activation and maturation of DC. DMF can also exert direct effects on T cells, such as inducing apoptosis in CD4 and CD8 T cells \textit{in vitro} (Treumer et al., 2003a), and it was demonstrated in MS patients that memory T cells were more susceptible than naïve T cells to apoptosis when patients were treated with DMF (Ghadiri et al., 2017). Many studies have shown an increased frequency of Th1 and Th17 cells in lesional skin of psoriasis patients (Annunziato et al., 2009, Kryczek et al., 2008, Lowes et al., 2008, Kagami, 2011). It has also been demonstrated that DMF can modulate T cell proliferation and cytokine production \textit{in vitro} (Tahvili et al., 2015, Ockenfels et al., 1998, Lehmann et al., 2007, Diebold et al., 2017, Gross et al., 2016, Wu et al., 2017), and \textit{in vivo} in RRMS patients treated with DMF (Gross, Schulte-Mecklenbeck et al. 2016, Diebold, Sievers et al. 2017, Wu, Wang et al. 2017).

Treg cells are important immunosuppressive cells that are critical for the prevention of autoimmunity. However, it is not yet fully understood how DMF affects Treg cells in psoriasis patients, and to date all of the studies on the effect of DMF on Treg cells \textit{in vivo} are in MS patients. A study demonstrated that the proportion of Treg cells in RRMS patients increased after treatment with DMF \textit{in vivo} (Gross et al., 2016). In contrast, other studies in RRMS patients showed that DMF did not affect Treg cells but reduced Th1 and Th17 cells (Wu et al., 2017, Longbrake et al., 2016). Treg cells from HC also seemed to be more resistant to apoptosis when cultured with DMF \textit{in vitro} compared with Tconv cells (Ghadiri et al., 2017). In the same study the effects of DMF \textit{in vivo} in RRMS patients was investigated and showed that there was a relative reduction in effector T cells expressing proinflammatory cytokines compared with Treg cells that were unchanged \textit{in vivo}.

It has been shown that Treg cells are more resilient to oxidative stress induced by H$_2$O$_2$ \textit{in vitro} compared with Tconv cells (Mougiakakos et al., 2009). The same group also demonstrated that Treg cells express and secrete higher levels of thioredoxin-1, which is an enzyme that counteracts oxidative stress by scavenging for ROS and can also prevent apoptosis (Mougiakakos et al., 2011, Holmgren and Lu, 2010). Thioredoxin-1 and glutathione are important in sustaining and maintaining the expression of reduced surface thiols which help to protect cells from oxidative stress (Sahaf et al., 2003, Mougiakakos et al., 2009, Thoren et al., 2007a, Thoren et al., 2007b). Thus, it is possible that the increased
frequency in Treg cells that was observed in MS patients treated with DMF could be due to the ability of Treg cells to counteract oxidative stress induced by DMF more efficiently than Tconv cells.

There are many studies and trials on the clinical effects of DMF on MS patients. In contrast there are limited number of studies and trials on DMF in psoriasis patients as a result of the ad hoc way which the drug was originally introduced in Germany, however a recent phase III randomised placebo-controlled trial confirmed that monotherapy with DMF is clinically effective in psoriasis and that the safety of the new formulation of DMF (LAS41008) monotherapy is equivalent to that of Fumaderm™ (Mrowietz et al., 2017). The current mechanism of action of DMF in psoriasis patients is unclear and investigation into the effects of DMF on T cell subsets in vitro and in vivo would provide important insights into the immunomodulatory effects of DMF.

### 4.1.1 Aims and hypothesis

**Hypothesis:**

The anti-inflammatory effects of DMF in vitro and in vivo results in part from differential effects on T cell subsets. The oxidative stress induced by DMF may provide a relative advantage to Treg cells which have a greater capacity to counteract oxidative stress.

The experiments in this chapter aimed to determine the immunomodulatory effects of DMF in vitro and in vivo in psoriasis patients. The specific aims were as follows:

- To examine the effects of DMF on LPS-induced DC maturation markers and cytokines.
- To investigate the ability of DMF to modulate T cell survival, proliferation, cytokine production and the frequency of Treg cells within PBMC and sorted CD3+ T cells.
- To examine the frequency of Th cell subsets, Treg cells and T cell cytokine production in psoriasis patients that were either untreated or treated with Fumaderm™.
4.2 Results

4.2.1 The Immunomodulatory effects of DMF on DC

DC play a key role in the immune response where they integrate the innate and adaptive responses. Activation of DC via their PRR results in upregulation of co-stimulatory and maturation markers that provide co-stimulatory signals, which together with TCR activation, are required for the full activation of T cells. In addition, activated DC secrete cytokines that drive the differentiation of naïve T cells into specific subsets. The DC cytokine profile and subsequent T cell response depends on the PRRs that were activated. Given the importance of DC in orchestrating T cell responses, the effect of DMF on the activation and cytokine production by DC was examined in monocyte derived DC.

4.2.1.1 DMF does not exert toxic effects on DC

Before examining the effects of DMF on DC phenotype and function, it was necessary to determine whether DMF was toxic to DC. Monocytes were isolated from healthy donor PBMC by positive selection using MACS CD14 magnetic beads as described in section 2.8 and the purity was analysed by flow cytometry as shown in Fig 2.1 and cultured in the presence of GM-CSF and IL-4 for 6 days to generate immature DC as described in section 2.9 and the purity of immature DC were analysed by flow cytometry as shown in Fig 2.2. Immature DC were pre-treated with DMF (0, 25, 50, 100 µM) or curcumin (10 µM) for 6 hr and then left unstimulated or stimulated with TLR4 agonist LPS. Curcumin has previously been shown to inhibit DC maturation and cytokine production and was therefore used as an experimental control (Campbell et al., 2018). After 24 hr cells were harvested and stained with viability dye and analysed by flow cytometry. Fig 4.1A, shows a representative dot plot of FSC vs SSC staining in DC, where the gate indicates the percentage of DC. These cells were further assessed for viability of DC (Fig 4.1B). The effect of DMF or curcumin on the frequency of viable immature or LPS-matured DC is shown in Fig 4.1C. There were no statistical differences in the frequency of viable immature or mature DC after treatment with DMF or curcumin, however there was a slight but non-significant reduction in both immature and LPS-matured DC when treated with 100 µM of DMF (Fig 4.1C). In summary, neither DMF nor curcumin at the doses tested exerted any significant toxic effect on human monocyte derived immature or mature DC.
Figure 4.1. The effect of DMF and curcumin on the viability of DC

Monocyte derived DC generated from healthy donors were pre-treated with DMF (0, 25, 50, 100 µM) or curcumin (10 µM) for 6 hr and then left unstimulated or stimulated with LPS (100 ng/ml). After 24 hr cells were harvested and stained with viability dye and analysed by flow cytometry. Cells were initially gated on the basis of FSC vs SSC to exclude debris (A) and then the percentage of viable cells was determined as shown in representative dot plots (B). The effect of DMF or curcumin on the frequency of viable immature or LPS-matured DC is shown (n=7) (C). Statistical differences were determined by one-way ANOVA with Tukey’s multiple comparison test. Data expressed as mean (+/- SEM).
4.2.1.2 The effect of DMF on the expression of co-stimulatory and maturation markers by DC

Having established that DMF at a range of concentrations was not toxic to DC, the effects of DMF on DC phenotype and function was examined next. The aim of initial experiments was to determine the effect of DMF on the expression of CD80, CD83 and CD86 on unstimulated or LPS matured DC. CD80 and CD86 provide the essential signal 2 that is required to provide co-stimulation for full T cell activation and CD83 is a maturation marker for DC. Curcumin has previously been shown to inhibit DC maturation (Campbell et al., 2018) and was therefore included as a positive control. Immature monocyte-derived DC were pre-treated with DMF (0, 25, 50, 100 µM) or curcumin (10 µM) for 6 hr and then left unstimulated or stimulated with LPS. After 24 hr cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD80, CD83, CD86 and viability dye and analysed by flow cytometry.

Representative histograms show the effect of DMF on CD80 MFI in immature (Fig 4.2A) or LPS-matured (Fig. 4.2B) DC. Figure 4.2C shows the mean (+/- SEM) of CD80 MFI, expressed as a percentage of the LPS stimulated control for immature or LPS-matured DC treated with different doses of DMF (n=7). Although there was a trend towards a dose dependent increase of CD80 expression on both immature and mature DC with DMF treatment, the differences were not significant. Figure 4.2 D shows representative histograms of the expression of CD80 in unstimulated DC, or LPS stimulated DC pre-treated with curcumin. In contrast to the results for DMF, curcumin inhibited the expression of CD80 as shown previously (Campbell et al., 2018), although the effect was not significant (Fig. 4.2E).

Representative histograms show the effect of DMF on CD86 MFI in immature (Fig 4.3A) or LPS-matured (Fig 4.3B) DC. Figure 4.3C shows the mean (+/- SEM) of CD86 MFI, expressed as a percentage of the LPS stimulated control for immature or LPS-matured DC treated with different doses of DMF (n=7). In Figure 4.3C the mean (+/- SEM) of CD86 MFI increased significantly when pre-treated with 100 µM of DMF compared with the LPS stimulated control (p≤0.01). Figure 4.3D shows representative histograms of the expression of CD86 in unstimulated DC, or LPS stimulated DC pre-treated with curcumin. In contrast to the results for DMF, curcumin significantly inhibited the expression of CD86 (p≤0.01) (Fig 4.3E).

Representative histograms show the effect of DMF on CD83 MFI in immature (Fig 4.4A) or LPS-matured (Fig 4.4B) DC. Figure 4.4C shows the mean (+/- SEM) of CD83 MFI, expressed as a percentage of the LPS stimulated control for immature or LPS-matured DC
treated with different doses of DMF (n=7). No significant differences in the MFI of CD83 were observed between different concentrations of DMF when left unstimulated or stimulated with LPS. Figure 4.4D shows representative histogram of the expression of CD83 in unstimulated DC, or LPS stimulated DC pre-treated with curcumin. In contrast to the results for DMF, curcumin significantly inhibited the expression of CD83 (p≤0.01) (Fig 4.4E).

In summary, 100 µM DMF significantly increased the expression of CD86, but did not significantly alter the expression of CD80 or CD83 on DC. Curcumin, which was used as a positive control, significantly decreased the expression of CD86 and CD83, with a trend towards decreased expression of CD80, in agreement with published data (Campbell et al., 2018).
Figure 4.2 The effect of DMF and curcumin on the expression of CD80 by DC
Monocyte derived DC generated from healthy donors were pre-treated with DMF (0, 25, 50, 100 µM) or curcumin (10 µM) for 6 hr and then left unstimulated or stimulated with LPS (100 ng/ml). After 24 hr cells were harvested and stained with fluorochrome-conjugated antibody specific for CD80 and a viability dye and analysed by flow cytometry. The representative histograms show expression of CD80 in immature DC (A) and in LPS matured DC pre-treated with DMF (B). The graph shows the mean (+/- SEM) of CD80 MFI, expressed as a percentage of the LPS stimulated control (n=7) (C). The representative histogram shows expression of CD80 in immature DC, or LPS-matured DC pre-treated with curcumin (D). The graph shows MFI values for CD80 in curcumin treated DC, expressed as a percentage of the LPS stimulated control (n=7) (E). Statistical differences between LPS matured DC that were untreated vs those treated with different concentrations of DMF or curcumin were determined by one-way ANOVA with Tukey’s multiple comparison test.
Figure 4.3 The effect of DMF and curcumin on the expression of CD86 by DC
Monocyte derived DC generated from healthy donors were pre-treated with DMF (0, 25, 50, 100 μM) or curcumin (10 μM) for 6 hr and then left unstimulated or stimulated with LPS (100 ng/ml). After 24 hr cells were harvested and stained with fluorochrome-conjugated antibody specific for CD86 and a viability dye and analysed by flow cytometry. The representative histograms show expression of CD86 in DMF treated immature DC (A) and in LPS matured DC pre-treated with DMF (B). The graph shows the mean (+/− SEM) of CD86 MFI, expressed as a percentage of the LPS stimulated control (n=7) (C). The representative histogram shows expression of CD86 in immature DC, or LPS-matured DC pre-treated with curcumin (D). The graph shows MFI values for CD86 in curcumin treated DC, expressed as a percentage of the LPS stimulated control (n=7) (E). Statistical differences between LPS matured DC that were untreated vs those treated with different concentrations of DMF or curcumin were determined by one-way ANOVA with Tukey’s multiple comparison test: **p≤0.01.
Figure 4.4 The effect of DMF and curcumin on the expression of CD83 by DC
Monocyte derived DC generated from healthy donors were pre-treated with DMF (0, 25, 50, 100 µM) or curcumin (10 µM) for 6 hr and then left unstimulated or stimulated with LPS (100 ng/ml). After 24 hr cells were harvested and stained with fluorochrome-conjugated antibody specific for CD83 and a viability dye and analysed by flow cytometry. The representative histograms show expression of CD83 in DMF treated immature DC (A) and in LPS matured DC pre-treated with DMF (B). The graph shows the mean (+/- SEM) of CD83 MFI, expressed as a percentage of the LPS stimulated control (n=7) (C). The representative histogram shows expression of CD83 in immature DC, or LPS-matured DC pre-treated with curcumin (D). The graph shows MFI values for CD83 in curcumin treated DC, expressed as a percentage of the LPS stimulated control (n=7) (E). Statistical differences between LPS matured DC that were untreated vs those treated with different concentrations of DMF or curcumin were determined by one-way ANOVA with Tukey’s multiple comparison test; **p≤0.01.
4.2.1.3 The effect of DMF on cytokine production by DC

Cytokines produced by activated DC play a key role in polarising T cell responses, therefore the effect of DMF on DC cytokine production was determined next. Monocyte derived immature DC were pre-treated with DMF (0, 25, 50, 100 µM) or curcumin (10 µM) for 6 hr and then left unstimulated or stimulated with TLR4 agonist LPS. After 24 hr supernatants were harvested and the concentrations of IL-12p70 (Fig 4.5A), IL-23 (Fig 4.5B), TNFα (Fig 4.5C) and IL-10 (Fig 4.5D) were determined by ELISA. In contrast to immature DC which produced no detectable cytokines, all of the cytokines were detectable in the supernatants in the supernatants from LPS stimulated DC (Fig. 4.5A-D). However, pre-treatment of DC with DMF exerted no significant differences on the levels of cytokines secreted by either unstimulated or LPS-stimulated DC (Fig. 4.5A-D). Figure 4.6 shows the effect of curcumin on cytokine secretion by DC. Pre-treatment with curcumin did not exert any significant changes in the concentrations of IL-23 (Fig 4.6B), TNFα (Fig 4.6C) and IL-10 (Fig 4.6D) secreted by LPS stimulated DC. However, there was a trend towards a decrease in IL-23 and a significant decrease in IL-12p70, in agreement with published findings (Campbell et al., 2018) (Fig 4.6A) (p<0.05).

In summary, DMF did not alter the LPS-induced cytokine production by DC. However, curcumin inhibited IL-12p70 production by DC in response to LPS stimulation as expected.
Figure 4.5 The effect of DMF on the production of cytokines by DC

Monocyte derived DC generated from healthy donors (n=6) were pre-treated with DMF (0, 25, 50, 100 µM) for 6 hr and then left unstimulated or stimulated with LPS (100 ng/ml). After 24 hr supernatants were harvested and the concentrations of IL-12p70 (A), IL-23 (B), TNFα (C) and IL-10 (D) determined by ELISA. Statistical differences between LPS matured DC that were untreated vs those treated with vs different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test. Data expressed as mean (+/- SEM).
Figure 4.6 The effect of curcumin on the production of cytokines by DC
Monocyte derived DC generated from healthy donors (n=6) were pre-treated with curcumin (10µM) for 6 hr and then left unstimulated or stimulated with LPS (100 ng/ml). After 24 hr supernatants were harvested and the concentrations of IL-12p70 (A), IL-23 (B), TNFα (C) and IL-10 (D) determined by ELISA. Statistical differences between LPS stimulated cells that were untreated vs those treated with curcumin were determined by one-way ANOVA with Tukey’s multiple comparison test; *p≤0.05. Data expressed as mean (+/- SEM).
4.2.2 The immunomodulatory effects of DMF on T cells

Having demonstrated the effects of DMF on DC, its immunomodulatory effects on PBMC were examined next. The aim of these experiments was to investigate the ability of DMF to modulate T cell survival, proliferation, cytokine production and the frequency of Treg cells within PBMC.

4.2.2.1 Effect of DMF on the viability of PBMC

DMF is known to induce T cell death, therefore initially it was important to determine the effect of DMF on the viability of PBMC at the doses used in this study. PBMC were isolated from healthy donors and left unstimulated or stimulated with anti-CD3 in the absence or presence of different concentrations of DMF (5, 10, 25, 50 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr for the purpose of subsequent cytokine analysis. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, and viability dye and analysed by flow cytometry. Cells were gated as shown in Fig 4.7. Representative dot plots show the viability of stimulated total PBMC, or gated CD4⁺ and CD8⁺ T cells that were untreated (Fig. 4.8A) or treated with 25 µM DMF (Fig. 4.8B), where the gate indicates the percentage of live cells. The viability of stimulated PBMC, CD4⁺ and CD8⁺ T cells that were untreated or treated with DMF is shown in Fig 4.8C (n=4). There was a dose dependent decrease in the frequency of viable cells within PBMC, CD4⁺ T cells with increasing DMF concentrations (5, 10, 25, 50 µM), however this was not significant. There was a significant decrease in the frequency of viable cells within CD8⁺ T cells at 50 µM DMF.
Figure 4.7 Flow cytometry gating strategy to identify the viability of PBMC, CD4 and CD8 T cells

PBMC were isolated, stimulated with anti-CD3 and cultured for 5 days. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8 and viability dye and analysed by flow cytometry. Cells were initially gated to exclude debris and the population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). Viable total cells were gated from the singlet population on the basis of fixable viability dye and FSC-A (C). Within the singlet population, CD4+ T cells were gated on the basis of CD3+CD4+ and CD8+ T cells were gated on the basis of CD3+CD4+ (D). Viable CD4+ (E) and CD8+ (F) T cells were gated on the basis of fixable viability dye and FSC-A.
Figure 4.8 The effect of DMF on the viability of T cells within PBMC

PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (5, 10, 25, 50 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, and viability dye and analysed by flow cytometry. CD4+ and CD8+ T cells were gated as shown in Fig 4.7 for cells that were untreated (0 µM DMF) (A) or treated with 25 µM DMF (B) as shown in the representative dot plots. The graphs show the effect of DMF on the viability of stimulated PBMC, CD4+ and CD8+ T cells (C) (n=4). Statistical differences between cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; *p≤0.05.
4.2.2.2 The effect of DMF on T cell proliferation

The effect of DMF on the proliferation of T cells within PBMC was examined next. PBMC were isolated from healthy donors and labelled with cell tracer dye CTV in order to assess proliferation and either left unstimulated or stimulated with anti-CD3 in the presence of different concentrations of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr for simultaneous cytokine analysis. Cells were then stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD8, intranuclear Ki67 as an alternative method to assess proliferation and viability dye and analysed by flow cytometry. For flow cytometric analysis CD4$^+$ and CD8$^+$ T cells were defined according to the gating strategy outlined in Fig 4.9. Fig 4.10A shows a representative histogram of CTV dilution in CD4$^+$ T cells, where proliferated cells are gated as CTV$^{lo}$. Unstimulated cells exhibited virtually no proliferation, whereas 82.1% of CD4$^+$ T cells stimulated with anti-CD3 had proliferated and this was reduced to 56.3% in the presence of 25 µM DMF. Overall, there was no significant decrease in the proliferation of CD4$^+$ T cells when treated with DMF, although there was a dose dependent trend towards a decrease with higher concentrations (Fig 4.10B). Fig 4.10C, shows representative dot plots of CD4 vs Ki67 where the gates indicate the percentage of cycling Ki67$^+$ cells. Unstimulated cells exhibited virtually no proliferation, whereas 95.9% of CD4$^+$ T cells stimulated with anti-CD3 were proliferating and this was reduced to 70.1% in the presence of 25 µM DMF. There was a significant decrease in the frequency of proliferating CD4$^+$Ki67$^+$ cells when treated with DMF at 25 µM (Fig. 4.10D).

Fig 4.11A, shows a representative histograms of CTV dilution in gated CD8$^+$ T cells within PBMC. Unstimulated cells exhibited no proliferation, whereas 84.8% of CD8$^+$ T cells stimulated with anti-CD3 proliferated and this was reduced to 55.6% in the presence of 25 µM DMF. Overall there was no significant decrease in the proliferation of CD8$^+$ T cells when treated with DMF, however there was a dose dependant trend towards a decrease with higher concentrations of DMF as shown in Fig 4.11B. A representative dot plot of CD3 vs Ki67 is shown where the gate indicates the frequency of cycling Ki67$^+$ cells (Fig 4.11C). Unstimulated cells exhibited no proliferation, whereas 91.8% of CD8$^+$ T cells stimulated with anti-CD3 were proliferating and this was inhibited to 68.2% in the presence of 25 µM DMF. Overall, there was no significant decrease in the proliferation of CD8$^+$ T cells when treated with DMF, however there was a dose dependent trend towards a decrease with higher concentrations (Fig 4.11D).
In summary, the proliferation, as measured by CTV dilution or Ki67 expression, of both CD4 and CD8 T cells within PBMC was somewhat inhibited by DMF in a dose dependent manner, with varying effects in different donors.
Figure 4.9 Flow cytometry gating strategy to identify CD4 and CD8 T cells after stimulation with PMA and ionomycin

PBMC were isolated, stimulated with anti-CD3 and cultured for 5 days. Cells were re-stimulated with PMA and ionomycin, stained with fluorochrome-conjugated antibodies specific for CD3 and CD8 and analysed by flow cytometry. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). Viable cells were gated from the singlet population on the basis of fixable viability dye and FSC-A (C). Finally, CD4+ T cells were gated on the basis of CD8−CD3+, since CD4 was downregulated upon PMA/ionomycin stimulation and CD8+ T cells were gated on the basis of CD8−CD3+ (D).
Figure 4.10 The effect of DMF on anti-CD3 induced proliferation of CD4+ T cells within PBMC

PBMC were isolated from HC and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the presence of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD8, intranuclear Ki67 and viability dye and analysed by flow cytometry. For the flow cytometric analysis CD4+ T cells were gated as shown in Fig 4.9. The percentage of proliferated CD4+ T cells was measured by the dilution of CTV (CTVlo), as shown in the representative histograms for unstimulated, anti-CD3 stimulated and anti-CD3 stimulated CD4+ T cells in the presence of 25 µM DMF (A). The effect of DMF on the proliferation of anti-CD3 stimulated CD4+ T cells is shown in (B) (n=4). Proliferation was also measured by gating on actively cycling Ki67+ cells as shown in the representative dot plots for unstimulated, anti-CD3 stimulated and anti-CD3 stimulated CD4+ T cells in the presence of 25 µM DMF (C). The effect of DMF on the frequency of proliferating Ki67+ CD4+ T cells is shown in (D) (n=4). Statistical differences between anti-CD3 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; *p≤0.05.
Figure 4.11 The effect of DMF on anti-CD3 induced proliferation of CD8\(^+\) T cells within PBMC

PBMC were isolated from HC and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the presence of DMF (5, 10, 25 \(\mu\)M). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD8, intranuclear Ki67 and viability dye and analysed by flow cytometry. For the flow cytometric analysis CD8\(^+\) T cells were gated as shown in Fig 4.9. The percentage of proliferated CD8\(^+\) T cells was measured by the dilution of CTV (CTV\(^{lo}\)), as shown in the representative histograms for unstimulated, anti-CD3 stimulated and anti-CD3 stimulated CD8\(^+\) T cells in the presence of 25 \(\mu\)M DMF (A). The effect of DMF on the proliferation anti-CD3 stimulated CD8\(^+\) T cells is shown in (B) (n=4). Proliferation was also measured by gating on actively cycling CD8\(^+\)Ki67\(^+\) cells as shown in the representative dot plots for unstimulated, anti-CD3 stimulated and anti-CD3 stimulated CD8\(^+\) T cells in the presence of 25 \(\mu\)M DMF (C). The effect of DMF on the frequency of proliferating Ki67\(^+\)CD8\(^+\) T cells is shown in (D) (n=4). Statistical differences between anti-CD3 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
4.2.2.3 The effect of DMF on the production of cytokines by T cells within PBMC

The effect of DMF on the production of cytokines by T cells within PBMC was examined next. PBMC were isolated from healthy donors and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the absence or presence of different concentrations of DMF (5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/-ionomycin for 5 hr and stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFNg, GM-CSF, TNFα, IL-2 and viability dye, and analysed by flow cytometry. The population of CD4+ and CD8+ T cells within PBMC were gated as shown in Fig 4.9, and then the frequency of cytokine producing cells was determined as shown in Fig 4.12. The effect of DMF on the frequency of cytokine producing cells within CD4+ T cells is shown in Fig 4.13. There was no change in the frequency of CD4+IL-17+ T cells as a result of DMF treatment (Fig. 4.13A). However, treatment of PBMC with 5, 10 and 25 µM DMF resulted in a significant decrease in IFNg+ CD4+ T cells compared with the control (p ≤ 0.05) (Fig 4.13B). DMF did not have any significant effect on the frequency of GM-CSF (Fig 4.13C), IL-2 (Fig 4.13D) and TNFα (Fig 4.13E) producing CD4+ T cells.

The effect of DMF on the frequency of cytokine producing cells within CD8+ T cells is shown in Fig 4.14. There was no significant change in the frequency of CD8+ IL-17+ (Fig 4.14A), CD8+GM-CSF+ (Fig 4.14C) or CD8+TNFα+ (Fig 4.14E) T cells. However, there was a non-significant trend towards a decrease in CD8+ IFNg+ T cells when treated with DMF (Fig 4.14B). There was a non-significant trend towards an increase in the frequency of CD8+IL-2+ T cells after DMF treatment compared with anti-CD3 stimulated control (Fig 4.14D).

In summary, DMF exerted varied effects on cytokine production by CD4+ and CD8+ T cells within PBMC, with no effects on IL-17, GM-CSF, IL-2 and TNFα but inhibition of IFNg.
Figure 4.12 Flow cytometry gating strategy to identify cytokine production by total or proliferated T cells using CTV
PBMC were isolated from HC, labelled with cell tracer dye CTV and stimulated with anti-CD3 for 5 days. Cells were analysed by flow cytometry and the proliferation of CD4$^+$ or CD8$^+$ T cells was determined by the dilution of CTV. The representative dot plot (gated for example on CD4$^+$ T cells as shown in Fig 4.9) shows the proliferation of CD4$^+$ T cells vs IL-2, where the gate indicates the frequency of IL-2 producing cells within the total CD4 T cell population (A). Alternatively, cells that had proliferated (CTV$^{lo}$) were gated as shown in the representative histogram (B), and then the frequency of proliferated cells producing cytokine was identified as shown in (C) A similar gating strategy was used for CD8$^+$ T cells and other cytokines.
Figure 4.13 The effect of DMF on cytokine production by CD4$^+$ T cells within PBMC

PBMC were isolated from HC and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFN$\gamma$, GM-CSF, IL-2, TNF$\alpha$ and viability dye and analysed by flow cytometry. For the flow cytometric analysis, cytokine producing CD4$^+$ T cells were identified as shown in Fig 4.12. The graphs show the effect of DMF on the frequency of cytokine producing CD4$^+$ T cells in response to anti-CD3; IL-17 (A), IFN$\gamma$ (B), GM-CSF (C), IL-2 (D), TNF$\alpha$ (E) (n=4). Statistical differences between anti-CD3 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; *p≤0.05, **p≤0.01.
Figure 4.14 The effect of DMF on cytokine production by CD8$^+$ T cells within PBMC

PBMC were isolated from HC and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFN$\gamma$, GM-CSF, IL-2, TNF$\alpha$ and viability dye and analysed by flow cytometry. For the flow cytometric analysis, cytokine producing CD8$^+$ T cells were identified as shown in Fig 4.12. The graphs show the effect of DMF on the frequency of cytokine producing CD4$^+$ T cells in response to anti-CD3; IL-17 (A), IFN$\gamma$ (B), GM-CSF (C), IL-2 (D), TNF$\alpha$ (E) (n=4). Statistical differences between anti-CD3 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
4.2.2.4 The effect of DMF on the production of cytokines by proliferated T cells within PBMC

Since the majority of cytokines are produced by activated and proliferating cells, the reduced viability and inhibition of proliferation by DMF observed above was likely to impact on the frequency of cytokine producing cells in a non-specific manner. Therefore, in order to identify any specific effects of DMF on cytokine production that were distinct from its global suppressive activities, the effect of DMF on cytokine production by cells that had proliferated in response to anti-CD3 was assessed next. The population of CD4+ and CD8+ T cells (gated as shown in Fig 4.9) within PBMC that had proliferated (CTVlo) in response to anti-CD3 stimulation was first gated and then the frequency of cytokine producing cells within this gate was determined as shown in Fig. 4.12. The effect of DMF on the frequency of cytokine producing cells within proliferated CD4+ T cells is shown in Fig 4.15. There was no change in the frequency of proliferated CD4+IL-17+ T cells as a result of DMF treatment (Fig. 4.15A). However, treatment of PBMC with 10 µM DMF resulted in a significant decrease in proliferated IFNγ+ CD4+ T cells compared with the control (p≤0.05) (Fig 4.15B). DMF did not have any significant effect on the frequency of GM-CSF+ CD4+ T cells (Fig 4.15C), however there was a significant increase in the frequency of proliferated IL-2+ CD4+ T cells with 10 µM (p≤0.01) (Fig 4.15D) and 25 µM (p≤0.05) of DMF compared with the control. DMF had no significant effect on the frequency of proliferated TNFα+ CD4+ T cells (Fig 4.15E).

The effect of DMF on the frequency of cytokine producing cells within proliferated CD8+ T cells is shown in Fig 4.16. There was no significant change in the frequency of proliferated CD8+ IL-17 (Fig 4.16A), CD8+ IFNγ+ (Fig 4.16B) or CD8+GM-CSF+ (Fig 4.16C) T cells that were treated with DMF compared with the anti-CD3 stimulated control, however, there was a dose dependant increase in proliferated CD8+ IL-2+ T cells that were treated with DMF compared with anti-CD3 stimulated control, however this was not significant (Fig 4.16D). There was no significant change in proliferated CD8+TNFα+ T cells treated with different concentrations of DMF when compared with anti-CD3 stimulated control (Fig 4.16E).

In summary, DMF exerted variable effects on cytokine production by proliferated CD4+ and CD8+ T cells within PBMC, with no significant effects on IL-17, GM-CSF and TNFα but significant inhibition of CD4+IFNγ+ cells and a significant increase in the frequency of CD4+IL-2+ cells.
Figure 4.15 The effect of DMF on cytokine production by proliferated CD4+ T cells within PBMC

PBMC were isolated from HC and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFNγ, GM-CSF, IL-2, TNFα and viability dye and analysed by flow cytometry. For the flow cytometric analysis, cytokine producing proliferated CD4+ T cells were identified as shown in Fig 4.12. The graphs show the effect of DMF on the frequency of cytokine producing CD4+ T cells that had proliferated (CTVlo) in response to anti-CD3; IL-17 (A), IFNγ (B), GM-CSF (C), IL-2 (D), TNFα (E) (n=4). Statistical differences between anti-CD3 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; *p≤0.05, **p≤0.01.
Figure 4.16 The effect of DMF on cytokine production by proliferated CD8+ T cells within PBMC

PBMC were isolated from HC, and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFNγ, GM-CSF, IL-2, TNFα and viability dye and analysed by flow cytometry. For the flow cytometric analysis, cytokine producing proliferated CD8+ T cells were identified as shown in Fig 4.12. The graphs show the effect of DMF on the frequency of cytokine producing CD4+ T cells that had proliferated (CTVlo) in response to anti-CD3; IL-17 (A), IFNγ (B), GM-CSF (C), IL-2 (D), TNFα (E) (n=4). Statistical differences between anti-CD3 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
4.2.2.5 The effect of DMF on the frequency of Treg cells within PBMC

After investigating the effects of DMF on the production of cytokines, the effects of DMF on the frequency of Treg cells was examined next since Treg cells play an important role in preventing and controlling autoimmune disease. PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the presence of DMF (0, 5, 10, 25 µM). Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3, and viability dye and analysed by flow cytometry. Fig 4.17A shows a representative dot plot of the lymphocyte population identified by the FSC and SSC. Within the lymphocyte, singlets were gated on the basis of FSC-W and SSC-W (Fig 4.17B). After excluding doublets, viable cells were gated on the basis of fixable viability dye and FSC-A is shown in Fig 4.17C. Within viable cells CD3⁺CD4⁺ T cells were gated as shown in the representative dot plot Fig 4.17D. The expression of Foxp3 and CD127 examined within the CD4⁺ population, which were gated (Fig 4.17E) and then FoxP3⁺CD127lo cells were gated and assessed further for the expression of CD25 (Fig 4.17F). Treg cells were identified as being CD4⁺ Foxp3⁺CD25⁺CD127lo cells. Fig 4.18A shows a representative dot plot of the frequency of Treg in the absence or presence of 25 µM of DMF. The effect of DMF on the frequency of Treg cells is shown in Fig 4.18B (n=4), where DMF exerted a dose dependant trend towards an increase, however no statistical differences in the frequency of Treg cells within PBMC were observed.
Figure 4.17 Flow cytometry gating strategy to identify Treg and Tconv T cells within PBMC

PBMC stimulated with anti-CD3 for 5 days and then stained with fluorochrome-conjugated antibodies specific for Treg cell markers CD4, CD25, CD127, Foxp3 and viability dye. Treg cells were identified as CD4^+^CD25^+^CD127^lo^Foxp3^+^ and Tconv cells as CD4^+^CD25^+^CD127^hi^. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). Viable cells were gated from the singlet population on the basis of fixable viability dye and FSC-A (C). CD4^+^ T cells were gated on the basis of CD4^+^CD3^+^ (D). Treg cells (Foxp3^+^CD127^lo^) and Tconv (Foxp3^+^CD127^hi^) cells were identified on the basis of the expression of Foxp3 and CD127 (E). Treg cells were further gated on the basis of Foxp3^+^CD25^+^, Treg cells were defined as CD4^+^CD25^+^CD127^lo^Foxp3^+^ (F).
Figure 4.18 The effect of DMF on the frequency of Treg cells within PBMC

PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (5, 10, 25 µM). Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. Treg cells were gated as shown in Fig 4.17. The representative dot plot shows the frequency of Treg cells in the absence or presence of 25 µM of DMF. The graph shows the effect of DMF on the frequency of Treg cells (C) (n=4). Statistical differences between anti-CD3 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
4.2.2.6 The effects of DMF on the viability of memory Treg and Tconv cells

It was shown above that DMF reduced the viability of CD4⁺ T cells within PBMC and resulted in a trend towards an increase in the frequency of Treg cells. It was possible that an increase in Treg cells might be due relative resistance of Treg cells to DMF induced cell death. Therefore, it was next determined whether DMF exerted differential effects on the viability of memory Treg vs memory Tconv cells within PBMC. Since DMF has been shown to exert differential effects on naïve vs memory T cells, both Treg and Tconv populations were gated on the basis of memory (CD45RO⁺) to ensure that the populations were comparable. PBMC were isolated from HC and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the presence of DMF (0, 25, 50 µM). After 5 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD45RO, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. For flow cytometric analysis memory Tconv cells were defined as CD4⁺CD45RO⁺CD25⁻CD127hi T cells and memory Treg cells as CD4⁺CD45RO⁺CD25⁺CD127loFoxp3⁺ T cells as shown in Fig 4.19. Fig 4.20A shows a representative dot plot of the viability of stimulated memory Tconv cells in the absence or presence of DMF (25, 50 µM). Anti-CD3 stimulated memory Tconv cells in the absence of DMF were 87% viable and this was reduced to 77.6% in the presence of 25 µM DMF and further reduced to 59.6% in the presence of 50 µM DMF. Fig 4.20B shows a representative dot plot of the viability of stimulated memory Treg cells, in the absence or presence of DMF (25, 50 µM). Anti-CD3 stimulated memory Treg cells in the absence of DMF were 97.8% viable and this was reduced to 97.8% in the presence of 25 µM DMF and further reduced to 97.6% in the presence of 50 µM DMF. Fig 4.20C shows the viability anti-CD3 stimulated memory Treg vs memory Tconv in the absence or the presence of DMF (25, 50 µM) (n=7). In the absence of DMF, memory Treg cells exhibited significantly higher viability than memory Tconv (p≤0.01). However, in the presence of 25 µM and 50 µM DMF there was a more substantial, although less significant decrease in the viability of Tconv cells relative to Treg cells (p≤0.05). In summary, the viability memory Treg cells was maintained in the presence of DMF, whereas the viability of memory Tconv cells decreased in the presence of DMF.
Figure 4.19 Flow cytometry gating strategy to identify the viability of memory Treg and memory Tconv T cells within PBMC

PBMC were isolated and stimulated with anti-CD3 for 5 days, cells were then stained with fluorochrome-conjugated antibodies specific for Treg cell markers CD4, CD25, CD127, Foxp3 and viability dye. Treg cells were identified as CD4^+CD45RO^+CD25^+CD127^loFoxp3^+. Memory Tconv cells were identified as CD4^+CD45RO^+CD25^+CD127^hi. Cells were initially gated to exclude debris on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B) and CD4^+T cells were gated as CD4^+CD3^+ (C). Memory CD4 T cells were gated as CD4^+CD45RO^+ within the CD4 population (D) and memory Treg and memory Tconv cells were identified on the basis of the expression of Foxp3 and CD127 within the memory population; memory Tconv were identified as CD4^+CD45RO^+Foxp3^-CD127^hi (E). Memory Treg cells were first gated as CD45RO^-Foxp3^-CD127^hi cells (D), and then subsequently gated further on the basis of CD25^+ (F); memory Treg cells were defined as CD4^+CD45RO^-CD25^-CD127^loFoxp3^-Viable memory Treg (G) or memory T conv (G) cells were then gated on the basis of fixable viability dye and FSC-A (H).
Figure 4.20 The effect of DMF on the viability of memory Treg and memory Tconv cells

PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the presence of DMF (0, 25, 50 µM). After 5 days cells were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD45RO, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. Treg and Tconv cells were gated as shown in Fig 4.19. The representative dot plots show the viability of memory Tconv (A) and memory Treg (B) cells in the absence or presence of DMF. The graphs show the relative frequency of viable memory Treg vs memory Tconv cells in the absence or presence of 25 or 50 µM DMF(C). Statistical differences between stimulated Treg cells vs Tconv cells in the absence or presence of DMF were determined by a paired, two tailed t test; *p≤0.05, **p≤0.01.
4.2.2.7 The effects of DMF on the proliferation of Treg and Tconv cells

Having established that there was a trend towards an increase in the frequency of Treg cells in the presence of DMF, it was important to next determine if this was due to increased Treg cell proliferation or due to a relative decrease in Tconv cells. PBMC were isolated from HC and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the presence of DMF (0, 25, 50 µM). After 5 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. For flow cytometric analysis Tconv cells were defined as CD4⁺CD25⁻CD127hi T cells and Treg cells as CD4⁺CD25⁺CD127loFoxp3⁺ as shown in the gating strategy in Fig 4.17. Fig 4.21A shows a representative histogram of CTV dilution within Tconv cells, where Tconv cells that had proliferated during culture are gated as CTVlo. Tconv cells stimulated with anti-CD3 had proliferated by 76.7% and this was reduced to 43.7% in the presence of 25 µM DMF and further reduced to 3% in the presence of 50 µM DMF. Fig 4.21B shows a representative histogram of CTV dilution within Treg cells. Treg cells stimulated with anti-CD3 had proliferated by 89.8% and this was reduced to 82.9% in the presence of 25 µM DMF and reduced to 28.6% in the presence of 50 µM DMF. Fig 4.21C shows anti-CD3 induced proliferation in Treg vs Tconv cells in the absence or the presence of DMF (0, 25, 50 µM). No differences were observed in anti-CD3 induced proliferation in Treg vs Tconv cells in the absence of DMF (Fig 4.21C). However, in the presence of 25 µM DMF there was a non-significant decrease in the proliferation of Tconv cells compared with Treg cells. In the presence of 50 µM of DMF there was a significant decrease observed in the proliferation of Tconv compared with Treg cells (Fig 4.21C) (p≤0.05). In summary, the proliferation of both Treg and Tconv cells decreased in the presence of DMF, however, the proliferation of Tconv cells decreased significantly more than that of Treg cells.
Figure 4.21 The effect of DMF on the proliferation of Treg and Tconv cells within PBMC

PBMC were isolated from HC and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (25, 50 µM). After 5 days cells were harvested, stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. Treg and Tconv cells were gated as described in Fig 4.17. The percentage of proliferated cells was measured by the dilution of CTV (CTVlo), as shown in the representative histograms; proliferation of Tconv cells (A) or Treg cells (B) cells stimulated with anti-CD3 alone or in the presence of 25 µM or 50 µM DMF. The graphs show the effect of DMF on the proliferation of stimulated Treg vs Tconv cells in the absence or presence of 25 µM or 50 µM DMF (C). Statistical differences between stimulated Treg vs Tconv cells that were untreated or treated with 25µM or 50 µM DMF were determined by a paired, two tailed t test; *p≤0.05.
4.2.2.8 The effects of DMF on the expression of Ki67 in Treg and Tconv cells

Since it was clear that DMF did not inhibit the proliferation of Treg cells to the same extent as that of Tconv cells, it was important to examine an alternative method of assessing the proliferation in Treg and Tconv cells. Therefore, staining to measure the expression of the cell cycle associated factor Ki67 was used in order to assess the frequency of proliferating cells at the time of analysis. In contrast, the CTV staining used above assessed the cumulative proliferation during the culture period. PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the presence of DMF (0, 25, 50 µM). After 5 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and Ki67 and viability dye and analysed by flow cytometry. For flow cytometric analysis Tconv cells were defined as CD4⁺CD25⁻CD127^{hi} T cells and Treg cells as CD4⁺CD25⁺CD127^{lo}Foxp3⁺ as shown in Fig 4.17. Fig 4.22A shows a representative dot plot of Ki67 staining in Tconv cells, where proliferating Tconv cells were gated as CD4⁺CD25⁻CD127^{hi}Ki67⁺ cells. Tconv cells stimulated with anti-CD3 had proliferated by 93.3% and this was reduced to 40.4% in the presence of 25 µM DMF and further reduced to 4.05% in the presence of 50 µM DMF. Treg cells stimulated with anti-CD3 had proliferated by 97.8% and this was reduced to 88.4% in the presence of 25 µM DMF and further reduced to 46.7% in the presence of 50 µM DMF (Fig 4.22B). Fig 4.22C shows anti-CD3 induced proliferation in Treg vs Tconv cells in the absence or the presence of DMF (25, 50 µM). No differences were observed in anti-CD3 induced proliferation in Treg vs Tconv cells in the absence of DMF. In the presence of 25 µM DMF the proliferation of Tconv cells decreased compared with that of Treg cells, however this was not significant. In the presence of 50 µM DMF there was a significant decrease observed in the proliferation of Tconv cells compared with that of Treg cells (p≤0.05). Overall, the proliferation of both Treg and Tconv cells was inhibited by DMF, however, the proliferation of Tconv cells decreased significantly more than that of Treg cells. These data using Ki67 as a measure of proliferation were in broad agreement with those measured by CTV dilution.
Figure 4.22 The effect of DMF on the frequency of Ki67+ cells in Treg and Tconv cells within PBMC

PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the presence of DMF (0, 25, 50 µM). After 5 days cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3, intranuclear Ki67 and viability dye and analysed by flow cytometry. Treg and Tconv cells were gated as shown in Fig 4.17. The percentage of proliferating cells was measured by gating on actively cycling Ki67+ cells within each population; CD4+CD25−CD127+Ki67+ (Tconv) or CD4+CD25+CD127−Foxp3+Ki67+ (Treg). The representative dot plots show the frequency of proliferating Tconv (A) or Treg (B) cells stimulated with anti-CD3 alone or in the presence of 25 µM or 50 µM DMF. The graphs show the effect of DMF on frequency of proliferating Treg vs Tconv cells stimulated in the absence or presence of 25 µM or 50 µM DMF (C). Statistical differences between Treg vs Tconv cells that were stimulated in the absence or presence of DMF were determined by a paired, two tailed t test; *p≤0.05.
4.2.2.9 The effect of DMF on the anti-CD3 induced expression of activation markers on CD4+ and CD8+ T cells within PBMC

Having shown that DMF inhibited the proliferation of CD4 and CD8 T cells within PBMC it was important to determine whether these effects might be due to reduced activation of T cells in the presence of DMF. Therefore, next it was determined whether DMF had an effect on T cell activation markers such as CD69 and CD25. CD69 is an early activation marker that is detectable within hours of TCR ligation and expression is lost after 48-72 hr. CD25 is the alpha chain of the IL-2 receptor, that is required for IL-2 driven T cell proliferation, and is upregulated later than CD69. The aim of this experiment was to determine the effects of DMF on the activation markers CD69 and CD25 in CD4+ and CD8+ T cells after activation with anti-CD3. PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (1, 5, 10, 50 µM). After 1, 4, 6 or 24 hr cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD4, CD8, CD69, CD25 and viability dye and analysed by flow cytometry. CD4 and CD8 T cells were gated and the frequency of CD69+ or CD25+ cells was determined as defined in Fig 4.23.

The expression of CD69 was significantly induced on CD4+ T cells from 4-24 hr post activation (Fig 4.24A) (p<0.01). After 4 hr post activation, the frequency of CD4+CD69+ cells were significantly decreased in the presence of 25 µM (p≤0.01) and 50 µM DMF (p≤0.01) (Fig 4.24A) with a dose dependent trend towards a decrease at 25 µM and 50 µM of DMF at 6 hr (Fig 4.24A). After 24 hr the frequency of CD4+CD69+ cells decreased significantly when treated with 25 µM (p≤0.05) and 50 µM (p≤0.01) DMF (Fig 4.24A).

Significant induction of CD69 expression in CD8 T cells only occurred after 24 hr. Nonetheless after 4 hr there was a significant decrease in CD69 expression in the presence of 50 µM DMF (Fig 4.24B) (p≤0.05). After 6 hr and 24 hr, DMF treatment did not result in a significant decrease in the frequency of CD8+CD69+ cells, however, there was a dose dependent trend towards a decrease in the presence of DMF (Fig 4.24B).

There was no significant induction of CD25 expression on CD4 T cells until 24 hr post activation (Fig 4.25A) at which point there was a significant decrease in the frequency of CD4+CD25+ in the presence of 5 µM (p≤0.05), 10 µM (p≤0.05), 25 µM (p≤0.001) and 50 µM (p≤0.01) DMF (Fig 4.25A). In CD8 T cells there was a non-significant increase in the frequency of CD8+CD25+ cells by 24 hr (Fig 4.25B), with a dose dependant trend towards a decrease in the frequency of CD8+CD25+ cells after DMF treatment (Fig 4.25B).

In summary, the expression of the early activation marker CD69 was upregulated from 4 hr after activation of CD4 T cells, with slower induction in CD8 T cells. DMF inhibited the
anti-CD3 induced expression CD69 in both CD4\(^+\) and CD8\(^+\) T cells, with a more significant effect on CD4 \(T\) cells. The expression of CD25 was upregulated somewhat later at 24 hr, at which point DMF inhibited its expression on CD4\(^+\) T cells but not in CD8\(^+\) T cells. These data suggest that DMF inhibits the early activation of T cells.
Figure 4.23 Flow cytometry gating strategy to identify anti-CD3 induced expression of CD25 and CD69 on T cells

PBMC were isolated from HC and stimulated with anti-CD3 for 24 hr and stained with fluorochrome-conjugated antibodies specific for CD4, CD8, CD25, CD69 and viability dye. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). Viable cells were gated from the singlet population on the basis of fixable viability dye and FSC-A (C). CD4+ T cells were gated on the basis of CD4+CD8- and CD8+ T cells were gated on the basis of CD8+CD4- (D). Anti-CD3 induced expression of CD25 in CD4+ (E) or CD8+ (F) T cells was identified on the basis of CD4/CD8 and CD25. Anti-CD3 induced expression of CD69 in CD4+ (G) or CD8+ (H) T cells was then identified.
Figure 4.24 The effect of DMF on the anti-CD3 induced expression of CD69 on CD4\(^+\) and CD8\(^+\) T cells

PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (1, 5, 10, 50 \(\mu\)M). After 1, 4, 6 or 24 hr cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD4, CD8, CD69 and viability dye and analysed by flow cytometry. The expression of CD69 was gated as described in Fig 4.23. CD4\(^+\) and CD8\(^+\) T cells within the live lymphocyte population were gated and the frequency of CD4\(^+\)CD69\(^+\) (A) or CD8\(^+\)CD69\(^+\) (B) cells after 1, 4, 6, 24 hr is shown (n=3). Statistical differences between unstimulated vs anti-CD3 stimulated cells (\#p\(\leq\)0.05, \##p\(\leq\)0.01, ### p\(\leq\)0.001, #### p\(\leq\)0.0001) or anti-CD3 stimulated cells that were untreated vs different concentrations of DMF (*p\(\leq\)0.05, **p\(\leq\)0.01) were determined by one-way ANOVA with Tukey’s multiple comparison test. Data expressed as mean (+/- SEM).
Figure 4.25 The effect of DMF on the anti-CD3 induced expression of CD25 on CD4^+ and CD8^+ T cells

PBMC were isolated from healthy controls and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (1, 5, 10, 50 µM). After 1, 4, 6, or 24 hr cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD4, CD8, CD25, and viability dye and analysed by flow cytometry. The expression of CD25 was gated as described in Fig 4.23. CD4^+ and CD8^+ T cells within the live lymphocyte population were gated and the frequency of CD4^+CD25^+ (A) or CD8^+CD25^+ (B) cells after 1, 4, 6, 24 hr is shown (n=3). Statistical differences between unstimulated vs anti-CD3 stimulated cells (#p≤0.05) or anti-CD3 stimulated cells that were untreated vs different concentrations of DMF (*p≤0.05, **p≤0.01, *** p≤0.001) were determined by one-way ANOVA with Tukey’s multiple comparison test. Data expressed as mean (+/- SEM).
4.2.2.10 The effect of DMF on the anti-CD3 induced expression of pS6 in CD4+ and CD8+ T cells

Having examined the effect of DMF on T cell activation, its effects on mTOR activation were examined next by measuring pS6 which is downstream of mammalian target of rapamycin (mTOR). mTOR is a protein kinase that regulates cell growth, cell proliferation, cell survival, protein synthesis and transcription. The validation for pS6 staining is shown in Fig 2.4. PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (1, 10, 25, 50 µM). After 1, 4, 6 or 24 hr cells were harvested and stained with fluorochrome-conjugated when antibodies specific for CD4, CD8, pS6 and viability dye and analysed by flow cytometry. For the flow cytometric analysis, the frequency of pS6+ CD4 and CD8 T cells was determined as shown in Fig 4.26, and the detection of pS6 was validated as described in Fig 2.4 Significant induction of pS6 expression occurred only after 6 hr in CD4 T cells (p<0.05), although there was a non-significant induction after 1, 4 and 24 hr. At 1 hr there was actually a significant induction of pS6 expression in the presence of 50 µM DMF (p<0.05 (Fig. 4.27A). At 4, 6, and 24 hr there was a non-significant dose dependent decrease in pS6+CD4+ T cells in the presence of DMF (Fig 4.27A). In CD8 T cells, pS6 was significantly induced after 24 hr, with a significant reduction in the presence of 10 µM (p<0.01), 25 µM (p<0.05) and 50 µM (p<0.05) DMF (Fig 4.27B). Interestingly, at early time points of 1 and 4 hr DMF appeared to increase the expression of pS6 in CD8 T cells (Fig. 4.27B), as was the case for CD4 T cells after 1 hr, however this was not significant.

In summary, DMF appeared to increase pS6 in both CD4+ and CD8+ T cells after 1 and 4 hrs, suggesting that DMF may promote the early activation of mTOR. In contrast however, after 6 hr and 24 hr DMF inhibited pS6 expression in CD4+ and CD8+ T cells.
Figure 4.26 Flow cytometry gating strategy to identify the expression of pS6 in T cells

PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 for 24 hr. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). Viable cells were gated from the singlet population on the basis of fixable viability dye and FSC-A (C). CD4+ T cells were gated on the basis of CD4+CD8- and CD8+ T cells were gated on the basis of CD8+CD4- (D). Anti-CD3 induced pS6 expression in stimulated CD4+ T (E) or CD8+ (F) T cells was identified on the basis of CD4/CD8 and pS6.
Figure 4.27 The effect of DMF on the anti-CD3 induced expression of pS6 in CD4+ and CD8+ T cells

PBMC were isolated from HC and left unstimulated or stimulated with anti-CD3 in the absence or presence of DMF (1, 5, 10, 50 µM). After 1, 4, 6 or 24 hr cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD4, CD8, pS6 and viability dye, and analysed by flow cytometry. The expression of pS6 was gated as described in Fig 4.26. CD4+ and CD8+ T cells within the live lymphocyte population were gated and the frequency of CD4+ pS6+ (A) or CD8+ pS6+ (B) cells after 1, 4, 6, 24 hr is shown (n=3). Statistical differences between unstimulated vs anti-CD3 stimulated cells (#p≤0.05) or anti-CD3 stimulated cells that were untreated vs different concentrations of DMF (*p≤0.05, **p≤0.01) were determined by one-way ANOVA with Tukey’s multiple comparison test. Data expressed as mean (+/- SEM).
4.2.3 The immunomodulatory effects of DMF on T cells

After establishing the effects of DMF on T cells within PBMC, the immunomodulatory effects on sorted CD3 T cells were examined next, since the effects previously observed in PBMC may have been due to the direct effects of DMF on T cells or indirect effects mediated by other cells within PBMC. The aim of these experiments was to investigate the direct effects of DMF on T cell survival, cytokine production and the frequency of Treg cells within sorted CD3 T cells.

4.2.3.1 The effect of DMF on the viability of sorted CD3 T cells

DMF induced T cell death within PBMC, therefore it was important to also determine the direct effects of DMF on the viability of sorted CD3 T cells at the doses used in this study. CD3+ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3+ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3+ T cells were left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of different concentrations of DMF (0, 5, 10, 25, 50 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr for the purpose of subsequent cytokine analysis. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8 and viability dye and analysed by flow cytometry. Cells were gated as shown in Fig 4.28. Representative dot plots show the viability of stimulated total sorted CD3 T cells, or gated CD4+ and CD8+ T cells that were untreated (Fig. 4.29A) or treated with 25 µM DMF (Fig. 4.29B), where the gate indicates the percentage of live cells. The viability of stimulated sorted CD3 T cells, CD4+ and CD8+ T cells that were untreated or treated with DMF is shown in Fig 4.29C (n=7). There was a significant decrease in the viability of stimulated sorted CD3 T cells (p≤0.05), CD4+ (p≤0.01) and CD8+ (p≤0.05) T cells that were treated with 50 µM DMF.
Figure 4.28 Flow cytometry gating strategy to identify the viability of sorted CD3 T cells, CD4 and CD8 T cells

CD3⁺ T cells were isolated from HC PBMC using magnetic beads and stimulated with anti-CD3 and anti-CD28 and cultured for 5 days. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4 and viability dye and analysed by flow cytometry. Cells were initially gated to exclude debris and the population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). Viable cells were gated from the singlet population on the basis of fixable viability dye and FSC-A (C). To identify the viability of CD4 and CD8 T cells, within the singlets population, CD4⁺ T cells were gated on the basis of CD3⁺CD4⁺ and CD8⁺ T cells were gated on the basis of CD3⁺CD4⁺ (D). Viable CD4⁺ (E) and CD8⁺ (F) T cells were then gated on the basis of fixable viability dye and FSC-A.
Figure 4.29 The effect of DMF on the viability of T cells within sorted CD3 T cells
CD3+ T cells were isolated from HC PBMC using magnetic beads, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 5, 10, 25, 50 µM). After 5 days cells were stained with fluorochrome-conjugated antibodies specific for CD3 and CD8, and viability dye, and analysed by flow cytometry. CD4+ and CD8+ were gated as shown in Fig 4.28 for cells that were untreated (0 µM DMF) (A) or treated with 25 µM DMF (B) as shown in the representative dot plots. The graphs show the effect of DMF on the viability of stimulated sorted CD3 T cells, CD4+ and CD8+ T cells (C) (n=7). Statistical differences between stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; *p<0.05, ** p<0.01.
4.2.3.2 The effect of DMF on the apoptosis of T cells

DMF was shown above to induce T cell death, therefore it was important to determine whether this death was due to apoptosis. CD3+ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3+ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3+ T cells were left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of different concentrations of DMF (0, 10, 25, 50 µM). After 24 hr or 3 days cells were harvested and were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD8, annexin and 7AAD and analysed by flow cytometry. For the flow cytometric analysis, the frequency of CD4 and CD8 were determined as shown in Fig 4.30. Representative dot plots show the apoptosis of stimulated CD4+ T cells after 24 hr (Fig. 4.31A) or after 3 days (Fig. 4.31E), where the gates indicate the percentage of live, early apoptosis and late apoptosis in CD4+ T cells. The effect of DMF on early apoptosis (Fig 4.31B), late apoptosis (Fig 4.31C), and total apoptosis (Fig 4.31D) in CD4+ T cells following anti-CD3 and anti-CD28 stimulation after 24 hr is shown (n=3). Treatment with DMF did not result in a significant increase in early apoptosis in CD4+ T cells after 24 hr (Fig 4.31B), however there was a significant increase in late apoptosis CD4+ T cells treated with 25 µM DMF (p≤0.05) (Fig 4.31C), and in total apoptosis in CD4+ T cells treated with 10 µM DMF (p≤0.05) (Fig 4.31D). There was no significant increase in early apoptosis in CD4+ T cells treated with different concentrations of DMF for 3 days (Fig 4.31F), however there was a significant increase late apoptosis (Fig 4.31G), and total apoptosis (Fig 4.31H) in CD4+ T cells treated with 50 µM after 24 hr (p≤0.05).

Representative dot plots show the apoptosis of stimulated CD8+ T cells after 24 hr (Fig. 4.32A) or after 3 days (Fig. 4.32E), where the gate indicates the percentage of live, early apoptosis and late apoptosis of CD8+ T cells. There was no significant increase in early apoptosis (Fig 4.32B), late apoptosis (Fig 4.32C) or total apoptosis (Fig 4.32D) in CD8+ T cells treated with different concentrations of DMF for 24 hr. After 3 days, there was a non-significant increase in early apoptosis in CD8+ T cells (Fig 4.32F), however there was a significant increase in late apoptosis in CD8+ T cells when treated with 10 µM DMF (p≤0.05) (Fig 4.32G), and a significant increase in total apoptosis CD8+ T cells when treated with 25 µM DMF (p≤0.05) (Fig 4.32H). In summary, stimulation of T cells in the presence of DMF induced apoptosis in both CD4 and CD8 T cells with increased levels observed after 3 days compared with 24 hr.
Figure 4.30 Flow cytometry gating strategy to identify apoptotic CD4 and CD8 within sorted CD3+ T cells
Sorted CD3+ T cells were stimulated for 24 hr or 3 days with anti-CD3/CD28 and then stained with fluorochrome-conjugated antibodies specific for CD4, CD8, annexin V and 7AAD. Cells were initially gated to exclude debris on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). CD4+ T cells were gated on the basis of CD3+CD4+ and CD8+ T cells were gated on the basis of CD3+CD4+. CD4+ T cells were gated on live, early apoptotic, late apoptotic CD4+ T cells on the basis of 7AAD and annexin V.
Figure 4.31 The effect of DMF on the apoptosis of CD4 T cells

CD3+ T cells were isolated from HC PBMC using magnetic beads, and stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 10, 25, 50 µM). After 24 hr or 3 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD3, CD4 and annexin V and 7AAD and analysed by flow cytometry. CD4+ T cells were gated as shown in Fig 4.30, and the percentage of apoptotic cells was measured using annexin and 7AAD as shown in the representative dot plots for anti-CD3/anti-CD28 stimulated cells for 24 hr (A) or 3 days (E). The graphs show the effect of DMF on the early (B), apoptosis (C) and total (D) apoptosis within stimulated CD4+ T cells after 24 hr (n=3), or on the early (E), late (F) and total (G) apoptosis after 3 days (n=3). Statistical differences between cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; *p≤0.05. Data expressed as mean (+/− SEM).
Figure 4.32 The effect of DMF on the apoptosis of CD8 T cells

CD3+ T cells were isolated from HC PBMC using magnetic beads, and stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 10, 25, 50 µM). After 24 hr or 3 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD3, CD8 and annexin and 7AAD and analysed by flow cytometry. CD8+ T cells were gated as shown in Fig 4.30, and the percentage of apoptotic cells was measured using annexin and 7AAD as shown in the representative dot plots for anti-CD3/CD28 stimulated cells for 24 hr (A) or 3 days (E). The graphs show the effect of DMF on the early (B), late (C) and total (D) apoptosis within stimulated CD8+ T cells after 24 hr (n=3), or the early (F), late (G) and total (H) apoptosis after 3 days (n=3). Statistical differences between cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; *p≤0.05. Data expressed as mean (+/- SEM).
4.2.3.3 The effect of glutathione on DMF induced death of sorted CD3 T cells

Having shown that DMF induced apoptosis in T cells, it was important to determine next whether DMF induced T cell death via oxidative stress. Therefore, next it was determined whether DMF-induced death in T cells could be reversed with an antioxidant such as glutathione. CD3+ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3+ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3+ T cells were stimulated with anti-CD3 and anti-CD28 in the absence or presence of DMF (50 µM) and/or glutathione (1 µM). After 5 days cells were harvested and were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD8 and viability dye and analysed by flow cytometry. For flow cytometric analysis, CD4+ and CD8+ T cells were defined as shown in Fig 4.28. There was no change in the viability of CD3 T cells cultured in the presence of glutathione without DMF, however in the presence of 50 µM DMF, addition of glutathione resulted in a significant decrease in non-viable CD3 T cells (p≤0.05) (Fig 4.3A). Similarly, there was no significant decrease observed in non-viable CD4+ (Fig 4.3B) or CD8+ (Fig 4.3C) T cells treated with glutathione in the absence of DMF, however there was a non-significant decrease in non-viable CD4+ and CD8+ T cells treated with glutathione in the presence of 50 µM DMF. In summary, glutathione appeared to rescue T cells from DMF-induced death, suggesting that death was induced by oxidative stress.
Figure 4.33 The effect of glutathione on DMF induced death of T cells
CD3+ T cells were isolated from HC PBMC using magnetic beads, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 50 µM) or and glutathione (1 µM). After 5 days cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD8 and viability dye, and analysed by flow cytometry. Cells were initially gated to exclude debris and the percentage of non-viable cells within total CD3+, CD4+ or CD8+ T cells were determined as shown in Fig 4.28. The graphs show the effect of 0 µM and 50 µM DMF on the viability of stimulated sorted CD3 T cells (A) CD4+ T cells (B) or CD8+ T cells (C) in the presence or absence of glutathione (n=3). Statistical differences between cells that that were untreated or treated with 1 µM glutathione were determined by a paired, two tailed t test; *p≤0.05.
4.2.3.4 The effect of DMF on T cell proliferation within sorted CD3 T cells

The effect of DMF on the proliferation of T cells within sorted CD3 T cells was examined next. CD3⁺ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3⁺ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3⁺ T cells were labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of different concentrations of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr for simultaneous cytokine analysis. Cells were then stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD8, intranuclear Ki67 as an alternative method to assess proliferation and viability dye and analysed by flow cytometry. For flow cytometric analysis CD4⁺ and CD8⁺ T cells were defined as shown in Fig 4.34. Fig 4.35A shows a representative histogram of CTV dilution in CD4⁺ T cells, where proliferated cells are gated as CTV<sub>lo</sub>. Unstimulated cells exhibited virtually no proliferation, whereas 80.1% of CD4⁺ T cells stimulated with anti-CD3 and anti-CD28 had proliferated and this was reduced to 30.3% in the presence of 25 µM DMF. Overall, there was a significant decrease in the proliferation of CD4⁺ T cells treated with 25 µM DMF (p<0.01) (Fig 4.35B) (n=9). Fig 4.35C, shows representative dot plots of CD4 vs Ki67 where the gates indicate the percentage of cycling Ki67⁺ cells. Unstimulated cells exhibited virtually no proliferation, whereas 80.8% of CD4⁺ T cells stimulated with anti-CD3 and anti-CD28 were proliferating and this was reduced to 58.2% in the presence of 25 µM DMF. There was a significant decrease in the frequency of proliferating CD4⁺Ki67⁺ cells when treated with DMF at 25 µM (p<0.01) (Fig. 4.35D) (n=9).

Fig 4.36A, shows a representative histograms of CTV dilution in gated CD8⁺ T cells within sorted CD3 T cells. Unstimulated cells exhibited virtually no proliferation, whereas 81% of CD8⁺ T cells stimulated with anti-CD3 and anti-CD28 proliferated and this was reduced to 29.5% in the presence of 25 µM DMF. Overall there was a significant decrease in the proliferation of CD8⁺ T cells when treated with 25 µM DMF (p<0.001) (Fig 4.36B) (n=9). A representative dot plot of CD3 vs Ki67 where the gate indicates the frequency of cycling Ki67⁺ cells (Fig 4.36C). Unstimulated cells exhibited no proliferation, whereas 84.7% of CD8⁺ T cells stimulated with anti-CD3 and anti-CD28 were proliferating and this was inhibited to 57.6% in the presence of 25 µM DMF. Overall, there was significant decrease in the proliferation of CD8⁺ T cells when treated with 25 µM DMF (p<0.001) as shown in Fig 4.36D (n=9).
In summary, the proliferation, as measured by CTV dilution or Ki67 expression, of both CD4 and CD8 T cells within CD3 sorted T cells was significantly inhibited by DMF at 25 \( \mu \text{M} \) DMF.
Figure 4.34 Flow cytometry gating strategy to identify CD4 and CD8 T cells after stimulation with PMA

CD3⁺ T cells were isolated from HC PBMC using magnetic beads and stimulated with anti-CD3 and cultured for 5 days respectively. Cells were re-stimulated with PMA and ionomycin, and stained with fluorochrome-conjugated antibodies specific for CD3 and CD8 and analysed by flow cytometry. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). Viable cells were gated from the singlet’s population on the basis of fixable viability dye and FSC-A (C). Finally, CD4⁺ T cells were gated on the basis of CD8⁺CD3⁺, since CD4 was downregulated upon PMA stimulation and CD8⁺ T cells were gated on the basis of CD8⁺CD3⁻ (D).
Figure 4.35 The effect of DMF on the proliferation of CD4⁺ T cells within sorted CD3 T cells

CD3⁺ T cells were isolated from HC PBMC using magnetic beads, and labelled with cell tracer dye CTV, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, intranuclear Ki67 and viability dye, and analysed by flow cytometry. CD4⁺ T cells were gated as shown in Fig 4.34 and the percentage of proliferated cells was measured by the dilution of CTV (CTV<sup>lo</sup>), as shown in the representative histograms for unstimulated, anti-CD3/anti-CD28 stimulated and anti-CD3/anti-CD28 stimulated CD4⁺ T cells in the presence of 25 µM DMF (A). The effect of DMF on the proliferation of anti-CD3/anti-CD28 stimulated CD4⁺ T cells is shown in (B) (n=9). Proliferation was also measured by gating on actively cycling Ki67<sup>+</sup> cells as shown in the representative dot plots for unstimulated, anti-CD3/anti-CD28 stimulated and anti-CD3 and stimulated CD4⁺ T cells in the presence of 25 µM DMF (C). The effect of DMF on the frequency of proliferating Ki67<sup>+</sup> CD4⁺ T cells is shown in (D) (n=9). Statistical differences between anti-CD3/anti-CD28 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; **p≤0.01.
Figure 4.36 The effect of DMF on the proliferation of CD8+ T cells within sorted CD3 T cells

CD8+ T cells were isolated from HC PBMC using magnetic beads, and labelled with cell tracer dye CTV, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, intranuclear Ki67 and viability dye, and analysed by flow cytometry. CD8+ T cells were gated as shown in Fig 4.34 and the percentage of proliferated cells was measured by the dilution of CTV (CTVlo), as shown in the representative histograms for unstimulated, anti-CD3/anti-CD28 stimulated and anti-CD3/anti-CD28 stimulated CD8+ T cells in the presence of 25 µM DMF (A). The effect of DMF on the proliferation of anti-CD3/anti-CD28 stimulated CD8+ T cells is shown in (B) (n=9). Proliferation was also measured by gating on actively cycling Ki67+ cells as shown in the representative dot plots for unstimulated, anti-CD3/anti-CD28 stimulated and anti-CD3 and stimulated CD8+ T cells in the presence of 25 µM DMF (C). The effect of DMF on the frequency of proliferating Ki67+ CD8+ T cells is shown in (D) (n=9). Statistical differences between anti-CD3/anti-CD28 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; ***p≤0.001.
4.2.3.5 The effect of DMF on the production of cytokines by T cells within sorted CD3 T cells

The effect of DMF on the production of cytokines by T cells within sorted CD3 T cells was examined next. CD3⁺ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3⁺ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3⁺ T cells were labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the absence or presence of different concentrations of DMF (5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr and stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFNγ, GM-CSF, TNFα, IL-2 and viability dye and analysed by flow cytometry. The population of CD4⁺ and CD8⁺ T cells within sorted CD3⁺ T cells were gated as shown in Fig 4.34, and then the frequency of cytokine producing cells was determined (Fig 4.37). The effect of DMF on the frequency of cytokine producing cells within CD4⁺ T cells is shown in Fig 4.38. There was a non-significant trend towards a decrease in the frequency of CD4⁺IL-17⁺ T cells when treated with 25 µM of DMF treatment (Fig. 4.38A). However, no significant change was observed in the frequency of CD4⁺IFNγ⁺ (Fig 4.38B), CD4⁺GM-CSF⁺ (Fig 4.38C), CD4⁺IL-2⁺ (Fig 4.38D) or CD4⁺TNFα⁺ (Fig 4.38E) T cells.

The effect of DMF on the frequency of cytokine producing cells within CD8⁺ T cells is shown in Fig 4.39. There was no significant change in the frequency of CD8⁺ IL-17 (Fig 4.39A), CD8⁺ IFNγ⁺ (Fig 4.39B), CD8⁺GM-CSF⁺ (Fig 4.39C) or CD8⁺TNFα⁺ cells (Fig 4.39E). There was a non-significant trend towards an increase in the frequency of CD8⁺IL-2⁺ T cells that were treated with DMF compared with anti-CD3 stimulated control (Fig 4.39D).

In summary, DMF exerted varied effects on cytokine production by total CD4⁺ and CD8⁺ T cells within sorted CD3⁺ T cells, with no effects on IFNγ, GM-CSF, IL-2 and TNFα but a trend towards inhibition of the frequency of CD4⁺IL-17⁺ and a trend towards an increase in the frequency of CD8⁺IL-2⁺ cells.
Figure 4.37 Flow cytometry gating strategy to identify cytokine production by total or proliferated T cells using CTV

CD3⁺ T cells were isolated from HC PBMC using magnetic beads, labelled with cell tracer dye CTV and stimulated with anti-CD3 and anti-CD28 for 5 days. Cells were analysed by flow cytometry and the proliferation of CD4⁺ or CD8⁺ T cells was determined by the dilution of CTV. The representative dot plot (example gated on CD4⁺ T cells) shows the proliferation of CD4⁺ T cells vs IL-2, where the gate indicates the frequency of IL-2 producing cells within the total CD4 T cell population (A). Alternatively, cells that had proliferated (CTVlo) were gated as shown in the representative histogram (B), and then the frequency of proliferated cells producing cytokine was identified as shown in (C). A similar gating strategy was used for CD8⁺ T cells and other cytokines.
Figure 4.38 The effect of DMF on cytokine production by CD4+ T cells within sorted CD3 T cells

CD3+ T cells were isolated from HC PBMC using magnetic beads, and labelled with cell tracer dye CTV, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the absence or presence of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFNγ, GM-CSF, IL-2, TNFα and viability dye, and analysed by flow cytometry. CD4+ T cells were gated as shown in Fig 4.34 and the frequency of cytokine producing cells was determined as shown in Fig 4.37. The graphs show the effect of DMF on the frequency of cytokine producing CD4+ T cells in response to anti-CD3/anti-CD28; IL-17 (A), IFNγ (B), GM-CSF (C), IL-2 (D), TNFα (E) (n=6-9). Statistical differences between anti-CD3/anti-CD28 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
Figure 4.39 The effect of DMF on cytokine production by CD8+ T cells within sorted CD3 T cells

CD3+ T cells were isolated from HC PBMC using magnetic beads, and labelled with cell tracer dye CTV, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the absence or presence of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFNγ, GM-CSF, IL-2, TNFα and viability dye, and analysed by flow cytometry. CD8+ T cells were gated as shown in Fig 4.34 and the frequency of cytokine producing cells was determined as shown in Fig 4.37. The graphs show the effect of DMF on the frequency of cytokine producing CD8+ T cells in response to anti-CD3/anti-CD28; IL-17 (A), IFNγ (B), GM-CSF (C), IL-2 (D), TNFα (E) (n=6-9). Statistical differences between anti-CD3/anti-CD28 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
4.2.3.6 The effect of DMF on the production of cytokines by proliferated T cells within sorted CD3 T cells

Since the majority of cytokines are produced by activated and proliferating cells, the reduced viability and inhibition of proliferation by DMF observed above was likely to impact on the frequency of cytokine producing cells in a non-specific manner. Therefore, in order to identify any specific effects of DMF on cytokine production that were distinct from its global suppressive activities, the effect of DMF on cytokine production by cells that had proliferated in response to anti-CD3 and anti CD28 was assessed next.

The population of CD4$^+$ and CD8$^+$ T cells within sorted CD3 T cells that had proliferated (CTV$^{lo}$) in response to anti-CD3 and anti-CD28 stimulation was first gated as shown in Fig 4.34 and then the frequency of cytokine producing cells within this gate was determined as shown in Fig. 4.37. The effect of DMF on the frequency of cytokine producing cells within proliferated CD4$^+$ T cells is shown in Fig 4.40. There was no change in the frequency of proliferated CD4$^+$IL-17$^+$ T cells as a result of DMF treatment (Fig. 4.40A). However, treatment of CD4$^+$ T cells with increasing concentrations of DMF resulted in a trend towards a decrease in proliferated IFN$^\gamma$+ CD4$^+$ T cells compared with the control, however this was not significant (Fig 4.40B). DMF did not have any significant effect on the frequency of GM-CSF$^+$ CD4 T cells (Fig 4.40C), however there was a dose dependant decrease in the frequency of proliferated IL-2$^+$ CD4$^+$ T cells when treated with DMF compared with the control (Fig 4.40C). DMF had no significant effect on the frequency of proliferated TNF$^\alpha$+ CD4$^+$ T cells (Fig 4.40E). The effect of DMF on the frequency of cytokine producing cells within proliferated CD8$^+$ T cells is shown in Fig 4.41. There was no significant change in the frequency of proliferated CD8$^+$ IL-17 T cells (Fig 4.41A), CD8$^+$ IFN$^\gamma$+ T cell (Fig 4.41B), CD8$^+$GM-CSF$^+$ (Fig 4.41C), CD8$^+$ IL-2$^+$ T cells (Fig 4.41D), TNF$^\alpha$+ CD4$^+$ T cells (Fig 4.41E) that were treated with DMF compared with anti-CD3 and anti-CD28 stimulated control.

In summary, DMF exerted variable effects on cytokine production by proliferated CD4 T cells within sorted CD3 T cells, with no effects on IL-17, GM-CSF and TNF$^\alpha$, but slight inhibition was observed in the production of both IFN$^\gamma$ and IL-2. DMF exerted no significant effects on the frequency of proliferated CD8$^+$ T cells that produced IL-17, IFN$^\gamma$, GM-CSF, IL-2 or TNF$^\alpha$. 
Figure 4.40 The effect of DMF on cytokine production by proliferated CD4+ T cells within sorted CD3 T cells

CD3+ T cells were isolated from HCPBMC using magnetic beads, and labelled with cell tracer dye CTV, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the absence or presence of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFNγ, GM-CSF, IL-2, TNFα and viability dye, and analysed by flow cytometry. CD4+ T cells were gated as shown in Fig 4.34 and the frequency of cytokine producing cells was determined as shown in Fig 4.37. The graphs show the effect of DMF on the frequency of cytokine producing CD4+ T cells that had proliferated (CTVlo) in response to anti-CD3/anti-CD28; IL-17 (A), IFNγ (B), GM-CSF (C), IL-2 (D), TNFα (E) (n=6-9). Statistical differences between anti-CD3/anti-CD28 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
Figure 4.41 The effect of DMF on cytokine production by proliferated CD8+ T cells within sorted CD3 T cells

CD3+ T cells were isolated from HC PBMC using magnetic beads, and labelled with cell tracer dye CTV, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the absence or presence of DMF (0, 5, 10, 25 µM). After 5 days cells were harvested and stimulated with PMA/ionomycin for 5 hr. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-17, IFNγ, GM-CSF, IL-2, TNFα and viability dye, and analysed by flow cytometry. CD8+ T cells were gated as shown in Fig 4.34 and the frequency of cytokine producing cells was determined as shown in Fig 4.37. The graphs show the effect of DMF on the frequency of cytokine producing CD8+ T cells that had proliferated (CTVlo) in response to anti-CD3/anti-CD28; IL-17 (A), IFNγ (B), GM-CSF (C), IL-2 (D), TNFα (E) (n=6-9). Statistical differences between anti-CD3/anti-CD28 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
4.2.3.7 The effect of DMF on the frequency of Treg cells within sorted CD3 T cells

After investigating the effects of DMF on the production of cytokines, the effects of DMF on the frequency of Treg cells within sorted CD3$^+$ T cells was examined next since Treg cells play an important role in preventing and controlling autoimmune disease. CD3$^+$ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3$^+$ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3$^+$ T cells were left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 5, 10, 25 µM). Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. The gating strategy is shown in Fig 4.42, where the lymphocyte population was identified by FSC and SSC (Fig 4.42A). Within the lymphocyte population singlets were gated on the basis of FSC-W and SSC-W (Fig 4.42B). After excluding doublets, viable cells were assessed using dot plots of viability dye vs FSC-A (Fig 4.42C), and within viable cells, CD3$^+$CD4$^+$ cells were gated (Fig 4.42D). The expression of Foxp3 and CD127 was examined within the CD4$^+$ population, and FoxP3$^+$CD127$^{lo}$ cells were gated (Fig 4.42E) and then assessed further for the expression of CD25 (Fig 4.42F). Treg cells were identified as being CD4$^+$Foxp3$^+$CD25$^+$CD127$^{lo}$. Fig 4.43A shows a representative dot plot of Treg staining in absence or presence of 25 µM of DMF. The effect of DMF on the frequency of Treg cells is shown in Fig 4.43B (n=9), where DMF significantly increased the frequency of Treg cells at 10 µM (p≤0.01) and 25 µM (p≤0.001) of DMF.
Figure 4.42 Flow cytometry gating strategy to identify Treg and Tconv T cells within sorted CD3+ T cells

Sorted CD3+ T cells were stimulated for 5 days with anti-CD3/CD28 and then stained with fluorochrome-conjugated antibodies specific for Treg cell markers CD4, CD25, CD127 and Foxp3. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). Viable cells were gated from the singlet population on the basis of fixable viability dye and FSC-A (C). CD4+ T cells were gated on the basis of CD4+CD3 (D). Treg and Tconv cells were identified on the basis of the expression of Foxp3 and CD127 within the CD4 population, where Tconv were identified as Foxp3+CD127hi (E). Treg cells were identified as Foxp3+CD127lo cells, and subsequently gated on the basis of CD25+, Treg cells were defined as CD4+CD25+CD127loFoxp3+ (F).
Figure 4.43 The effect of DMF on the frequency of Treg cells within sorted CD3 T cells

CD3⁺ T cells were isolated from HC PBMC using magnetic beads and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 5, 10, 25 µM). Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye, and analysed by flow cytometry. Treg were gated as described in Fig 4.42. The representative dot plots show the frequency of Treg cells in the absence or presence of 25 µM DMF (A). The graph shows the effect of DMF on the frequency of Treg cells (B) (n=9). Statistical differences between anti-CD3/anti-CD28 stimulated cells that were untreated vs those treated with different concentrations of DMF were determined by one-way ANOVA with Tukey’s multiple comparison test; * p≤0.05, ** p≤0.01, ***p≤0.001.
4.2.3.8 The effect of glutathione on the DMF-increased frequency of Treg cells within sorted CD3 T cells

Having shown that DMF increased the frequency of Treg cells, it was important to next determine whether the increased frequency of Treg cells might be mediated via the oxidative stress induced by DMF, and whether this could be reversed with the antioxidant glutathione. CD3+ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3+ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3+ T cells were left unstimulated or stimulated with anti-CD3 and anti-CD28 in the absence or presence of DMF (25 µM) or/and glutathione (1 µM). Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. The purity of CD3+ T cells were described as shown in Fig 2.3. For flow cytometric analysis Treg cells were gated as shown in Fig 4.42. The effect of glutathione on the frequency of Treg cells in the absence or presence of DMF is shown in Fig 4.44 (n=3), whereas shown previously DMF significantly increased the frequency of Treg cells in the absence of glutathione. In the absence of DMF, glutathione had no effect on the frequency of Treg cells. However, the addition of glutathione plus DMF resulted in a non-significant decrease in the frequency of Treg cells compared with DMF alone, similar to the frequency of Treg cells in the absence of DMF. These data suggest that DMF may increase the frequency of Treg cells via the induction of oxidative stress.
Figure 4.44 The effect of glutathione on the DMF-induced increase in frequency of Treg cells

CD3⁺ T cells were isolated from HC PBMC using magnetic beads and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the absence or presence of DMF (25 μM) or/and glutathione (1 μM). Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye, and analysed by flow cytometry. Treg cells were gated as described in Fig 4.42. The graph shows the effect of DMF and glutathione on the frequency of Treg cells (n=3). Statistical differences between anti-CD3/anti-CD28 stimulated cells that were untreated vs those treated with different concentrations of DMF and/or glutathione were determined by one-way ANOVA with Tukey’s multiple comparison test; * p≤0.05. Data expressed as mean (+/- SEM).
4.2.3.9 The effects of DMF on the viability memory Treg and memory Tconv cells

Having shown that DMF increased the frequency of Treg cells, it was necessary to determine whether this increase might be due to differential effects of DMF on the viability of Treg and Tconv cells. Memory Tconv and memory Treg were gated for this experiment to ensure that the populations were comparable. For flow cytometric analysis memory Tconv cells were defined as CD4\(^+\) CD45RO\(^-\)CD25 CD127\(^{hi}\) cells and memory Treg cells as CD4\(^+\)CD45RO\(^+\)CD25\(^-\)CD127\(^{lo}\)Foxp3\(^+\) cells as shown in Fig 4.45. CD3\(^+\) T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3\(^+\) T cells were analysed by flow cytometry as shown in Fig 2.3. CD3\(^+\) T cells were left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25, 50 \(\mu\)M). After 5 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD45RO, CD25, CD127, Foxp3 and viability dye, and analysed by flow cytometry according to the gating strategy shown in Fig 4.45. Fig 4.46A shows a representative dot plot of the viability of stimulated memory Tconv cells in the absence or presence of 25\(\mu\)M or 50 \(\mu\)M DMF. Anti-CD3 and anti-CD28 stimulated memory Tconv cells in the absence of DMF were 88.5\% viable and this was reduced to 50.3\% in the presence of 25 \(\mu\)M DMF and further reduced to 14.4\% in the presence of 50 \(\mu\)M DMF. Anti-CD3 and anti-CD28 stimulated memory Treg cells in the absence of DMF were 98.8\% viable and this was reduced to 96\% in the presence of 25 \(\mu\)M DMF and further reduced to 69.2\% in the presence of 50 \(\mu\)M (Fig 4.46B). Fig 4.46C shows the viability anti-CD3/anti-CD28 stimulated memory Treg vs memory Tconv cells in the absence or the presence of DMF (25, 50 \(\mu\)M) (n=7). There was a significantly higher viability of memory Treg vs memory Tconv cells in the absence of DMF (p≤0.05) (Fig 4.46C). However, in the presence of 25 \(\mu\)M or 50 \(\mu\)M of DMF the viability of memory Tconv cells decreased significantly compared with that of memory Treg cells (p≤0.01, p≤0.05 respectively). In summary, the viability of both memory Treg and memory Tconv decreased in the presence of DMF, however, the viability of memory Tconv cells decreased significantly more than that of memory Treg cells.
Figure 4.45 Flow cytometry gating strategy to identify the viability of memory Treg and memory Tconv T cells within sorted CD3⁺ T cells

CD3⁺ T cells were isolated from HC PBMC using magnetic beads, and stimulated with anti-CD3 and anti-CD28. After 5 days cells were stained with fluorochrome-conjugated antibodies specific Treg cell markers CD4, CD45RO, CD25, CD127, Foxp3 and viability dye. Memory Treg cells were identified as CD4⁺ CD45RO⁺CD25⁺CD127loFoxp³⁺ and memory Tconv cells as CD4⁺ CD45RO⁺CD25⁺CD127hi. Cells were initially gated to exclude debris on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-W and FSC-A (B). CD4⁺ T cells were gated on the basis of CD4⁺CD3⁺ (C). Memory CD4⁺ T cells were gated on the basis of CD4⁺CD45RO⁺ (D). Memory Treg cells and Memory Tconv cells were identified on the basis of the expression of Foxp3 and CD127 within the CD4 population, where Memory Tconv were defined as CD45RO⁻Foxp3⁺CD127hi (E). Memory Treg cells were identified as CD45RO⁻CD25⁻Foxp3⁺CD127lo cells, which were subsequently gated on the basis of CD25⁻; memory Treg cells were defined as CD4⁺ CD45RO CD25⁻CD127loFoxp³⁺ (F). Viable memory Treg (G) or memory Tconv (H) cells were gated on the basis of fixable viability dye and FSC-A.
Figure 4.46 The effect of DMF on the viability of memory Treg and memory Tconv T cells

CD3⁺ T cells were isolated from HC PBMC using magnetic beads, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25, 50 µM). After 5 days cells were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD45RO, CD25, CD127 Foxp3 and viability dye and analysed by flow cytometry. Memory Treg and memory Tconv cells were gated as described in Fig 4.45. The representative dot plots show the viability of memory Tconv (A) and memory Treg (B) cells in the absence or presence of 25 or 50 µM DMF (A). The graphs show the frequency of viable memory Treg vs memory Tconv cells in the absence or presence of 25 or 50 µM DMF (C) (n=7). Statistical differences between stimulated memory Treg cells vs memory Tconv cells that were untreated or treated with 25 µM or 50 µM DMF were determined by a paired, two tailed t test; *p≤0.05, **p≤0.01.
The effects of DMF on the proliferation of Treg and Tconv cells

The question of whether the increased Treg cell frequency in the presence of DMF observed above could be accounted for by an increased susceptibility of Tconv cells to the anti-proliferative effects of DMF was examined next. Therefore, the effect of DMF on proliferation of both Treg and Tconv was compared. CD3+ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3+ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3+ T cells were labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25, 50 µM). After 5 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. For flow cytometric analysis Tconv cells were defined as CD4+CD25+CD127hi and Treg cells as CD4+CD25+CD127loFoxp3+ as shown in Fig 4.42. Fig 4.47A shows a representative histogram of CTV dilution of Tconv cells, where Tconv cells that had proliferated during culture are gated as CTVlo. Tconv cells stimulated with anti-CD3 and anti-CD28 had proliferated by 80.5% and this was reduced to 9.8% in the presence of 25 µM DMF and further reduced to 1.92% in the presence of 50 µM DMF. Fig 4.47B shows a representative histogram of CTV dilution of Treg, where Treg cells stimulated with anti-CD3 and anti-CD28 had proliferated by 66.9% and this was reduced to 22.9% in the presence of 25 µM DMF and reduced to 7.02% in the presence of 50 µM DMF. Fig 4.47C shows anti-CD3/anti-CD28 induced proliferation in Treg vs Tconv in the absence or the presence of DMF (25, 50 µM) (n=7). No differences were observed in anti-CD3/anti-CD28 induced proliferation in Treg vs Tconv in the absence of DMF. However, in the presence of 25 µM of DMF the proliferation of Tconv cells decreased significantly compared with Treg cells (p≤0.05). In the presence of 50 µM of DMF there was a non-significant decrease observed in the proliferation of Tconv compared with Treg cells. In summary, the proliferation of both Treg and Tconv decreased in the presence of DMF, however, that of Tconv cells decreased significantly more than that of Treg cells.
Figure 4.47 The effect of DMF on the proliferation of Treg and Tconv cells within sorted CD3 T cells

CD3⁺ T cells were isolated from HC PBMC using magnetic beads and labelled with cell tracer dye CTV and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25, 50 µM). After 5 days cells were harvested stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye, and analysed by flow cytometry. Treg and Tconv cells were gated as described in Fig 4.42. The percentage of proliferated cells was measured by the dilution of CTV (CTV⁺), as shown in the representative histograms; proliferation of Tconv (A) or Treg (B) cells stimulated with anti-CD3/anti-CD28 alone or in the presence of 25 µM or 50 µM DMF. The graphs show the effect of DMF on the proliferation of Treg vs Tconv cells stimulated in the absence or presence of 25 µM or 50 µM DMF (C) (n=7). Statistical differences between stimulated Treg vs Tconv cells that were untreated or treated with 25 µM 50 µM DMF were determined by a paired, two tailed t test; *p≤0.05.
4.2.3.11 The effects of DMF on the expression of Ki67 in Treg and Tconv cells

After determining that DMF did not inhibit the proliferation of Treg cells to the same extent as that of Tconv cells, it was important to confirm these results by examining an alternative method of assessing the proliferation. CD3⁺ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3⁺ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3⁺ T cells were left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25, 50 µM). After 5 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3, Ki67 and viability dye and analysed by flow cytometry. For flow cytometric analysis Tconv cells were defined as CD4⁺CD25⁻CD127⁺ and Treg cells as CD4⁺CD25⁺CD127⁻Foxp3⁺ as shown in Fig 4.42. Fig 4.48A shows a representative dot plot of Ki67 staining in Tconv cells, where proliferating T conv cells were gated as CD4⁺CD25⁻CD127⁺Ki67⁺ cells. Tconv cells stimulated with anti-CD3 and anti-CD28 had proliferated by 80.3% and this was reduced to 20.8% in the presence of 25 µM DMF and further reduced to 3.8% in the presence of 50 µM DMF. Fig 4.48B shows a representative dot plot of Ki67 staining in Treg cells, where Treg cells stimulated with anti-CD3 and anti-CD28 had proliferated by 71.8% and this was reduced to 45% in the presence of 25 µM DMF and further reduced to 21.1% in the presence of 50 µM DMF. Fig 4.48C shows anti-CD3/anti-CD28 induced proliferation in Treg vs Tconv cells in the absence or the presence of DMF (25, 50 µM) (n=7). No differences were observed in anti-CD3/anti-CD28 induced proliferation in Treg vs Tconv cells in the absence of DMF. In the presence of 25 µM and 50 µM DMF the proliferation of Tconv cells decreased significantly compared with Treg cells (p≤0.05). Overall, the proliferation of both Treg and Tconv cells decreased in the presence of DMF, however, the proliferation of Tconv cells decreased significantly more than that of Treg cells.
Figure 4.48 The effect of DMF on the frequency of Ki67+ cells in Treg and Tconv populations within sorted CD3 T cells

CD3+ T cells were isolated from HC PBMC using magnetic beads, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25, 50 µM). After 5 days cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3, intranuclear Ki67 and viability dye, and analysed by flow cytometry. Treg and Tconv cells were gated as described in Fig 4.42. The percentage of proliferating cells was measured by gating on actively cycling CD4+CD25+CD127hiKi67+ (Tconv) or CD4+CD25+CD127loFoxp3+Ki67+ (Treg). The representative dot plots show the proliferating Tconv (A) or Treg (B) cells stimulated with anti-CD3/anti-CD28 alone or in the presence of 25 µM or 50 µM DMF. The graphs show the effect of DMF on the frequency of proliferating Treg vs Tconv stimulated in the absence or presence of 25 µM or 50 µM DMF (C) (n=7). Statistical differences between Treg vs Tconv cells that were stimulated in the absence or presence of DMF were determined by a paired, two tailed t test; *p<0.05.
4.2.3.12 The effect of DMF on thiols on the surface of CD4+ T cells

DMF is an electrophile that is known to have oxidative capacity. Furthermore, it was shown above that DMF induced T cell death, and oxidative stress is known to have detrimental effects on T cells. Since cell surface thiols, together with other mechanisms, serve to protect cells from oxidative stress, the ability of DMF to oxidise thiols was determined next. Staining with ALM 633 C Maleimide (ALM633) binds to reduced thiols on the cell surface by forming a stable thioether linkage, which allows the reduced thiol groups be detected by flow cytometry. The validation for ALM633 staining is shown in Fig 2.8. CD3+ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3+ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3+ T cells were left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25, 50 µM). After 5 days cells were harvested and stained with ALM633, fluorochrome-conjugated antibodies specific for CD3 and CD4, and viability dye and analysed by flow cytometry. Fig 4.49A shows representative histograms of the effect of stimulation and DMF on ALM633 MFI and Fig 4.49B shows the mean (+/- SEM) of ALM633 MFI (n=3). Reduced thiols were present at low levels on unstimulated CD4 T cells and were increased upon activation (Fig 4.49B). Furthermore, in the presence of DMF, there was non-significant dose dependent decrease in ALM633 MFI in stimulated CD4 T cells. These data validated the use of ALM633 to detect cell surface thiols since these are known to increase upon activation of T cells, and also suggested that DMF oxidised the cell surface thiols.
Figure 4.49 The effect of DMF on thiols on the surface of CD4⁺ T cells

CD3⁺ T cells were isolated from HC PBMC using magnetic beads, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 10, 25 µM). After 5 days cells were stained with ALM633 and fluorochrome-conjugated antibody specific for CD4 and viability dye, and analysed by flow cytometry. CD4⁺ T cells were gated and reduced cell surface thiols were detected with ALM633 as shown in the representative histograms for unstimulated, or stimulated cells in the presence or absence of DMF (A). The graph shows the mean (+/- SEM) of ALM633 MFI (% of CD4 T cells) (B). Statistical differences between stimulated cells that were untreated vs those stimulated cells that were treated with DMF were determined by one-way ANOVA with Tukey’s multiple comparison test.
4.2.3.13 The effect of DMF on thiols on the surface of Treg and Tconv T cells

It has been demonstrated that Treg cells contain higher thioredoxin-1, which plays a role as an antioxidant and is essential in maintaining reduced thiols (Mougiakakos et al., 2011). Therefore, having shown that DMF oxidised thiols on the surface of CD4⁺ T cells, it was next important to examine the effects of DMF on the surface thiols of both Treg and Tconv cells. CD3⁺ T cells were isolated from HC PBMC using magnetic beads as described in section 2.8 and the purity of CD3⁺ T cells were analysed by flow cytometry as shown in Fig 2.3. CD3⁺ T cells were left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25, 50 µM). After 5 days cells were harvested and stained with ALM633, fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127 and viability dye and analysed by flow cytometry. For flow cytometric analysis Tconv cells were defined as CD4⁺CD25⁺CD127 vibrant T cells and Treg cells as CD4⁺CD25⁺CD127 dull since the intracellular staining required for Foxp3 could not be used in conjunction with ALM633. Fig 4.50A shows representative histograms of the effect of DMF on ALM633 MFI in both Treg and Tconv T cells (Fig 4.50A) and figure 4.50B shows the mean (+/- SEM) of ALM633 MFI in Treg cells (n=3). ALM633 staining on Treg cells was markedly (but not significantly) increased upon activation, with only a slight reduction in the presence of 25 µM DMF (Figure 4.49B). Upon activation of Tconv cells, there was an increase in ALM633 MFI, but to a much lesser extent than that observed on Treg cells (Figure 4.50C). Furthermore, there was a significant decrease in ALM633 staining when Tconv cells were treated with 25 µM DMF compared with the stimulated control (p≤0.01). When ALM633 staining on Treg vs Tconv cells was compared, Treg expressed significantly more reduced thiols than Tconv cells in the absence of DMF (p<0.05) (Figure 4.50D) and also in the presence of 25µM DMF (not significant) (Figure 4.50E). In summary, Treg cells had higher induction of reduced thiols after stimulation compared with Tconv cells. Treg cells surface thiols were not affected by DMF however, Tconv reduced surface thiols were significantly decreased when treated with DMF. Taken together with the data above, this suggested that the increased capacity of Treg cells to maintain reduced cell surface thiols may confer protection from the oxidative effects of DMF.
Figure 4.50 The effect of DMF on thiols on the surface of Treg and Tconv T cells
CD3+ T cells were isolated from HC PBMC using magnetic beads, and left unstimulated or stimulated with anti-CD3 and anti-CD28 in the presence of DMF (0, 25 µM). After 5 days cells were stained with ALM633 and fluorochrome-conjugated antibodies specific for CD4, CD25 and CD127 and viability dye, and analysed by flow cytometry. Treg (CD4+CD25+CD127lo) and Tconv (CD4+CD25-CD127hi) cells were gated and thiols detected with ALM633 as shown in the representative histograms for unstimulated or stimulated Treg and Tconv in the presence or absence of DMF (A). The graphs show the mean (+/- SEM) of ALM633 MFI in Treg (B) and Tconv (C) cells (n=3). The graph shows the mean (+/- SEM) of ALM633 MFI in Treg vs Tconv cells stimulated in the absence (D) or the presence of 25 µM DMF (E). Statistical differences between stimulated Treg or Tconv that were untreated vs stimulated that were treated with DMF (B, C) were determined by one-way ANOVA with Tukey’s multiple comparison test; **p≤0.01. Statistical differences between stimulated Treg vs Tconv in the presence or absence of DMF (D, E) were determined by a paired, two tailed t test; *p≤0.05.
4.2.4 The *in vivo* immunomodulatory effects of Fumaderm™ on T cells from psoriasis patients

Having establishing the effects of DMF on T cells *in vitro*, it was of interest to determine next whether DMF exerted similar effects *in vivo*. Therefore, the frequency of T cell subsets, Treg cells and T cell cytokine production was examined in psoriasis patients that were either untreated or treated with Fumaderm™, in which the active ingredient is DMF. The details of the two groups of psoriasis patients is shown in Table 4.1. Untreated patients received no systemic therapy and blood samples were taken prior to phototherapy, which is usually the first line treatment for psoriasis. The group of patients that were treated with Fumaderm™ were either on low (30 mg per day) or high (120 mg per day) dose regimen.

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Table 4.1 Details of psoriasis patient groups

1 Psoriasis area and severity index

2 Normal range 1000-4000 cells/µl
4.2.4.1 The effect of Fumaderm™ treatment on the frequency of memory CD4+ T cell subsets in psoriasis patients

Initially the effects of Fumaderm™ on memory CD4+ T cell subsets in psoriasis patients was examined. PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD45RO, CCR7 and viability dye and analysed by flow cytometry. CD4+ T cells were gated as shown in Fig 4.51 and Fig 4.52A shows a representative dot plot of the gating strategy for identifying the CD4+CD45RO+ memory T cell population, CD4+CD45RO+CCR7+ CM and CD4+CD45RO−CCR7− EM populations. Fig 4.52B shows the frequency of total memory, CM and EM CD4+ T cells in psoriasis patients that were untreated (n=15) or treated with Fumaderm™ (n=30). There was a significant decrease in total memory T cells in the patient group treated with Fumaderm™ relative to untreated psoriasis patients (p≤0.05) (Figure 4.52B). However, there was no significant decrease in the frequency of either CM or EM CD4+ T cells in the Fumaderm™ treated group (Fig 4.52B). When the Fumaderm™ treated patients were divided into the low (n=11) and high (n=19) dose subgroups, there were no significant differences in total memory, CM or EM CD4 T cells between groups (Fig 4.52C).

In summary, psoriasis patients treated with Fumaderm™ exhibited a reduced frequency of total memory CD4+ T cells relative to patients that were untreated.
Figure 4.51 Flow cytometry gating strategy to identify CD4$^+$ and CD8$^+$ T cells in psoriasis patients
PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4, CD3 together with other relevant markers and viability dye and analysed by flow cytometry. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC) (A). Singlets were gated to exclude doublets from the lymphocyte population on the basis of FSC-H and FSC-A (B). Viable cells were gated from the singlet population on the basis of fixable viability dye and FSC-A (C). CD4$^+$ T cells were gated on the basis of CD4$^+$CD3$^+$ and CD8$^+$ T cells were gated on the basis of CD4$^+$CD3$^+$ (D). CD3$^-$CD4$^+$ are possibly monocytes.
Figure 4.52 The effect of Fumaderm™ treatment on the frequency of memory CD4⁺ T cell subsets in psoriasis patients

PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD45RO, CCR7 and viability dye and analysed by flow cytometry. CD4⁺ T cells were gated as shown in Fig 4.51. A representative dot plot shows the gating strategy for identifying the total CD4⁺CD45RO⁺ memory T cell population, CD4⁺CD45RO⁺CCR7⁺ central memory T cells (CM) and CD4⁺CD45RO⁺CCR7⁻ effector memory T cells (EM) (A). The frequency of total memory, CM, and EM T cells in psoriasis patients that were untreated or treated with Fumaderm™ is shown in (B), where statistical differences between untreated vs Fumaderm™ treated patients were determined by an unpaired, two tailed t test; **p≤0.01. The frequency of memory, CM, and EM T cells in psoriasis patients that were untreated or treated with low or high dose Fumaderm™ is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm™ treatment were determined by one-way ANOVA with Bonferroni post-test; **p≤0.01. A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
4.2.4.2 The effect of Fumaderm™ treatment on the frequency of memory CD8⁺ T cell subsets in psoriasis patients

The effects of Fumaderm™ on memory CD8⁺ T cell subsets in psoriasis patients was examined. PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD45RO, CCR7 and viability dye and analysed by flow cytometry. CD8⁺ T cells were gated as shown in Fig 4.51 and Fig 4.53A shows a representative dot plot of the gating strategy for identifying the CD8⁺CD45RO⁺ memory T cell population, CD4⁺CD85RO⁻CCR7⁺ CM and CD8⁺CD45RO⁻CCR7⁻ EM populations. Fig 4.53B shows the frequency of total memory, CM and EM CD4⁺ T cells in psoriasis patients that were untreated (n=15) or treated with Fumaderm™ (n=30). There was a non-significant decrease in total memory T cells in the patient group treated with Fumaderm™ relative to untreated psoriasis patients (Figure 4.53B). However, there was no significant decrease in the frequency of either CM or EM CD8⁺ T cells in the Fumaderm™ treated group (Fig 4.53B). When the Fumaderm™ treated patients were divided into the low (n=11) and high (n=19) dose subgroups, there were no significant differences in total memory, CM or EM CD4 T cells between groups (Fig 4.53C). In summary, psoriasis patients treated with Fumaderm™ exhibited a trend towards a reduction in the frequency of total memory CD8⁺ T cells relative to patients that were untreated.
Figure 4.53 The effect of Fumaderm™ treatment on the frequency of memory CD8\(^+\) T cell subsets in psoriasis patients

PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD45RO, CCR7 and viability dye and analysed by flow cytometry. CD8\(^+\) T cells were gated as shown in Fig 4.51. A representative dot plot shows the gating strategy for identifying the total CD8\(^+\)CD45RO\(^+\) memory T cell population, CD8\(^+\)CD45RO\(^+\)CCR7\(^+\) central memory T cells (CM) and CD8\(^+\)CD45RO\(^+\)CCR7\(^-\) effector memory T cells (EM) (A). The frequency of total memory, CM, and EM T cells in psoriasis patients that were untreated or treated with Fumaderm™ is shown in (B), where statistical differences between untreated vs Fumaderm™ treated patients were determined by an unpaired, two tailed t test. The frequency of memory, CM, and EM T cells in psoriasis patients that were untreated or treated with low or high dose Fumaderm™ is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm™ treatment were determined by one-way ANOVA with Bonferroni post-test; A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
Pro-inflammatory cytokines play a key role in the pathogenesis of psoriasis by activating keratinocytes. Therefore, the production of cytokines by T cells from psoriasis patients that were untreated or treated with Fumaderm™ was examined next. PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stimulated with PMA and ionomycin for 5 hr and were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IFNγ, IL-17, GM-CSF, IL-22, TNFα and IL-2 and viability dye and analysed by flow cytometry. CD4+ T cells were gated as shown in Fig 4.54 and Fig 4.55A shows a representative dot plot of the gating strategy for identifying IL-17 and IFNγ. Fig 4.55B shows the frequency of IFNγ+CD4+, IL-17+CD4+ and IFNγ+IL-17+CD4+ T cells in psoriasis patients that were untreated (n=15) or treated with Fumaderm™ (n=30). There was a significant reduction in IFNγ+CD4+ (p≤0.05), IL-17+CD4+ (p≤0.05) and IFNγ+IL-17+CD4+ (p≤0.0001) T cells in the Fumaderm™ treated group relative to untreated patients. Fig 4.55C shows the frequency of IFNγ+CD4+, IL-17+CD4+ and IFNγ+IL-17+CD4+ T cells in psoriasis patients that were untreated or treated with low (n=11) or high dose (n=19) Fumaderm™, however, there were no significant differences between groups. Fig 4.56A shows a representative dot plot of the gating strategy for identifying GM-CSF+ and IL-22+ CD4+ T cells and TNFα+ and IL-2+ CD4+ T cells. Fig 4.56B shows the frequency of IL-22+CD4+, GM-CSF+CD4+, IL-2+CD4+ and TNFα+ CD4+ T cells in psoriasis patients that were untreated (n=15) or treated with Fumaderm™ (n=30). There was a significant reduction in the frequency of IL-22+CD4+ (p≤0.001) and GM-CSF+CD4+ (p≤0.05) in the Fumaderm™ treated group compared with untreated patients. However, no significant reduction was observed between groups for IL-2+CD4+ and TNFα+ CD4+ T cells. Fig 4.56C shows the frequency of cytokine producing CD4+ T cells in psoriasis patients that were untreated or treated with low (n=11) or high dose (n=19) Fumaderm™. There was a significant decrease in IL-22+CD4+ T cells in both low dose (p≤0.05) and high dose (p≤0.01) Fumaderm™ treated groups. However, no significant decrease was observed in GM-CSF+CD4+, IL-2+CD4+ and TNFα+ CD4+ T cells in psoriasis patients that were treated with low or high dose Fumaderm™. In summary, psoriasis patients treated with Fumaderm™ exhibited varied effects on cytokine production by CD4+ T cells with no effects on IL-2 and TNFα but significant inhibition of IL-22 and GM-CSF.
**Figure 4.54 Flow cytometry gating strategy to identify cytokines in CD4+ and CD8+ T cells after stimulation with PMA/ionomycin**

PBMC were stimulated with PMA and ionomycin for 5 hr and stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IFNγ, IL-17, IL-22, GM-CSF, IL-2 and TNFα and viability dye and analysed by flow cytometry. The lymphocyte population was identified on the basis of size (FSC) and granularity (SSC). Lymphocytes were then gated on singlets to exclude doublets from the population on the basis of FSC-W and FSC-A. Within singlets viable cells were gated on the basis of fixable viability dye and FSC-A. Within viable cells CD4+ T cells were gated on the basis of CD8−CD3+, since CD4 was downregulated upon PMA stimulation, and CD8+ T cells were gated on the basis of CD8+CD3+. Finally, within the CD4 (or CD8) gate, cytokines were gated on the basis of for example IL-2 and IL-22.
Figure 4.55 The effect of Fumaderm™ treatment on the frequency of CD4+ T cell cytokines IL-17 and IFNγ in psoriasis patients.

PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stimulated with PMA and ionomycin for 5 hr and stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IFNγ, IL-17 and viability dye and analysed by flow cytometry. Cells were gated as described in Fig 4.54. A representative dot plot shows the gating strategy for identifying cytokines IL-17 and IFNγ within the CD4+ T cell population (A). The frequency of IFNγ+CD4+, IL-17+CD4+, and IFNγ+IL-17+CD4+ T cells in psoriasis patients that were untreated or treated with Fumaderm™ is shown in (B), where statistical differences between untreated vs Fumaderm™ treated patients were determined by an unpaired, two tailed t test; *p≤0.05, **** p≤0.0001. The frequency of IFNγ+CD4+, IL-17+CD4+, and IFNγ+IL-17+CD4+ T cells in psoriasis patients that were untreated or treated with low or high dose Fumaderm™ is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm™ treated patients were determined by one-way ANOVA with Bonferroni post-test; *p≤0.05, **p≤0.01. A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
Figure 4.56 The effect of Fumaderm™ treatment on the frequency of CD4⁺ T cell cytokines IL-22, GM-CSF, IL-2, and TNFα in psoriasis patients.

PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stimulated with PMA and ionomycin for 5 hr and stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-22, GM-CSF, IL-2, and TNFα and viability dye and analysed by flow cytometry. Cells were gated as described in Fig 4.54. A representative dot plot shows the gating strategy for identifying cytokines IL-22, GM-CSF vs IL-2 and TNFα vs IL-2 within the CD4⁺ T cell population (A). The frequency of IL-22⁺CD4⁺, GM-CSF⁺CD4⁺, TNFα⁺CD4⁺, and IL-2⁺CD4⁺ T cells in psoriasis patients that were untreated or treated with Fumaderm™ is shown in (B), where statistical differences between untreated vs Fumaderm™ treated patients were determined by an unpaired, two tailed t test; *p≤0.05, ***p≤0.001. The frequency of IL-22⁺CD4⁺, GM-CSF⁺CD4⁺, TNFα⁺CD4⁺, and IL-2⁺CD4⁺ T cells in psoriasis patients that were untreated or treated with low or high dose Fumaderm™ is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm™ treatment were determined by one-way ANOVA with Bonferroni post-test; *p≤0.05, ***p≤0.001. A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
4.2.4.4 The effect of Fumaderm™ treatment on the frequency of CD8⁺ T cell cytokines psoriasis patients

Pro-inflammatory cytokines play a key role in the pathogenesis of psoriasis by activating keratinocytes. Therefore, the production of cytokines by T cells from psoriasis patients that were untreated or treated with Fumaderm™ was examined next. PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stimulated with PMA and ionomycin for 5 hr and were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IFNγ, IL-17, GM-CSF, IL-22, TNFα and IL-2 and viability dye and analysed by flow cytometry. CD8⁺ T cells were gated as shown in Fig 4.54 and Fig 4.57A shows a representative dot plot of the gating strategy for identifying IL-17 and IFNγ. Fig 4.57B shows the frequency of IFNγ⁺CD8⁺, IL-17⁺CD8⁺ and IFNγ⁺IL-17⁺CD8⁺ T cells in psoriasis patients that were untreated (n=15) or treated with Fumaderm™ (n=30). There was no significant reduction in IFNγ⁺CD8⁺, IL-17⁺CD8⁺ and IFNγ⁺IL-17⁺CD8⁺ T cells in the Fumaderm™ treated group relative to untreated patients. Fig 4.57C shows the frequency of IFNγ⁺CD8⁺, IL-17⁺CD8⁺ and IFNγ⁺IL-17⁺CD8⁺ T cells in psoriasis patients that were untreated or treated with low (n=11) or high dose (n=19) Fumaderm™, however, there were no significant differences between groups. Fig 4.58A shows a representative dot plot of the gating strategy for identifying GM-CSF⁺ and IL-22⁺ CD8⁺ T cells and TNFα⁺ and IL-2⁺ CD8⁺ T cells. Fig 4.58B shows the frequency of IL-22⁺CD8⁺, GM-CSF⁺CD8⁺, IL-2⁺CD8⁺ and TNFα⁺ CD8⁺ T cells in psoriasis patients that were untreated (n=15) or treated with Fumaderm™ (n=30). There was no significant reduction in the frequency IL-22⁺CD8⁺, GM-CSF⁺CD8⁺, IL-2⁺CD8⁺ and TNFα⁺ CD8⁺ T cells in the Fumaderm™ treated group compared with untreated patients. Fig 4.58C shows the frequency of cytokine producing CD4⁺ T cells in psoriasis patients that were untreated or treated with low (n=11) or high dose (n=19) Fumaderm™. There was no significant decrease IL-22⁺CD8⁺, GM-CSF⁺CD8⁺, IL-2⁺CD8⁺ and TNFα⁺ CD8⁺ T cells in Fumaderm™ treated groups. In summary, psoriasis patients treated with Fumaderm™ exhibited no effects on cytokine production by CD8⁺ T cells.
Figure 4.57 The effect of Fumaderm™ treatment on the frequency of CD8⁺ T cell cytokines IL-17 and IFNγ in psoriasis patients.
PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stimulated with PMA and ionomycin for 5 hr and were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IFNγ, IL-17 and viability dye and analysed by flow cytometry. Cells were gated as described in Fig 4.54. A representative dot plot shows the gating strategy for identifying cytokines IL-17 and IFNγ within the CD8⁺ T cell population (A). The frequency of IFNγ⁺CD8⁺, IL-17⁺CD8⁺, and IFNγ⁺IL-17⁺CD8⁺ T cells in psoriasis patients that were untreated or treated with Fumaderm™ is shown in (B), where statistical differences between untreated vs Fumaderm™ treated patients were determined by an unpaired, two tailed t test; The frequency of IFNγ⁺CD8⁺, IL-17⁺CD8⁺, and IFNγ⁺IL-17⁺CD8⁺ T cells in psoriasis patients that were untreated or treated with low or high dose Fumaderm™ is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm™ treated patients were determined by one-way ANOVA with Bonferroni post-test; A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
Figure 4.58 The effect of Fumaderm™ treatment on the frequency of CD8⁺ T cells cytokines IL-22, GM-CSF, IL-2, and TNFα in psoriasis patients.

PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stimulated with PMA and ionomycin for 5 hr and were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IL-22, GM-CSF, IL-2, and TNFα and viability dye and analysed by flow cytometry. Cells were gated as described in Fig 4.54. A representative dot plot shows the gating strategy for identifying cytokines IL-22, GM-CSF vs IL-2 and TNFα vs IL-2 within the CD8⁺ T cell population (A). The frequency of IL-22⁺CD8⁺, GM-CSF⁺CD8⁺, TNFα⁺CD8⁺ and IL-2⁺CD8⁺ T cells in psoriasis patients that were untreated or treated with Fumaderm™ is shown in (B), where statistical differences between untreated vs Fumaderm™ treated patients were determined by an unpaired, two tailed t test. The frequency of IL-22⁺CD8⁺, GM-CSF⁺CD8⁺, TNFα⁺CD8⁺ and IL-2⁺CD8⁺ T cells in psoriasis patients that were untreated or treated with low or high dose Fumaderm™ is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm™ treatment were determined by one-way ANOVA with Bonferroni post-test. A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
4.2.4.5 The effect of Fumaderm™ treatment on the frequency of CD161⁺CD4⁺ Th17 lineage cells in psoriasis patients

Having established the effect of Fumaderm™ on the frequency of cytokine producing CD4⁺ T cells, the effects of Fumaderm™ on specific T cell subsets was examined next. Th17 cells play a key role in the pathogenesis of psoriasis (Blauvelt, 2007). CD161 is a lineage marker for Th17 lineage cells, and CD161⁺ CD4 T cells include Th17 cells which produce IL-17 as well as exTh17 cells which have undergone plasticity and lost the expression of IL-17, but produce IFNγ (Basdeo et al., 2017). Therefore, the effects of Fumaderm™ treatment on memory CD161⁺CD4⁺ Th17 lineage cells in psoriasis patients was examined next. PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD161 and viability dye and analysed by flow cytometry. CD4⁺ T cells were gated as shown in Fig 4.51 and Fig 4.59A shows a representative dot plot of the gating strategy for identifying CD4⁺CD161⁺ T cells. Overall, there was a highly significant decrease in CD4⁺CD161⁺ Th17 lineage cells in the Fumaderm™ treated group compared with untreated psoriasis patients (p≤0.001) Fig 4.59B. Fig 4.59C shows the frequency of CD4⁺CD161⁺ Th17 lineage cells in psoriasis patients that were untreated or treated with low (n=11) or high dose (n=19) Fumaderm™, where there was a significant decrease in the frequency of CD161⁺CD4⁺ T cells in both the low dose (p≤0.05) and high dose (p≤0.001) Fumaderm™ treated group Fig 4.59C. These data suggest that Fumaderm™ inhibits Th17 lineage cells in vivo, and given that Th17 cells are pathogenic in psoriasis this is likely to be of therapeutic significance.
Figure 4.59 The effect of Fumaderm™ treatment on the frequency of CD161⁺CD4⁺ Th17 lineage cells in psoriasis patients

PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD161 and viability dye and analysed by flow cytometry. Lymphocytes were gated on the basis of FSC and SSC, and CD4⁺ T cells within the lymphocyte gate were gated on the basis of CD4⁺CD3⁺ as shown in Fig 4.51. A representative dot plot shows the gating strategy for identifying the CD4⁺CD161⁺ T cell population (A). The frequency of CD161⁺CD4⁺ T cells in psoriasis patients that were untreated or treated with Fumaderm™ is shown in (B). Statistical differences between untreated vs Fumaderm™ treated patients were determined by an unpaired, two tailed t test; ***p ≤ 0.001. The frequency of CD161⁺CD4⁺ T cells in psoriasis patients that were untreated or treated with low or high Fumaderm™ is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm™ treated patients were determined by one-way ANOVA with Bonferroni post-test; *p ≤ 0.05 **p ≤ 0.01. A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
4.2.4.6 The effect of Fumaderm™ treatment on the frequency of Th1, Th17, and exTh17 lineage cells in psoriasis patients

Having showed that CD161⁺ Th17 lineage cells were reduced in psoriasis patients that were treated with Fumaderm™, it was important to next to determine the effects of Fumaderm™ on specific T cell subsets. Th1 lineage cells (CD4⁺CD161⁻IFNγ⁺), exTh17 (CD4⁺CD161⁺IFNγ⁺) and Th17 (CD4⁺CD161⁺IL-17⁺) cells were examined in the untreated psoriasis patients and Fumaderm™ treated patients. PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stimulated with PMA and ionomycin for 5 hr and were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, CD161, IFNγ, IL-17 and viability dye and analysed by flow cytometry. CD4⁺ T cells were gated as shown in Fig 4.51 and Fig 4.60A shows a representative dot plot of the gating strategy for identifying CD4⁺CD161⁻IFNγ⁺ and CD4⁺CD161⁺IFNγ⁺) T cells. Fig 4.60B shows the frequency of Th1, exTh17 and Th17 CD4⁺ T cells in psoriasis patients that were untreated (n=15) or treated with Fumaderm™ (n=30). There was no significant change in the frequency of Th1 cells in the Fumaderm™ treated group vs untreated psoriasis patients. However, both exTh17 and Th17 cells were significantly reduced in the patients treated with Fumaderm™ vs untreated patients (p<0.05). Fig 4.60C shows the frequency of Th1, exTh17 and Th17 T cells in psoriasis patients that were untreated or treated with low (n=11) or high dose (n=19) Fumaderm™. There was no significant difference in Th1 and exTh17 cells between the groups. However, there was a significant decrease in Th17 cells in the high dose Fumaderm™ treated group relative to untreated patients (Fig 4.60C). Taken together, these data indicate that Fumaderm™ treatment has a specific inhibitory effect on Th17 and exTh17 cells, but not on Th1 cells in psoriasis.
Figure 4.60 The effect of Fumaderm™ treatment on the frequency of Th1, Th17, and exTh17 lineage cells in psoriasis patients

PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stimulated with PMA and ionomycin for 5 hr and were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, CD161, IFNγ, IL-17 and viability dye and analysed by flow cytometry. Lymphocytes were gated on the basis of FSC and SSC, and CD4+ T cells within the lymphocyte gate were gated on the basis of CD3+CD8+ as shown in Fig 4.51. A representative dot plot shows the gating strategy for identifying the Th1 (CD161–IFNγ+), Th17 (CD161–IL-17+), and exTh17 (CD161+IFNγ+) T cell population (A). The frequency of Th1, Th17, and exTh17 T cells in psoriasis patients that were untreated or treated with Fumaderm™ is shown in (B), where statistical differences between untreated vs Fumaderm™ treated patients were determined by an unpaired, two tailed t test; *p≤0.05. The frequency of Th1, Th17, and exTh17 T cells in psoriasis patients that were untreated or treated with low or high Fumaderm™ is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm™ treated patients were determined by one-way ANOVA with Bonferroni post-test; *p≤0.05. A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
4.2.4.7 The effect of Fumaderm™ treatment on the frequency of Treg cells in psoriasis patients.

Treg cells play an important role in preventing and controlling autoimmune disease and it has been shown that Treg cells have impaired suppressive capacity in psoriasis patients (Kagen et al., 2006). Therefore, it was of interest to investigate the effects of Fumaderm™ in vivo on the frequency of Treg cells in psoriasis patients. PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm™. PBMC were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. The lymphocyte population was identified by FSC and SSC (Fig 4.61A). Fig 4.61B shows a representative dot plot of FSC-W and SSC-W to exclude doublets and Fig 4.61C shows a representative dot plot for viable cells on the basis of fixable viability dye and FSC-A. Within viable cells CD4+ T cells were gated on the basis of CD3+CD4+ as shown in the representative dot plot (Fig 4.61D). Foxp3+CD127lo cells were gated within the CD4+ population (Fig 4.61E) and then assessed further for the expression of CD25 (Fig 4.61F); Treg cells were identified as being CD4+ Foxp3+CD25+CD127lo. Fig 4.61G shows the frequency of Treg cells in psoriasis patients that were untreated (n=15) or treated with Fumaderm™ (n=30), where there was a significant increase in the frequency of Treg cells in the Fumaderm™ treated group relative to the untreated group (p≤0.01). Although, there was no significant increase in the frequency of Treg cells in psoriasis patients that were treated with low dose Fumaderm™, there was a significant increase in the high dose Fumaderm™ group vs untreated patients (p<0.05) (Fig 4.9H). These data together with the in vitro data above, suggests that DMF treatment selectively targets Tconv over Treg cells, since Treg cells have the capacity to resist the apoptotic and anti-proliferative effects of DMF.
Figure 4.61 The effect of Fumaderm\textsuperscript{TM} treatment on the frequency Treg cells in psoriasis patients.

PBMC were isolated from psoriasis patients that were either untreated (n=15) or administered low (n=11) or high (n=19) dose Fumaderm\textsuperscript{TM}. PBMC were stained with fluorochrome-conjugated antibodies specific for CD4, CD3, CD25, CD127, Foxp3 and viability dye and analysed by flow cytometry. Lymphocytes were identified on the basis of FSC vs SSC (A), followed by gating on singlets (B) and then viable cells (C). Within the viable cells CD4\textsuperscript{+}CD3\textsuperscript{+} cells were gated (D). Within the CD3\textsuperscript{+}CD4\textsuperscript{+} gate, Foxp3 and CD127 were examined, and CD4 \textsuperscript{+}Foxp3\textsuperscript{+}CD127\textsuperscript{lo} were gated and then assessed further for the expression of CD25 (A). The frequency of Treg cells in psoriasis patients that were untreated or treated with Fumaderm\textsuperscript{TM} is shown in (B), where statistical differences between untreated vs Fumaderm\textsuperscript{TM} treated patients were determined by an unpaired, two tailed t test; **p≤0.01. The frequency of Treg cells in psoriasis patients that were untreated or treated with low or high dose Fumaderm\textsuperscript{TM} is shown in (C), where statistical differences between untreated vs low and high dose Fumaderm\textsuperscript{TM} treated patients were determined by one-way ANOVA with Bonferroni post-test; *p≤0.05. A proportion of patient samples (23/48) were analysed by Dr Deborah Cluxton.
4.3 Discussion

DMF is an electrophile and cysteine modifier that has been shown to have a range of immunomodulatory effects in vitro (Scannevin et al., 2012, Blewett et al., 2016). However, the precise mechanism of action of DMF in vitro and in vivo in psoriasis patients is still unclear. In this study, the immunomodulatory effects of DMF in vitro and in vivo in psoriasis patients were investigated, and its anti-inflammatory effects on T cell subsets were examined. The data in this chapter has demonstrated the effects of DMF in vitro in DC, PBMC and for the first time in sorted CD3+ T cells, and also in vivo in psoriasis patients treated with Fumaderm™.

DC play a key role in the immune response where they integrate the innate and adaptive responses. Activation of DC via their PRR results in upregulation of co-stimulatory and maturation markers that provide co-stimulatory signals, which together with TCR activation, are required for the full activation of T cells. In addition, activated DC secrete polarising cytokines that drive the differentiation of naïve T cells into specific subsets. The DC cytokine profile and subsequent T cell response depends on the PRR/s that were activated. Given the importance of DC in orchestrating T cell responses, the effect of DMF on the activation and cytokine production by DC was investigated in monocyte derived DC. The data in this chapter revealed the surprising finding that DMF upregulated DC maturation marker CD86, with a trend towards an increase in CD80; this was in contrast to the working hypothesis that DMF would inhibit DC activation and maturation. In order to allay concerns that the results might be artefactual, curcumin was used as a positive control as it is a known inducer of HO-1 (Martin et al., 2004, Scapagnini et al., 2002) and has recently been shown to inhibit DC maturation markers and cytokine production (Campbell et al., 2018). However our data was not in agreement with a previous study that revealed that DMF inhibited slanDC maturation markers CD83 and CD86 when DC were pre-treated with DMF for 6 hr and stimulated with TLR7/8 ligand R848 (Oehrl et al., 2017). Data from this chapter also contrasts with a study that showed that DMF inhibited maturation markers CD80 and CD86 in LPS stimulated BMDC from mice (Peng et al., 2012). Another study showed that mDC from DMF-treated RRMS patients displayed a less mature phenotype with low expression of CD86, CD83, CD40, CCR7 and HLADRB (Mazzola et al., 2017). These discrepancies could be due to the fact that different DC types that were used in the previous studies, and it is possible that there are differences between monocyte derived DC, dermal slanDC, and BMDC. A possible explanation for the upregulation of surface markers observed here is that DMF is a known contact sensitisier (Schad et al., 2010, D'Erme et al., 2012) and it has also been shown that
the treatment of monocyte derived DC with sensitisers can upregulate the maturation markers CD83 and CD86 in monocyte derived DC (Kagatani et al., 2010). Another possible explanation is that DMF is an inducer of oxidative stress (Gill and Kolson, 2013, Ghoreschi et al., 2011), and a study showed that oxidative stress induced by H2O2 can induce maturation markers CD80 and CD40 in BMDC from mice (Batal et al., 2014).

The effect of DMF on LPS-induced DC cytokine production was also investigated. However, overall DMF did not alter the LPS induced cytokine production by DC, with variable results between donors. This was not consistent with several other studies; one such study indicated that DMF increased IL-10 from BMDC and impaired their capacity to produce IL-12 and IL-23 in response to LPS stimulation (Ghoreschi et al., 2011). Another study also demonstrated that DMF inhibited IL-12 and IL-6 in LPS-stimulated BMDC (Peng et al., 2012). Furthermore, it was also shown that DMF decreased the production of IL-12, IL-23, TNFα and IL-10 in slanDC that were stimulated with TLR7/8 ligand R848 (Oehrl et al., 2017). Similarly, a study showed that MMF inhibited IL-10, IL-12p70 and TNFα from monocyte derived DC that were stimulated with LPS. (Litjens et al., 2006). However, an important experimental difference between the latter study and the current one was that monocytes were actually differentiated into immature DC in the presence or absence of MMF, whereas in the current study DMF was added to immature DC prior to activation with LPS. In summary, the data presented here showed that DMF increased rather than decreased the expression of maturation markers by DC and did not result in inhibition of pro-inflammatory cytokine production and this broadly contrasts with the literature. Nonetheless, the experimental system was validated by the use of curcumin as an internal control, and it is possible that the differences in cells and experimental conditions could account for the contradictory results. Interestingly, both curcumin and DMF are inducers of HO-1 which has been shown to exert anti-inflammatory effects in DC, yet these compounds exerted opposite effects in this study (Lehmann et al., 2007, McNally et al., 2007, Scapagnini et al., 2002). In line with the original hypothesis that DMF would inhibit the activation and maturation of DC, the plan had been to next examine the effects of DMF on the ability of DC to activate and polarise T cells. However, given the lack of effect of DMF on LPS-induced cytokine production, these experiments were not pursued further and instead the effect of DMF on PBMC and T cells was examined.

It was important to investigate the effects of DMF on both PBMC and sorted T cells, since the effects of DMF on T cells might be mediated either directly or indirectly via other cells within PBMC. A dose dependent decrease in the viability of total PBMC, and CD4+ and
CD8+ T cells within PBMC was observed as consequence of DMF treatment. Similarly, increasing concentrations of DMF decreased the viability of sorted CD3+ T cells, and CD4+ and CD8+ T cells within sorted CD3+ T cells in this study. Interestingly, DMF had more of a toxic effect on sorted T cells than on T cells within PBMC, suggesting that other cells within PBMC may exert a protective effect. It was also important to establish whether the DMF induced T cell death was due to apoptosis, and it was clear from the data that DMF induced apoptosis in both CD4+ and CD8+ T cells. DMF is known to cause T cell death, and this data is consistent with that from other studies that showed that DMF causes apoptosis in anti-CD3 stimulated CD4+ and CD8+ T cells in vitro (Treumer et al., 2003a). It was also demonstrated that DMF induced apoptosis in unstimulated CD4+ and CD8+ T cells, where CD8+ T cells were more susceptible to apoptosis than CD4+ T cells (Ghadiri et al., 2017). The study by Ghadiri et al. 2017 showed that unstimulated CD8+ T cells were more susceptible to apoptosis compared with CD4+ T cells. Similarly to the study by Ghadiri, another group demonstrated that DMF induced apoptosis in unstimulated CD4+ and CD8+ T cells with more DMF induced apoptosis in CD8+ T cells (Wu et al., 2017). The current study did not observe any obvious differences in apoptosis between CD4+ and CD8+ T cells, but this could have been due to the fact that T cells were stimulated with anti-CD3 whereas the study by Ghadiri used unstimulated T cells. This suggests that stimulation may alter the susceptibility of at least some T cell subsets to DMF-induced apoptosis.

The mechanism by which DMF induces apoptosis has not yet been investigated, however the study by Treumer et al. 2003 suggested that in activated T cells, DMF induced apoptosis may be through inhibition of NF-κB, which has been shown to cause cell death (Treumer et al., 2003a). However, the study by Ghadiri et al. 2017, used unstimulated T cells which raises the possibility that DMF induced death is not through NF-κB inhibition since NF-κB would not be activated in unstimulated T cells (Ghadiri et al., 2017). Since DMF is known to be a highly reactive oxidative molecule, the current study investigated whether the DMF induced apoptosis in T cells was due to oxidative stress, and if it could be reversed by the antioxidant glutathione. This study demonstrated for the first time that addition of glutathione could rescue T cells from DMF induced death. DMF is known to deplete and modify glutathione (Lehmann et al., 2007, Dibbert et al., 2013, Brennan et al., 2015, Schmidt et al., 2007a), and depletion of glutathione has also been shown to induce the antioxidant pathway via Nrf2 (Lehmann et al., 2007, Brennan et al., 2015). This would suggest that the apoptosis observed in the current study is due to oxidative stress.
Investigating the effects of DMF on T cell proliferation within PBMC and sorted CD3+ T cells revealed that DMF inhibited T cell proliferation as measured by Ki67 and CTV dilution in both CD4+ and CD8+ T cells. This is consistent with another study that showed that DMF inhibited the proliferation of T cells within PBMC (Diebold et al., 2017). Similarly, another study also observed that DMF inhibited the proliferation of T cells in vitro (Wang et al. 2017).

It was possible that the DMF-induced apoptosis discussed above could account for the reduced T cell proliferation observed in the presence of DMF. However it is possible that DMF could also specifically inhibit the activation of T cells. Indeed, the data showed that DMF inhibited expression of the early activation marker CD69 in both CD4+ and CD8+ T cells and inhibited the expression of CD25 only in CD4+ T cells. This data agrees with a study that was conducted in primary human T cells which showed that DMF inhibited the expression of activation markers CD69 and CD25 after 8 hr (Blewett et al., 2016). DMF-treated RRMS patients also exhibited an inhibition of CD69 expression in total CD4+ T cells and the CM and EM compartments within CD4+ T cells (Wu et al., 2017). The activation of CD69 in response to T cell activation is correlated with the recruitment and activation of PKCθ (Diaz-Flores et al., 2003). Additionally, PKC seems to regulate the expression of CD25 genes through NF-κB (Sun et al., 2000). Interestingly, DMF has been shown to inhibit PKCθ recruitment to the TCR signalling complex upon activation of CD4+ T cells (Blewett et al., 2016). Thus, the ability of DMF to inhibit the activation/recruitment of PKCθ could explain the inhibition of both CD69 and CD25 by DMF treatment. In summary, it appears that in addition to inducing apoptosis in a proportion of T cells, DMF can also inhibit the activation and subsequent proliferation of the surviving T cells, possibly via modification of PKCθ.

Inflammatory cytokines produced by Th1 and Th17 cells have been implicated in the pathogenesis of psoriasis and therefore it was important to examine the effects of DMF on cytokines produced by T cells. This chapter examined the effects of DMF on cytokine production by both CD4+ and CD8+ T cells within CD3 sorted T cells and within PBMC. The majority of cytokines are produced by activated and proliferating T cells, therefore the reduction in viability and inhibition of proliferation by DMF was likely to inhibit cytokines in a non-specific manner. Therefore, not only the total cytokine production, but also the cytokine production by cells that had proliferated was measured in this study using intracellular staining and flow cytometry. Intracellular staining was considered preferable to ELISA for the measurement of cytokines in this study, since the concentrations of cytokines
in the supernatants would reflect the suppressive effects of DMF on T cell survival and proliferation, whereas intracellular cytokine staining would be more likely to reveal potential specific effects that DMF might have on cytokine production.

Inhibition of the frequency of total and proliferated IFN\(\gamma\)\(^+\)CD4\(^+\) T cells by DMF was observed in PBMC. However, this inhibition was not observed in sorted CD3 T cells. This suggested that DMF did not inhibit IFN\(\gamma\)\(^+\)CD4\(^+\) T cells directly possibly via indirect effects mediated by other cells within PBMC. Given the importance of IL-17 in the pathogenesis of psoriasis, DMF might have been expected to inhibit IL-17 and indeed a dose dependent trend towards IL-17 inhibition was observed in sorted T cells but not PBMC. This suggested that DMF might exert a direct inhibitory effect on IL-17 production/Th17 cells. It would have been of interest to analyse T cell subsets in more detail by identifying Th1, Th17 and ex-Th17 cells using their expression of CD161 and cytokine profile. However, under the experimental conditions used the analysis of CD161 expression was unreliable. Interestingly DMF increased the frequency of proliferated IL-2\(^+\)CD4\(^+\) T cells within PBMC and not sorted CD3 T cells, which again suggests that the effect of DMF on IL-2 production was mediated indirectly. It is possible that the increase in IL-2 could be due to the ability to upregulate activation markers on APC as was described earlier in DC. CD86 is a costimulatory signal essential for T cell activation and CD28 signalling has been shown to increase IL-2 (Umlauf et al., 1995). An alternative explanation could be that since DMF induces oxidative stress, a paper showed that high ROS increased the production of IL-2 (Kaminski et al., 2010). Overall total cytokines produced by CD8\(^+\) T cells and cytokines from proliferated CD8\(^+\) T cells were not affected by DMF.

All of the other studies investigating the effects of DMF on T cell cytokine production are not directly comparable to the current study since they measured cytokines in supernatants by ELISA/multiplex assay. In agreement with the current study, a study by Lehmann et al. 2007 demonstrated that DMF inhibited IFN\(\gamma\) production by anti-CD3/anti-CD28 stimulated purified T cells. However, in contrast to the current study they also showed that DMF inhibited IL-2, IL-4, IL-5, and IL-10, suggesting that DMF inhibited both Th1 and Th2 cytokines, although Th2 cells were not investigated in the current study (Lehmann et al., 2007). The same study revealed inhibition of IL-10, IL-6 and IFN\(\gamma\) by DMF within PHA-stimulated PBMC (Lehmann et al., 2007). However, the study by Lehmann et al. 2007 used higher concentrations of up to 100 \(\mu\)M DMF compared to the current study which used up to 25 \(\mu\)M (Lehmann et al., 2007). In addition, cytokines were measured in supernatants by ELISA assay, rather than by intracellular cytokine staining in the current study. Thus, it is
likely that the inhibition of cytokines observed in the Lehmann et al. 2007 study may simply reflect DMF induced death and inhibition of proliferation rather than specific effects on cytokine production. In contrast to the latter study, a paper showed that PBMC from RRMS patients and controls treated with or without 10 μM DMF and/or anti-CD3/CD28 stimulation for 24 hr, exhibited no significant change in cytokines IFNγ, GM-CSF, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNFα as measured by multiplex assay (Diebold et al., 2017). However, it was possible that the shorter time and relatively low concentration of DMF used in this study was insufficient to observe an effect.

Overall, the data in this study revealed that at a concentration of 25 μM, DMF inhibited IFNγ producing T cells, and may have also had an effect on IL-17 producing T cells. The more extensive inhibition of cytokines observed in other studies may have been an artefact of the DMF-induced death and inhibition of proliferation that would have been reflected in cytokine concentrations in the supernatants.

Treg cells play a crucial role in preventing and controlling autoimmune disease, thus therapeutic strategies that promote Treg cells or their function would be desirable for the treatment of autoimmune diseases such as MS and psoriasis. However, it is yet not fully understood how DMF affects Treg cells in vitro. Importantly, the data in this chapter showed that DMF treatment of T cells resulted in a relative increase in the frequency of Treg cells. Interestingly the increase was significant in sorted CD3 cells, but not within PBMC. It is also possible that DMF favoured Treg cells via a direct effect on T cells that was somewhat negated by other cells within PBMC or that the lack of significance was due to low number of HC in this experiment and that the frequency of Treg cells in the presence of DMF within PBMC might have been significantly increased with higher HC. The relative increase in Treg cells prompted the investigation of the mechanism by which DMF exerted this effect, which could have been due to relatively more inhibition of Tconv cells vs Treg cells, or via by active induction of Treg cells.

It was important to determine if the observed increase in the frequency of Treg cells was due to a relative decrease in Tconv cells that could be mediated by DMF-induced oxidative stress. In order to investigate this, the antioxidant glutathione was used to reverse the oxidative stress in T cell cultures with DMF. This suggested that DMF increased the frequency of Treg cells via the induction of oxidative stress, since the effect was largely reversed in the presence of glutathione. However, additional data using other antioxidants would be needed to confirm these findings. To date other studies have not investigated the
effects of antioxidants in T cell cultures with DMF, although, it has been shown that DMF is an inducer of oxidative stress (Ghoreschi et al., 2011, Gill and Kolson, 2013).

In order to further elucidate the mechanism by which DMF increased the frequency of Treg cells, it was investigated whether the increase was due to differential effects of DMF on the viability of Treg cells vs Tconv cells. The data showed that DMF affected the viability of both Treg and Tconv cells, however, the viability of Tconv cells was reduced to a greater extent than that of Treg cells in both PBMC and sorted CD3 T cells. However, it is important to note that Treg cells within PBMC were relatively more protected from DMF induced death compared with Treg cells within sorted CD3 T cells. This suggested that perhaps other cell types within PBMC exerted a protective effect on Treg cells, possibly by enhancing their ability to withstand the oxidative stress induced by DMF. Taken together the data suggested that Treg cells have a greater capacity to withstand oxidative stress than Tconv cells and are further protected by the presence of other cell types within PBMC. Interestingly, Treg cells have been shown to have the capacity to compete with Tconv cells for cysteine uptake, and cysteine is required for the synthesis of glutathione, which in turn counteracts oxidative stress (Yan et al., 2010b). It was also shown that APC induce a cysteine pool that is readily available for T cell activation and proliferation (Edinger and Thompson, 2002). T cell activation and proliferation requires a reduced microenvironment which is provided by APC (Angelini et al., 2002). The cysteine pool that is generated by APC is required for the synthesis of glutathione, which aids the proliferation of T cells (Messina and Lawrence, 1989, Yan et al., 2010b, Suthanthiran et al., 1990). Furthermore, a study showed that DC are capable of synthesising the enzyme thioredoxin which is released into the extracellular pool and used by T cells to maintain reduced cell surface thiols which provide protection from oxidative stress (Yan et al., 2010b). Thioredoxin was also shown to counteract oxidative stress (Mougiakakos et al., 2011) similarly to glutathione (Yan et al., 2010b). Thus, in the context of the data presented in this study, it seems likely that Treg cells deplete the cysteine pool and thioredoxin produced both by T cells but also other cells in the microenvironment, thus favouring their own ability to survive oxidative stress at the expense of Tconv cells. This may therefore represent an additional mechanism by which Treg cells can exert their suppressive effects, in addition to the multiple other mechanisms that have been described previously. This may be an important Treg mechanism at sites of inflammation where oxidative stress is common. Furthermore, this favouring of Treg cells under conditions of oxidative stress may in part explain the fact that their frequency is often observed to be increased at such inflammatory sites.
Since DMF had been shown to inhibit overall proliferation of T cells, it was also important to determine whether the increase that was observed in the frequency of Treg cells was due in part to differential effects of DMF on the proliferation of Treg vs Tconv cells, in addition to their increased survival described above. Indeed, although the proliferation of both Treg cells and Tconv cells decreased in the presence of DMF, the proliferation of Tconv cells decreased more than that of Treg cells in both PBMC and sorted CD3 T cells. Since it was shown earlier that DMF inhibited the early activation of T cells, and effective activation precedes T cell proliferation, it is likely that the inhibition of T cell proliferation was a consequence of this. Furthermore, although any differential effects that DMF might have had on the early activation of Treg vs Tconv cells were not investigated in the current study, it is possible that the ability of DMF to inhibit the recruitment of PKC\(\theta\) to the TCR signalling complex could account for this (Blewett et al., 2016). Interestingly, it was shown that although activation of Tconv cells requires the recruitment of PKC\(\theta\), the activation of Treg cells does not (Zanin-Zhorov et al., 2010). This suggests that the inhibition of PKC\(\theta\) by DMF might inhibit the activation and subsequent proliferation of Tconv cells more than that of Treg cells and may account in part for the more pronounced inhibitory effect of DMF on Tconv cells that was observed in the present study.

Similarly, to the Treg vs Tconv viability data, the proliferation of both Treg and Tconv cells was reduced more in sorted CD3 T cells than in PBMC. This could possibly be explained by the increased availability of cysteine from APC within PBMC compared with sorted CD3 cells, since cysteine is needed for T cell proliferation (Messina and Lawrence, 1989, Yan et al., 2010b, Suthanthiran et al., 1990). Alternatively, it is possible the increased reducing capacity of APC could have quenched the oxidative capacity of DMF in the PBMC cultures. Since it has been shown that DC can reduce the extracellular microenvironment for T cell activation (Angelini et al., 2002), it is plausible that other APC within PBMC could reduce the oxidation caused by DMF.

Having shown that Treg cells were protected from DMF induced death and inhibition of proliferation, it was interest to investigate how such protection might be mediated. It has been shown that cell surface thiols, together with antioxidants are capable of protecting Treg cells from oxidative stress (Mougiakakos et al., 2011). Furthermore, it has been shown that Treg cells express relatively high levels of thioredoxin-1, which is an important antioxidant in maintaining reduced surface thiols (Mougiakakos et al., 2011). These studies therefore prompted the hypothesis that the ability of Treg cells to withstand DMF-induced oxidative stress as described above, might be due to increased expression of reduced thiols on their
cell surface. Indeed, the data in this study demonstrated a novel role for Treg cells in counteracting DMF induced oxidative stress. Resting Treg cells did not express high levels of reduced cell surface thiols as detected using ALM 633. However, once activated with anti-CD3/CD28, reduced thiols were upregulated on both Treg and Tconv cells, but to a much greater extent on Treg cells. This suggested that Treg cells have a greater capacity to reduce their cell surface thiols. Furthermore, the data showed that DMF did not oxidise surface thiols on Treg cells to the same extent as in Tconv cells. However, an important limitation of these experiments was that intracellular staining with FoxP3 could not be used in conjunction with ALM staining to detect thiols. Therefore, Treg cells were defined as CD4⁺CD25⁺CD127lo cells which may not have been sufficiently specific to identify Treg cells. In summary, the in vitro data indicated that DMF exerted a negative effect on T cell viability and proliferation, but that Treg cells were relatively protected from these effects, thereby accounting for the increased frequency of Treg cells that was induced by DMF.

After examining the effects of DMF in vitro it was important to determine whether DMF could exert similar effects in vivo. There are a very limited number of studies that have investigated the immunomodulatory effects of DMF in psoriasis patients. Therefore, the frequencies of T cell subsets, Treg cells and T cell cytokine production in psoriasis patients that were either untreated or treated with Fumaderm™ was determined.

Initially the effect of Fumaderm™ treatment on the frequency of memory vs naïve CD4⁺ and CD8⁺ T cells in psoriasis patients was investigated. The data showed that Fumaderm™ treated patients had a decrease in the frequency of total memory CD4⁺ T cell subsets with a trend towards a decrease in total memory CD8⁺ T cells compared with untreated psoriasis patients. There was a reciprocal increase in naïve T cells (data not shown). To date there are no comparable studies in psoriasis patients, however the results are broadly consistent with studies in RRMS patients which indicate a selective reduction in memory vs naïve T cell subsets in response to DMF treatment. A study in RRMS patients showed that after 6 months of DMF treatment, the frequency of both CD4 and CD8 memory T cells was reduced (Gross et al., 2016). This is in agreement with a study which showed that DMF reduced EM cells within both CD4⁺ and CD8⁺ T cells in RRMS patients after 3 months of treatment (Diebold et al., 2017). However, in the current study the total memory population was only decreased in CD4⁺ T cells and not in CD8⁺ T cells. Another study also showed that DMF treatment reduced the frequency of EM and CM CD4⁺ and CD8⁺ T cells with a relative increase in naïve CD4⁺ and CD8⁺ T cells (Wu et al., 2017). Similarly, a study showed that DMF reduced the frequency of CM and EM CD4⁺ and CD8⁺ T cells in both non-lymphopenic and
lymphopenic RRMS patients (Longbrake et al., 2016). The mechanism by which DMF selectively depletes memory over naïve T cells in vivo is unclear. However, in a study by Diebold et al. 2017 they showed that DMF induced cell death and oxidative stress in CD3+ T cells from RRMS patients, which could suggest that the T cell depletion observed in vivo may be due to oxidative stress induced cell death (Diebold et al., 2017). Alternatively, an in vitro study showed that DMF induced apoptosis in T cells (Ghadiri et al., 2017).

Th17 cells play a key role in the pathogenesis of psoriasis (Blauvelt, 2007). Th17 cells are characterised by their expression of both the Th17 lineage marker CD161 and their signature cytokine IL-17 (Cosmi et al., 2008). In this study CD161+CD4+ T cells, which represent all Th17 lineage cells, were reduced in the total Fumaderm™ treated patients, as well as in the low and high dose Fumaderm™ groups compared with untreated patients. IL-17+CD4+ T cells were reduced in Fumaderm™ treated psoriasis patients relative to untreated patients. Furthermore, Th17 cells more precisely defined as CD161+IL-17+CD4+ T cells were also inhibited in Fumaderm™ treated patients compared with untreated patients. CD161+IL-17+CD4+ T cells were also reduced in high dose Fumaderm™ treated patients compared with untreated patients. This data revealed the novel finding that Fumaderm™ treatment appeared to exert an inhibitory effect on Th17 cells in vivo. Given the importance of IL-17 in psoriasis as demonstrated by the success of anti-IL-17 therapy (van de Kerkhof et al., 2016, Krueger et al., 2012), this finding has important clinical relevance and suggests that the beneficial effect of Fumaderm™ may be mediated at least in part via inhibition of Th17 cells/IL-17 although there are no comparable studies to date in psoriasis patients, this data from the present study was in agreement with another that showed that IL-17-expressing CD4+ T cells were inhibited in DMF treated RRMS patients after 12 months of treatment (Montes Diaz et al., 2018) and after 18 months (Wu et al., 2017). However, a conflicting study revealed no change in CD4+IL-17+ T cells in DMF treated RRMS patients after 6 months, possibly as a result of the shorter treatment period (Gross et al., 2016). It is possible that the inhibition in IL-17 by DMF in vivo is due to a direct effect on T cells or indirectly via APC; however, the exact mechanism in which DMF inhibits proinflammatory cytokine IL-17 is still unknown.

The data is broadly consistent with the in vitro data which showed a dose dependent reduction in IL-17 producing T cells cultured in the presence of DMF. A possible mechanism whereby DMF might specifically inhibit Th17 cells is via its reported inhibition of glycolysis (Kornberg et al., 2018). DMF inhibited glycolysis via modification of cysteine molecules in GAPDH, and a paper recently published by our group has shown that Th17 cells depended upon glycolysis (Cluxton et al., 2019). IL-22 is another downstream effector cytokine for
Th17 cells which has been implicated in psoriasis. IL-22 mRNA is increased in psoriatic lesions (Liu et al., 2007a) and serum IL-22 has been shown to be higher in psoriasis patients vs controls (Caproni et al., 2009). The data from this chapter demonstrated that Fumaderm™ treated patients had significantly decreased IL-22 expressing CD4+ T cells compared with untreated patients. IL-22 was also inhibited in both the low and high dose Fumaderm™ treated groups. Another Th17 associated cytokine is GM-CSF and a significant decrease in GM-CSF expressing CD4+ T cells in Fumaderm™ treated patients compared with untreated patients was observed in this study.

Th17 cells are plastic and can switch to a phenotype that no longer produces IL-17 but produce IFNγ, these are called ex-Th17 or non-classical Th1 cells that are defined as CD161+IFNγ+IL-17+ cells (Basdeo et al., 2017, Annunziato et al., 2013). Ex-Th17 cells are polyfunctional cytokine producing cells that accumulate in the joints of RA patients, and are resistant to Treg cell suppression (Basdeo et al., 2017). However, the role of ex-Th17 cells in psoriasis has not yet been established. The current data showed that transitioning Th17/ex-Th17 cells that are characterised by expressing both IFNγ+ and IL-17+ are reduced in Fumaderm™ treated psoriasis patients as well as in the low and high dose Fumaderm™ groups compared with untreated patients. These transitioning Th17 can express both IFNγ+ and IL-17+ and are increased in synovial fluid mononucleocytes, however this result was non-significant (Basdeo et al., 2017). It is possible that these transitioning Th17 cells are polyfunctional and are resistant to Treg mediated suppression, however this is unknown in psoriasis. It is also possible that these transitioning Th17 cells also produce IL-22, however this is also unknown in psoriasis. The reduction in the frequency of IL-22 could be due to the reduced frequency of Th17, transitioning Th17 and ExTh17 cells, however this would need to be investigated further. Ex-Th17 cells were also reduced in Fumaderm™ treated patients compared with untreated patients. This data which showed that Fumaderm™ treatment was associated with reduced frequencies of both Th17 and ex-Th17 cells, suggests that ex-Th17 cells may also contribute to the pathogenesis of psoriasis.

Prior to the discovery of the role of Th17 cells in psoriasis, it was previously thought that psoriasis was a Th1 mediated disease, in which both IFNγ and TNFα were the key proinflammatory cytokines (Lew et al., 2004). The current study revealed that the frequency of total IFNγ+CD4+ T cells was reduced in Fumaderm™ treated patients compared with untreated patients. Interestingly however, there was no reduction in Th1 cells specifically defined as CD161+IFNγ+CD4+ cells. This suggested that the reduction in total IFNγ producing CD4 T cells in DMF treated patients could be derived from inhibition of IFNγ
producing exTh17 cells rather than Th1 cells. This data provides further support for the idea that DMF treatment may specifically inhibit Th17 lineage cells, which include Th17 and exTh17 cells, but not Th1 cells.

This study also investigated the effect of Fumaderm™ treatment on the production of other cytokines by both CD4+ and CD8+ T cells. TNFα is produced by both T cells and APC and is a key mediator in the progression of psoriasis (Gottlieb et al., 2005). Indeed, anti-TNFα has been shown to be an effective treatment for psoriasis and has also been shown to inhibit Th17 cells (Zaba et al., 2007). Keratinocytes express receptors for IL-17 and TNFα; and both cytokines combined have a synergistic effect in inducing an increased proinflammatory environment in psoriasis (Chiricozzi et al., 2011). In this study however, Fumaderm™ did not reduce the expression of TNFα in CD4+ or CD8+ T cells. However, TNFα expression was only measured in T cells and it was possible that DMF could inhibit its expression by APC.

Finally, the frequency of Treg cells were examined in Fumaderm™ treated psoriasis patients. It was observed that there was an increased frequency of Treg cells in Fumaderm™ treated psoriasis patients compared with those that were untreated. In addition, there was also an increase in the frequency of Treg cells in high dose Fumaderm™ treated patients. This in vivo data correlates with the in vitro data that showed increased Treg cell frequency in the presence of DMF. Thus, it is possible that the increase in Treg cells that was observed in vivo may be due to the increased capacity of Treg cells to withstand DMF induced death and inhibition of proliferation. It is also possible that the relative increase in the frequency of Treg cells is due to their ability to counteract DMF induced oxidative stress in vivo. However, further studies on the ability of Treg cells to counteract oxidative stress in vivo need to be performed. Other studies on the effect of DMF in vivo on Treg cells are conflicting and have been in RRMS rather than psoriasis patients. Two studies showed that Treg cells increased following DMF treatment in RRMS patients (Diebold et al., 2017, Gross et al., 2016). Although conflicting studies showed that DMF treatment in RRMS patients did not alter the frequencies of Treg cells, effector T cells were reduced (Longbrake et al., 2016, Wu et al., 2017). Thus, it is possible that Treg cells were relatively protected from DMF induced death in vivo in the latter studies as was described in the current study in vitro.

The appropriate balance of the Treg:Th17 axis is crucial for the maintenance of the immune system, and dysregulation of this axis is associated with autoimmune inflammation. Treg cells play a role in maintaining self-tolerance, while Th17 cells contribute to inflammation.
in psoriasis. Therefore understanding how the Treg:Th17 axis is modulated can provide an insight into therapies for psoriasis in the future. Importantly, this data in this study has demonstrated for the first time that DMF can modulate the Treg:Th17 axis in favour of regulation, since it inhibited Th17 lineage cells and increased Treg cells both \textit{in vitro} and \textit{in vivo}. This suggests that the therapeutic effect of DMF in psoriasis may be mediated at least in part, through the modulation of the Treg:Th17 axis.
Chapter 5:

General discussion and future work
5.1 General Discussion

The data in this study provided new insights into immunomodulatory therapies for both RRMS and psoriasis. Both RRMS and psoriasis are autoimmune inflammatory disorders, in which Th17 cells are thought to play a key role in the pathogenesis of these and other autoimmune and inflammatory diseases (Pittock and Lucchinetti, 2007, Kim and Krueger, 2017). On the other hand, Treg cells help to prevent autoimmunity and regulate pathogenic Th17 cells. Thus the balance between Th17 cells and Treg cells is critical in the context of inflammation and autoimmunity, and the Treg:Th17 axis has been a key therapeutic target for autoimmune and inflammatory disease. In this study the effect of vitamin D3 supplementation in CIS patients and HC and the effect of DMF in vitro and in vivo in psoriasis patients was examined. The common themes between these two studies are that firstly, autoreactive Th17 cells and an imbalance of the Treg:Th17 cell axis are thought to be involved in both RRMS and psoriasis. Secondly, both vitamin D3 and DMF are small, orally administered molecules that exert a broad range of immunomodulatory effects, however their immunomodulatory effects in humans are still poorly understood. Finally, both vitamin D3 and DMF have been used to treat both MS and psoriasis, suggesting that they may exert a common mechanism of action in the two diseases. The aim of these studies therefore was to gain a greater insight into the immunomodulatory effects of both vitamin D3 and DMF, with a particular focus on how they affect T cell subsets.

5.1.1 Modulation of Th17 and Th1 cells in MS and psoriasis

Given that both Th17 and Th1 cells are thought to play a pathogenic role in RRMS (Amedei et al., 2012, Atkins et al., 2012), and that vitamin D3 has been shown to directly and indirectly inhibit Th1 and Th17 cells, and also based on the preliminary data from a pilot study, it was hypothesised vitamin D3 supplementation in CIS patients or HC might be associated with inhibition of these T cell subsets. However, vitamin D3 supplementation had no effect on Th1 or Th17 cells or their cytokines in HC or CIS patients. This was in contrast to the findings from a small pilot study where vitamin D3 supplementation had been shown to inhibit IL-17 responses upon restimulation of T cells in vitro. It is also possible that stimulating T cells in the presence of 1, 25(OH)2D3 could have had anti-inflammatory effects on Th1 or Th17. It was also assumed that vitamin D supplementation increased the active form of vitamin D thus influencing inflammatory T cell responses. A direct comparison between the presence of 1, 25(OH)2D3 on T cells and vitamin D supplementation could have explained why there was no effect seen in the vitamin D trial. As discussed previously in Chapter 3, it is possible that issues relating to trial design or execution may have accounted
for the trial not reaching its clinical or immunological endpoints. In addition, other trials conducted since the trial presented in this study have failed to reach a consensus on whether vitamin D₃ supplementation could inhibit Th17 cells (Smolders et al., 2010, Muris et al., 2016). Therefore, the question of whether vitamin D₃ supplementation can modulate T cell responses remains unanswered.

Based on the rationale that Th17 cells have been implicated in the pathogenesis of MS, IL-12/23 p40 antibodies have been used in clinical trials to target the upstream signalling of Th17 differentiation, but they did not reduce RRMS lesions (Segal et al., 2008, Vollmer et al., 2011). However, it was recently shown in a small proof of concept trial that secukinumab reduced the number of cumulative T1 lesions in RRMS patients (Havrdova et al., 2016). This suggests that targeting Th17 cells/IL-17 in RRMS may indeed be a viable therapeutic strategy, and that if vitamin D₃ supplementation can indeed be shown to reduce Th17 responses then it may still be an option to investigate further in future.

It was previously thought that psoriasis was a Th1 mediated disease but more recently it has been shown that the IL-23/Th17 signalling pathways play a crucial role in the pathogenies of psoriasis (Kim and Krueger, 2017). The data in this study showed that the frequency of total Th17 lineage cells as well as Th17 cells and their associated cytokines were reduced in Fumaderm™ treated psoriasis patients compared to untreated psoriasis patients. This suggested that DMF has the ability to modulate Th17 lineage cells in vivo. Data in this study also demonstrated that DMF reduced the frequency of exTh17 cells and transitioning exTh17 cells and associated cytokines IL-17 and IFNγ. The data collectively suggests that not only Th17 but also exTh17 may play a role in the pathogenesis of psoriasis and targeting exTh17 as well as IL-17 cells could potentially be therapeutic over targeting IL-17 alone. Secukinumab has shown to be extremely effective in the treatment of moderate to severe psoriasis (van de Kerkhof et al., 2016). Taken together with the data in the current study, these findings suggest that the clinical efficacy of DMF in RRMS and psoriasis is likely to be mediated at least in part by its ability to inhibit Th17 and possibly exTh17 cells. However, the precise mechanism by which DMF inhibits Th17 cells remains to be elucidated.

5.1.2 Modulation of Treg cells in MS and psoriasis
It has been demonstrated that the frequency of Treg cells and their suppressive capacity were impaired in RRMS patients and in psoriatic patients (Viglietta et al., 2004, Sugiyama et al., 2005). Given the importance of Treg cells in autoimmunity, strategies to increase the frequency or suppressive function of Treg cells are desirable. A number of studies have
provided evidence to suggest that vitamin D₃ supplementation might promote Treg cells. It has been shown that high serum 25(OH)D correlated with improved Treg function (Smolders et al., 2009b), and vitamin D₃ supplementation increased the frequency of Treg cells (Prietl et al., 2010). However, the data presented in this study demonstrated that vitamin D₃ supplementation had no effect on Treg cells in HC or CIS patients. Therefore, further studies are needed to determine whether vitamin D₃ supplementation can induce Treg cells. Interestingly, strategies to administer vitamin D₃ tolerised DC to RRMS patients have been proposed (Phillips et al., 2017), and it is possible that this might prove beneficial in future. Currently approved drugs for RRMS that have been shown to promote Treg cells and/or enhance their function include IFNβ (Chen et al., 2012), GA (Haas et al., 2009) and alemtuzumab (Havari et al., 2014), suggesting that promoting Treg cells in RRMS is likely to translate to clinical benefit. Given the fact that DMF was shown to induce Treg cells in vitro and in vivo in psoriasis patients, this suggests that the clinical benefit of DMF in RRMS patients may be mediated in part via its effects on Treg cells.

It has also been shown that Treg cells are functionally and numerically deficient in psoriasis patients (Soler et al., 2013, Yang et al., 2016), which suggests that enhancing Treg cell numbers or function via therapies such as DMF would be beneficial, yet the effects of DMF on Treg cells are poorly understood. This data demonstrated that DMF increased the frequency of Treg cells in vitro and in vivo in psoriasis patients. Interestingly, this increase observed in Treg cells in vitro was due to their relatively increased capacity to counteract oxidative stress induced by DMF and this may also be the mechanism whereby Treg cells were increased in vivo. Taken together with other studies that have shown that the frequency and suppressive function of Treg cells are promoted under conditions of oxidative stress (Mougiakakos et al., 2009, Mougiakakos et al., 2011) this suggests that Treg cells are superior at maintaining their survival even under oxidative stress compared with Tconv cells and could possibly also suppress better under oxidative stress. Indeed Treg cells were shown to function better under oxidative stress in psoriatic dermatitis (Kim et al., 2014). This indicates that Treg cells can withstand high oxidative stress commonly found in inflamed sites, and this may be an important mechanism that facilitates Treg function at the sites where their suppressive capacity is most needed. On the other hand, Treg cells had higher HO-1 expression and could tolerate the oxidative stress in hypoxic tumours, suggesting that the ability of Treg cells to withstand oxidative stress may also promote the suppressive microenvironment and thereby promote tumour growth (Dey et al., 2014). Thus a more
detailed understanding of how T cell subsets are regulated by oxidative stress is important in order to target Treg dysfunction in human disease.

5.1.3 Role of vitamin D in MS and psoriasis

It has been revealed that the role of vitamin D goes beyond the regulation of calcium homeostasis and bone health. Vitamin D deficiency has been linked to several autoimmune diseases such as type I diabetes mellitus, IBD, rheumatoid arthritis, systemic lupus erythematosus, MS and psoriasis although evidence of causality is lacking. The majority of people of in Ireland and in similar latitudinal locations are insufficient or deficient in vitamin D during winter/spring (Lonergan et al., 2011). This raises the issue of whether vitamin D should be supplemented in the general population. There is no clear consensus on the RDA for vitamin D and interestingly the majority of European countries have their own specific vitamin D recommendations (Doets et al., 2008). However, these recommendations are to maintain adequate serum 25(OH)D levels to support bone health, and may not be sufficient for immunomodulatory effects (Del Valle et al., 2011).

Even though there is conflicting evidence regarding vitamin D supplementation trials in RRMS, it would be of interest to use vitamin D supplementation during pregnancy as a preventative for autoimmunity, and this might decrease the susceptibility to developing autoimmune diseases that are influenced by vitamin D status. To date there are currently no long-term studies on the effect of early exposure to vitamin D on the development of MS in susceptible individuals. Interestingly, it has been shown that HLA-DRB1*15:01 is an MS risk allele in northern Europeans and that vitamin D can influence the expression of HLA-DRB1*15:01, thereby possibly altering thymic selection in utero (Ramagopalan et al., 2009).

The utility of the much higher doses required to exert immunomodulatory effects in individuals who have developed RRMS has still not been unequivocally established, and although this study showed that the doses used were safe and resulted in increased serum 25(OH)D no clinical benefit was observed.

25(OH)D serum levels are generally low in psoriasis patients, however serum 25(OH)D levels have not been associated with disease severity (Hambly and Kirby, 2017). Interestingly topical vitamin D analogues are used to treat psoriatic lesions, and are even more effective in combination with steroids (Devaux et al., 2012). It has also been demonstrated that the loss of VDR via deletion, or mutations in CYP27B1 which prevents the ability to generate the active form of vitamin D causes hyperproliferation of keratinocytes.
(Bikle, 2011), suggesting that active vitamin D may act to constrain keratinocyte proliferation which is the hallmark of psoriasis.

5.1.4 DMF therapy in MS and psoriasis
DMF is a non-specific oral drug that has multiple mechanisms of action and currently it is not yet fully understood how DMF exerts its effects in vivo. DMF is currently used to treat both MS and psoriasis. However, despite the relatively recent introduction of DMF as a treatment for MS, there are many more studies on the effect of DMF in MS compared with psoriasis. This limited availability of studies in psoriasis is a result of the non-traditional way in which the drug was introduced in Germany. Interestingly, the rationale for using DMF in MS came from psoriasis patients who also had MS, and treatment of their psoriasis resulted in improvement of their MS. This suggests that DMF may target common pathogenic processes in both psoriasis and MS, via similar mechanism/s of action. Although multiple mechanisms of action in variety of cell types has been described for DMF, they all would appear to arise from the ability of DMF to act as an electrophile and to modify thiol groups. These mechanisms include the induction of the Nrf2 antioxidant pathway via modification of cysteines in Keap1, inhibition of NF-κB, PKCθ and GAPDH, also via modification of cysteines within these proteins and the activation HCA2 signalling pathway. This seems paradoxical, since while DMF has been shown to exert antioxidant and anti-inflammatory effects it also induces significant oxidative stress within cells. Interestingly, the data in this study provides new information as to how the oxidative stress induced by DMF can favour Treg cells over Tconv cells.

It is not yet clear which of the multiple mechanisms described for DMF are the most important in mediating its effects in RRMS and psoriasis. Until recently, induction of the Nrf2 pathway had been considered the key anti-inflammatory mechanism of DMF, however recently DMF was shown to still exert therapeutic effects in Nrf2 deficient mice suggesting that this is not the case (Gillard et al., 2015). Also, importantly data from mouse models do not always translate and therefore mechanistic studies in humans are essential. As discussed above, T cells are thought to play a key role of both MS and psoriasis, therefore it seems likely that the ability of DMF to inhibit Th17 cells and increase Treg cells as shown in this study may be of key importance in mediating its therapeutic effects in both diseases.

The induction of oxidative stress and T cell apoptosis appears to play an integral role in the therapeutic effect of DMF. In vitro, increasing doses of DMF have a dose dependent effect in inducing T cell death, however it is difficult to determine the physiologically relevant
dose of DMF. Indeed a limitation of studying DMF and attempting to translate in vitro findings, is that it is not easily detected in vivo as it metabolises quite rapidly into MMF (Rostami-Yazdi et al., 2010, Dibbert et al., 2013). Therefore, it is difficult to determine whether the concentrations used in vitro are physiologically relevant in terms of those used in vivo in RRMS or psoriasis patients.

The induction of apoptosis by DMF leads to lymphopenia in some patients, which has been associated with the development of PML in both RRMS and psoriasis patients (Ermis et al., 2013b, Ermis et al., 2013a, van Oosten et al., 2013, Rosenkranz et al., 2015). Given the seriousness of PML, would be of great interest to have a better understanding of how DMF exerts its effects and to potentially stratify patients for different doses of DMF. Currently, the Fumaderm™ dose administered to psoriasis patients is determined empirically, a low dose of 30 mg per day is administered with increasing doses for three wk up to 120 mg. Interestingly, the low dose is highly effective in some psoriasis patients, whereas others require a much higher dose and the reasons for this are not understood. Once the psoriasis has cleared, the dose is then decreased to the lowest dose at which the psoriasis remains clear (Mrowietz et al., 2018). In this study few differences in the frequencies of T cell subsets were seen between the low and high dose groups, suggesting that similar immunomodulatory effects are exerted in both dosage groups. This would therefore suggest that either the high dose group has less effective absorption/metabolism of DMF, or that their cells are more resistant to its effects. In RRMS the dose regimens for Tecfidera are low dose 120 mg and a high dose 240 mg. The low dose is usually administered first in order to reduce the impact of side effects, and then increased to 240 mg. High dose Tecfidera™ can be reduced if patients cannot tolerate the recommended maintenance dose of 240 mg. Tecfidera is seen to be more effective than other first line therapies for RRMS, and Tecfidera™ 240 mg reduced the number of new gadolinium enhanced lesions in RRMS by 85% (Kappos et al., 2012).

It has been shown that severe lymphopenia <500 cells/µl is associated with the development of PML (Longbrake and Cross, 2015, van Oosten et al., 2013). However, it has been documented that a case of PML was reported in a non-severe lymphopenic patient that had 792 cells/µl (Nieuwkamp et al., 2015), indicating that overall lymphocyte counts may not be the key factor that determines susceptibility to PML. Since preferential loss of CD8+ T cells in RRMS patients following DMF treatment has been demonstrated (Ghadiri et al., 2017), and CD8+ T cells are critical for defending against JCV, it has been suggested that continued low CD8+ T cell counts may allow re-activation of the JCV and development of PML (Du Pasquier et al., 2004, Spencer et al., 2015a, Spinelli and Bagert, 2017).
It is not yet understood why a higher dose of DMF is required in RRMS compared with psoriasis or why some patients are more susceptible to lymphopenia. It is possible that some patients’ immune cells can tolerate oxidative stress better than others, or that the drug is absorbed or metabolised more efficiently than others. There is a need to understand this better in order to individualise treatment. Careful monitoring of patients for DMF induced lymphopenia, specifically CD8+ T cells could prevent future PML cases in JCV+ individuals, since PML is caused by the activation of the JCV when the immune system is compromised. It was shown that the anti-JCV antibody index prior to the use of DMT was higher in those that subsequently developed PML (Plavina et al., 2014). This would suggest that measuring anti-JCV antibodies could potentially be a method of screening patients that are more susceptible to PML.

5.1.5 Balancing safety and efficacy of therapies for autoimmune disease

Any therapy can be associated with undesirable side effects and therefore it is important to consider both the efficacy and risk associated with therapy. It may be acceptable to accept higher risk if disease is deemed to be more serious, or if other therapies have failed. For example given the progressive and disabling nature of RRMS, it may be necessary to use drugs with higher efficacy even though risks are more serious, whereas such risks may not be acceptable to clinicians or patients in the case of psoriasis. DMF is a first line therapy for moderate to severe psoriasis, and cases of PML have been reported (Ermis et al., 2013a), which have caused great concern amongst dermatologists. This possibility of PML, even though cases are very rare, may restrict the use of DMF in psoriasis even though overall it is seen as a valuable therapeutic tool. Therefore, it is important to understand the mechanism of action of DMF and how this relates to its leukopenia inducing effects. The data in this study suggests that the ability of DMF to induce T cell inhibition/apoptosis may be integral to its therapeutic effects mediated by T cells, and therefore successful therapy with DMF is likely to require at least a degree of T cell death. Interestingly, although lymphopenia is a risk factor for the development of PML (van Oosten et al., 2013, Rosenkranz et al., 2015), PML has been described in cases where lymphopenia was not severe, raising the question of whether selective depletion of specific immune subsets confers risk of developing PML. A better understanding of these mechanisms will aid in the introduction of screening for patients to avoid severe cases of PML in both psoriasis and MS. For example, future studies could examine the effect of DMF therapy on JCV-specific T cell responses and also attempt to predict which patients might be most susceptible to DMF-induced leukopenia.
The recent induction of DMF as the only oral first line therapy for RRMS is a valuable addition since it has been shown to be more effective than both IFNβ and GA, which have the added disadvantage of being injectable. However, DMF therapy in RRMS patients has also been shown to cause PML in rare cases, which has raised concerns and could lead to its use being restricted to the second line. Indeed, natalizumab, which was originally a first line therapy, was shown to induce PML in some patients and it is now restricted to second line use. Therefore, there is still an unmet need for more safe and effective therapies for MS.

The obvious advantage of vitamin D₃ as a potential immunomodulatory drug for RRMS/CIS is its safety profile since even high doses are well tolerated. However, as discussed above there is still no convincing evidence from the current study or others, of the clinical efficacy of vitamin D₃ in RRMS or CIS. The lack of evidence could indicate that vitamin D₃ does not in fact exert the necessary immunomodulatory effects, or it is possible that the trials to date have not been sufficiently robust. An obvious limitation to studying the role of a generic compound such as vitamin D₃ in a clinical trial setting is that these studies are generally investigator led trials with relatively limited resources. Trial design and execution, including trial duration and group numbers, plays a critical role in the outcome of clinical trials, however, this can be restricted to by resources and lack of clinical trial experience. Since our study showed that vitamin D₃ had no clinical benefit in CIS patients it is possible that vitamin D₃ could be beneficial as an add-on therapy. It is also possible that in genetically susceptible individuals that vitamin D₃ supplementation could serve as a preventative for the development of MS. However further studies will be required to investigate this.

5.1.6 Conclusions
This work has provided insights into the immunomodulatory therapies of autoimmune disease, in vitro, in vivo and in a clinical trial setting. This study demonstrated the overall 10,000 IU of vitamin D₃ is safe and tolerable in HC and CIS patients and increased serum 25(OH)D, however primary endpoints were not met. The study also did not show any immunomodulatory effects on T cell responses. Additionally, in vitro studies using DMF revealed that it increases the relative frequency of Treg cells and demonstrated that Treg cells have an advantage through their ability to counteract DMF induced oxidative stress. This increased frequency in Treg cells was also observed in Fumaderm™ treated psoriasis patients in addition to a relative in the frequency of Th17 lineage cells. Thus this study showed for the first time that DMF can modulate the Treg:Th17 axis in vitro and in vivo. A summary of these findings are outlined in Fig 5.1
Figure 5.1 Regulation of the Treg:Th17 axis by DMF in vitro and in vivo

DMF in vitro inhibits proliferation, reduced viability and oxidises surface thiols in both Treg and Tconv cells. However, Treg cells had a higher capacity to upregulate reduced thiols compared with Tconv cells, making Treg cells more resistant to DMF induced death and inhibition of proliferation compared with Tconv cells (A). Untreated psoriasis patients have a dysregulated Treg:Th17 axis (B). In Fumaderm™ treated psoriasis patients the Treg:Th17 cell axis was modulated in favour of Treg cells, with a relative decrease in Th17 and exTh17 cells and an increase in Treg cells (C).
5.2 Future work

The findings from this study could be expanded further as outlined below:

5.2.1 Future vitamin D₃ supplementation studies

It remains unclear as to whether vitamin D₃ supplementation has any effects on the clinical outcomes in CIS patients or modulates T cell responses in either CIS patients or HC. Although a follow up trial at SVUH is unlikely due to lack of funding, possible future trials could further address the effects of vitamin D₃ on T cell responses in CIS patients. It would of interest to increase the duration of the study, as 24 wk might not be a sufficient duration to observe the possible clinical and immunomodulatory effects of vitamin D₃ supplementation. In addition, increasing the number of participants in both CIS and HC groups might increase the chances of observing an effect. It would also be of interest to recruit RRMS patients and HC in addition to CIS patients. This would allow for a direct comparison between all three groups. It is possible that a modified trial design would reveal any possible effects of vitamin D₃ on T cell responses and on annual relapse rate/progression/MRI lesions.

5.2.2 Further investigation of antioxidant capacity of Treg vs Tconv cells

This study showed that, relative to Tconv cells, Treg cells could better maintain their survival and proliferation in the presence of DMF. This study also demonstrated that the maintenance of survival observed in Treg cells in the presence of DMF could be due to the increased reduced surface thiols on Treg cells compared with Tconv. However, a limitation of the current study into effect of DMF on surface thiols was that Treg cells were defined as CD4⁺CD25⁺CD127lo since Foxp3 staining could not be used in conjunction with the ALM633 stain. This definition of Treg cells is not ideal when comparing to Tconv cells that are stimulated for 5 days, since activated Tconv cells may have been incorrectly included in the Treg cell gate. Thus ideally, the effect of DMF on cell surface thiols on FACS sorted Treg and Tconv populations would be examined. The populations would be sorted, stimulated in vitro for 5 days followed by ALM633 staining. This would then unequivocally confirm that activated Treg cells defined as CD4⁺CD25⁺CD127loFoxp3⁺ express higher cell surface thiols compared with activated Tconv cells.

A study has shown that Treg have increased thioredoxin-1 and this enhances the capacity of Treg cells to counteract oxidative stress (Mougiakakos et al., 2011). In light of this, investigating the levels of thioredoxin and glutathione in Treg vs Tconv cells both in vitro and in Fumaderm™ treated patients would be informative. Comparing Treg cells from the
peripheral blood vs Treg cells from psoriatic lesions could also demonstrate that Treg cells from different locations could possibly have altered antioxidant capacity.

5.2.3 Does DMF exert differential effects on the activation of Treg cells vs Tconv cells via PKCθ?

The data in this study indicated that DMF inhibited the early activation of the total T cell population and furthermore, DMF was shown to inhibit the proliferation of Tconv cells to a greater extent than that of Treg cells. This suggests that DMF may exert differential effects on the activation of Treg vs Tconv cells however this question was not addressed in the current study. Recruitment of PKCθ to the TCR signalling complex is essential for T cell activation, and DMF has been shown to inhibit PKCθ signalling in T cells (Blewett et al., 2016). Interestingly however, a separate study indicated that the PKCθ inhibitor sotrastaurin prevented T cell activation and proinflammatory cytokine production, but preserved Treg cell activation and suppressor function (He et al., 2014) Taken together, these studies suggest that since Tconv cells have a greater requirement for PKCθ for their activation, that PKCθ inhibition by DMF may inhibit the activation of Tconv cells more than that of Treg cells. Therefore, examining the effect of DMF on PKCθ activation/recruitment in both Treg and Tconv cells could potentially shed light on the mechanism by which Treg cells are favoured in DMF treated cells in vitro and in Fumaderm™ treated patients.
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