

Central and peripheral characterisation of TLR7/8 and endocannabinoid signalling in multiple sclerosis



Trinity College Dublin

Coláiste na Tríonóide, Baile Átha Cliath

The University of Dublin

Thesis submitted to the University of Dublin, Trinity
College for the degree of Masters of Science

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April 2022

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Declaration of authorship

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Dr John-Mark Fitzpatrick contributed to data in Fig. 4.1, 4.2, 4.3, 4.4, 4.5, 4.6 and 4.7 (responsible for drawing blood, isolating plasma); Fig. 4.8 and 4.9 (isolated PBMCs from whole blood); Fig. 7.1 (assessed *CNR1/2* mRNA expression in PBMCs isolated from pwMS and HC cases); Fig. 7.6 (assessment of samples of whole blood via Sysmex haematology analyser); Table 4 and Table 5 (gathered patient data and medication information at time of blood draw); Table 6 (assessment of samples of whole blood via Sysmex haematology analyser following blood draw).

Dr Carol Murray and Dr Eva Jiminez-Mateos contributed to data in Fig. 5.4, 5.5, 5.6, 5.7 and 5.8.

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Abstract

Toll-like receptors (TLRs) are a class of pattern-recognition receptors (PRRs) involved in initiating an immune response to infection or endogenous damage. These receptors are expressed on cells of both the innate and adaptive immune system and recognise conserved pathogenic motifs of bacterial, fungal and viral origins, as well as endogenous danger signals. The activation of TLRs results in downstream activation of intermediate adaptor proteins which culminate in the expression of a variety of cytokines, chemokines and interferons (IFNs), as well as priming of a specific adaptive immune response. A wide body of literature indicates that TLRs have roles to play in the pathophysiology of several diseases. Indeed, many studies have implicated TLR signalling in the pathophysiology of multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). Endosomal TLRs (comprising TLR3, TLR7, TLR8 and TLR9) play a pivotal role in the recognition of viruses as well as clearing of viral infection. The Epstein-Barr virus (EBV) has strong association with the risk of developing MS. Thus dysregulation of viral TLRs may prove key in the pathophysiology of MS. Indeed, our laboratory has previously identified key roles for both viral TLRs (TLR3) and bacterial TLRs (TLR4) in immune cells associated with MS. Thus, we sought to assess the potential functional role of TLR7 and TLR8, two closely-related endosomal TLRs, in the pathophysiology of MS, both centrally and peripherally. Both TLR7 and TLR8 signal through the myeloid differentiation primary response protein 88 (MyD88) adaptor, which has been shown to be instrumental in the development of EAE. Thus targeting TLR7/8 signalling may prove beneficial in MS, and offer insight on the role(s) of endosomal TLRs in MS pathogenesis.

Phytocannabinoids (pCBs) are cannabinoid compounds isolated from the dioecious plant *Cannabis L. sativa*. The use of medicinal cannabis has spanned many centuries in the treatment of a plethora of ailments. Indeed, the pCB Δ^9 -tetrahydrocannabinol (THC), has been shown to exert neuroprotective and anti-inflammatory properties in the context of neurodegenerative disease. This is likely a result of its actions through the cannabinoid receptor 1 (CB₁) that is present at high levels in the brain. THC however, has also been associated with euphoria and psychosis, and is responsible for the 'high' associated with recreational cannabis use. Cannabidiol (CBD), another major pCB isolated from the cannabis plant, has shown no euphoric or psychotic symptoms, and furthermore can mitigate the psychoactive side effects associated with THC. CBD possesses neuroprotective, as well as anti-inflammatory, propensity. Indeed, previous experiments in our laboratory have shown that pre-treatment of human macrophages

with CBD can inhibit TLR3/4-dependent induction of inflammatory cytokines and chemokines. TLR4 classically signals via the MyD88 pathway, while TLR3 signals via the TIR-domain containing adapter-inducing interferon- β (TRIF)-dependent pathway, independent of MyD88. TLR4 can also signal via TRIF. Little evidence currently exists regarding the impact of CBD on MyD88-dependent endosomal TLR signalling, particularly in inflammatory diseases such as MS. Hence, a key goal of this study was to determine if endosomal TLR signalling via MyD88 is targeted by CBD.

This project first set out to characterise TLR7 and TLR8 inflammatory signalling pathways in THP-1 macrophages. Data presented herein indicates that TLR7/8-induced inflammatory signalling pathways are operative in THP-1 macrophages, as indicated by induction of C-X-C motif chemokine ligand 10 (CXCL10) and tumor necrosis factor alpha (TNF- α). However, the TLR7/8 agonist CL075 failed to induce production of the type I IFN, IFN- β , in THP-1 macrophages. Data presented herein also indicates that treatment of THP-1 macrophages with CL075 promoted an increase in the expression of *TLR8* mRNA. Components of the endocannabinoid system (eCS) were also identified in THP-1 macrophages, and our findings indicate that TLR7/8 activation has the proclivity to alter the expression profile of components of the eCS in macrophages. CBD treatment did not impact TLR7/8-induced pro-inflammatory signalling, or impact on expression levels of the eCS, in macrophages. This suggests that CBD does not target inflammatory signalling via the endosomal MyD88-dependent TLR signalling pathway in THP-1 macrophages.

In this study we also set out to determine if the expression profile of endosomal TLR7 and TLR8, in addition to components of the eCS, are altered in immune cells and brain tissue in MS. For primary immune cell analysis informed consent was obtained from each participant and the study received ethical approval from Beaumont Hospital Ethics and the Faculty of Health Sciences Research Ethics Committee, TCD. Post-mortem brain samples were provided by the UK MS Society Tissue Bank and were collected following informed consent by the donors via a prospective donor scheme according to Ethics committee approval. We characterised the expression profile of TLR7 and TLR8, and components of the eCS, including cannabinoid receptors (CB_{1/2}), FAAH (responsible for hydrolysing anandamide) and MAGL (hydrolyses 2-AG), in peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers and people with MS, and in post-mortem human cortical brain samples from primary progressive MS, secondary progressive MS, and non-MS control cases. Our findings indicate that TLR7/8, and the eCS, are expressed

in immune and CNS tissue, and provide evidence of alterations in TLR7/8 expression, and the eCS, in the cortex of MS cases.

Overall, this study indicates a role for endosomal TLR7/8 in central pathophysiology of MS and this may impact signalling via the eCS. In addition, data presented in this project suggest that CBD does not impact classical MyD88-dependent signalling via TLR7/8. This may have implications in terms of the cellular effects of cannabinoid therapeutics in MS.

Acknowledgements

There are so many people to thank. Of course a massive thank you to my supervisor, Eric Downer, for all the advice and guidance he gave me during the course of this project.

To Noreen, for all her patience and your kindness to me over the past two years. Thanks to the Physiology Department as well, you've all been fantastic, especially Aiden for all your help with ordering. I must also thank the consultant neurologist involved in this project, Lisa Costelloe for her assistance with patient recruitment and for provision of EDSS scores. My deepest gratitude also goes to the study participants themselves. Without you this research would not have been possible. A big thank you has to go out to the Fearon lab as well, for all of their help and support during my time in the laboratory!

To Aaron, for listening to me, even when you really, really didn't want to!

The biggest thank you has to go out to my parents, for their unwavering faith in me.

Publications

Peer-reviewed published papers:

1. Pereira, SR., **Hackett, B.**, O Driscoll, DN., Cui Sun, M. and Downer, EJ. (2021) Cannabidiol modulation of oxidative stress and signalling. *J.Neuronal Signal.* 5 NS20200080. DOI: 10.1042/NS20200080.

Conference publications:

1. Neuroscience Ireland Conference 2021: John-Mark Fitzpatrick, **Becky Hackett**, Lisa Costelloe, William Hind and Eric J. Downer: Botanically-derived purified cannabidiol and D⁹-tetrahydrocannabinol, and their 1:1 combination, modulate toll-like receptor 3 and 4 signalling in immune cells from people with multiple sclerosis.
2. Neuroscience Ireland Conference, 2021: **Becky Hackett**, John-Mark Fitzpatrick, Carol L. Murray, Noreen T. Boyle, Lisa Costelloe and Eric J. Downer: Central and peripheral characterisation of TLR7/8 and endocannabinoid signalling in immune cells and CNS tissue with relevance to multiple sclerosis.
3. Neuroscience Ireland Conference, 2021: Melody Cui Sun, **Becky Hackett**, Carol L. Murray, Noreen T. Boyle, Siew Mei Yap, Richard J. Magee, Yvonne M. Nolan and Eric J. Downer: Profiling microglial activation and mitogen activated protein kinases phosphatase (MKP)-1 expression in normal appearing white matter and multiple sclerosis cortical lesions.

Courses

1. Research Integrity module (10 ECTs)
2. Tangent's Creative Thinking and Innovation module (30 ECTs)
3. Venepuncture phlebotomy course completed

List of abbreviations

APC - antigen presenting cell

BBB - blood brain barrier

CAL - chronic active lesion

CAM - cell adhesion molecule

CB1 - cannabinoid receptor 1

CB2 - cannabinoid receptor 2

CBC - cannabichrome

CBD cannabidiol

CBDA - cannabidiolic acid

CBDV - cannabidivarin

CBG - cannabigerol

CBN - cannabinol

CCL - C-C motif chemokine ligand

cDNA - complementary DNA

CLR - C-type lectin receptor

CNS - central nervous system

CNR1 - cannabinoid receptor 1 (specifically referring to mRNA expression in this project)

CNR2 - cannabinoid receptor2 (specifically referring to mRNA expression in this project)

COX-2 - cyclooxygenase-2

CpG ODNs - CpG oligodeoxynucleotides

CRP - C reactive protein

CSF -cerebrospinal fluid

Δ CT - delta cycle threshold

CT -cycle threshold

CXCL10 - C-X-C motif chemokine ligand 10

DC - dendritic cell

DMSO - dimethyl sulfoxide

DMT - disease-modifying therapy

DNA - deoxyribonucleic acid

EAE - experimental autoimmune encephalomyelitis

EBV - epstein-barr virus

EBVNA1 - epstein barr virus nuclear antigen 1

eCB - endocannabinoid

eCS - endocannabinoid system

EDSS - expanded disability status scale

ELISA - enzyme-linked immunosorbant assay

FAAH - fatty acid amide hydrolase

FBS - fetal bovine serum

GA - glatiramer acetate

GM - grey matter

GPCR - g-protein-coupled receptor

GPR55 - g-protein coupled receptor 55

HC - healthy control

HCMV - human cytomegalovirus

HCT - haematocrit

HGB - haemoglobin

HHV-6 - human herpesvirus 6

HLA - human leukocyte antigen

IFN - interferon

IKK α - IKappaB kinase-alpha

IL - interleukin

iNOS - inducible nitric oxide synthase

IRAK - interleukin-1 receptor associated kinase

IRF - interferon regulatory factor

LFB - Luxol Fast Blue

LPS - lipopolysaccharide

LRR - leucine rich repeat

LTA - lipoteichoic acid

LYM - lymphocyte number

MAGL - monoacylglycerol lipase

MAIT cells - mucosa-associated invariant T cells

MAL - MyD88-adaptor-like protein

MAPK - mitogen-activated protein kinase

MCH - mean corpuscular haemoglobin

MCHC - mean corpuscular haemoglobin concentration

M-CSF - macrophage colony-stimulating factor

MCV - mean corpuscular volume

mDC - myeloid dendritic cells

MHC - major histocompatibility complex

MIP-1- α - macrophage inflammatory protein 1-alpha

MMP - matrix metalloproteinase

MOG - myelin oligodendrocyte glycoprotein

MS - multiple sclerosis

MSQoL- MS Quality of Life

MTT - thiazolyl blue tetrazolium bromide

MV - measles virus

MXD - mixed cell number

MyD88 - myeloid differentiation primary response 88

NAWM - normal-appearing white matter

NAGM - normal-appearing grey matter

NED - N-1-naphylethylenediamene dihydrochloride

NEU - neutrophil count

NF- κ B - nuclear factor kappa-light-chain-enhancer of activated B cells

NLRs - NOD-like receptors

NO - nitric oxide

oxLDLs - oxidised low density lipoproteins

p38 MAPK - p38 mitogen-activated protein kinase

PBS - phosphate buffered saline

pCB - phytocannabinoid

PBMCs - peripheral blood mononuclear cells

PCR - polymerase chain reaction

PD – Parkinson’s disease

pDC - plasmacytoid dendritic cells

PEA - palmitoylethanolamide

PLT - platelet number

PMA - phorbol myristate acetate

PML - progressive multifocal leukoencephalopathy

Poly I:C - polyinosinic:polycytidylic acid

PPAR - peroxisome proliferator-activated receptors

PPMS -primary progressive MS

PRD - positive regulatory domain

pwMS - people with multiple sclerosis

pwPPMS - people with primary progressive multiple sclerosis

pwSPMS - people with secondary progressive multiple sclerosis

RANTES - Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted

RBC - red blood cell

RLR - RIG-I like receptor

RNA - ribonucleic acid

ROS - reactive oxygen species

RRMS -relapsing-remitting MS

rRNA - ribosomal ribonucleic acid

RT-qPCR - quantitative real-time polymerase chain reaction

SEM - standard error of the mean

SNP - single nucleotide polymorphism

SPMS - secondary progressive MS

ssRNA - single-stranded ribonucleic acid

T1D - type 1 diabetes

THC - Δ^9 -tetrahydrocannabinol

THCA – tetrahydrocannabinolic acid

TIR - Toll-IL-1 receptor

TLR - toll-like receptor

TMB - tetramethylbenzidine

TMEV-IDD - Theiler's murine encephalomyelitis virus-induced demyelinating disease

TNF - tumor necrosis actor

TRAF - tumor necrosis factor receptor-associated factor

TRAM - TRIF-related adaptor molecule

Treg - regulatory T cell

TRIF - TIR-domain-containing adapter-inducing interferon- β

TRPV1 - transient receptor potential cation channel subfamily V receptor 1

WBC - white blood cell

WM - white matter

WT - wild type

YB-1 - Y-box protein-1

ZO-1 - zonula occludens 1

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Chapter 1 Introduction

Introduction

1.1 Innate and adaptive immunity

The immune system is a complicated and multi-faceted response mechanism to injury and infection. Two distinct branches of the immune system exist in mammals, the innate and adaptive immune systems. The innate immune system, initially thought to be a simplistic and non-specific first-line of protection against infection, was initially disregarded by the research community. However, more recently it has come to light as an extremely important aspect of immunity that is not only capable of acting in a specific manner, but also has a key role in priming and informing cells of adaptive immunity. Innate immunity has sophisticated mechanisms of identifying and defending against pathogens to enhance adaptive immunity. Cells of the innate immune system contain pattern-recognition receptors (PRRs) on their surface, which are capable of binding conserved peptide sequences/hallmarks of viral, bacterial and(or) fungal infection known as damage/pathogen-associated molecular pattern (DAMPs/PAMPs), and thus initiate an immune response (1). Many cells of the innate immune system circulate in the periphery and include monocytes, macrophages, dendritic cells (DCs) and granulocytes (eosinophils, basophils, NK cells, mast cells and neutrophils). Cells of the adaptive immune system consist mainly of B and T lymphocytes. B cells are capable of becoming antibody-secreting cells and memory cells. In addition, B cells are deemed professional antigen-presenting cells (APCs) as a result of their ability to present antigen to T cells in the context of the major-histocompatibility complex class II (MHC II), thus inducing T cell activation and differentiation. T cells, when activated, can differentiate into one of several classes of T cells, including Th1, Th2, Th17, regulatory T cells (Treg) and CD8⁺ cytotoxic T cells (2). Such adaptive cells recognise specific pathogenic antigens and stimulate an appropriate response via release of several cytokines and chemokines to activate different factors and pathways to polarise the immune system towards a specific immune response. More recently, PRRs have been shown to have a significant impact on influencing adaptive immunity, an idea first put forward by Charles Janeway in 1989 (3). Thus far, several classes of PRRs have been discovered, with a number of distinct families of PRRs identified in mammals. These include the Toll-like receptor (TLR) family, the NOD-like receptors (NLRs), C-Type Lectin receptors (CLRs) and RIG-I like receptors (RLRs) (4). The TLR family is the primary focus of this thesis.

1.2 TLRs and TLR signalling

The TLRs were the first class of PRRs to be discovered and thus far are the most well-characterised. To date, 10 functional TLRs have been identified in humans (TLR1-10), with 12 TLRs identified in mice (5). TLRs are present in many different cell types of the immune system, including innate immune cells such as DCs, as well as non-immune cells such as epithelial cells (5). TLRs are also expressed on T cells and B cells (6, 7). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are present on the cell membrane, while TLR3, TLR7, TLR8 and TLR9 are expressed within the endosome of the cell. TLRs play an essential role in innate immunity and its ability to inform the adaptive immune response to combat infection and damage. Their discovery followed the identification of the interleukin (IL)-1R receptor (8) that shares sequence homology with the *Toll* receptor initially identified in *Drosophila Melanogaster* (9). The *Toll* receptor, initially believed only to have roles in embryonic development (10), was found to act through proteins with close homology to the transcription factor, nuclear factor (NF)- κ B (11). It was then discovered to play a role in immunity via production of antimicrobial peptides in response to infection (12, 13). Thus it was speculated that such *Toll* homologues played a part in the initiation of the immune response in humans. This was verified when a human *Toll* homologue, now known as TLR4, was demonstrated to activate NF- κ B and ultimately result in T cell activation (14). This was the first clear instance of innate immunity initiating adaptive immunity in humans.

TLRs are type I transmembrane receptors and share a degree of structural homology. Indeed, three structures are common to all TLRs. Typically, TLRs consist of an extracellular domain rich in leucine-rich-repeats (LRRs); the motif is generally 24 residues in length, interspersed with hydrophobic residues and forms part of the ligand binding domain. The LRRs of TLRs tend to conform to the shape of a horseshoe (15). All TLRs also have a cytoplasmic Toll-IL-1 receptor (TIR) signalling domain (16), and a helical transmembrane domain (17). The mechanism(s) by which ligands bind to TLRs has not been fully elucidated. TLRs respond to both distinct and overlapping ligands via dimerization (homodimerisation or heterodimerisation) (18). Some members of the TLR family such as TLR1, can only function when the receptor dimerises with the TLR2 receptor chain (18). Other TLRs can form both heterodimers and homodimers, dimerising with a separate TLR, or with another copy of the same TLR, to facilitate signal transduction.

TLRs recognise a wide variety of conserved peptides from microorganisms. For example, TLR4 has evolved to recognise and respond mainly to lipopolysaccharide (LPS) of gram-negative bacteria (19-21), as well as heat shock protein hsp60 (22). TLR1 acts to form a heterodimer with TLR2 to recognise gram negative bacterial PAMPs such as peptidoglycan (23). The main ligand for TLR2 is peptidoglycan (24). TLR2 also recognises a functional equivalent of LPS in gram positive bacteria known as lipoteichoic acid (24, 25), in addition to triacyl lipopeptides (26) and the fungal ligand zymosan (27), among others (28). Recently it was determined that TLR2 was in fact necessary for LPS-induced TLR4 signalling (29). TLR5 recognises the flagellin protein from bacteria (30). TLR3, TLR7, TLR8 and TLR9 are viral TLRs and respond to viral DNA or RNA. Viral TLRs are also known as endosomal TLRs and are present on the surface of endosomal vesicles within the cytosol of mammalian cells. Viral TLRs can act to induce proinflammatory cytokines, but the main class of proteins they induce are interferons (IFNs), which are anti-viral peptides (31). The induction of IFNs activate the Janus kinase/signal transducers activators of transcription (JAK/STAT) pathways (32), thus activating a multitude of IFN-stimulated genes (33, 34) that play a role in anti-viral immunity. TLR3 recognises double stranded (ds)RNA facilitating its detection of a multitude of viruses (35). Upon recognition of dsRNA, TLR3 signals through the TIR-domain containing adapter-inducing interferon- β (TRIF) adaptor to stimulate a variety of IFNs, in addition to inflammatory cytokines and chemokines, via NF- κ B and IFN regulatory factor (IRF)3 (35-37). Similarly, TLR4 can signal through TRIF via a bridging adaptor (38). Both TLR7 and TLR8 are present within the endosomal compartments of the cell. TLR7 predominantly promotes signalling to produce type I IFNs and the expression of pro-inflammatory cytokines (39, 40). TLR8 has a similar structure to TLR7, however TLR8 is polarised towards the induction of a more robust inflammatory response (41). Akin to TLR7, TLR8 also recognises single stranded (ss)RNA viruses within the endosome. In response to stimulation by its ligand, TLR8 induces the production of cytokines such as IL-12 and tumor necrosis factor alpha (TNF- α), with little induction of IFNs, in contrast to TLR7 (41, 42). Classically, TLR9 is activated by unmethylated CpG dinucleotide motifs common to viruses and bacteria (43, 44). TLR9 is also sensitive to stimulation via CpG oligodeoxynucleotides (ODNs), and can be stimulated via three separate classes of synthetic CpG ODNs, with each eliciting a distinct cytokine profile from separate immune cell populations (45).

To date, the full mechanism of ligand-binding to TLRs has not been elucidated, however it is thought that ligand binding triggers a conformational change in the receptor whereby the TIR domains dimerise, allowing TIR domain-containing adaptor proteins, such as the myeloid differentiation primary response protein 88 (MyD88), to interact with the dimerised TLR via the conserved TIR domain (15). This induces downstream activation of transcription factors NF- κ B and IRFs, with subsequent activation of the mitogen-activated protein (MAP) kinase family members (46). Such signalling results in the production of cytokines, DC maturation, T cell activation and cell proliferation (16, 47). MyD88 attracts the interleukin-1 receptor-associated kinase (IRAK) family of proteins via its death domain into a complex which becomes activated as a result of both cross and autophosphorylation, followed by ubiquitination. These interactions result in the activation of NF- κ B (48). Both TLR2 and TLR4 signalling require the MyD88 adaptor-like (MAL) protein to recruit MyD88 and activate NF- κ B (49, 50).

All TLRs act through the MyD88 signalling pathway, apart from TLR3, which signals via the MyD88-independent signalling pathway involving TRIF, allowing the subsequent activation of IRF3 and induction of type I IFNs, in addition to NF- κ B (51). TLR4 can also signal through this MyD88-independent pathway, with the adaptor protein TRIF-related adaptor molecule (TRAM) acting as a bridge to recruit TRIF and activate IRFs (52, 53). Activation of TLRs present on immune cells results in downstream induction of both inflammatory and anti-inflammatory cytokines to play a role in innate defence. TLRs activated on professional APCs (such as DCs) result in the priming of adaptive immunity via antigen presentation, subsequent activation of antigen-specific T cells, and the differentiation of antibody-secreting plasma cells (54, 55).

1.3 Focus on TLR7/8 signalling

The recognition of viral ligands within the endosome by TLR7 and TLR8 is dependent on the acidification of the endosomal compartment (56). Both TLRs operate on the basis of recognising ssRNA viruses within the endosome and both TLRs share a signalling pathway dependent on the recruitment of the MyD88 adaptor. The MyD88 adaptor protein interacts with IRAK-4 and subsequently IRAK-1, which interact with TNF receptor associated factor 6 (TRAF-6) to induce transforming growth factor- β -activated kinase 1 (TAK1) activation and subsequent downstream inflammatory signalling via activation of NF- κ B and MAPKs (5, 57) and induction of cytokines (58-61). IRAK-1 has been identified

as dispensable in this complex, however (57). TLR7 signalling also promotes the expression of anti-inflammatory IFN- α , particularly in plasmacytoid DCs (pDCs) (58), via IRF7 activation in a complex involving MyD88, IRAK-4 and TRAF6 (62, 63). Recent experiments have highlighted the importance of IRAK-1, TRAF3 and IKK α in this complex for the induction of IFN- α in pDCs via IRF7 (64-66).

Both TLR7 and TLR8 share significant structural homology, although their expression varies in different cells of the immune system. TLR8 is predominantly expressed in endosomal compartments of macrophages and monocytes (67), and is also present in myeloid DCs (mDCs). TLR7 is expressed largely in pDCs (68) and B cells (69), and to a much lesser extent in myeloid cells. Both TLR7 and TLR8 are also expressed in NK cells (70). TLR7 signalling is involved in anti-inflammatory responses via production of the class I IFNs, IFN- α and IFN- β , with lesser production of inflammatory cytokines and mediators (68, 71). TLR8, on the other hand, is polarised towards promoting a pro-inflammatory response, with emphasis on the production of cytokines such as TNF- α , IL-6 and IL-12, with minimal induction of IFNs (41). Interestingly, IL-12p70 induction in human monocytes was achieved only through synergy of TLR4 and TLR8, while ligation of either TLR4 or TLR8 alone induced production of IL-12p40 (60). Overall, the precise cytokine profile induced by both TLR7 and TLR8 generally depends on the viral RNA recognised (72). Synthetic agonists for TLR7 and TLR8 are generally non-specific and activate both receptors, but recently more specific agonists have become available, allowing for assessment of TLR7 and TLR8 signalling alone or together. These ligands include synthetic imidazoquinolines and thiazoquinolone derivatives such as imiquimod (R848) (73) and CL075 (3M-002) (41).

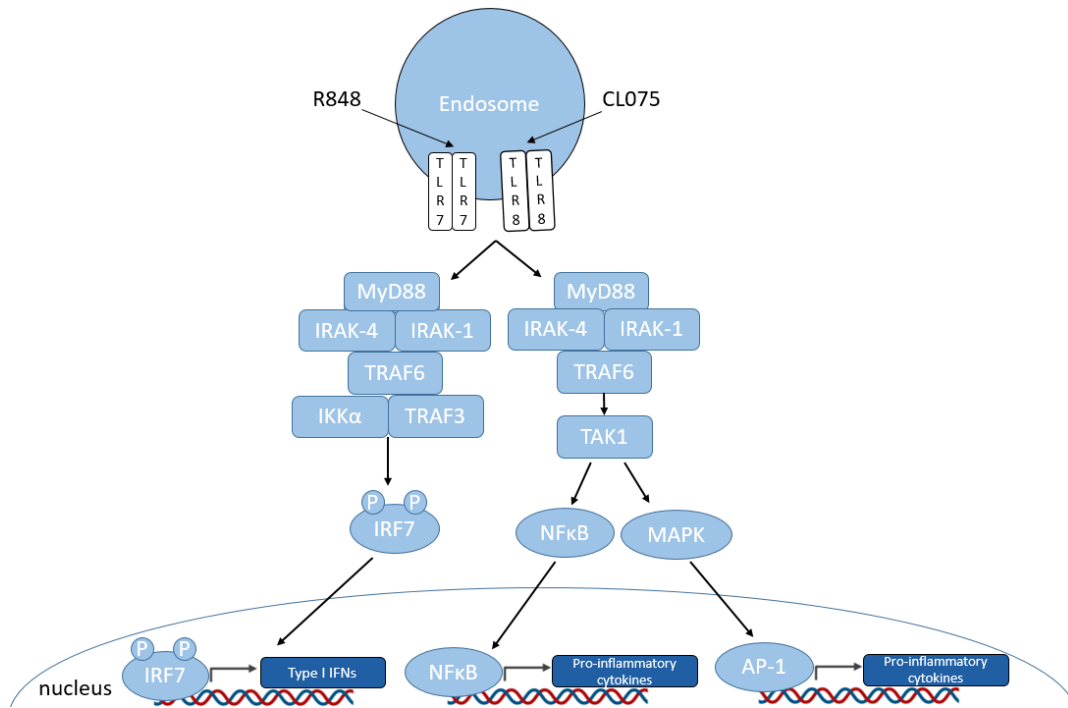


Figure 1.1 Endosomal TLR signalling pathways and downstream activation of NF-κB and IRFs. When endosomal TLR7/8 are activated by their respective ligands, TLRs recruit a range of adaptors to facilitate downstream signalling. Type I IFN induction by TLR7 involves activation of IRF7. Stimulation of TLR7 and TLR8 within the endosome results in recruitment of MyD88. IRAK-4, IRAK-1, TRAF6, TRAF3 and IKKα are also recruited, forming a complex. IRF7 is recruited to this complex and phosphorylated by IKKα and/or IRAK-1, resulting in its activation and translocation into the nucleus where it promotes the induction of IFN-inducible genes. To induce production of inflammatory cytokines through endosomal TLR7/8, MyD88 is recruited and attracts IRAK-4. IRAK-4 proceeds to activate IRAK-1 and leaves the complex. TRAF6 is recruited to the complex where it facilitates activation of TAK1. TAK-1 phosphorylation allows activation of MAPK as well as release of NF-κB from the cytoplasmic IKK complex, allowing it to translocate into the nucleus and induce production of pro-inflammatory cytokines. MAPK allows for activation of the activator protein-1 (AP-1) family, which also translocates to the nucleus and initiates induction of pro-inflammatory cytokines.

1.4 THP-1 macrophages as a model for TLR signalling assessment

For this project, THP-1-monocyte-derived macrophages were used as a model of human macrophages during *in vitro* culture experiments. THP-1 monocytes are an immortalised monocyte-like cell line, initially isolated from the peripheral blood of a childhood case of acute monocytic leukaemia (74). THP-1 macrophages are differentiated from THP-1 monocytes via incubation with phorbol-12 myristate-13 acetate (PMA) (also referred to as 12-O-tetradecanoylphorbol-13-acetate) (75). Differentiation of THP-1 monocytes to macrophages using PMA can result in a variety of inflammatory changes within the differentiated macrophage, depending on the dose of PMA used and a period of rest following differentiation. Based on data from the host laboratory, for the studies presented herein, 48 h stimulation with PMA followed by 24 h rest, was favoured (76).

Several studies have assessed the suitability of THP-1 cells as cell models for assessing TLR signalling. THP-1 cells express both the Fc receptor and the c3b complement receptor, essential for binding and opsonising pathogens (74), making this cell line an appropriate culture model for assessing ligation of PAMPs by TLRs. THP-1 macrophages stimulated with a variety of TLR ligands induce heightened expression of the TRIM family of ubiquitin ligases (77). These proteins have a wide range of functions in the body, including restricting viral replication. Both TLR7 and TLR8 expression have been identified previously in THP-1-monocyte-derived macrophages (67). Indeed, differentiation of THP-1 monocytes to macrophages results in increased expression of both TLR7 and TLR8 (78). In support of the literature, this cell type has previously been used to assess TLR signalling in our laboratory, with data indicating that both TLR3 and TLR4 signalling are operative in THP-1 macrophages (79). Further data elsewhere indicate that TLR8 signalling (via stimulation with CL075) is operative in THP-1 monocytes (80). CL075 has also been used in separate studies, identifying functional differences between TLR7 and TLR8 activation in THP-1 monocytes and THP-1 macrophages induced by PMA (81). It is clear that the THP-1 cell line is a suitable *in vitro* model for assessment of TLR signalling mechanisms.

1.5 An introduction to Multiple Sclerosis (MS)

MS is a chronic inflammatory autoimmune disease of the CNS, characterised by neuroinflammation, glial scarring, demyelination, chronic pain and neuronal damage (82). In terms of disease pathogenesis, MS is defined by infiltration of the CNS by leukocytes, resulting in loss of myelin throughout the CNS. This leads to axonal damage and subsequent cognitive and behavioural alterations, as well as physical disability including visual and mobility impairment (83). Such disabling symptoms include limb ataxia, neuropathic pain, blurred vision, numbness of limbs, spasticity, fatigue, depression, bladder issues, as well as cognitive deficits and impaired speech (83). Three separate forms of MS have been classified: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS) (84). Most commonly, MS presents as a relapsing-remitting form of disease, characterised by periods of demyelination and formation of lesions within the CNS, followed by subsequent recovery. After a prolonged period of the relapsing-remitting form of the disease, usually a decade or more, many patients convert to a more progressive form of the disease, SPMS. This clinical course, consisting of two phases, RRMS and SPMS, has been reasoned to have two separate disease hallmarks. In RRMS the major pathological contributor is inflammatory demyelination, whereas axonal loss is a more prominent feature of disease in SPMS (85). Approximately 15% of people present with PPMS from onset. PPMS has the worst prognosis of the three sub-types of disease, and is generally characterised by chronic disease progression from outset (86).

Progression of MS is characterised by increased disability as defined by the expanded disability status scale (EDSS), a scale that denotes an individual's physical and neurological disability as a number between 0 and 10 (87). A score of 0 is indicative of a normal physical and neurological examination, with no physical or sensory impairment observed. An individual's EDSS score is monitored by a Neurologist to determine the change in disability associated with MS over time. Those with an EDSS score of under 5 can generally function normally, with little aid in physical activities such as walking. More severe disability is noted by a score of between 5 and 6, where individuals require the use of a walking aid (87). A score of 7 denotes a person as having severe physical disability and are restricted to the use of a wheelchair full time (88). Paraplegia follows usually from a score of 8 onwards, and a score of 10 is indicative of death as a result of MS (87).

Clinical course of MS is not a straightforward story of disease progression. In separate stages of the disease there appear to be distinct hallmarks of pathology. For example, RRMS is classically identified by periods of inflammation and demyelination within the CNS, followed by a period of 'recovery'. The first signs of RRMS is visual in many cases, with individuals commonly presenting with unilateral optic neuritis as a result of a newly-formed lesion (89), or double vision. In terms of MS diagnosis, the McDonald criteria are most commonly used (90), which are based on the extent of dissemination of lesions throughout the CNS, as well as the occurrence of lesions over time. This method generally excludes most other neurodegenerative diseases. In addition, MRI scans are frequently used to determine an MS diagnosis clinically (via gadolinium-enhanced lesions), alongside tests on cerebrospinal fluid (CSF) (90).

MS is classically associated with demyelination and axonal loss, along with an inflammatory cellular infiltrate entering the CNS. Usually the disease presents as periods of relapse, thus identifying as RRMS, however in some cases the disease can be progressive from the outset. There is no exact point identified where a switch between relapsing-remitting and secondary progressive forms of the disease occurs (91), although it is suggested to represent a period when axonal loss surpasses compensation by the CNS, thus contributing to irreversible progressive neurological disability (92). However this may not be the case as disability occurs at similar ages across all MS subtypes, thereby suggesting a separate process governing MS progression beyond irreparable demyelination (85). According to studies performed assessing disease progression, both PPMS and SPMS proceed at similar rates with similar clinical characteristics (93, 94). In individuals who first present clinically with RRMS, 50% become wheelchair-bound some 25-30 years post disease onset (95). Individuals with PPMS commonly become wheelchair-bound at a similar age, due to the fact that although PPMS is a more aggressive and faster-acting form of MS, the onset of PPMS is usually later in life, when compared to the onset of RRMS. Indeed, the average age of onset of RRMS is generally 30 years, whereas progressive forms manifest at 39 years, on average (85).

1.6 Epidemiology of MS and risk factors

MS affects up to 2.8 million individuals globally (96), it is the leading cause of non-traumatic disability in young adults (97) and affects between 2-3 times more women than men (83, 98). The number of individuals with MS is increasing exponentially, with

up to 1,000,000 people living with MS in Europe alone, with an increase of 35% in MS prevalence in the three years between 2017 and 2020 (96). In Ireland, the incidence of MS in women and men is 2.7:1, with an overall prevalence of 6/100,000 (99). Risk factors associated with the development of MS are multi-fold, and include smoking, viral infection, diet, vitamin D exposure, and geographical location. In particular, conditions associated with inflammation are considered risk factors, such as obesity and smoking (100). In fact, data from Zhang *et al.*, (2016), indicate that smoking can increase the risk of developing MS by 83% (100). Obesity in children is a significant risk factor in the development of paediatric-onset MS (101), increasing the risk two-fold in both males and females (102). It was found that obese children that have developed paediatric MS had significantly more relapses when compared to non-obese children (102). In addition, MS prevalence increases with distance from the equator, and this is associated with reduced sunlight exposure and vitamin D production (103). This latitude factor may be addressed via the use of supplements as well as certain food intake, such as a seafood rich diet (104). For many years there has been a strong association between Epstein-Barr virus (EBV) infection and the development of MS (105). The exact reason for MS development in individuals with latent EBV has not yet been elucidated in full, although there are links between infection and altered cellular immune responses. Indeed, individuals with EBV are two-fold more likely to develop MS if the infection is symptomatic in the form of infectious mononucleosis (106). Almost 100% of individuals with MS are seropositive for EBV (107). Individuals with MS have a greater CD4⁺ T cell (predominantly Th1) recognition and response to EBV nuclear antigen 1 (EBNA1), when compared to healthy controls (HCs) carrying the virus with the same HLA risk alleles for MS. These antigen-specific T cells are more likely to recognise myelin antigens than other autoantigens (108). There was speculation that MS risk alleles, such as HLADRB1*1501, may be involved in the increased presence of EBNA1-specific T cells, although this has not been proven (108). In addition, MS genetic risk factors are multitudinous. A recent study showed that several low frequency variants, or single nucleotide polymorphisms (SNPs), in genes outside of the HLA region on chromosome 6 were linked to MS development (109), however the HLA region on chromosome 6 has the highest risk alleles and are commonly associated with the development of MS. The allele HLA-DRB1*1501 has the strongest association with the development of MS (110). In addition, there are several alleles associated with protection against MS, including HLA-A*02:01, HLA-A*02:05 and HLA-A*02:06 (110).

1.7 MS pathophysiology

MS disease pathology is associated with the formation of lesions within the CNS, along with gliosis and inflammation (111). These lesions are areas of demyelination and occur in several distinct areas within the CNS, including the optic nerves, spinal cord, areas of the cerebellum, periventricular white matter and the brain stem (83). Several factors drive lesion formation in the context of MS including inflammation, breakdown of myelin, astrogliosis, axonal loss, neurodegeneration, remyelination and oligodendrocyte injury (112).

As discussed previously, initial presentation of the disease usually follows a relapse in RRMS cases (113). Features of early MS lesions include demyelination, macrophage invasion, T cell infiltrates (both perivascular and parenchymal), alongside axonal preservation and relatively low numbers of infiltrating B cells (114). T cell infiltrates within early MS lesions promote a large degree of inflammation accompanied by heavy involvement of foamy macrophages, in addition to astrogliosis (114). These lesions are responsible for some clinical symptoms, including impaired vision and muscle weakness (113). Lesions occur mainly in the white matter (WM) but also in grey matter (GM) (115, 116) of people with MS (pwMS). It was initially believed that MS was predominantly a disease of the WM, involving atrophy, axonal damage and loss of neurons, however, recent studies have shown the extensive involvement of the GM in several areas of pathology (117, 118). Indeed, there is a strong link between the atrophy of GM in MS and long-term disability (119).

In the early stages of RRMS, bouts of demyelination occur within the CNS, and this is followed by repair and remyelination involving astrocytes (85), although complete remyelination is rare. Astrocytes found within WM lesions form sclerotic glial scars and eventually sclerotic plaques around the exposed axon following repeated demyelination and breakdown of the myelin sheath. While these prevent further tissue damage in the area, they also prevent neural repair and remyelination by oligodendrocytes. This results in the formation of sclerotic plaques and subsequent axonal loss (120, 121). The progression from RRMS to progressive MS, and the worsening of symptoms in both SPMS and PPMS, has been linked to axonal loss (85, 122). In fact axonal damage and loss has been identified as the major cause of permanent neurologic disability among pwMS

(85). Cognitive impairments also result from this loss, most commonly deficits in short-term memory, learning, visuospatial perception, information processing speed and the ability to focus (123, 124).

MS is also associated with psychological impairment, which also correlates with MS duration (125). Indeed, atrophy/lesion burden has been linked with depression (126). In addition, a 2004 study identified that depressed pwMS have higher CSF volume and lower GM volume in the left anterior temporal lobe of the brain, when compared to pwMS who were not depressed (127). Several other studies have assessed the impact of brain atrophy and demyelination on neurological behaviour, and have found links to heightened withheld anger (128) as well as apathy (129).

The extensive pathophysiology associated with MS is linked to blood-brain-barrier (BBB) dysfunction, immune cell activation and infiltration of the CNS, with subsequent inflammation and axonal loss. In animal models, dephosphorylation of occludin, a major component of tight junctions within the BBB, has been observed prior to visible signs of disease in murine MS, namely experimental autoimmune encephalomyelitis (EAE) (130). No single cause has been pinpointed as the initiator of BBB breakdown in MS. However, this breakdown facilitates leukocyte infiltration of the CNS, inflammation, lesion formation, demyelination and axonal loss.

Involvement of mitochondria and inflammation

Mitochondrial genes are upregulated within MS lesions (131), and post-mortem assessment has determined altered mitochondrial gene expression in newly formed lesions in MS which can lead to oxidative damage in CNS tissue (131). Oxidative damage plays a larger role in progressive stages of MS, contributing to demyelination and neurodegeneration (131, 132). As shown in post-mortem immunopathological studies, the greatest density of infiltrating T cells, and accompanied inflammation, is most evident in active lesions, which is common in RRMS and early stages of MS (133). Inflammation has been determined within lesions in PPMS, and to a lesser extent SPMS, but this was less pronounced in SPMS (133). However, one study found that progressive MS axonal damage still has strong links with inflammation (133). This suggests that while inflammation is not necessarily the main factor driving pathology in progressive stages of MS, it still has a key role to play.

The role of T cells

In MS, initial infiltrates of the CNS across the BBB primarily consist of macrophages and cytotoxic CD8⁺ T cells, in addition to smaller numbers of autoreactive CD4⁺ T cells and B cells. As disease progresses, higher numbers of B cells and antibody-producing plasma cells accumulate within the CNS (133). The number of CD8⁺ T cells infiltrating the CNS and within lesions is substantially larger than CD4⁺ T cells (133), and their presence within the CNS has been linked to axonal damage (134). A separate study also noted that the number of macrophages and CD8⁺ T cells within lesions is associated with acute axonal damage as a result of the accumulation of amyloid precursor protein, which is linked to the formation of plaques (135). It has long been established that subsets of CD8⁺ T cells that are autoreactive for myelin proteins exist in pwMS (and HCs) (136). The ratio of CD8⁺ to CD4⁺ T cells in MS can be as pronounced as 10:1 (137). Both activated and memory type CD8⁺ T cells have been identified in the CSF and CNS of pwMS (138). There is a potential role for CD8⁺ Tregs in protection against MS, although these cells may be present in lower numbers in pwMS (139). However, disease modifying therapies (DMTs) such as glatiramer acetate, may target Tregs to exert therapeutic efficacy (140). A substantial portion of autoreactive CD8⁺ T cells in MS lesions have been redefined as mucosa-associated invariant T cells (MAIT) cells, as a result of their ability to produce the IL-17 cytokine. One study found CD8⁺ MAIT cells were capable of infiltrating the CNS and subsequently identified CD8⁺ MAIT cells in brain lesions of pwMS (141). Low levels of MAIT cells have also been identified in the CSF of pwMS (141). A significant amelioration of MS following MAIT cell depletion, pointed to their role in disease pathology (142). It also supports evidence that IL-17 plays a key role in MS. Indeed, it was recently determined that MAIT cells in pwMS produce more IL-17 than MAIT cells in HCs (143).

Autoreactive CD4⁺ T cells are expressed early in disease, and evidence suggests that autoreactive CD4⁺ T cells, recognising myelin antigens, are responsible for initial demyelination seen within the CNS. Indeed, autoreactive CD4⁺ T cells have been identified in blood and CSF of pwMS, and such T cells were not observed in HCs (144). The initial concept of MS as a T cell-driven autoimmune disease stemmed from experiments in animal models. In rodents it was found that removal of the thymus prevented EAE development in rats (145). In EAE, both Th1 and Th17 autoreactive T cells infiltrate the CNS to drive disease progression (146). Both Th1 and Th17 cells have been

presented as the primary subsets responsible for disease pathogenesis in the CNS, and have been identified in the CSF and CNS of pwMS (147). The level of Th1 cells in peripheral blood and CSF are higher than Th17, and interestingly, only Th17 cells within the CSF were found to be increased in relapse in MS (147). Interestingly, CD8⁺ T cells dominate chronic lesions in MS, while CD4⁺ T cells are prevalent in acute lesions (148). The frequency of Th1 and Th17 cells can be altered in different subtypes of MS. For example, Th1 cells are elevated in peripheral blood mononuclear cells (PBMCs) of individuals with RRMS, when compared to PBMCs from individuals with SPMS or PPMS (149). In recent studies, T cells that do not fit the criteria of either Th1 or Th17 cells secreting both IFN- γ and IL-17A have been identified within CNS lesions in individuals with MS (150).

The role of DCs

DCs are one of the many cell types that gain access to the CNS from the periphery in MS, likely attracted by $\alpha 4\beta 1$ integrin (151). DCs are important in MS disease progression mainly due to their antigen-presenting ability, presenting auto-antigens to immune cells, thus causing autoreactivity. DCs with altered function have been identified in MS lesions (152). pDCs have a complicated role in MS pathology, and the number of pDCs are upregulated in the CSF of pwMS during relapse (153). However, in murine EAE, depletion of pDCs can act to exacerbate disease severity, with increased CD4⁺ T cell secretion of IFN- γ and IL-17 in the CNS following their depletion (154). This suggests pDCs have a pleiotropic and complex role to play in the pathology associated with MS. In addition, pDC subsets, and their relevant ratios, are skewed in pwMS. Indeed, the pDC1 subset (noted to induce IL-10 producing T cells) was significantly downregulated in peripheral blood of pwMS, whereas pDC2 (which favourably induces IL-17 producing Th17 cells) in peripheral blood was significantly upregulated in pwMS, when compared to HCs (155). This suggests an altered expression profile of pDCs in MS. Indeed, 1,213 abhorrently expressed genes in pDCs have been identified in MS, although 60 of these can be corrected with three months of IFN- β therapy (156). One study has also shown that it is likely that resident DCs within the brain promote homing of T cells into the CNS during neuroinflammation (157). In addition, DCs that differentiated from CD14⁺ monocytes that have migrated across the BBB have the capacity to polarise T cells to either a Th1 or Th17 phenotype within the CNS (158). CD83 (a marker of mature DCs) is expressed at

lower levels on DCs in peripheral blood of individuals with SPMS and PPMS, when compared to blood samples from HCs, indicating a reduction in mature DCs in people with progressive MS (159). In the same study, a reduction in the expression of the costimulatory molecules CD80 and CD86 on mDCs from individuals with PPMS and SPMS was observed, when compared to mDCs from HCs (159).

The role of B cells

B cells, while initially thought to have little impact on disease progression, have been shown to play a key role in the pathogenesis of MS. The extent of their involvement was only fully realised following the success of the anti-CD20 monoclonal antibody treatment, showing significant amelioration in MS symptoms (160). Post-mortem studies have revealed that in progressive forms of MS, the frequency of antibody-secreting plasma cells increases, and can persist after lymphocytic infiltration of the CNS ceases (133). B cells from pwMS demonstrate increased expression of both MHC II and CD40, suggesting increased antigen-presenting ability and proclivity to activate autoreactive T cells (161). The main role of B cells in MS is likely the activation of autoreactive T cells within the CNS, which in turn drives inflammation and disease progression. The expression of CD20⁺ B cells is higher in active lesions of RRMS patients, when compared to other forms of MS, suggesting they may contribute to inflammation within active lesions (133).

The role of NK cells

NK cells have been identified in MS lesions and overall much data indicate that NK cells have altered/dysregulated function in pwMS. Indeed, one study identified an increase in immature circulating NK cells in stable MS, which was not observed in active MS, suggesting that NK cells have a role to play in both relapse and remission in the disease (162).

The role of macrophages and glial cells

Both microglia and macrophages have significant roles to play in MS pathology, with profuse macrophage and microglial activation seen in lesions in WM (163). In early stages of MS macrophages are known to have M1 and M2 phenotypes within the normal-appearing white matter (NAWM) of MS individuals, and this

expression/distribution is comparable to non-MS control brains. Interestingly, the majority of macrophages in active lesions have an intermediate phenotype, which facilitates their inflammatory, as well as neuroprotective, roles in the CNS of pwMS (163). Acute lesions are identified by having a high macrophage burden, where the phagocytes contain internalised myelin (111). Macrophages are a double-edged sword in the context of MS, as they have been shown to be both neuroprotective, as well as producing detrimental pro-inflammatory mediators (164). In the early stages of the disease, macrophages and microglia exhibit a more M1 phenotype during relapse, resulting in the production of inflammatory cytokines and subsequent demyelination within the CNS (165). Later in the disease, this profile changes to an M2 phenotype, resulting in release of anti-inflammatory mediators and facilitating a potential return to homeostatic conditions (165). This is responsible for the relapsing-remitting nature of the disease, and the resolution of relapse. Interestingly, post-mortem studies have identified macrophages as the main cell type in active lesions within the brain of pwMS, whereas microglia have been shown to dominate lesions in the NAWM and the cortex, as well as inactive lesions (133). Microglia have been determined as the main cell type in inactive lesions of the brain (133). It has been suggested that initial onset of neuropathology in MS, as indicated by the formation of lesions, may be associated with activation of microglia prior to lymphocyte infiltration of the CNS (166). Astrocytes and microglia may potentiate disease progression via axonal damage as a result of toxic mediators they produce, including cytokines, glutamate and reactive oxygen species (ROS) (131, 167). This neuroaxonal damage in the initial relapsing phases of the disease, accompanied by chronic activation of resident cells of the CNS such as microglia and astrocytes, may culminate in long-term propagation of neurodegeneration in the CNS in progressive MS.

It is clear that the pathogenesis of MS involves complex interplay between cells of the immune and nervous systems. Histopathological studies demonstrate that the pattern of lesions, their cellular composition, as well as the extent of myelin loss, varies significantly between cases of MS (168). Data from Lucchinetti *et al.*, (2000), indicate that four demyelination patterns are identified in MS (168). Once inactive, plaques undergo remyelination, with sharp demarcation of the plaque that typically form around veins. Deposition of IgG are identified in lesions of pattern two. Patterns three and four

are characterised by a certain amount of inflammatory infiltrate involving T lymphocytes and macrophages, but with no antibody deposition identified. Furthermore, pattern three plaques are generally diffuse within tissue, with poorly defined plaque borders, in contrast to well-defined plaques of pattern four. Remyelination was found to be very poor in both pattern three and four inactive lesions. It was suggested by the authors that patterns three and four appear to be more indicative of virally-induced demyelination and inflammation within the CNS (168). The method of myelin damage was different in all four patterns studied. This suggests that although MS shares many common pathological features, the agents responsible for development of MS lesions may vary between individuals. This, however, has not been proven to date. Active lesions are generally identified by reduced degrees of myelination and inflammation, as remyelination occurs to a certain extent in inactive lesions. It is noted that individuals with SPMS have reduced ability to perform extensive remyelination of inactive lesions, when compared with PPMS cases (169). The exact mechanism behind the trigger of these immunopathological events is yet to be discovered, although it is likely that TLRs have a major role to play. The pathophysiological features of MS are summarised in Figure 1.2, below.

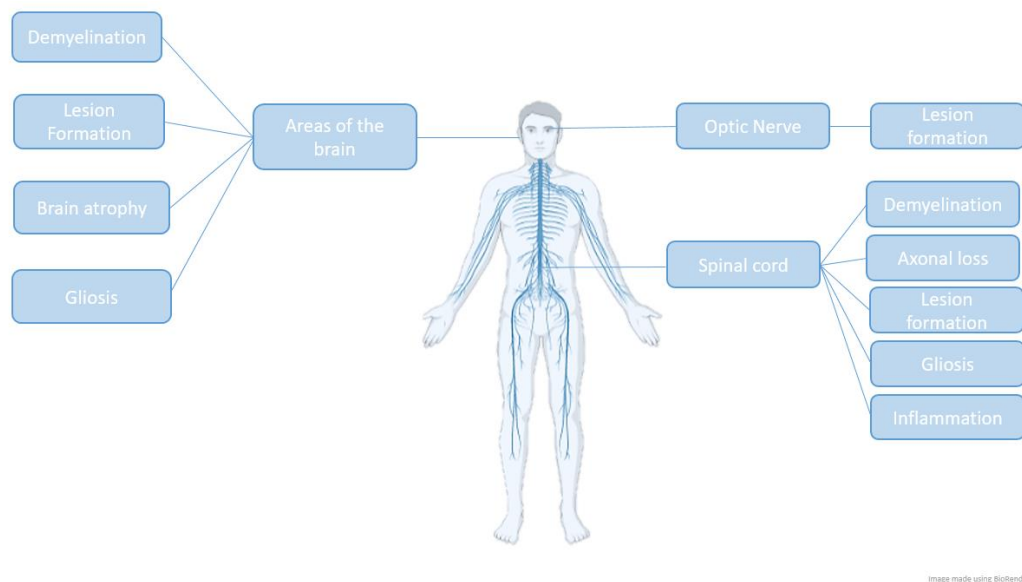


Figure 1.2 Pathophysiology of MS. This diagram summarises the pathophysiological features of MS, in the spinal cord, the optic nerve, and distinct areas of the brain. These pathophysiological features are described in detail in section 1.7 above.

1.8 TLR signalling in MS with focus on viral TLR signalling

TLRs have a significant role to play in the onset and progression of MS. Indeed, viral TLR signalling may be dysregulated in MS, and thus may account for a certain degree of pathology. Hansen *et al.*, (2006), indicate that activation of TLR signalling may induce autoreactive T cells in EAE (170). Interestingly, MyD88^{-/-} mice are resistant to EAE development, suggesting onset of disease is mediated through TLR signalling (171). Agonists for TLR1, TLR2 and TLR4, used as adjuvants with myelin oligodendrocyte glycoprotein (MOG) 35-55, result in proliferation of autoreactive T cells in mice. However, the TLR3 agonist poly(I:C), when used as adjuvant, does not induce autoreactive encephalitogenic T cells (170). Indeed poly(I:C) reduces disease activity and demyelination via the induction of CCL2 and IFN- β in EAE (172). In addition, TLR4^{-/-} mice exhibit an exacerbation in EAE severity (173), with a similar EAE pathogenesis seen in mice who are deficient in the TRIF adaptor (174). Overall these data suggests that TLRs, and their signalling adaptors, are intricately involved in EAE pathogenesis.

Interestingly, the TLR9 ligand CpG DNA, has the ability to induce activation of CD4⁺ T cells, while simultaneously suppressing Treg cells (175). This may result in significantly increased levels of inflammation in autoimmunity. TLR7 deficient mice demonstrate reduced disease severity in EAE due to a decrease in autoreactive T cell activation (176). However, stimulation of murine TLR7 via imiquimod has been shown to ameliorate EAE severity (177). O'Brien *et al.*, (2014) have also shown that IRF3 is essential in the development of murine EAE, with IRF3 knockout mice developing less severe forms of the disease, further implicating the role of TLR signalling in EAE development (178). Elsewhere, data indicate that IFN- β 1a treatment inhibits Th17-polarising cytokine release (including IL-17F, IL-1 β and IL-1R1, among others) by monocyte-derived DCs isolated from individuals with RRMS via upregulation of TLR7 *in vitro*, as well as increasing the expression of TLR3 (179). IFN- β treatment also inhibits the expression of various chemokines in PBMCs (179). More recently a deficiency in TLR7-induced immunoglobulins IgG and IgM has been identified in PBMCs from pwMS, and TLR7 mRNA expression is significantly reduced in PBMCs and monocytes isolated from pwMS, when compared to immune cells from HCs (180). This may highlight the importance of TLR7 in informing an effective adaptive immune response in MS.

The exact role of TLR8 in MS and EAE is poorly understood and requires further study. Data indicate that the expression of TLR8 is elevated in the spinal cord of EAE mice (181,

182), and that the expression of TLR8 is reduced (and demonstrates altered function) in PBMCs from pwMS (183). Interestingly, treatment of THP-1 monocytes *in vitro* with 1,25-Dihydroxyvitamin D3 inhibits TLR8 signalling, as evident by reduced production of the inflammatory cytokines IL-1 β and TNF- α (182). TLR8 expression is increased in the SC of EAE mice (182) and treatment with 1,25-Dihydroxyvitamin D3 reduces TLR8 expression, alongside the production of inflammatory cytokines, in the SC (182). This suggests that TLR8 may have significant roles in infiltration of the CNS by inflammatory cells. In *in vitro* experiments in THP-1 monocytes, 1,25-Dihydroxyvitamin D3 has also been shown to suppress inflammatory TLR8 signalling induced by CL075 (182).

TLR activation can induce B cell activation and proliferation, thereby contributing to autoreactivity. B cell activation and proliferation is not sustained by autoreactive T cells alone (184) in the absence of TLR agonists. TLR signalling may contribute to B cell involvement in MS by sustaining autoantibody responses within serum. The TLR9 agonist CpG has been shown to induce the formation of memory B cells (185) and this has been suggested to be important in both MS and EAE. In addition, TLR9 may be a double-edged sword in autoimmunity. Mice deficient in TLR9 develop EAE with reduced clinical symptoms of disease (including attenuated inflammation and demyelination), when compared to wild type (WT) mice (171). In contrast, a separate study showed that TLR4^{-/-} and TLR9^{-/-} mice developed more severe EAE, when compared to WT mice (173). In terms of TLR2, TLR2 is expressed at higher levels in Tregs isolated from peripheral blood of pwMS, when compared to controls (186), and TLR2^{-/-} mice demonstrate a reduction in BBB breakdown as a result of reduced matrix metalloproteinase-9 (MMP9) expression in astrocytes (187).

It is likely that bystander activation within the CNS may be responsible for initiation of autoimmunity in murine models of MS, where DCs present endogenous antigens and induce activation of autoreactive T cells in the CNS (188). It has also been speculated that bystander activation mediated by TLRs in the CNS may result in tissue damage, thus uncovering myelin antigens, allowing for antigenic/epitope spread and initiation of autoimmunity within the CNS (189). Overall, much data indicates that there are many ways in which TLRs are involved in initiating an autoreactive immune response in the CNS, and this is intricately associated with the pathogenesis of MS. A summary of the contributions of viral TLRs to MS pathogenesis is outlined in Table 1 below.

Table 1. Viral TLR contribution to MS pathogenesis

TLR	Roles in the pathogenesis of MS	Roles in the pathogenesis of murine EAE
TLR3	TLR3-induced CXCL10 in PBMCs isolated from pwMS are desensitised, when compare to PBMCs from HCs (unpublished data, Downer laboratory).	TLR3 activation does not induce encephalitogenic T cells when administered as an adjuvant along with MOG 35-55 to mice (170). TLR3 agonist reduces disease activity and demyelination via induction of CCL2 and IFN- β in EAE mice (172).
TLR7	mRNA expression reduced in PBMCs and monocytes isolated from pwMS (180). Expression upregulated by IFN- β treatment, inhibiting Th17-polarising cytokine production (179).	Stimulation with imiquimod ameliorates disease severity in EAE (177).
TLR8	Expression reduced in PBMCs from pwMS (183). Function altered in PBMCs from pwMS (183).	Expression upregulated in SC in EAE (181, 182). Likely has a role in lymphocytic infiltration into the CNS in EAE and release of inflammatory IL-17, TNF- α and IFN- γ (182).
TLR9	CpG induces formation of memory B cells in pwMS, which may be important in MS (185).	CpG DNA activates CD4 ⁺ T cells and reduces Tregs in EAE (175). Mice with TLR9 deficiency have reduced disease severity in EAE (171). TLR9 ^{-/-} mice develop a more severe form of EAE (173).

1.9 DMT treatments in MS

There is no cure for MS, however, several effective drug therapies, or DMTs, that are used to treat symptoms and slow clinical progress of the disease, have been developed. These therapies act mainly to reduce long-term disability and slow disease activity. As MS is a pleiotropic disease, several different forms of therapy exist to combat symptoms, focusing on different aspects of the autoimmune response. Not every therapy works for each individual with MS, and in some cases there is trial and error involved in selection of the appropriate therapy. If chosen correctly, these drug treatments can, in some cases, effectively stop the course of disease for long periods of time, preventing relapses and staving off disease progression. There are several classes of DMTs used in MS, referred to as first-line, second-line and third-line drug treatments. Patients are started on first-line treatments, mainly to combat inflammation and to alter the balance of autoreactive cells within the immune system in early stages of disease. These DMTs are first-line owing to their proven clinical effectiveness and generally mild side effects. If an individual fails to respond to first-line treatment (indicated by increased MRI lesion load, worsening neurological disability) or side effects become too great, a progression to second-line treatment is made, and subsequently third-line.

DMTs approved for use currently include immunomodulators, monoclonal antibodies and immunosuppressants. Immunomodulatory drugs are first-line treatment DMTs and mainly act to alter the balance of autoreactive and inflammatory immune cells in pwMS, by skewing T cells towards Th2 phenotypes, reducing T cell proliferation and preventing lymphocytic infiltration of the BBB. Such DMTs are effective in RRMS by reducing inflammation in the CNS. IFN- β is a first-line DMT and remains one of the most widely used first-line treatments in MS to date (190). IFN- β therapy has been shown to be neuroprotective in MS (191). Despite its widespread use, its mechanism of action to ameliorate disease activity and clinical symptoms is yet to be fully elucidated. The mechanism of action of IFN- β is mainly immunomodulatory, acting to reduce the expression of inflammatory cytokines and polarise differentiation towards an anti-inflammatory Th2 subtype (192) by promoting IL-10 (193), while downregulating IFN- γ (194), expression. IFN- β can also act to inhibit T cell proliferation, including CD8⁺ memory T cells (192). Evidence also suggests that IFN- β therapy reduces autoreactivity of T lymphocytes to MOG (192). IFN- β therapy acts most effectively in the earliest inflammatory stages of disease in RRMS, and as MS disease course progresses, the

therapeutic efficacy of IFN- β declines (195). This is likely due to the proclivity of this IFN to suppress inflammation which is superseded by progressive neurodegeneration and axonal loss in later stages of disease.

Glatiramer acetate (GA), also referred to as Copaxone, is another injectable first-line immunomodulatory DMT used commonly in the treatment of MS. It has been approved for use in the treatment of RRMS in the USA since 1996. Significant reduction in relapse rate is observed with the use of GA in MS (196, 197). Initially created as a synthetic analogue of myelin protein, it was shown to inhibit demyelination in EAE (198). In pwMS, GA increases the expression of the anti-inflammatory cytokine IL-10 in serum, as well as suppressing TNF- α expression (196). It has also been shown to polarise T cells towards a Th2 response (199). Similar to IFN- β , GA shows greater efficacy when treatment is commenced early in the course of MS development (200). Dimethylfumarate and fingolimod are also considered first-line treatments for MS, although fingolimod is usually reserved for use only in individuals with high disease activity and can also be classified as a second-line treatment. In a 2020 study, both treatments were shown to display similar ability to reduce relapse rate in pwMS (201).

Rituximab is in use as an anti-CD20 chimeric monoclonal antibody which actively depletes CD20⁺ B lymphocytes for the treatment of MS as both a first- and second-line treatment. It has proven a successful treatment via reduction of inflammation and a reduction in gadolinium-enhancing lesions on MRI in RRMS (202, 203). Rituximab has also been shown to significantly reduce relapse rates and disability progression in people with RRMS and progressive MS (160). Reduction in B cells is thought to lead to a depletion in T cell activation and may potentially deplete circulating numbers of latent EBV which is found sequestered in B cells (204, 205) and potentially impact cellular signalling through expression of microRNAs (miRNAs) (206). Rituximab is generally considered a safe DMT for use in MS, with a relatively low number of severe adverse side effects reported with the use of this DMT (207).

Second-line DMTs for MS include Natalizumab and Alemtazumab, both are humanised monoclonal antibodies designed initially for use in other autoimmune diseases. Natalizumab is a humanised monoclonal antibody specific for the α -4 integrin cell adhesion molecule (CAM) and was initially used as a treatment for Crohn's (208, 209). Its repurposing as an MS therapy originally derived from experiments in EAE mice whereby blocking the integrin resulted in prevention of leukocyte accumulation in the

CNS (210). In addition, Natalizumab may prevent interactions between α -4 integrin and extracellular matrix proteins that promote inflammation in the CNS (211). In the context of RRMS, Natalizumab was been shown to reduce the annual relapse rate by 68% following one year of treatment, and this study noted an 83% reduction in the average quantity of enlarged or new hypertense lesions, with 92% fewer lesions (gadolinium-enhanced) overall in individuals with RRMS treated with natalizumab (212). Alemtazumab is a humanised monoclonal antibody specific for CD52, with its initial use intended for B-cell chronic lymphocytic leukemia, targeting most lymphocyte subsets for depletion (213). Alemtazumab has proven very efficacious in the treatment of RRMS, likely as a result of reduced inflammation and BBB infiltration following depletion of lymphocytes. Lymphocytic repopulation subsequently follows alemtazumab therapy (214). Interestingly, repopulation of CD4⁺ T cells is particularly delayed (215), perhaps this is partially responsible for the amelioration of symptoms in pwMS. Data from Coles and colleagues (2004) demonstrate that Alemtazumab reduces relapse rate in SPMS by 97%, and reduces relapse rate by 94% in RRMS individuals who had poor response to other treatments, or very high relapse rate prior to alemtazumab therapy (216). However, progression of neurological disability was maintained in individuals with SPMS, as defined by the EDSS (216). Alemtazumab therapy is, however, associated with a high risk of developing a secondary autoimmune condition (217-219). Despite the efficacy and promise of humanised monoclonal antibodies as treatments, there are side effects associated with DMT use, including progressive multifocal leukoencephalopathy (PML), which can occur following significant immunosuppression (220). Steps are being taken to reduce the likelihood of developing such conditions. Furthermore, in the study above using Natalizumab, five individuals from a study cohort of 627 with RRMS receiving natalizumab developed cancer, compared to one in the placebo group (212).

Third-line treatments for MS are rarely used in MS and include DMTs such as mitoxantrone, in addition to alemtazumab in the US, which has already been discussed. Mitoxantrone is a chemotherapy drug and its mechanism of action in pwMS has yet to be fully elucidated, although it is believed to act as an immunomodulator. Mitoxantrone reduces the number of relapses in pwMS and ameliorates disease progression in all three subtypes of MS (221). Mitoxantrone is considered a third-line treatment as a result of its severe side effects, which includes leukemia (222) and cardiotoxicity (223).

1.10 Cannabinoids

Cannabinoids are at the forefront of modern medicine, but are also a cause of widespread debate. Their development as therapeutics has been hampered due to the use of cannabis as a recreational drug. However, historical records have dated the earliest evidence of medicinal cannabis as far back as the 4th century (224), where it was used as a treatment for a range of ailments. Egyptian papyrus scrolls have detailed its use as far back as the 16th century BC (224). Despite concerns, cannabinoids, compounds extracted from the plant *Cannabis L. sativa*, have shown real promise as immunotherapeutics in the clinical setting, particularly in the context of neurodegenerative diseases such as MS. Among their many uses, cannabinoids have been shown to reduce spasticity (225, 226), alleviate central and peripheral neuropathic pain (225, 227, 228), and reduce inflammation (as well as associated pain) (228). Several studies have also indicated that cannabinoids are neuroprotective following insult or inflammation within the CNS (229-231). In an online survey on the use of cannabis to alleviate pain associated with neurodegenerative disease, 66% of individuals with MS admitted to using cannabis for alleviation of symptoms. In this study these individuals self-reported a significant reduction in fatigue as well as improvements in mood (232).

The major cannabinoids clinically associated with amelioration of pain, spasticity and neurodegeneration, are phytocannabinoids (pCBs), with particular emphasis on Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD). THC has been shown to alleviate several symptoms of inflammation, spasticity and pain (233), but has potentially detrimental side effects due to its psychoactivity, including sensations of euphoria and psychosis (234). CBD is a non-euphoric cannabinoid and has been shown to ameliorate several symptoms associated with neurodegenerative disease, with mild side effects (235, 236). An entourage effect, whereby a mixture of both THC and CBD are used in conjunction in medication, has shown promising beneficial effects in a clinical setting, as CBD can mitigate the psychotropic effect(s) of THC (237, 238). Several other non-euphoric pCBs have been isolated from the plant such as cannabigerol (CBG) and cannabidivarin (CBDV), and research into their potential use in therapeutics is ongoing (239-241). These pCBs comprise some of the major cannabinoids isolated from *Cannabis L. sativa*, although over 150 minor pCBs have also been isolated from the plant to date (242).

In addition, endocannabinoid (eCB) compounds may contribute significantly to symptom amelioration in MS. eCBs are endogenous lipid molecules produced within the body which are involved in the maintenance of several homeostatic processes (243, 244). The most well-studied eCBs to date include anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG) (245). These lipid messengers are produced only when needed in the body and exert their function through a variety of G-protein-coupled receptors (GPCRs), in particular cannabinoid receptors 1 and 2 (CB₁ and CB₂) (246). eCBs and the cannabinoid receptors are collectively referred to as the endocannabinoid system (eCS).

1.11 pCBs and eCBs

Cannabinoids produced by the dioecious plant *Cannabis L. sativa* are referred to as pCBs. THC and CBD are the most extensively studied in a clinical setting, but a myriad of other pCBs have been identified. Other pCBs such as cannabinol (CBN), CBG, CBDV and cannabichrome (CBC) are also currently under investigation for their therapeutic potential. Recently, CBG was noted to have potential anti-inflammatory and neuroprotective effects (241), and CBDV has proven to be anti-inflammatory in models of ulcerative colitis (247). Biosynthesis of pCBs in the cannabis plant involves a complex process. Initially, pCBs are formed as an acidic form of the compound such as tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), with poor oxidative stability. The acidified cannabinoids are converted to a more stable compound via decarboxylation or oxidation, as a result of heat or naturally over time (248). This reaction yields stable THC and CBD as end-products (248). The most prominent site of cannabinoid production is thought to be the glandular trichomes of the plant (248). In fresh cuttings, significantly higher levels of the acidic cannabinoids are present in plant tissue compared to the decarboxylated form (249). For example, THCA is likely formed in the glandular trichome of the plant, whereas THC and other cannabinoids accumulate in the secretory cavity only (250). Other organic compounds isolated from the cannabis plant include terpenes and organic aromatic hydrocarbon compounds, which are also produced in glandular trichomes (251). Terpenes make up much of the essential oils in the plant and are responsible for its aroma (252), as well as acting as a form of insecticide to protect the plant from damage (251, 253). The aromatic properties of terpenes have led to their inclusion in food-additives, fragrances and essential oils (251). In addition, terpenes have also been shown to have a degree of anti-inflammatory and analgesic

activity (254). Female flowers of the cannabis plant contain the highest quantities of pCBs and terpenes within their secretory cavity (255). THC has been shown to act via the CB₁ and CB₂ receptors, with euphoric and psychotic side effects associated with its interaction with CB₁ (233). CBD has low affinity for either CB₁ or CB₂ (256). Both of these pCBs act to mitigate inflammation and exert neuroprotective effects (257, 258).

THC and CBD are lipophilic, it was initially thought that their activity resulted from a proclivity to fluidise membranes. However, this was proven to be incorrect following the identification of the cannabinoid receptors CB₁ and CB₂ in the 1990s (259, 260). CB₁ and CB₂ are expressed differentially in different areas of the body, and on different cell types. For example, CB₁ is expressed mostly in the CNS and is the most abundantly expressed GPCR in the brain (261), being widely expressed in areas of the brain such as the cerebellum, neocortex and brainstem, as well as in cells such as astrocytes, neurons, microglia and oligodendrocytes (262, 263). It is expressed to a lesser degree in the peripheral immune system and within tissues including the heart, lungs, liver, blood vessels and digestive system (264). CB₁ is involved in regulating several processes in the body, including cognitive function, memory, appetite, learning, motor functions and synaptic plasticity (265, 266). The majority of CB₁ is expressed within the cell membrane, though recently it has been identified within endosomes and lysosomes in neurons (267). CB₁ has also been identified in mitochondria, with CB₁ having a role to play in cellular respiration (268). CB₂, first isolated in the spleen (260), was initially believed to be absent from the CNS, however the receptor has now been identified in microglia in the brain (269), in astrocytes following inflammation or damage, as well as in populations of neurons in the brainstem (270). Although not prevalent in large numbers centrally, CB₂ has important roles in neuroinflammation and pain (271-273). It is also present on cells of the GI tract and liver, the reproductive system, adipose tissue, bone, tonsils and the cardiovascular system (233). It is speculated that CBD, due to its poor affinity for CB₁/CB₂, may act through other receptors such as the transient receptor potential cation channel subfamily V member 1 (TRPV1). There is evidence to suggest that eCBs also act as agonists for the orphan receptor G-protein coupled receptor 55 (GPR55), TRPV1 and peroxisome proliferator-activated receptors (PPARs) (274-276).

With the discovery of the eCS, scientists began the hunt for endogenous molecules that acted as ligands for this system, subsequently identifying the eCBs, AEA and 2-AG (277-279). eCBs also vary in terms of their affinity for the cannabinoid receptors. AEA

demonstrates partial affinity for both CB₁ and CB₂ (280). In contrast, 2-AG exhibits high affinity for both receptors (278, 279). Both eCBs are produced on demand in response to increased concentrations of intracellular Ca²⁺ (261, 281), and subsequently act to control neurotransmission (282). 2-AG acts mainly through GABAergic retrograde neurotransmission (281) across the synapse, and acting via CB₁, controls the release of neurotransmitters (281). In contrast, AEA acts at TRPV1 (275) which is suggested to have involvement in eCB signalling (283). AEA has also been shown to regulate the biosynthesis of 2-AG (284), thus controlling synaptic transmission. Following their synthesis and release, both AEA and 2-AG are hydrolysed by the enzyme fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (285, 286). Basal concentrations of 2-AG in brain tissue are 170 times greater than that of AEA (287).

1.12 The eCS in MS

Alterations in the expression of components of the eCS in the CSF of pwMS have been linked to disease-associated dysregulation of eCB signalling (288, 289). Indeed, AEA was shown to be increased in CSF from individuals with RRMS, at a 6-fold higher concentration than that identified in HCs (289). In the same study, AEA levels were also increased in peripheral lymphocytes of pwMS, and this correlated with reduced presence of FAAH and its enzymatic activity in peripheral lymphocytes (289). Such an increase was not observed for 2-AG (289). Similarly, the levels of AEA in plasma of individuals with RRMS, SPMS and PPMS is significantly elevated, when compared to HCs, while no difference in 2-AG was observed in MS plasma compared to HCs (290). Data elsewhere identified an increase in AEA levels, but not 2-AG levels, in T lymphocytes and NK cells in individuals with MS, when compared to HCs (291). This elevation was significantly attenuated following one year of clinical IFN- β therapy, with final AEA levels similar to that of HCs in B, T and NK cells (291). Evidence also indicates that eCBs are present at reduced concentrations in CSF of pwMS when compared to controls, but the eCB levels are increased in CSF when individuals are in relapse in RRMS (288). Importantly, the eCB-like molecule, palmitoylethanolamide (PEA), is significantly upregulated in plasma of pwMS, when compared to plasma from HCs (290). In addition, the concentrations of both AEA and PEA are significantly higher in plasma isolated from SPMS individuals, compared to control individuals. In the same study, a significant reduction in FAAH expression in blood samples from individuals with SPMS was also

observed (290). Interestingly, FAAH expression was not shown to differ significantly in NK, T or B cells from pwMS and HCs, and suggests that the observed increase in AEA levels in these cells was not solely due to decreased FAAH activity or reduced levels of its expression (291). This is in contrast to a separate study that determined that individuals with SPMS had significantly decreased expression of FAAH in whole blood, when compared to whole blood from HCs (290).

In murine studies, a reduction in FAAH activity, and a subsequent increase in the levels of AEA, were also observed in the forebrain of animals in murine models with EAE (289). Interestingly, deletion of the FAAH gene ameliorates motor dysfunction in an animal model of chronic EAE (292). Data elsewhere indicates that use of PEA in a viral-induced model of MS in mice, Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), ameliorates motor impairment and reduced inflammation (293).

Interestingly, altered expression of the main cannabinoid receptors, CB₁ and CB₂, have been observed in pwMS. Indeed, individuals with PPMS have significantly elevated expression of both CB₁ and CB₂ mRNA in whole blood, in comparison to control individuals (290). People with SPMS have increased expression of CB₂ mRNA in whole blood, when compared to whole blood from HCs (290). PwMS had an observed increase in CB₂ expression in B cells compared to healthy donors, although this was reduced to normal levels following twelve months of IFN- β therapy (291). In the same study, NK and T cells from pwMS expressed similar levels of CB₂, when compared to cells from HCs (291). Furthermore, T cells from pwMS were also noted to have increased expression of CB₁, when compared to T cells from HCs (291). 2-AG levels were comparable in both B and T cells in pwMS and control individuals in this study. Interestingly, chronic cannabis use in healthy individuals can also affect the eCS, and this is associated with increased CB₁ and CB₂ expression in peripheral blood monocytes (294).

There are clear alterations in eCB signalling in pwMS, particularly CB₁/CB₂ expression and the expression of AEA. It is likely that there is a functional role for the eCS in MS pathogenesis, in terms of symptom development. This suggests that the eCS should be considered as a novel therapeutic target to protect against neuroinflammation and several other clinical symptoms of the disease.

1.13 Cannabinoids as therapeutics in MS

Cannabinoids are considered as add on therapeutics in MS in cases where an individual demonstrates little or no improvement with the use of conventional DMTs. Cannabinoids are known to alter immune functions, including their ability to suppress T cell function (295), as well as reducing IFN- γ production (296), which have proven beneficial in the context of RRMS. This is supported by a wealth of pre-clinical and clinical studies exploring the use of THC and CBD. The use of THC, although associated with euphoria and other side effects, acts via CB₁ to produce neuroprotective effects (257). CBD has been shown to counteract the negative effects of THC administration (237, 238), and this effect has been harnessed in the form of drugs that are available for the treatment of spasticity in MS.

Sativex has been approved for use in certain countries for the treatment of spasticity in pwMS (297). Sativex is an oromucosal spray, consisting of a 1:1 mixture of THC and CBD with other minor components (298). One study found that sativex was capable of reducing muscle spasticity in MS by up to 20% at its peak, while increasing the dosage of sativex two-fold resulted in a 40% reduction in MS spasticity, reducing limb stiffness within 10 minutes of administration (299). According to a study carried out by Al-Ghezi *et al.*, (2019), a 1:1 mixture of THC and CBD reduced disease severity in EAE, with observed increases in the expression of anti-inflammatory cytokines such as IL-10 in serum (300). Furthermore, there was a noticeable reduction in infiltrating cells and associated damage within the brain, with an overall reduction in neuroinflammation (300). In terms of clinical data, a double-blind study of 160 patients similarly concluded that treatment with sativex resulted in a significant attenuation of spasticity, as well as improved quality of sleep (226). A THC:CBD oromucosal spray has also been shown to reduce neuropathic pain and improve quality of sleep in pwMS (227). Significant improvements in MS-associated spasticity have been reported following administration of Sativex (301). The use of sativex in treating MS symptoms is commonly part of an adjunctive therapy, rather than a stand-alone therapy. Interestingly, in pwMS who commenced medicinal cannabis therapy, the reported use of opioids for the treatment of sleep, anxiety and migraines, was reduced (302). This may result from the impact of eCB signalling on endogenous opioid levels, as THC has been shown to regulate the release of endogenous opioids such as dynorphin A (303, 304).

CBD has neuroprotective and anti-inflammatory properties (258), with administration of CBD ameliorating EAE and delaying disease onset and progression in murine models (305). In addition, administration of CBD has anti-depressant effects in animal models of MS (306), and this may be attributable to the neuroprotective effects of CBD given the links between depression and atrophy within specific regions of the brain (127). A brief schematic to summarise this section may be seen below (Fig. 1.3).

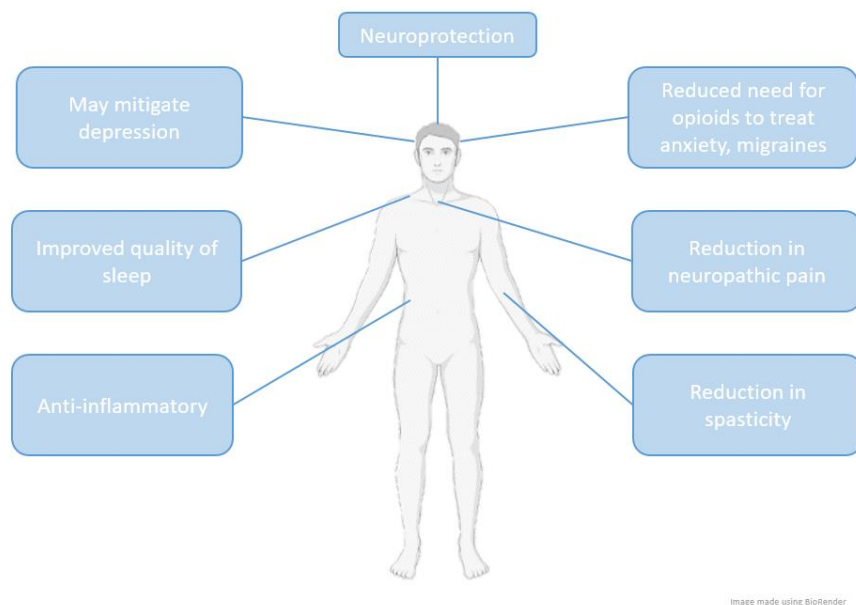


Figure 1.3 Impact of cannabinoids in MS. This schematic summarises the known impact of cannabinoids on various aspects of MS. Evidence indicates that cannabinoids can improve quality of sleep, reduce spasticity and neuropathic pain, in addition to acting as neuroprotectants and anti-inflammatories. Cannabinoids also reduce the dependence of opioids for symptom relief for migraines and anxiety, among others. These are discussed in further detail in section 1.13 above.

1.14 Cannabinoids targeting TLR signalling

Cannabinoid targeting the regulation of TLR signalling is a new area of research, although there is substantial evidence to support this. In cell culture experiments, cannabinoids have been shown to impair the activation of monocytes in a TLR-dependent manner, by reducing TLR-induced secretion of inflammatory cytokines IL-1 β and TNF- α (307). THC has also been shown to impair monocytic differentiation to DCs (308). Most research assessing the impact of cannabinoids on TLR signalling has been carried out using the TLR4 ligand, LPS, given that LPS promotes a classic model of bacterial inflammation. Indeed, LPS induction of IL-6, IL-1 β and TNF- α in the blood/brain of a rodent model of inflammation was reduced via exposure to a synthetic antagonist of CB₁, SR141716A (309). This indicates that CB₁ has an important role to play in LPS-activated TLR4 inflammatory signalling. THC has also been shown to inhibit TLR4-induced nitric oxide (NO) production in macrophages (310), as well as inducing apoptosis (311). THC affects post-translational processing and secretion of TNF- α in murine macrophages activated by LPS (312, 313). The cannabinoid derivative, CD-101, has also been shown to suppress the production of inflammatory cytokines IL-1 β , IL-6 and TNF- α , as well as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), in murine microglia when activated by LPS (314). The CB₂ agonist, JWH-133 was also found to downregulate TLR4 expression on DCs isolated from mice, suppressing antigen presentation and reducing cellular responses to LPS (315). The authors suggest that this may mediate increased infection in chronic cannabis users (315). 2-AG and the aminoalkylindole WIN55,212-2, were also shown to inhibit TLR2-induced NF- κ B phosphorylation via CB₁ in human glioma cells (316).

The proclivity of cannabinoids to target TLR signalling has also been investigated with relevance to MS pathogenesis. In a study conducted by Downer *et al.*, (2011), R(+)-WIN55,212-2, a synthetic cannabinoid, was shown to inhibit endosomal TLR3- and TLR4-induction of NF- κ B in *in vitro* experiments in HEK-293 cells expressing TLR3 and TLR4 (317). Pre-treatment with this synthetic cannabinoid also resulted in significant dose-dependent attenuation of downstream TNF- α induction through TLR3 and TLR4 stimulation in primary murine astrocytes (317). In addition, R(+)-WIN55,212-2 increased poly(I:C)-dependent IRF3 activation and subsequent activation of the IFN- β promoter in HEK293 cells expressing TLR3, while inhibiting LPS-dependent IRF3 activation in HEK293 cells expressing TLR4 (317). R(+)-WIN55,212-2 also enhanced poly(I:C) induced

expression of *IFN-β* mRNA in murine bone marrow-derived macrophages (BMDMs) (317). Interestingly, TRIF-deficient BMDMs had reduced proclivity for *IFN-β* expression following stimulation with poly(I:C), and R(+)WIN55,212-2 failed to augment this expression, indicating that the TRIF pathway may be a therapeutic target of R(+)WIN55,212-2 (317). It was subsequently confirmed that IRF3 phosphorylation and nuclear translocation, which is essential for induction of type I IFNs, was promoted by R(+)WIN55,212-2, in the presence or absence of poly(I:C) (317). This indicates that R(+)WIN55,212-2 has a direct effect on IRF3 of the TRIF pathway, which impacts on *IFN-β* induction (317). The use of CB₁ and CB₂ antagonists did not affect the proclivity of the synthetic cannabinoid to potentiate poly(I:C)-induced expression of *IFN-β* mRNA or IRF3 activation *in vitro* in HEK-293 cells, however (317). It was subsequently determined that the effects of R(+)WIN55,212-2 on *IFN-β* expression were PPARα-dependent manner, involving the phosphorylation of c-Jun N-terminal Kinase (JNK) and activation of activator protein-1 (AP-1), which in turn activates the positive regulatory domain (PRD) IV region of the *IFN-β* promoter (318). R(+)WIN55,212-2 was also capable of inducing heightened activity at separate regions of the *IFN-β* promoter through a PPARα-independent mechanism (318). Together, this resulted in heightened induction of *IFN-β* (318). It was also confirmed that administration of R(+)WIN55,212-2 proved beneficial in murine models of EAE, by reducing lymphocytic infiltration and demyelination of the spinal cord, and that the protective effects of R(+)WIN55,212-2 in EAE were *IFN-β* dependent (317).

In addition, TLR3-induced CXCL10 expression in PBMCs isolated from pwMS are desensitised, when compared to PBMCs from HCs (unpublished data from Downer laboratory). In contrast, published data from our laboratory indicates that PBMCs from pwMS are hypersensitive to TLR4 treatment in terms of TNF-α expression, when compared to PBMCs from HCs (319). Interestingly, THC and CBD, when administered alone and in a 1:1 mixture, inhibit TLR3-induced expression of *IFN-β* and CXCL10 in a human-derived macrophage cell line (79). Similarly, THC and CBD mitigated TLR4-induced CXCL10 expression in THP-1 macrophages (79). Overall it is clear that cannabinoid signalling cross-talks with TLR signalling, both in animal models and human studies. Given their role in inflammation, TLRs are clinical targets, and cannabinoid compounds may be used to target inflammatory TLR signalling events associated with MS pathogenesis. A summary table of what is known on the impact of cannabinoids on TLR signalling has been included below in Table 2.

Table 2. Impact of cannabinoids on TLR signalling

TLR	Impact of cannabinoids
TLR2	-TLR2-dependent NFκB phosphorylation via CB ₁ inhibited by 2-AG and the synthetic cannabinoid, WIN55,212-2 (316)
TLR3	-TLR3-dependent induction of NFκB in TLR3-expressing HEK293 cells nullified by the synthetic cannabinoid, R(+)WIN55,212-2 (317) -TLR3-dependent IRF3 activation enhanced by the synthetic cannabinoid R(+)WIN55,212-2 in HEK293 cells (317) -TLR3-dependent IFN-β mRNA induction in murine BMDMs enhanced by the synthetic cannabinoid R(+)WIN55,212-2 through TRIF pathway (317) -TLR3-dependent induction of IFN-β and CXCL10 are inhibited by THC, CBD and THC:CBD (1:1 mixture) in THP-1 macrophages (79) -Inhibition of FAAH via URB597 demonstrated a reduction in activation of both microglia and macrophages by TLR3 as demonstrated by reduced expression of CD11 and CD68 (320) -FAAH inhibition by URB597 reduced fever associated with TLR3 agonist administration via injection in rats, as well as increased nociceptive behaviour (320) -N-acylethanolamines, OEA and PEA, mitigate TLR3-induced hyperthermia in the hypothalamus in rats, while OEA also attenuates CXCL10, iNOS, COX2, IRF7, IL-1β and TNF-α genes among others in the hypothalamus (321)
TLR4	-Reduced TLR4-dependent IL-6, IL-1β, TNF-α induction in rodent brains/blood following treatment with synthetic CB₁ antagonist SR141716A (309) -THC reduces TLR4-dependent induction of NO in macrophages (310)

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- THC affects murine post-translational processing of TNF- α and its secretion following TLR stimulation (312, 313)
 - Synthetic CD-101 reduces TLR4-dependent induction of IL-6, TNF- α , IL-1 β , COX-2 and iNOS in murine microglia (314)
 - The CB₂ agonist, JWH-133, reduces TLR4 expression on murine DCs (315)
 - The FAAH inhibitor PF3845 significantly enhances expression of AEA, and *N*-acylethanolamines induced by the TLR4 agonist LPS in the frontal cortex of rats when delivered systemically, while direct injection of PF3845 into the frontal cortex resulted in attenuated expression of several NF κ B-inducible genes including IL-10, IL-6, TNF- α and IL-1 β induced by TLR4 (322)
 - Pretreatment with THC and CBD reduce TLR4-dependent induction of IL-6 and IL-1 β , as well as IFN- β in BV2 microglial cells, both at the level of the protein and mRNA (323)
 - CBD pretreatment attenuates TLR4-dependent IRKA-1 degradation in BV2 microglial cells, thereby reducing activation of the NF κ B pathway (323)
 - TLR4-dependent induction of NF κ B in HEK293 cells stably expressing TLR4 was nullified by R(+)-WIN55,212-2, a synthetic cannabinoid (317)
 - TLR4-dependent IRF3 activation was inhibited by the synthetic cannabinoid R(+)-WIN55,212-2 in HEK293 cells (317)
 - TLR4-dependent induction of CXCL10 was inhibited by THC and CBD in THP-1 macrophages (79)
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TLR7	-In TLR7-stimulated co-culture of monocytes and astrocytes, production of IL-6 and monocyte chemoattractant protein-1 by astrocytes (mediated by monocytes) was significantly attenuated following incubation with THC for 24 h (324)
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	-IL-1β production induced by TLR7 in monocytes of a co-culture of monocytes and astrocytes was significantly downregulated following incubation with THC or CB₂ agonist JWH-015 for 24 h (324) -THC at a concentration of [10 μM] can induce apoptosis in TLR7-activated monocytes (324) -pDCs activated through TLR7 and pretreated with AEA had significantly attenuated induction of IFN-α and TNF-α (325) -In human peripheral blood monocytes, secretion of TNF-α, IL-1β and IL-6 induced by TLR7 agonist R837 was significantly reduced following incubation with CBD (326)
TLR7/8	-Pre-treatment of mDCs stimulated through TLR7/8, with AEA or the CB₂ agonist JWH-015, significantly attenuated IL-6, TNF-α and IL-12p40 production by these cells (325) -Pre-treatment of pDCs with AEA attenuated IFN-α and TNF-α production following activation through TLR7/8 (325) -pDC/mDC activation marker expression increased following incubation with TLR7/8 agonist R848 and was not inhibited by pretreatment with AEA (325)
TLR8	-Secretion of TNF-α and IL-10 was induced in TLR8-activated human peripheral blood monocytes when treated with CBD, while IL-6 secretion was mitigated (326)
TLR9	-Human peripheral blood monocytes activated through TLR9 experienced a reduction in IL-6, TGF-β1 and IL-1β secretion following treatment of the cells with CBD (326)

Overall hypothesis and aims

Hypothesis

The study hypothesis is that TLR7 and TLR8 signalling, in addition to eCB signalling, are altered both centrally (in the CNS) and peripherally (in immune cells) in MS. This offers potential for future therapeutics by targeting such cellular signalling pathways using components of the cannabis plant.

Aims

- To characterise TLR7/8 signalling in human THP-1-monocyte-derived macrophages.
- To characterise eCS signalling in THP-1-monocyte-derived macrophages.
- To assess the impact of the pCB CBD on TLR7/8-dependent induction of inflammatory cytokines and chemokines in macrophages.
- To characterise the expression of inflammatory cytokines and chemokines in plasma from pwMS and HCs.
- To characterise the expression of components of the eCS, and relative TLR7 and TLR8 expression, in PBMCs isolated from pwMS and HCs.
- To characterise the expression of components of the eCS, and relative TLR7 and TLR8 expression, in cortical brain samples from MS cases and non-MS control cases.

Chapter 2 Materials and Methods

Materials and Methods

2.1 Cell Line Cultures

The THP1 monocyte-like cell line was used as a cell culture model in this project. THP-1 cells were kindly gifted by Prof. Andrew Bowie and Prof. Marina Lynch, or were purchased from ATCC (Maryland, USA). The THP-1 cell line is a well-characterised acute monocytic cell line that can be differentiated to macrophages by external stimuli such as phorbol esters (327), and these cells are an appropriate *in vitro* model in which to study TLR signalling (328, 329). Indeed, data published from our laboratory indicate that both the TLR3-IRF3-IFN- β /CXCL10 and TLR4-NF- κ B-TNF- α signalling axes are operative in THP-1-differentiated macrophages (79). The THP1 monocyte cell line was originally derived from a childhood case of acute monocytic leukemia. Monocytes were maintained in culture with RPMI 1640 media (Gibco, Life Technologies) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Sigma-Aldrich, Dorset, UK) and 1% penicillin streptomycin solution (Sigma). The cells were incubated at a temperature of 37°C at 5% CO₂ and were passaged every 2-3 days. For passaging, the cells were seeded at a density of 3×10^6 cells per flask.

2.2 PMA-induced differentiation of THP-1-monocyte-derived macrophages

PMA (Sigma-Aldrich) was used to differentiate THP-1 monocytes to macrophages at a final concentration of 10ng/ml. PMA was reconstituted in sterile ethanol (Sigma-Aldrich) at a stock concentration of 0.2 mg/ml, aliquoted and stored in the dark at -20°C until use. To differentiate THP-1 monocytes to THP-1-derived macrophages, THP1 monocytes were resuspended in a flask containing RPMI 1640 media (Gibco, Life Technologies) supplemented with 10ng/ml PMA solution, resuspended in RPMI 1640 from 0.2mg/ml stock. The cells were immediately added to sterile cell culture plates at a desired density of 0.25×10^6 cells per well (or 0.04×10^6 cells per well for Thiazoyl Blue cytotoxicity assay) and were maintained in culture to differentiate at 37°C for 48 h, as is consistent with the literature available (76). Following 48 h incubation, all media was removed from the wells and cells were washed three times in sterile RPMI 1640 media. The cells were then left to 'rest' for a further 24 h at 37°C to return to a less pro-inflammatory state, as is consistent with other literature/various publications (76) and unpublished data from the Downer laboratory.

2.3 Drug Treatments

2.31 CL075 Treatment

To assess TLR7/8 signalling in THP-1 macrophages, CL075, a TLR7/8 agonist, was purchased from Invivogen. THP-1-derived macrophages were seeded at a density of 0.25×10^6 cells/ml in 24-well sterile cell culture plates (Biosciences). Stock CL075 (Invivogen), prepared at 1mg/ml in sterile H₂O, was diluted separately in RPMI 1640 to the desired working concentrations (0.625-10 µg/ml). Cells were stimulated with CL075 for time points between 10 min to 24 h. Vehicle control wells in duplicate were included on the plate, containing RPMI and 0.1% H₂O. Following incubation, all supernatants were harvested and stored at -20°C for protein assessment by ELISA and nitrite assessment via the griess reaction. Cells were harvested for RNA extraction in a 1% lysis buffer of β-mercaptoethanol (Sigma) in RA1 buffer (Nucleospin® RNA II isolation kit (Machery-Nagel Inc., Geschäftsführer, Germany)).

2.32 LPS Treatment

In some cases, to assess TLR4 signalling in THP-1 macrophages, we employed the use of LPS (ALEXIS Biochemicals, USA). THP-1-monocyte derived macrophages were seeded at a density of 0.25×10^6 cells per well in 24-well sterile cell culture plates (Biosciences). Stock LPS (10 µg/ml) was diluted in RPMI 1640 to the desired concentration of 100 ng/ml. Cells were stimulated with LPS for time points ranging between 10 min to 24 h, while control wells containing RPMI alone were incubated for the same time points, containing the same media volume as LPS-treated wells. Upon completion of the incubation, all supernatants were harvested and stored at -20°C for assessment of cytokine, chemokine, IFN and nitrite production using ELISA and griess reactions (data included in appendices). Cells were harvested in RA1 buffer solution (Nucleospin® RNA II isolation kit (Machery-Nagel Inc., Geschäftsführer, Germany)) containing 1% β-mercaptoethanol (Sigma). These cell samples were stored at -80°C to avoid protein degradation prior to RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR).

2.33 Treatment with CBD

THP-1-derived macrophages were seeded at a density of 0.25×10^6 cells per well in 24-well sterile cell culture plates. The pCB, CBD (Tocris, Abingdon, UK), was dissolved in sterile ethanol and stored, protected from light, at -20°C at a stock concentration of 10 mM. CBD was then dissolved separately in RPMI 1640 to the desired working concentration (0.001-10 μM). To assess the impact of CBD on cytokine/chemokine, IFN and nitrite production, ELISA, griess assays and RT-qPCR were performed. THP-1-derived macrophages were pre-treated with CBD for 30-45 min prior to addition of the TLR7/8 agonist CL075 for 6 and 24 h. In some cases, cells were pre-treated with CBD (10 μM) for 30-45 min prior to addition of the TLR4 agonist LPS for 6 h (data included in appendices). In all phytocannabinoid experiments, control wells included cells treated with both RPMI 1640 (in duplicate) alone and vehicle controls consisting of 0.1% sterile ethanol (Sigma Aldrich) in RPMI 1640 in the same volume as the TLR agonist-treated wells (500 μl).

2.4 Thiazoyl Blue (MTT) Cytotoxicity Assay

THP-1-derived macrophages were seeded at a density of 0.04×10^6 cells per well in 96-well sterile cell culture plates (Thermo Scientific). Macrophages were differentiated from THP-1 monocytes as described above. Cells were stimulated with CL075 (dissolved in RPMI 1640) at a working concentration of 2.5 $\mu\text{g}/\text{ml}$ for either 6 or 24 h. Control cells were treated with 0.1% H_2O in RPMI 1640 in triplicate. Dimethyl sulfoxide (DMSO) (10%; Sigma Aldrich) was employed as a positive control in MTT assays, and was added to control wells at the beginning of the incubation, to allow for membrane permeabilisation. MTT (5mg/ml stock dissolved in PBS, filtered sterilised; final working concentration of 1mg/ml; Merck) was added to every well 3.5 h prior to the end of the incubation. At the end of the incubation, all media was removed and DMSO was added to each well. MTT (yellow) is metabolised by viable cells to yield a purple formazan end product. DMSO was added at the end of the incubation to dissolve the purple formazan product. The DMSO was triturated gently to ensure colour homogenisation in each well. Each plate included several wells containing DMSO alone as blank controls for analysis. The sample absorbance was measured at a wavelength of 560nm in a multiwell plate reader.

2.5 Griess Assay

The griess reaction was used to determine cell viability by assessing NO formation by measuring production of the stable compound NO^-2 (nitrite), a product formed from the breakdown of NO. This experiment was run on cell culture supernatants to determine the impact of drug treatment on cell viability. NO is a biomarker for inflammation and cell death resulting from oxidative stress and DNA damage, amongst other factors (330). Standards of NaNO_2 were prepared from the 0.1 M stock of NaNO_2 to a concentration of 100 μM in RPMI 1640. Standards were prepared in duplicate in RPMI 1640 in 96-well plates at concentrations of 100 μM , 50 μM , 20 μM , 10 μM , 5 μM , 2 μM and 0 μM NaNO_2 . Each sample (100 μl) was added to the plate and 1% sulphanilamide (TCI; 100 μl) was added to all wells. The plate was left to incubate for 5 min, protected from the light. N-1-naphylethylenediamene dihydrochloride (NED) (VWR) (100 μl of a 0.1% solution) was then added to each well and the plate left to incubate, covered from the light, for 5 min. The absorbance of the samples was measured at 530 nm using a plate reader.

2.6 Blood samples from HC subjects and pwMS

HC cases and pwMS attending the Neurology clinics at Beaumont Hospital Dublin were recruited to this study. HC individuals were mainly recruited as volunteers from Trinity College Dublin. The cohort of subjects contributing data to this project consisted of 35 pwMS (30 female/5 male) and 43 HC subjects (28 female/ 15 male). Written informed consent was obtained from each participant and the study received ethical approval from the School of Medicine Research Ethics committee, Trinity College Dublin, and the Beaumont Hospital, Ethics (Medical Research) Committee (Appendix 2). The EDSS scores were determined by a Neurologist and all pwMS had a RR form of MS with minimal to moderate disability, as defined by the revised McDonald criteria (90). All pwMS had to meet the revised MacDonald diagnostic criteria for clinically defined MS including patient history, clinical signs and symptoms, physical examination, and adjunctive diagnostic tools including MRI (90).

Peripheral blood was drawn via venepuncture, with up to 50 ml being drawn from each individual. The blood was stored at room temperature for up to 4 h. To assess the cellular composition of the whole blood, 25 μl of the blood sample was sampled via a Symsex Haematology Analyser. The following cell readouts were automatically generated: white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), platelet number

(PLT), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), lymphocyte number (LYM), neutrophil number (NEU), mixed cell number (MXD) (lymphocyte, neutrophil and other immune cells number). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient over Lymphoprep (Axis-Shield, Oslo, Norway) as previously described (319). Plasma was aliquoted and stored at -80°C until analysis and PBMCs were harvested for RNA extraction in a 1% lysis buffer of β -mercaptoethanol in RA1 buffer. The recruitment of participants, sysmex analysis and PBMC/plasma isolations were conducted by Dr. John-Mark Fitzpatrick (PhD student, Dr. Downer laboratory). PBMCs isolated from whole blood in this study have also been used in a variety of ELISA and PCR experiments to assess TLR3/4 signalling, in addition to the impact of phytocannabinoids on TLR signalling, which have recently been published (331).

At the time of blood draw, study participants completed the self-reporting MSQoL-54 and QIDS-SR₁₆ questionnaires. MSQoL-54 is tailored specifically towards understanding the impact of MS on an individual's quality of life (QoL), with focus on physical and mental health. This questionnaire has been used previously by the laboratory and has been widely used elsewhere (332, 333). The QIDS-SR₁₆ questionnaire focuses on severity of depressive symptoms experienced by an individual (334). Scores of each individual as determined by each questionnaire were collated and used to determine any differences between HCs and individuals with MS in terms of depression, physical and mental health, according to a number of categories.

2.7 Acquisition of post-mortem brain tissue

CNS tissue from non-MS control cases, PPMS and SPMS cases were sourced from the UK MS tissue bank. In addition to tissue samples, neuropathological case reports were also obtained for each case. In total, tissue samples and reports from 36 cases were sourced (24 MS cases and 12 non-MS control cases). Ethics for the project was approved by the Trinity School of Medicine Research Ethics Committee and by the St James' Hospital Research Ethics Committee (Appendix 2). Data from the reports consisted of details such as age, sex, cause of death, brain weight, CSF pH, tissue preservation interval, and clinical history. Furthermore, the reports contained details on the macroscopic and microscopic post-mortem findings. For certain donors, snap frozen blocks of cortical tissue and

paraffin embedded cortical brain sections were provided. During the course of this project, snap frozen sections were used for RT-qPCR analysis, while demyelination was assessed in sections stained previously with Luxol Fast Blue (LFB). Male/female stratification for brain weight in control cases and MS cases (PPMS and SPMS) was carried out for further investigation.

2.8 Demyelination assessment in post-mortem tissue

Each MS case included in the tissue donation from the UK MS Brain Bank included both a section of NAWM and a section of brain tissue including a chronic active lesion (CAL). Overall 30 tissue samples were assessed from the brain bank (6 controls, 6 PPMS NAWM, 6 PPMS CAL, 6 SPMS NAWM, 6 SPMS CAL) in the course of this study. Each tissue sample was accompanied by an image of the formalin-fixed paraffin embedded sample stained with LFB, a common stain for myelin. This staining was previously performed by Mr. Richard Magee and Dr. Siew-Mei Yap (Dr. Downer laboratory). All sections of demyelination show on the image as white (Figure 2.1), thus allowing for quantification of demyelination that occurred in the tissue. Image J was used to determine the percentage of demyelination that had occurred in each sample. For demyelination assessment, each section was analysed in a blinded manner. For each sample, the measurement scale was set to 1mm by following the scale provided in the image and using the 'set scale' option in the analyse tab. The image J drawing tool was used to trace the full area of the tissue in the image, this was measured using the 'measure' function on Image J and fixed in place. Subsequently, each area of demyelination was measured and fixed onto the image using the same technique. These results were used to determine the percent demyelination in the tissue by inclusion in an excel sheet where the total area of demyelination in the image was divided by the total area of NAWM tissue, multiplied by 100. A map of the NAWM and NAGM of each image was then made using the Inkscape programme version 1.1.1. This was achieved by layering the image to remove all background stain, showing only the relevant tissue labelled as NAWM and NAGM.

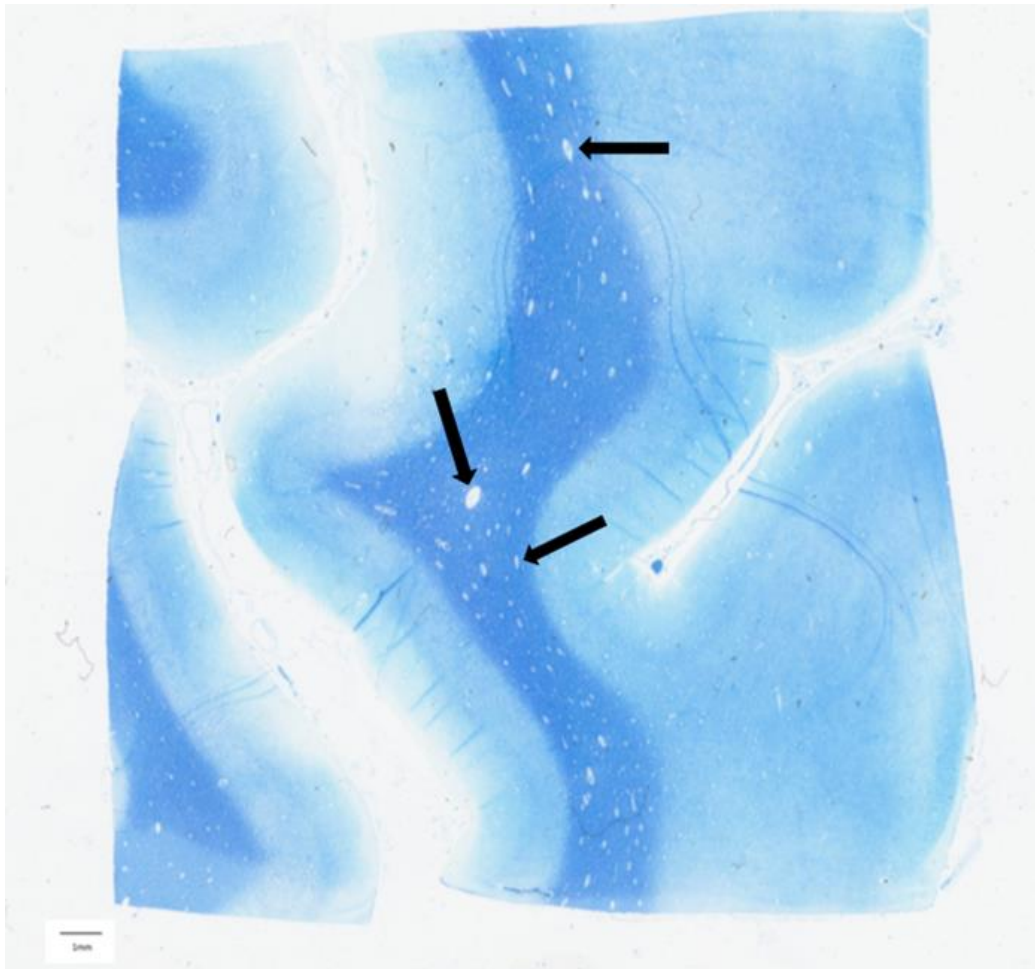


Figure 2.1 Areas of demyelination on a formaldehyde-fixed paraffin-embedded section of SPMS NAWM tissue (donor 044) stained with LFB. Above is the raw image sourced from the UK MS and Parkinson's Brain Bank stained with LFB. LFB stains myelin, thus any areas of demyelination appear as unstained areas. Different examples of areas of demyelination on the image are indicated with arrows above. Although not fully discernible here, the scale given in this image is 1mm.

2.9 Enzyme-Linked Immunosorbant Assays (ELISAs)

The expression of IL-1 β , TNF- α , IFN- β , RANTES, CXCL10 and IL-10 was assessed via ELISA in supernatants from THP1-derived macrophages. In addition the expression of IL-1 β , TNF- α , CXCL10, C-reactive protein (CRP), RANTES and IFN- β was assessed by ELISA in human plasma samples isolated from pwMS and HC subjects. IL-1 β , TNF- α , CRP, RANTES, IFN- β , CXCL10 and IL-10 antibodies were supplied by R&D Systems (DuoSet human

cytokine ELISA kit, biotechne). Standard concentrations of the target protein were prepared from the stock solutions and were resuspended in RPMI 1640 media/1% BSA to a working concentration. Supernatants and plasma samples were run as samples in duplicate. On day 1 the capture antibody was prepared, diluted in filtered PBS to the desired working concentration of 4 µg/ml and 50 µl was added to each well of a 96-well plate (Thermo Scientific). The plate was left covered overnight at room temperature. On day 2, the 96-well plate was washed (x3) in 200 µl PBS-Tween (0.05% Tween (Sigma-Aldrich) in PBS) to remove unbound capture antibody. Each well was blocked with 200 µl blocking buffer (1% BSA) (Sigma-Aldrich) for 1 h at room temperature. Each well was then washed (x3) in PBS-Tween (200 µl). Samples and the standards (50 µl each) were loaded onto the plate in duplicate, diluted as necessary in RPMI. The plate was left covered for 2 h at room temperature. The plate was washed (x3) in wash buffer (200 µl) and detection antibody (50 µl) was added to each well. The plate was left covered at room temperature for 2 h. The plate was again washed (x3), 50 µl Streptavidin-HRP (1:40 dilution in 1% BSA; R&D Systems) was added to each well, the plate was covered from the light and left for 20 min. The plate was washed (x3) in wash buffer and Tetramethylbenzidine (TMB) solution (50 µl; Sigma-Aldrich) was added to each well, the plate was covered from light and left for a maximum of 30 min. Sulfuric acid (0.18 M)(25 µl; Aldrich Chemistry) stop solution was added to each well to stop the reaction. The plate was then read at 450 nm using a spectrophotometer, giving the absorbance of each sample.

For each ELISA kit the recommended concentration of the top standard varied from 2,000 pg/ml-500 pg/ml. Thus the standard curve created on each plate was consistent with one of the following: In those with a top standard of 2,000 pg/ml, the following dilutions were made: 1,000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, and 31.25 pg/ml. Those with a top standard of 1,000 pg/ml the following dilutions made up the standard curve: 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, and 15.6 pg/ml. While those in which the top standard on the plate was at a concentration of 500 pg/ml the following dilutions of the standard were made: 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.5 pg/ml, 15.75 pg/ml, and 7.88 pg/ml. For IL-1β ELISAs the minimal detection level was reported as 3.9 pg/ml as per manufacturer's instructions. For TNF-α, RANTES and CRP ELISAs, the minimal detection level was noted as 15.6 pg/ml as per manufacturer's instructions. In CXCL10 ELISAs the minimum detection was 31.2 pg/ml,

and for IFN- β ELISAs minimal detection was 7.81 pg/ml as per manufacturer's instructions.

2.10 Quantitative real-time PCR

THP-1 macrophages were plated at 0.25×10^6 cells per well in 24-well sterile cell culture plates. Cells were treated with either CL075 (2.5 μ g/ml) for 6 or 24 h, or were pre-treated (30-45 min) with CBD (10 μ M) prior to addition of the TLR7/8 agonist CL075 (2.5 μ g/ml) for either 6 h or 24 h. At time zero, supernatants were removed and stored, the wells gently washed with sterile PBS, and 100 μ l of RA1 lysis buffer (RA1 buffer solution containing 1% β -mercaptoethanol) added to macrophages in all wells. As macrophages are adherent cells, the pipette tip was used to gently scrape the wells and dislodge cells in the buffer. These samples were stored at a -80°C until RNA extraction. For PBMC analysis, PBMCs from "control" treatments were previously harvested and stored in RA1 buffer by Dr John-Mark Fitzpatrick (Dr. Downer laboratory). For analysis of post mortem brain tissue, tissue blocks were placed on dry ice and a tissue puncher (Integra Miltex biopsy punch with plunger system 2.00 mm) was used to take a punch of brain tissue from each cortical brain sample (10-30 mg per sample). Dr Carol Murray and Dr. Eva Jimenez-Mateos assisted with this process. For post-mortem brain samples, tissue punches were taken of either NAWM, NAGM or CALs in WM. The tissue punched was placed into coded DNase/RNase free eppendorfs and the tissue weight determined prior to storage at -80°C. RNA was extracted from THP-1 macrophages, PBMCs (from HC and pwMS) and cortical tissue blocks (from non-MS control NAWM, PPMS NAWM, CALs and NAGM and SPMS NAWM, CALs and NAGM) using the Nucleospin[®] RNA II isolation kit (Machery-Nagel Inc., Geschäftsführer, Germany). For macrophages each sample was run in duplicate as part of the cell culture, for the extraction of the RNA the two individual samples were pooled, resulting in 200 μ l of lysis buffer containing RNA for each sample. For post mortem brain samples, tissue was manually disrupted using a pestle in lysis buffer. The tissue was further homogenised using a motorised pestle. This was undertaken by Dr Carol Murray. The RA1 lysis buffer containing the RNA from each sample was thawed and vortexed prior to addition to a Nucleospin filter in a 2 ml collection tube which was spun at 11,000 *g*, ensuring all the lysate was passed through the filter. The filter was discarded and 350 μ l of molecular grade ethanol (Sigma) was added to the lysate, trituration was used to ensure appropriate homogenisation of the solution was achieved. The homogenate was then added to a Nucleospin RNA column

and spun for 30 seconds at 11,000 *g*, this allowed RNA present to bind to the filter. 350 µl of MDB (Machery Nagel) was added to desalt the silica membrane and the sample was spun at 11,000 *g* for 1 min. 95 µl DNase reaction mixture (reconstituted 10 µl of the RDNase provided in 90 µl of reaction buffer for RDNase) was added to every sample and incubated for 15 min at room temperature. A series of washes were used to wash and dry the silica membrane. 200 µl RA2 buffer was then added to the Nucleospin filter and spun at 11,000 *g* for 30 seconds. This was followed by addition of 600 µl of buffer RAW3, which was spun at 11,000 *g* for 30 seconds. Finally, another 250 µl of RAW3 was added and the sample spun at 11,000 *g* for a further 2 min. The collection column was changed after each wash. The RNA was eluted in 40 µl RNase free H₂O spun for 1 min at 11,000 *g*. The RNA concentration was determined via addition of 1 µl of RNA to a UV-vis spectrophotometer. The purified extracted RNA was stored at -80°C.

Once RNA was extracted, complementary DNA (cDNA) synthesis was performed. RNA concentrations were quantified via addition of 1 µl of extracted RNA to a UV-vis spectrophotometer. cDNA synthesis was performed in samples of RNA (between 0.1-1 µg) using the high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, California) according to the manufacturer's instructions. Real-time PCR primers were delivered by Taqman[®] Gene Expression Assays (Applied Biosystems), containing both forward and reverse primers. Each expression assay contains a FAM-labelled MGB Taqman probe for the specific target gene. The following primers were used to assess gene activation in cultured THP-1-derived macrophages, PBMCs and post-mortem tissue: CXCL10: Hs00171042_m1; TNF-α: Hs01113624_g1; IFN-β: Hs01077958_s1; ccl5: Hs00982282_m1; TLR8: Hs00152972_m1; TLR7: Hs00152971_m1; CNR1: Hs00275634_m1; CNR2: Hs00361490_m1; FAAH: Hs01038664_m1; MGLL: Hs00996004_m1 gene expression assays. Equal amounts of cDNA (10 µl; 1:4 cDNA in H₂O) from each sample was added to the plate, as well as 15 µl Master Mix containing 12.5 µl Taqman Universal PCR Master Mix (Applied Biosystems), 1.25 µl of Taqman gene assay primer (Applied Biosystems) and 1.25 µl Eukaryotic 18S ribosomal RNA (rRNA) (20X) (catalog no. 4319413E) as an endogenous control primer labelled with a VIC antibody (Applied Biosystems). Non-template controls were also prepared in two wells. The PCR plate was loaded into the PCR machine (Applied Biosystems 7300 Real-time PCR system) and run for 40 cycles. Each cycle ran as follows: 2 min at 50°C, 10 min at 95°C, 15 s at 95°C and 1 min at 60°C. Gene expression was determined with respect to the

endogenous Eukaryotic 18S rRNA control. Analysis was performed using the $2^{-\Delta\Delta CT}$ method.

2.11 Statistical Analysis

All statistical analysis was performed using GraphPad Prism, version 8 and 9. ROUT method was used to identify outliers in all experiments apart from the assessment of plasma cytokines/chemokines presented in Table 8 and Fig. 4.2, 2.3, 4.4, 4.5 and 4.6. The reason for this was the inherent variability associated with plasma cytokine/chemokine assessment in human samples. All data was tested for normality using the Shapiro-Wilk test. If data were normally distributed, parametric testing was used via student's *t*-test or one-way ANOVA followed by *post hoc* Dunnett's multiple comparisons test. If data were not normally distributed non-parametric testing was employed using the Mann-Whitney U test or the Kruskal-Wallis test following by Dunn's multiple comparisons test. Data are expressed as means $\pm$ standard errors of the mean (SEM). Within each experiment, duplicate/triplicate determinations were performed for each condition/drug treatment. Correlations were assessed using simple linear regression. Significance in all experiments was defined as $p < 0.05$.

Chapter 3 Characterisation of TLR7/8 and eCS signalling in THP-1 macrophages

3.1 TLR7 and TLR8 are expressed in THP-1-derived macrophages

A major goal of this study was to characterise endosomal TLR7/8 signalling in immune cells with relevance to MS. Hence, initially we set out to determine whether the TLR7/8 signalling axis is operative in THP-1-derived macrophages. The expression profile of both TLR7 and TLR8 was first assessed using human THP-1 macrophages over four separate passages. THP-1 monocytes were cultured in media containing PMA (10 ng/ml) for 48 h, followed by incubation with PMA-free media for an additional 24 h. RNA was then harvested from THP-1 macrophages, and RT-qPCR performed to determine the relative expression profile of *TLR7* and *TLR8* mRNA. 18S rRNA was employed as an endogenous control. Data presented in Table 3 demonstrate that both TLR7 and TLR8 are expressed in THP-1 macrophages. This concurs with the available literature (78).

Table 3. TLR8 and TLR7 receptor expression in THP-1-derived macrophages

Target	Average basal expression (Ct) (n=4)
TLR8	34.16 ± 0.26
18S rRNA	6.97 ± 0.14
TLR7	32.35 ± 0.19
18S rRNA	6.90 ± 0.07

Data are expressed as mean ± SEM; Ct, cycle threshold; rRNA, ribosomal ribonucleic acid

3.2 The dose-dependent effect of the TLR7/8 agonist CL075 on the expression of CXCL10, TNF- α and RANTES in THP-1 macrophages

Following determination that both TLR7 and TLR8 are expressed in THP-1 macrophages, we next employed the use of a TLR7/8 agonist, CL075 (a thiazoquinoline), to assess the impact of TLR7/8 activation on the induction of pro-inflammatory cytokines and chemokines (Fig. 3.1). CL075 is an activator of both the TLR7 and TLR8 signalling networks, with a higher affinity for TLR8 (41). THP-1-derived macrophages were treated with CL075 at final concentrations of 0.625 $\mu\text{g/ml}$, 1.25 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ for 24 h. The supernatants were harvested and analysed by ELISA to determine the protein concentration of TLR7/8-associated chemokines/cytokines (Fig. 3.1). Data presented in Figure 3.1A indicates that CL075 treatment promoted an increase in the expression of the inflammatory chemokine CXCL10 at all doses tested, and this reached significance at a final concentration of 2.5 $\mu\text{g/ml}$ ($p < 0.01$; Fig. 3.1A), which is consistent with wider literature in THP-1 monocytes (80). Furthermore, CL075 promoted a dose-dependent increase in TNF- α protein expression in THP-1 macrophages, again reaching statistical significance at 2.5 $\mu\text{g/ml}$ ($p < 0.01$), 5 $\mu\text{g/ml}$ ($p < 0.05$) and 10 $\mu\text{g/ml}$ ($p < 0.01$) (Fig. 3.1B). The dose-dependent increase in TNF- α production was not mirrored by CXCL10. TNF- α is commonly induced by TLR signalling through the MyD88-dependent pathway and is known to be induced in response to viral infection through TLR7/8 signalling (335). In experiments carried out elsewhere on THP-1 macrophages it was demonstrated that TLR7/8 activation through CL075 induced NF κ B activation, with subsequent induction of TNF- α (78). Here, we demonstrated the most significant induction of TNF- α at a CL075 concentration of [2.5 $\mu\text{g/ml}$] which has been employed elsewhere (59). Interestingly, it was reported elsewhere that THP-1 macrophages incubated with CL075 had lost the ability to activate ISGs, although induction of CXCL10 specifically was not assessed in this instance (78). CXCL10 is an IFN-inducible gene (336), thus this may explain the lack of sensitivity demonstrated by CXCL10 in Fig. 3.1A to increasing doses of CL075. However, IFN-independent induction of CXCL10 has been previously demonstrated in the context of viral infection through the action of NF- κ B and IRF3 (337). Further experiments are required to determine the dose-dependent effects of CL075 exposure on MAPK, NF κ B and IRF signalling in terms of the regulation of cytokine/chemokine expression in THP-1 macrophages. Data presented in Figure 3.1C indicate that TLR7/8 activation had no significant effect on the expression of the chemokine RANTES in macrophages. It is noteworthy that in control cells, basal RANTES expression was high (within the 1000

pg/ml range) (Fig. 3.1C), and this may negate any stimulatory effect of CL075 on the expression of this chemokine in THP-1-derived macrophages (Fig. 3.1C). Indeed, high basal RANTES expression was recorded in THP-1 macrophages elsewhere (338). Data in Fig. 3.1 suggest that treatment of THP-1 macrophages with CL075 at [2.5 µg/ml] has the proclivity to promote TLR7/8-induced CXCL10 and TNF-α protein expression.

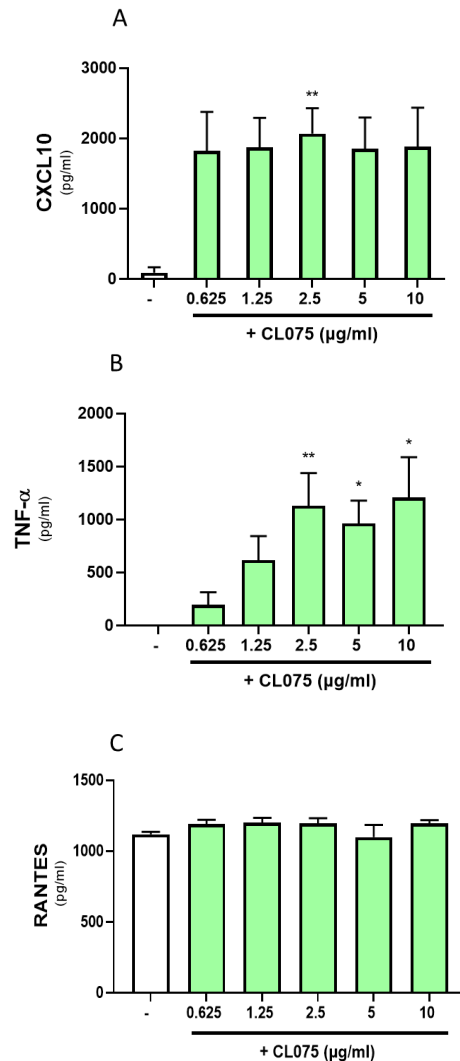


Figure 3.1 CL075 induces the expression of inflammatory cytokines and chemokines in a dose-dependent manner in THP-1 macrophages. THP-1-derived macrophages were treated with CL075 at concentrations of 0.625, 1.25, 2.5, 5 and 10 µg/ml for 24 h, and the expression of **(A)** CXCL10, **(B)** TNF-α and **(C)** RANTES determined by ELISA in supernatants. Data are presented as the mean ± SEM of duplicate determinations from 4 independent passages. For non-parametric data, Kruskal-Wallis followed by Dunn's multiple comparison test was performed. * $p < 0.05$ and ** $p < 0.01$ versus vehicle -treated cells.

3.3 CL075 time-dependently induces the expression of CXCL10 and TNF- α protein in THP-1-derived macrophages

Given that CL075 at a concentration of 2.5 $\mu\text{g/ml}$ promoted the expression of CXCL10 and TNF- α (Fig. 3.1), we next assessed the time-dependent (10 min-24 h) effect of CL075 treatment at this concentration on the production of chemokines (CXCL10, RANTES) and cytokines (TNF- α), in addition to the production of the type I IFN, IFN- β , in THP-1 macrophages (Fig. 3.2). THP-1 monocytes were differentiated to macrophages using PMA (10 ng/ml) for 48 h and then incubated with PMA-free media for a further 24 h. After 24 h, THP-1 macrophages were stimulated with 2.5 $\mu\text{g/ml}$ solution of CL075 for a range of time points (10 min-24 h). At time zero, the supernatants were harvested and analysed by ELISA to determine the relative protein concentration of the target cytokines/chemokines (Fig. 3.2). Data presented in Fig 3.2A indicates that CL075 induced CXCL10 protein expression most markedly at the 24 h time point, although this did not reach statistical significance (Fig. 3.2A). Data presented in Fig. 3.2B indicates that CL075 time-dependently promoted an increase in TNF- α protein expression in THP-1-macrophages, with a significant induction of the cytokine determined at 24 h ($p < 0.05$; Fig. 3.2B). This trend of enhanced TNF- α expression in THP-1-derived macrophages treated with CL075 at [2.5 $\mu\text{g/ml}$] was also noted by Odoardi *et al.*, (2021) (81). In addition, data presented in Fig. 3.2C indicates that RANTES protein expression was not significantly upregulated following TLR7/8 activation at all time points tested in macrophages (Fig. 3.2C). Again, a high basal expression of RANTES was determined in control supernatants (approx. 1,500 pg/ml). Interestingly, IFN- β protein was not detected in supernatants following TLR7/8 activation at all time points tested, suggesting that CL075 does not induce IFN- β expression at the protein level in THP-1 macrophages (Fig. 3.2D).

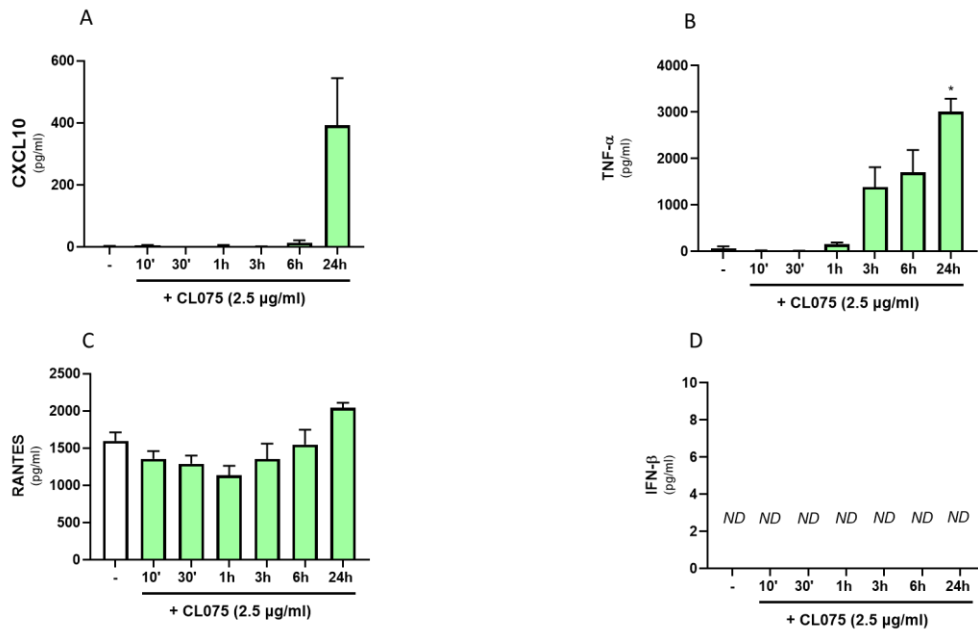


Figure 3.2 CL075 time-dependently induces the production of the inflammatory chemokine CXCL10, as well as the inflammatory cytokine, TNF- α , in THP-1 macrophages. THP-1 macrophages were treated with CL075 (2.5 μ g/ml) for 10 min, 30 min, 1 h, 3 h, 6 h and 24 h, and the concentrations of **(A)** CXCL10, **(B)** TNF- α , **(C)** RANTES and **(D)** IFN- β assessed in supernatants by ELISA. Data are presented as the mean $\pm$ S.E.M of triplicate determinations from 4 independent passages. Data were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test. * $p < 0.05$ versus untreated control cells.

3.4 Time-dependent effect of CL075 on cytokine/chemokine mRNA expression in THP-1-derived macrophages

Given that 2.5 µg/ml CL075 induced cytokine/chemokine expression in THP-1 macrophages at the protein level (Fig. 3.1, 3.2), we next assessed the impact of this ligand on cytokine and chemokine gene expression. THP-1 macrophages were differentiated from monocytes via incubation with PMA (10 ng/ml) for 48 h, followed by 24 h incubation with media in the absence of PMA. After 24 h, THP-1 macrophages were treated with CL075 at a working concentration of 2.5 µg/ml for a period of either 6 or 24 h. At time zero, RNA was harvested, cDNA synthesis was performed and RT-qPCR used to determine cytokine/chemokine gene expression in THP-1-macrophages (Fig. 3.3). The data below indicates that *CXCL10* mRNA was induced most markedly (albeit insignificantly) in THP-1 macrophages at 6 h post-stimulation with CL075 (Fig. 3.3A). The gene expression of TNF-α was significantly increased at both the 6 h and 24 h time points following treatment with CL075 ($p<0.05$; Fig. 3.3B). This is in agreement with published work suggesting that TNF-α is upregulated following incubation with CL075 at a 24 h time point (81). Interestingly, TLR7/8 activation with CL075 promoted a significant increase in *RANTES* mRNA at both the 6 h and 24 h time points ($p<0.01$; Fig. 3.3D). *RANTES* mRNA, but not protein expression, was enhanced following CL075 exposure at 6 h and 24 h. Basal *RANTES* protein expression was high in Fig 3.2C, which may mask any effect determined with the TLR ligand in terms of *RANTES* protein expression. Protein and mRNA differences may also reflect post-transcriptional modifications of *RANTES*, in addition to degradation of chemokine following its production. Given that CL075-induced *RANTES* mRNA at 24 h, it would be interesting to determine the temporal impact of CL075 treatment on *RANTES* protein expression at later time points of 36 - 48 h. Furthermore, CL075 treatment also induced *IFN-β* mRNA expression, albeit insignificant, at both 6 h and 24 h in THP-1 macrophages (Fig. 3.3C). Overall these findings suggest that TLR7/8 stimulation of THP-1 macrophages with CL075 promotes an increase in the expression of inflammatory genes in THP-1 macrophages.

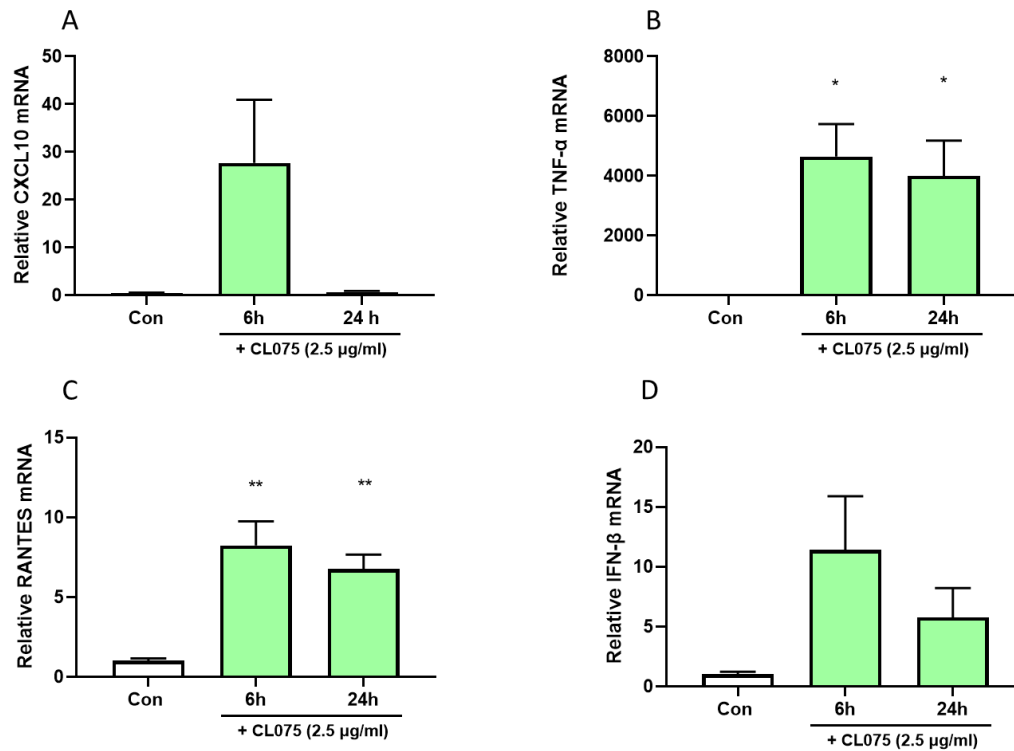


Figure 3.3 Effect of CL075 on *CXCL10*, *TNF-α*, *RANTES* and *IFN-β* mRNA expression in THP-1 macrophages. THP-1 macrophages were treated with CL075 (2.5 µg/ml) for 6 h and 24 h, and the expression of **(A) *CXCL10***, **(B) *TNF-α***, **(C) *RANTES*** and **(D) *IFN-β*** mRNA assessed by RT-qPCR. Data are presented as the mean $\pm$ S.E.M of triplicate determinations from 4 independent passages. Data were analysed using one-way ANOVA and Dunnett's multiple comparisons test. * $p < 0.05$ and ** $p < 0.01$ versus control cells.

3.5 CL075 is not toxic to THP-1 macrophages

Having determined that CL075 activates TLR7/8 signalling in THP-1-derived macrophages in culture, we set out to determine if incubation of differentiated THP-1 cells with 2.5 µg/ml CL075 resulted in cellular toxicity using MTT assays. THP-1 macrophages were differentiated from monocytes via incubation with PMA (10 ng/ml) for 48 h. This was followed by 24 h incubation with PMA-free media, after which THP-1 macrophages were incubated with 2.5 µg/ml CL075 for 6 or 24 h. Vehicle control cells were incubated with 0.1% H₂O in RPMI. Positive control cells were treated with sterile DMSO (10%) for 24h. The data presented in Fig. 3.4 clearly indicate that there was no difference in the viability of the control cells versus those that were treated with 2.5 µg/ml CL075 for both 6 h and 24 h. DMSO significantly reduced cell viability ($p<0.001$; Fig. 3.4). Overall, this suggests that the impact of CL075 on inflammatory cytokine/chemokine expression in Fig. 3.1, 3.2 and 3.3 was not due to toxicity associated with the use of this ligand.

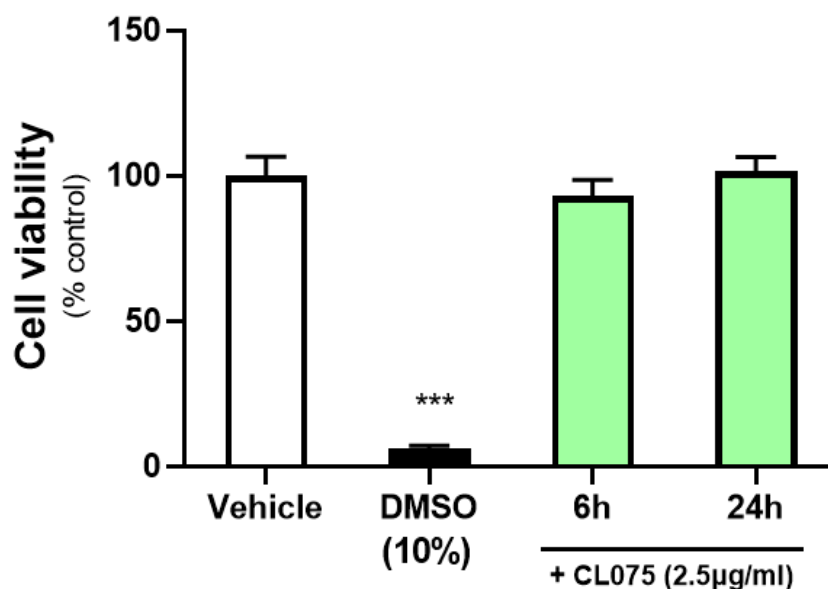


Figure 3.4 Effect of CL075 on the viability of THP-1 macrophages. THP-1-derived macrophages were treated with CL075 (2.5 µg/ml) for 6 h and 24 h, and an MTT assay performed to determine the impact of the TLR7/8 ligand on cell viability. Data are presented as the mean ± S.E.M of triplicate determinations from 4 independent passages. Data were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test. *** $p<0.001$ versus control cells.

3.6 Time-dependent effect of CL075 on nitrite production in THP-1 macrophages

Following determination by MTT that the viability of the THP-1 macrophages was not impacted by incubation with 2.5 $\mu\text{g/ml}$ CL075, we next assessed whether overproduction of nitrite, as a result of stimulation with the TLR agonist, was occurring. NO_2^- is a stable by-product of NO and thus gives an accurate measurement of NO released into the supernatants by cells. NO overproduction is linked to inflammation and cell death (339-341). The Griess assay was carried out in THP-1 macrophages incubated with 2.5 $\mu\text{g/ml}$ CL075 at time points ranging from 10 min-24 h (Fig. 3.5). As indicated in Fig. 3.5, no change in nitrite concentration was determined following incubation with CL075 at each time point tested. Indeed, the average concentration of nitrite detected in supernatants was 0.08 μM in both control cells and cells stimulated with the TLR agonist. This suggests that CL075 does not promote nitrite production in THP-1 macrophages.

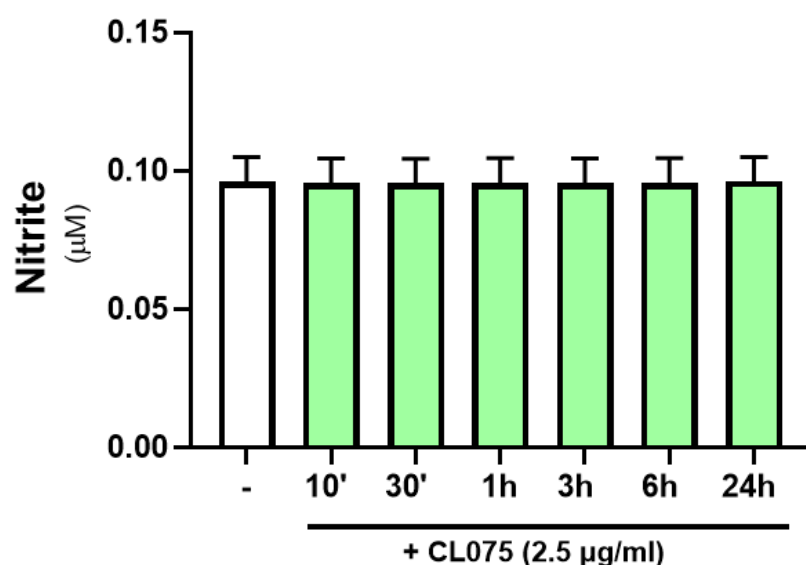


Figure 3.5 Effect of CL075 on nitrite production in THP-1-derived macrophages. THP-1-derived macrophages were treated with CL075 (2.5 $\mu\text{g/ml}$) for 10 min, 30 min, 1 h, 3 h, 6 h and 24 h, and the concentration of nitrite determined via the Griess reaction. Data are presented as the mean $\pm$ S.E.M of triplicate determinations from 4 independent passages. Data were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test.

3.7 Components of the eCS (CNR1, CNR2, FAAH and MAGL) are expressed in THP-1 macrophages

A key goal of this project was to assess the impact of cannabinoids on TLR7/8 signalling. We first set out to determine whether the primary constituents of the eCS were expressed at a basal level in cultured THP-1-derived macrophages, with focus on the main cannabinoid receptors, FAAH (responsible for hydrolysing AEA) (285) and MAGL (predominantly hydrolyses 2-AG) (286). THP-1 macrophages were differentiated from monocytes via incubation with PMA (10 ng/ml) for 48 h, followed by 24 h incubation with media in the absence of PMA. RNA was then harvested, cDNA synthesis performed and RT-qPCR used to determine the expression of *CNR1*, *CNR2*, *FAAH* and *MAGL* mRNA in THP-1 macrophages. The endogenous control employed was Eukaryotic 18s rRNA. As can be seen in Table 4 below, all four components of the eCS are expressed at the gene level in THP-1 macrophages. These data are in concurrence with previous data published in our laboratory demonstrating that both the CNR1 and CNR2 genes are expressed in THP-1-derived macrophages (79). This indicates that THP-1 macrophages express various components of the eCS, and will likely respond to exogenous addition of cannabinoids to macrophage cultures.

Table 4. *CNR1*, *CNR2*, *FAAH* and *MAGL* mRNA expression in THP-1-derived macrophages

Target	Average basal expression (Ct) (n=4)
CNR1	28.06 ± 0.24
18S rRNA	6.96 ± 0.07
CNR2	37.08 ± 0.33
18S rRNA	6.88 ± 0.10
FAAH	28.29 ± 0.11
18S rRNA	6.84 ± 0.14
MAGL	24.25 ± 0.16
18S rRNA	7.25 ± 0.15
Data are expressed as mean ± SEM; Ct, cycle threshold; rRNA, ribosomal ribonucleic acid	

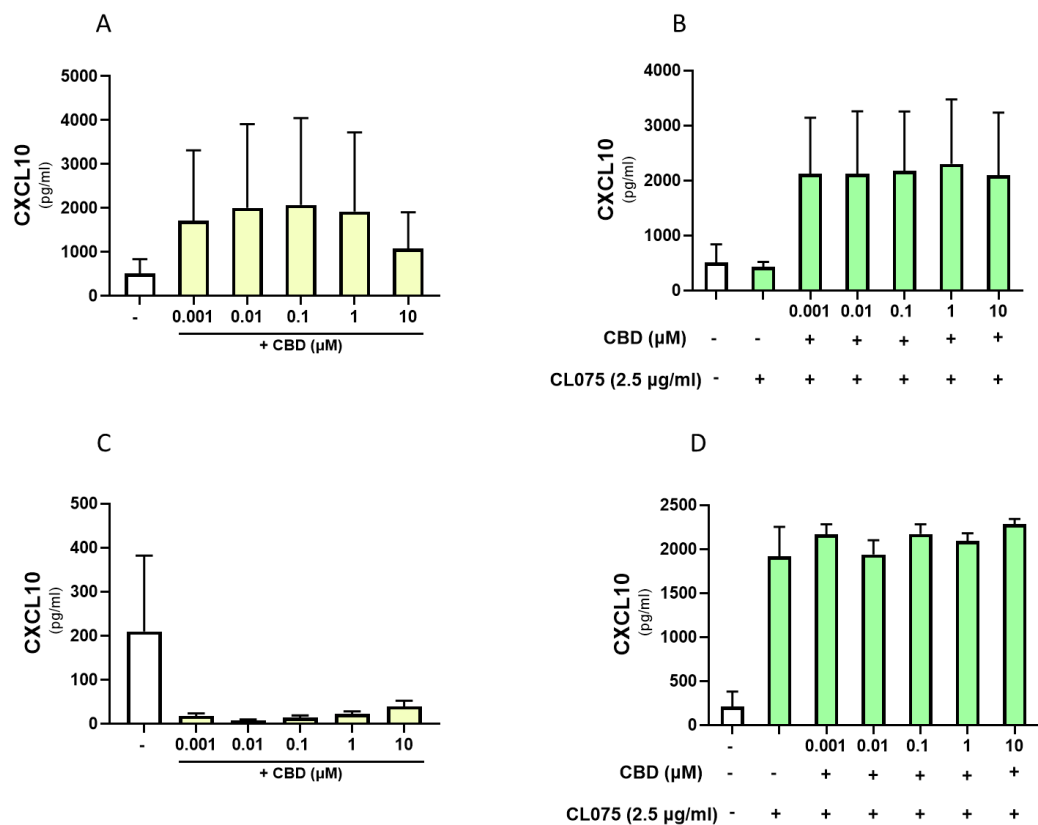
3.8 The impact of CBD on the CL075-dependent induction of cytokines and chemokines at 6 h and 24 h

Following the determination that components of the eCS are expressed in THP-1 macrophages (Table 3), we next set out to determine the impact of exogenous treatment with cannabinoids on the induction of CXCL10, TNF- α , RANTES and IFN- β in CL075-stimulated THP-1 macrophages. We selected the pCB CBD, given the recent clinical development of this cannabinoid (342-344). THP-1 monocytes were differentiated to macrophages following 48 h incubation with PMA (10 ng/ml). The cells were maintained for a period of 24 h, upon which they were incubated with various concentrations of CBD (0.001 - 10 μ M) alone for 6 or 24 h. THP-1 macrophages were also pre-treated for 30-45 min with CBD (0.001 - 10 μ M), followed by addition of CL075 at a working concentration of [2.5 μ g/ml] for both 6 and 24 h. At time zero, the supernatants were harvested and analysed for cytokine/chemokine expression by ELISA (Fig. 3.6). Data in Fig. 3.6A indicates that treatment of cells with CBD alone had no significant effect on CXCL10 expression at 6 h. Treatment with CL075 for 6 h failed to impact CXCL10 expression (Fig. 3.6B); CBD, when administered in the presence of CL075, had no significant impact on the induction of CXCL10 at any concentration tested at 6 h, although it did promote a trend towards increasing the concentration of CXCL10 when administered with CL075 (Fig. 3.6B). The failure of CL075 (2.5 μ g/ml) to promote CXCL10 protein expression at 6 h is consistent with previously reported data (Fig. 3.2A). Given these findings, this experiment was repeated at the 24h time point. Data presented in Fig. 3.6C indicates that following incubation of THP-1 macrophages with CBD for 24 h, CBD promoted a non-significant reduction in CXCL10 expression at all doses tested, when compared to the vehicle control (Fig. 3.6C). Pre-treatment with CBD at concentrations ranging from 0.001 μ M - 10 μ M, had no impact on CL075-induced CXCL10 expression at 24 h (Fig. 3.6D)

Next we assessed the impact of CBD on CL075-induced TNF- α protein expression in THP-1 macrophages. Firstly, CBD had no significant impact on TNF- α expression in THP-1 macrophages when delivered alone, although there was a trend towards an increase in the expression of this cytokine at a concentration of 0.1 μ M, when compared to the vehicle control, but this did not reach significance (Fig. 3.6E). Data presented in Fig. 3.6F indicates that treatment with CL075 at [2.5 μ g/ml] for 6 h failed to significantly promote TNF- α expression (Fig. 3.6F), however, treatment with CBD in the presence of CL075 potentiated TNF- α protein expression at 6 h, although this again did not reach statistical

significance at each dose of CBD tested (Fig. 3.6F). TNF- α protein was not detected in THP-1 macrophages following 24 h incubation with CBD at all doses tested (Fig. 3.6G). Pre-treatment with CBD at doses ranging from 0.001 μ M - 10 μ M failed to impact CL075-induced TNF- α expression at 24 h (Fig. 3.6H). Throughout the experiments, some variations in basal expression of cytokines and chemokines were determined in control treatments from experiment to experiment. This is likely due to variations in basal cytokine/chemokine production in this cell line that may occur with the passage number used in various batches of experiments.

Finally, the impact of CL075 and CBD on the expression profile of the chemokine RANTES was assessed at the 24 h time point. Data presented in Fig. 3.6I indicate that there was no difference between vehicle control-treated cells and THP-1 macrophages treated for 24 h with various concentrations of CBD. This is likely due to the high basal protein expression of RANTES in THP-1 macrophages (Fig. 3.6H). Surprisingly, treatment with CL075 at [2.5 μ g/ml] for 24 h significantly increased the expression of RANTES, when compared to vehicle controls ($p < 0.01$) (Fig. 3.6J). Pretreatment with CBD had no impact on CL075-dependent RANTES induction at the 24 h timepoint (Fig. 3.6J).



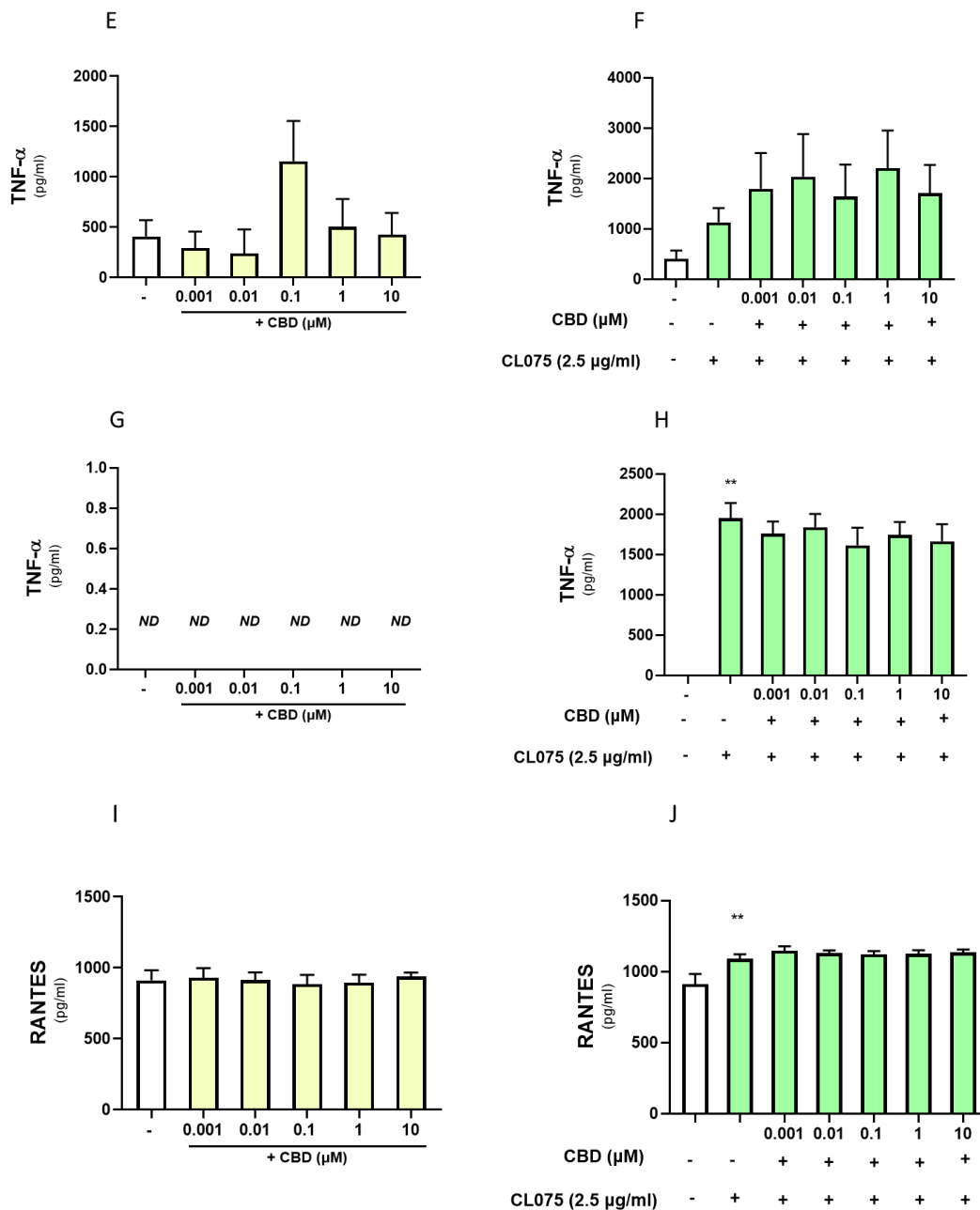


Figure 3.6 Effect of CBD on CL075-induced CXCL10, TNF-α and RANTES expression in THP-1-monocyte-derived macrophages at 6 h and 24 h. THP-1-derived macrophages were treated with different concentrations of CBD (1nM, 10 nM, 100nM, 1μM, 10 μM), in the absence or presence of CL075 (2.5 μg/ml) for (A, B, E, F,) 6 h or (C, D, G, H, I, J) 24 h. The supernatants were harvested and analysed for cytokine/chemokine expression by ELISA. Data are presented as the mean ± S.E.M of duplicate determinations from 4 independent passages. Data were analysed using (A, B, C, D, E, F, G, H) the Kruskal-Wallis test and Dunn's multiple comparisons test or (I, J) one-way ANOVA and Dunnett's multiple comparisons test. ** $p < 0.01$ versus control cells.

3.9 Impact of CBD on the CL075-dependent induction of cytokine/chemokine mRNA expression in THP-1-macrophages

Having assessed the impact of CBD on CL075-induced cytokine/chemokine expression at protein level, we set out to determine the effect of pCB treatment on CL075-induced cytokine/chemokine mRNA expression in macrophages. We focused on the impact of CBD (10 μ M) on CL075-dependent induction of cytokines and chemokines at 24 h. THP-1 monocytes were differentiated to macrophages following 48 h incubation with PMA (10 ng/ml). The cells were maintained for a period of 24 h, following which they were incubated with CL075 at [2.5 μ g/ml] or CBD (10 μ M) for 24 h. In addition, THP-1 macrophages were pre-treated with CBD (10 μ M) for 30-45 min prior to stimulation of cells with CL075 at [2.5 μ g/ml] for 24 h. At time zero, RNA was harvested, cDNA synthesis was performed and RT-qPCR used to determine the relative gene expression of the cytokines and chemokines produced by THP-1-macrophages. Data in Fig. 3.7A indicates that incubation with CL075 at [2.5 μ g/ml] promoted an induction of *CXCL10* gene expression at 24 h, although this did not reach significance (Fig. 3.7A). However, the expression of the *CXCL10* gene was significantly upregulated in THP-1 macrophages pre-treated with 10 μ M CBD prior to CL075 treatment for 24 h, when compared to vehicle-treated cells ($p < 0.05$; Fig. 3.7A). Incubation with CBD for 24 h did not result in induction of *CXCL10* mRNA expression (Fig. 3.7A). A significant increase in *TNF- α* gene expression was noted following 24 h incubation with CL075 at [2.5 μ g/ml] ($p < 0.001$), when compared to the vehicle control-treated cells (Fig. 3.7B). Pre-treatment with CBD had little impact on CL075-induced *TNF- α* mRNA expression (Fig. 3.7B). Treatment with CBD alone for 24 h had no impact on *TNF- α* mRNA expression in THP-1 macrophages (Fig. 3.7B).

Similarly, data presented in Fig. 3.7C demonstrates that incubation with CL075 (for 24 h) resulted in a significant induction of *RANTES* mRNA expression, when compared to controls ($p < 0.05$; Fig. 3.7C). Pre-treatment with CBD had no significant impact on the expression of *RANTES* mRNA (Fig. 3.7C). Finally, in this set of experiments, treatment with CL075 alone, and in the presence of CBD, had no impact on *IFN- β* mRNA expression (Fig. 3.7D). Treatment with CBD alone also had no significant impact on *IFN- β* mRNA expression (Fig. 3.7D).

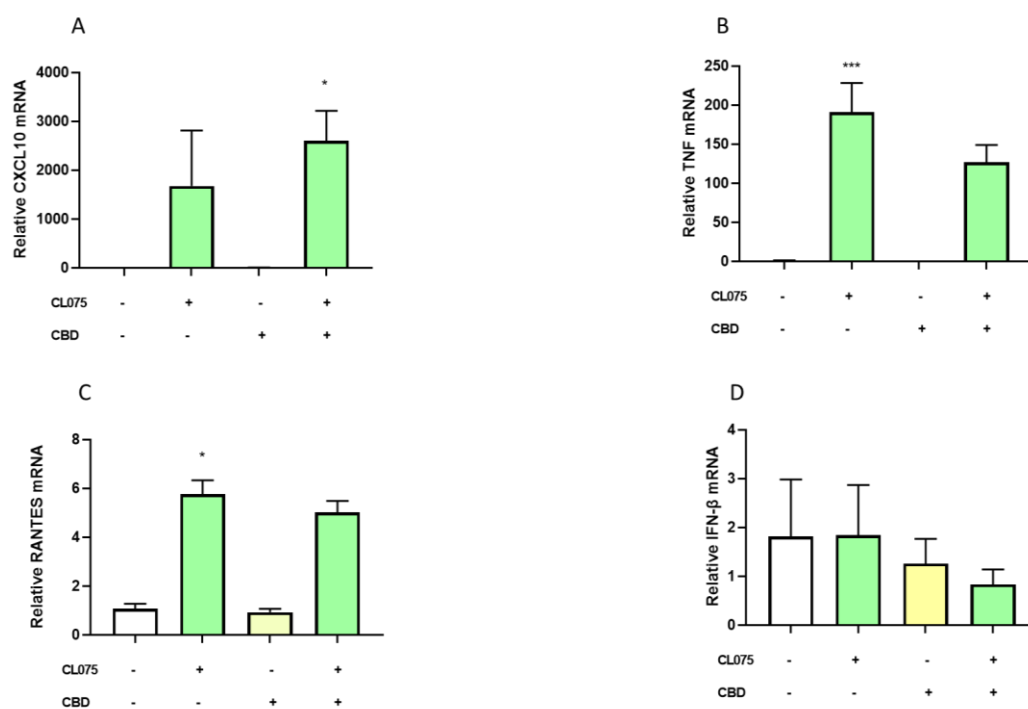


Figure 3.7 Impact of CBD on cytokine/chemokine mRNA expression in CL075-stimulated THP-1 macrophages. THP-1 macrophages were treated with CL075 (2.5 $\mu\text{g/ml}$) for 24 h, CBD (10 μM) for 24 h or were pre-treated with CBD (10 μM) for 30-45 mins prior to addition of CL075 (2.5 $\mu\text{g/ml}$) for 24 h, and the expression of **(A)** *CXCL10*, **(B)** *TNF- α* , **(C)** *RANTES*, **(D)** *IFN- β* mRNA determined by RT-qPCR. Data are presented as the mean $\pm$ S.E.M of triplicate determinations from 4 independent passages. Data were analysed using **(A,B)** the one-way ANOVA and Dunnett's multiple comparisons test or **(C,D)** the Kruskal-Wallis test followed by Dunn's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$ versus vehicle-treated control cells.

3.10 Impact of CL075 and CBD treatment on *TLR8*, *TLR7*, *CNR1*, *CNR2*, *FAAH* and *MAGL* mRNA expression in macrophages

Previously we identified that both TLR8 and TLR7 are expressed in THP-1 macrophages (Table 2), and furthermore, data presented in this Chapter indicate that stimulation of macrophages with the TLR7/8 ligand CL075 promotes inflammatory signalling in THP-1 macrophages. Evidence suggests that both TLR and cannabinoid ligands have the proclivity to modulate relative TLR expression in immune cells (315). Hence, we next set out to determine if CL075 treatment, in addition to CBD treatment/pre-treatment, impacts the relative expression of TLR7 and TLR8 in macrophages. Data presented in Fig. 3.8A indicates that incubation of macrophages with CL075 at [2.5 µg/ml] for 24 h resulted in a significant induction of *TLR8* mRNA expression ($p<0.05$; Fig. 3.8A); such effect was maintained upon pre-treatment with CBD. Treatment with CBD (10 µM) alone for 24 h had no significant impact on basal *TLR8* mRNA expression (Fig. 3.8A). In contrast, both CL075 (2.5 µg/ml) and CBD (10 µM) treatment (24 h) had no significant effect on relative *TLR7* mRNA expression (Fig. 3.8B).

Data presented in Table 3 demonstrates that components of the eCS are expressed in THP-1 macrophages. In the final set of experiments in this Chapter we set out to determine if stimulation of macrophages with the TLR7/8 ligand CL075 (2.5 µg/ml), and the pCB CBD (10 µM), alters the expression profile of components of the eCS in macrophages. Interestingly, the data shown in Fig. 3.8 indicates that CL075 treatment for 24 h significantly reduced the level of *CNR1* mRNA expressed in THP-1 macrophages, compared to control-treated cells ($p<0.05$; Fig. 3.8C), while it significantly enhanced *MAGL* mRNA expression ($p<0.05$; Fig. 3.8F). The level of *FAAH* mRNA expression was also attenuated following 24 h incubation with CL075, albeit insignificantly (Fig. 3.8E). Treatment with CBD (10 µM) alone at 24 h had no effect on the relative basal expression of *CNR1* mRNA (Fig. 3.8C), *CNR2* mRNA (Fig. 3.8D), *FAAH* mRNA (Fig. 3.8E) or *MAGL* mRNA (Fig. 3.8F), when compared to vehicle controls, while pre-treatment with CBD prior to CL075 had no impact on TLR7/8-induced modulation of *CNR1* mRNA (Fig. 3.8C) or *MAGL* mRNA (Fig. 3.8F). These findings suggest the CL075 can promote the transcription of the *TLR8* gene, while treatment of macrophages with CL075 can alter the expression of components of the eCS.

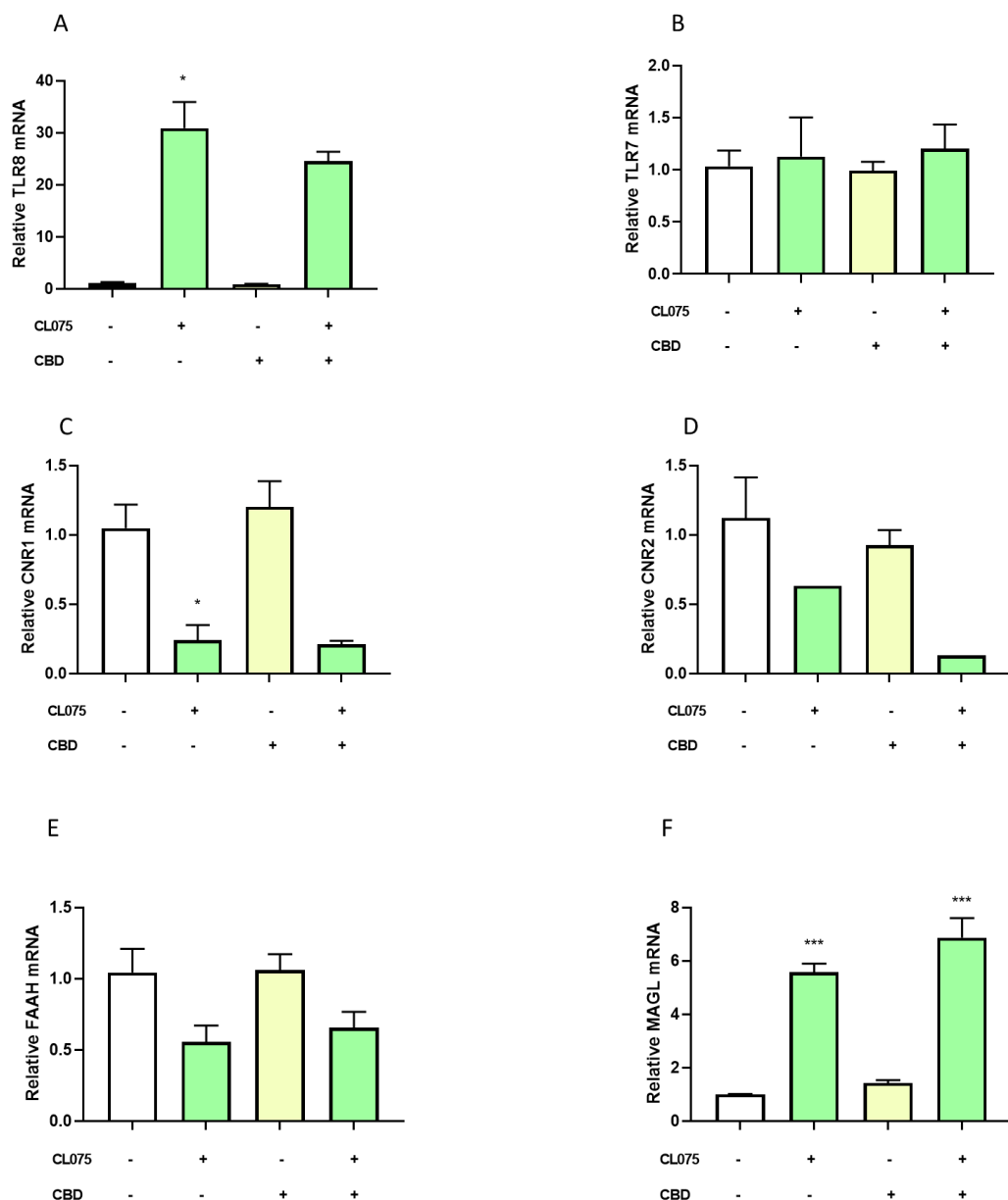


Figure 3.8 Impact of CL075 and CBD treatment (at 24 h) on *TLR8*, *TLR7*, *CNR1*, *CNR2*, *FAAH* and *MAGL* mRNA expression in THP-1-macrophages. THP-1-derived macrophages were treated with CL075 (2.5 µg/ml) for 24 h, CBD (10 µM) for 24 h or were pre-treated with CBD (10 µM) for 30-45 mins prior to incubation with CL075 (2.5 µg/ml) for 24 h. The expression of **(A) *TLR8***, **(B) *TLR7***, **(C) *CNR1***, **(D) *CNR2***, **(E) *FAAH*** and **(F) *MAGL*** mRNA was then assessed by RT-qPCR. Data are presented as the mean ± S.E.M of triplicate determinations from 4 independent passages. Data were analysed using **(A, C, D)** the Kruskal Wallis test followed by Dunn's multiple comparisons test or **(B, E, F)** one-way ANOVA and Dunnett's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$ versus vehicle-treated control cells.

Chapter 4 Characterisation of TLR7/8 and eCS signalling in immune cells from pwMS and in HC cases

4.1 Clinical characteristics and MSQoL of pwMS and HCs in the study cohort

Much data suggests that inflammation and TLR signalling are closely associated with MS pathogenesis (319), and TLR7/8 activation in the CNS in murine models has been shown to promote neuroinflammation (71). Furthermore, given evidence that TLR signalling is targeted by cannabinoids in cells from pwMS (317), the next series of experiments set out to characterise the expression profile of inflammatory cytokines, chemokines and type I IFNs (as assessed in Chapter 3) in plasma isolated from pwMS and HC cases, in addition to profiling the expression of TLR7, TLR8, and components of the eCS, in PBMCs isolated from pwMS and HCs. A total of 78 subjects were assessed in this study, consisting of HC participants ($n = 43$) and pwMS ($n = 35$) (Table 5). All pwMS included in the study had a RR form of MS. Mean disease symptom duration in the MS cohort was 5.28 ± 0.84 (mean $\pm$ SEM) years and subjects had mild to moderate disability as reflected by EDSS scores of 2.16 ± 0.40 (mean $\pm$ SEM) (Table 5). The mean age of the 35 participants in the MS cohort included in the study was $38.72 (\pm 1.55)$ years, compared to the average age of $35.50 (\pm 2.64)$ years in HCs. Overall, 74% of the study participants were female. The demographics of the participants are reported in Table 5. Of the pwMS included in this study, five individuals were actively treated with Tysabri, four with Gilenya, four with Plegridy, two with Rituximab, two with Tecfidera, two with Copaxone and one patient was treated with Avonex (Table 6). At the time of participation in the study one individual with MS was a cannabis user (Table 6), three were cigarette smokers, and three pwMS were taking vitamin D supplements (Table 6). The MS-Quality of Life questionnaire (MSQoL-54 questionnaire) (Appendix 3) consists of 54 self-reporting questions on an individual's physical and mental function. Both categories are split into several sub-categories such as energy, sexual function, social function, pain, emotional wellbeing, mental health, cognitive function, health distress and overall QoL. Each participant taking part in the study completed the MSQoL questionnaire, in addition to the QIDS-SR₁₆ questionnaire (Appendix 3). The QIDS-SR₁₆ questionnaire is a self-assessment of depressive symptomatology. Both questionnaires are widely used assessments for reporting QoL and depressive symptomatology in pwMS (332-334), as well as having been used previously in the Downer laboratory (345). The dichotomy observed in each of these categories of health and depression between pwMS and HCs are presented in Fig. 4.1. As is shown below, pwMS demonstrated significantly reduced scores across all categories, both in physical and mental health (Fig. 4.1A-K). In addition, pwMS demonstrated significantly elevated depressive symptomatology, with a highest

reading of 17, compared to a highest score of 10 in control individuals (Fig. 4.1L). This indicates the negative impact of MS on overall QoL indices in the study cohort included in this study.

Table 5. Clinical characteristics of pwMS and healthy controls

Clinical category	Healthy Control	MS
<i>n</i>	43	35
Sex (Female/Male)	28/15	30/5
Age (years)	35.50 ± 2.64	38.72 ± 1.55
EDSS	n/a	2.16 ± 0.40
Disease duration (years)	n/a	5.28 ± 0.84

Data expressed as Mean ± SEM; MS: Multiple sclerosis; EDSS: Expanded Disability Status Scale; n/a: not applicable

Table 6. Medications taken at time of blood draw

Clinical category	HC (<i>n</i>)	MS (<i>n</i>)
MS DMTs		
Tysabri	-	5
Gilenya	-	4
Plegridy	-	4
Rituximab	-	2
Tecfidera	-	2
Capaxone	-	2
Avonex	-	1
Other		
Cannabis use	1	1
Smoker	-	3
Vitamin D	-	3
Folic acid	-	2

HC: healthy control; MS: Multiple sclerosis;

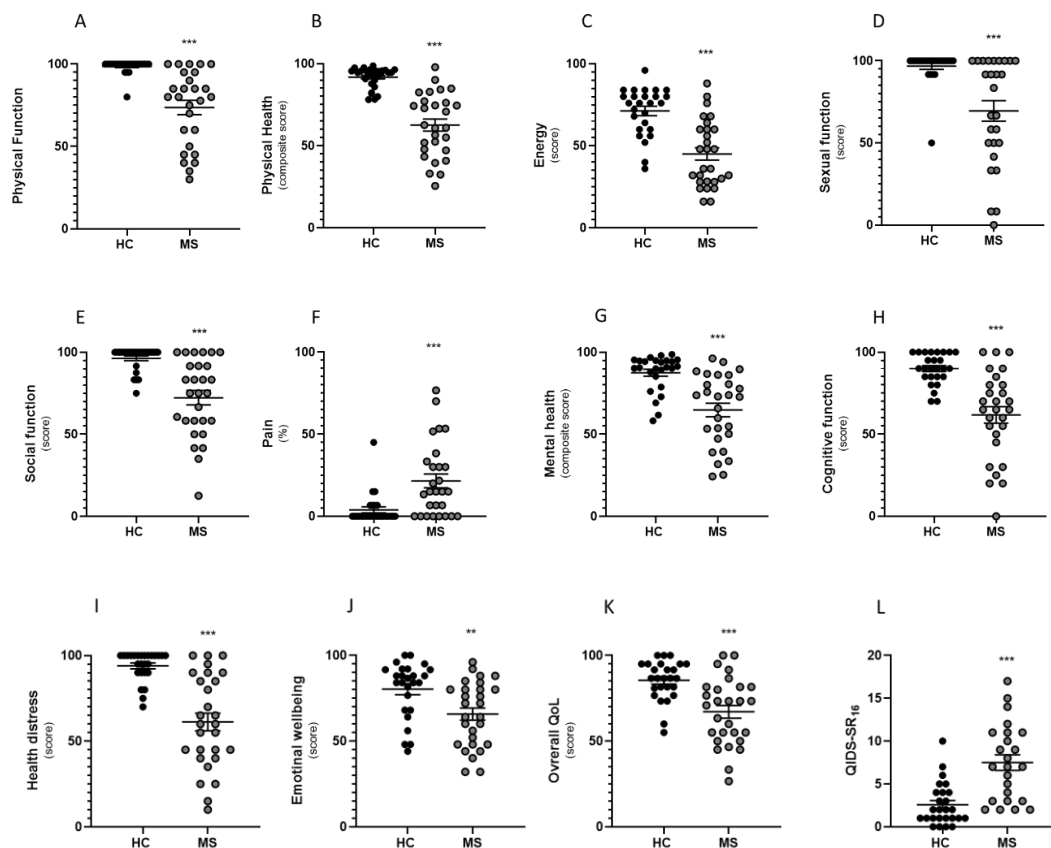


Figure 4.1 MS-QoL-54 and QIDS-SR₁₆ sub-categories in pwMS and HC individuals assessed in the study. Individuals completed the MS-QoL-54 and QIDS-SR₁₆ questionnaires at the time of venepuncture. The data provided within these questionnaires was collated to demonstrate **(A)** physical function, **(B)** physical health (composite score), **(C)** energy/fatigue, **(D)** sexual function, **(E)** social function, **(F)** pain, **(G)** mental health, **(H)** cognitive function, **(I)** health distress, **(J)** emotional wellbeing, **(K)** overall QoL and **(L)** QIDS-SR₁₆ depressive symptomatology values in pwMS and control volunteers. Data are presented as the mean $\pm$ SEM. Data was tested for significance using the Mann-Whitney test. ** $p<0.01$, *** $p<0.001$.

4.2 Sysmex data gathered from the whole blood of pwMS and HCs

Several clinical studies have noted alterations in immune cell activity between HC cases and pwMS, alongside altered expression of miRNAs (346) and elevated levels of inflammatory proteins, particularly in CSF (347, 348). Thus, to ascertain if there were marked differences in immune/blood parameters between cohorts assessed in the study, whole blood from each sample was profiled using a Sysmex haematology analyser. This provided a readout of haemoglobin content, the number of neutrophils, the number of lymphocytes, haematocrit and platelet number, among others indicated in Table 7. There was a significant difference between the mean corpuscular haemoglobin (MCH; pg) in blood samples obtained from pwMS, when compared to sample from HC volunteers ($p < 0.05$; Table 7). No further significant differences in the read-outs assessed was determined in samples from control cases and pwMS.

Table 7. Comparison of blood parameters in HC and MS subjects

Parameter	HC (n=20)	MS (n=22)	P value
WBC ($\times 10^3/\mu\text{L}$)	6.29 $\pm$ 0.39	6.25 $\pm$ 0.45	0.4251
RBC ($\times 10^6/\mu\text{L}$)	4.74 $\pm$ 0.16	4.64 $\pm$ 0.14	0.5674
HGB (g/dL)	14.23 $\pm$ 0.41	14.53 $\pm$ 0.36	0.7584
PLT ($\times 10^3/\mu\text{L}$)	258.85 $\pm$ 16.43	275.27 $\pm$ 16.60	0.8034
HCT (%)	39.94 $\pm$ 1.25	39.86 $\pm$ 1.07	0.6321
MCV (fL)	84.41 $\pm$ 0.70	86.04 $\pm$ 0.66	0.9638
MCH (pg)	30.16 $\pm$ 0.44	31.49 $\pm$ 0.52	0.0450*
MCHC (g/dL)	35.70 $\pm$ 0.33	36.58 $\pm$ 0.47	0.1204
LYM ($\times 10^3/\mu\text{L}$)	1.81 $\pm$ 0.12	1.75 $\pm$ 0.24	0.7988
NEUT ($\times 10^3/\mu\text{L}$)	4.10 $\pm$ 0.47	3.89 $\pm$ 0.32	0.9018

Data are expressed as mean $\pm$ SEM; HC: Healthy control; HCT: haematocrit; HGB: haemoglobin; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; MS: Multiple sclerosis; NEUT: neutrophil number; PLT: platelet number; RBC: red blood cell count; WBC: white blood cell count; * $p < 0.05$ compared to HCs

4.3 Cytokine, chemokine and CRP assessment in plasma from whole blood samples isolated from healthy volunteers and pwMS

To determine whether components of the TLR7/8-induced inflammatory signalling axis are detected and also perturbed in the context of MS, we assessed cytokine/chemokine concentrations within plasma isolated from HCs and pwMS by ELISA. Data presented in Table 8 indicates the average cytokine/chemokine and CRP concentration detected in plasma isolated from controls and pwMS (Table 8). CXCL10 was detected in the majority of samples, and the average concentration of CXCL10 was nearly ten-fold higher in HCs than in individuals with MS, although these values are reflected in research carried out elsewhere (349). As indicated in Table 8, the expression profile of TNF- α , an important inflammatory cytokine in the context of MS (350), was limited in plasma, and was not detected in the majority of plasma samples tested. Indeed, TNF- α protein was only detected in plasma isolated from four pwMS. Interestingly, in the HC cohort, mean plasma concentrations of TNF- α was 765.97 ($\pm$ 275.50) pg/ml. This is high; indeed data elsewhere indicates that the normal plasma concentration of TNF- α in healthy adults falls under 20 pg/ml (351-353). Such high levels of the cytokine could indicate underlying inflammation or infection within certain control subjects. Indeed, the values reported herein would align to concentrations recorded in serum of individuals with type 2 diabetes mellitus and sepsis (354, 355). However, there is a high degree of variability in the recorded concentration of cytokines such as TNF- α in plasma, and it is noteworthy that less than a third of control individuals assessed in our study had detectable levels of this cytokine in their plasma (Table 8). The chemokine RANTES was detected in all samples assessed, with similar concentrations determined in plasma isolated from both HCs and pwMS (1345.76 ($\pm$ 61.93) and 1185.23 ($\pm$ 87.70) pg/ml, respectively). The type I IFN, IFN- β , was not detected in the majority of plasma samples assessed; it was detected in plasma from 35% of controls and in 29% of pwMS. IFN- β is important in the context of MS treatment as it has been shown to be protective (191, 356) and is frequently employed as a DMT in pwMS. It should be noted that several patients within the MS cohort were on IFN- β therapy at the time of blood draw, and this may impact the circulating concentrations of the IFN. In this set of experiments we also assessed the expression profile of IL-1 β , a potent inflammatory cytokine linked to inflammasome formation (357). It is likely that continuous inflammasome activation within the CNS contributes to disease pathology in MS (358-360). It has been shown that even in the absence of inflammasome activation, IL-1 β can be produced by myeloid cells within the

brain, resulting in sterile inflammation within the CNS (361). Data presented in Table 8 indicates that IL-1 β was detected in plasma isolated from just over 50% of samples within each study cohort; average plasma concentrations of IL-1 β was 180.04 ($\pm$ 86.73) pg/ml in pwMS, in comparison to an average plasma concentration of 450.49 ($\pm$ 141.32) pg/ml in plasma from HCs (Table 8). Such concentrations seen within the HC cohort are high when compared to other studies, where plasma IL-1 β concentrations of less than 10 pg/ml have been reported (362). It should be noted that the majority of HC samples assessed herein did demonstrate low IL-1 β levels in plasma of less than 10 pg/ml. However, in certain samples a higher concentration of IL-1 β was determined in plasma, which may indicate inflammation or infection in certain subjects at the time of blood draw. Similarly in the MS cohort, the average concentration of IL-1 β is higher than reported elsewhere, which may reflect DMT use at the time of blood draw. Given that neurological symptoms are exacerbated by infection (363), the plasma expression profile of the inflammatory reactant CRP was next determined. CRP was detected in all plasma samples assessed, which is consistent with literature elsewhere (364). Importantly the expression profile of CRP was similar in plasma isolated from pwMS and HCs (3323.28 ($\pm$ 116.01) and 3259.48 ($\pm$ 142.03) pg/ml, respectively). Elevated levels of circulating CRP have been identified elsewhere in relapses of MS (364). It should be noted that ROUT analysis was not performed when assessing cytokine/chemokine concentrations in plasma samples from HC and MS groups presented in Table 8, in addition to Fig. 4.2, 4.3, 4.4, 4.5, and 4.6, as inclusion of all values was thought to represent the high degree of variability that occurs when conducting plasma cytokine/chemokine assessment in human samples.

Table 8. Detection rates and concentration (pg/ml) of cytokines, chemokines and CRP present in plasma

Clinical Category	Healthy Control	Mean ($\pm$ SEM)	MS	Mean ($\pm$ SEM)
	Detection Rate		Detection Rate	
CXCL10	30/43	1117.80 ($\pm$ 297.49)	19/35	304.95 ($\pm$ 72.32)
TNF- α	13/43	765.97 ($\pm$ 275.50)	4/35	73.75 ($\pm$ 38.88)
RANTES	43/43	1345.76 ($\pm$ 61.93)	33/33	1185.23 ($\pm$ 87.70)
IFN- β	15/43	58.10 ($\pm$ 24.06)	10/35	51.14 ($\pm$ 13.34)
IL-1 β	14/27	450.49 ($\pm$ 141.32)	12/19	180.04 ($\pm$ 86.73)
CRP	43/43	3259.48 ($\pm$ 142.03)	35/35	3323.28 ($\pm$ 116.01)

This table shows the mean values of cytokines, chemokines and CRP detected. MS: Multiple Sclerosis; SEM: Standard Error of Mean

4.4 Concentration of the chemokine CXCL10 in plasma isolated from HC and pwMS

The inflammatory chemokine CXCL10 plays a part in MS pathogenesis, with the expression profile of CXCL10 elevated in the CSF of pwMS (365, 366). In addition, the CXCL10 receptor, CXCR3⁺ T cells, accumulates in active lesions in pwMS, and CXCL10 is linked demyelination in MS (367). Data presented in Chapter 3 indicates that TLR7/8 stimulation via CL075 in macrophages promotes the expression of the CXCL10 chemokine, indicating that the production of this chemokine is associated with the activation of the TLR7/8 signalling axis. Hence, we assessed the expression of this chemokine in plasma isolated from both pwMS and HCs to determine if the expression profile of CXCL10 is altered in the periphery in disease. Whole blood samples were collected from pwMS and control individuals as previously described in the laboratory (319), and plasma isolated for analysis of CXCL10 expression by ELISA. Data presented in Fig. 4.2 indicates that the plasma expression of CXCL10 was significantly elevated on average in the plasma isolated from HC volunteers, when compared to plasma isolated from pwMS. This may reflect DMT usage in the MS cohort. In addition, CXCL10 is a chemokine that exhibits great variability in expression from individual to individual, with concentrations of up to 1,000 pg/ml being recorded in HC individuals elsewhere (349).

It is well known that MS affects between 2-3 times more females than males, and the X chromosome has been implicated in autoimmunity (368). Indeed, both males and females tend to respond differently to pathogens and infection (369). In addition, one study observed an altered cytokine response in blood samples from male and female pwMS (370). Given these findings, we stratified plasma CXCL10 expression based on gender. Data presented in Fig. 4.2B indicates that there was no significant difference in the concentration of CXCL10 found in plasma between males and females in the HC cohort. In addition, there was a trend towards an increase in CXCL10 expression in plasma isolated from female pwMS, when compared to males in the MS cohort, although this did not reach statistical significance (Fig. 4.2B). It is noteworthy that this trend may be impacted by the limited number of male participants recruited to the study. Overall, these data indicate that MS is associated with a reduction in circulating CXCL10 expression in plasma, and also suggest that females may have elevated levels of CXCL10 in their plasma, compared to their male counterparts. This may be linked to the dichotomy in immune responses noted between males and females, although males were noted to produce more CXCL10 than females in instances of infection (371, 372).

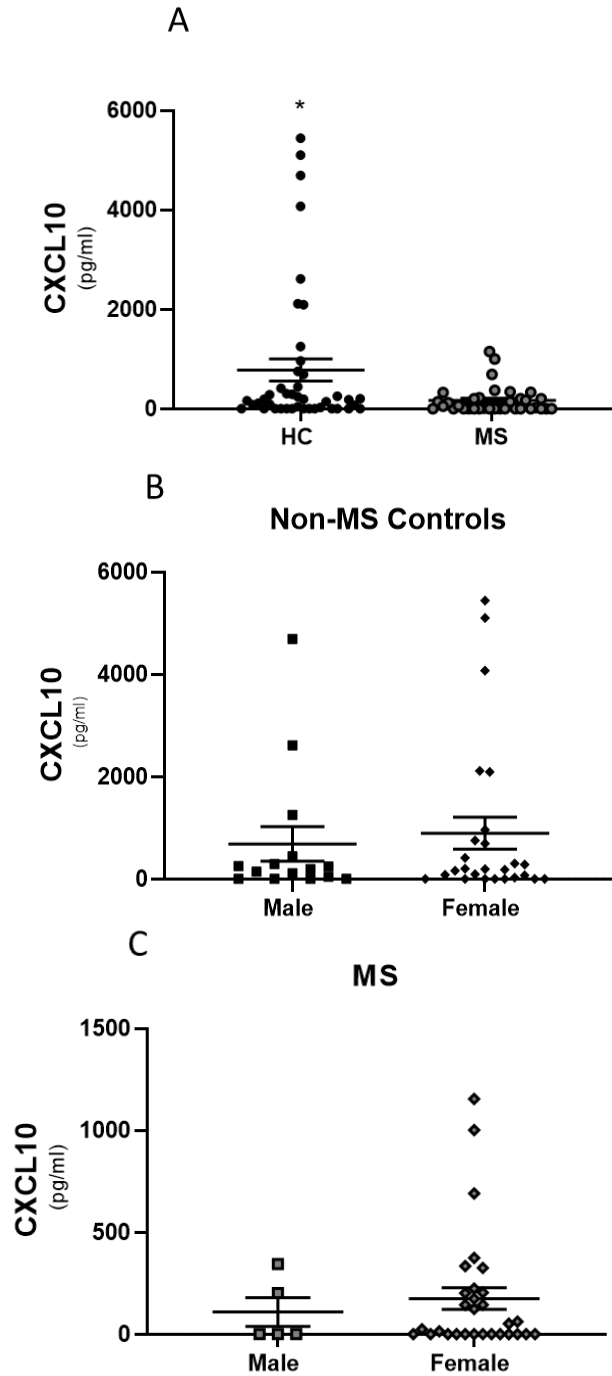


Figure 4.2 Expression of the chemokine CXCL10 is reduced in plasma isolated from pwMS, compared to HCs. Blood was drawn from both pwMS and HCs, plasma isolated and the concentration of the chemokine CXCL10 assessed in samples from **(A)** HCs and pwMS, **(B)** males and females within the control group and **(C)** males and females within the MS cohort. Data are presented as mean $\pm$ SEM. Data were analysed using Mann-Whitney tests. * $p < 0.05$ versus MS subjects.

4.5 Concentration of the inflammatory cytokine TNF- α in plasma from MS and HC cohorts

TNF- α is a proinflammatory cytokine largely produced by macrophages and DCs, although it can also play a role in reducing inflammation, as shown in murine models of disease (373, 374). Indeed, TNF- α has been shown to play a variety of roles in several models of inflammatory autoimmune disease including MS; however, the use of TNF blockers, such as etanercept, have resulted in onset of MS while being used to treat a separate autoimmune condition (375, 376). However, the detection of the cytokine in CSF has been linked to MS disease progression (350). It was determined in Chapter 3 (Fig. 3.1B, 3.2B) that the expression of TNF- α was upregulated in THP-1 macrophages following incubation with the TLR7/8 agonist CL075. Hence we next determined the expression of TNF- α protein in plasma isolated from pwMS and HCs (Fig. 4.3A), as well as determining the impact of sex on its expression profile (Fig. 4.3B, C). Whole blood samples were collected from pwMS and HCs, and plasma samples were isolated for analysis of TNF- α protein expression by ELISA. As indicated in Fig. 4.3A, TNF- α protein expression was significantly increased in plasma isolated from HCs compared to pwMS ($p < 0.05$); average concentration of 765.97 ($\pm$ 275.50) pg/ml in plasma from HCs, compared to 73.75 ($\pm$ 38.88) pg/ml in plasma from pwMS (Table 8). This is in agreement with findings from Obradović *et al.*, (2012) (377), suggesting that patients in remission have much lower levels of TNF- α in plasma. In addition, the use of DMTs among the MS group may have contributed to very low levels of TNF- α in plasma (Fig. 4.3 A). TNF- α concentration in HC plasma was significantly elevated, with an average plasma concentration comparable to that seen in individuals with sepsis (355). This may reflect an underlying infection in certain individuals at the time of blood draw, or indeed it may be partially attributable to a degree of variability occurring between individuals. Assay variability may also have played a factor in this result. It should also be noted that TNF- α expression was not detected in the majority of plasma samples assessed (Table 8).

Figure 4.3B indicates that in plasma from female HCs there was a trend towards elevated TNF- α expression, compared to their male counterparts, although this was not significant. This is in contrast to other literature suggesting that males have a higher number of CD3⁺ cells producing TNF- α in plasma, in the context of RRMS (370). Data presented in Figure 4.3C demonstrates that female pwMS have similar levels of TNF- α in plasma, compared to males included in the MS cohort. Again, this may be a result of low detection rates within the MS cohort (4/35), with only one male participant in this group having detectable levels of TNF- α protein in plasma. These results demonstrate that

although pwMS produce less TNF- α in plasma, females tend to have increased circulating TNF- α compared to males, although this was minimal in pwMS.

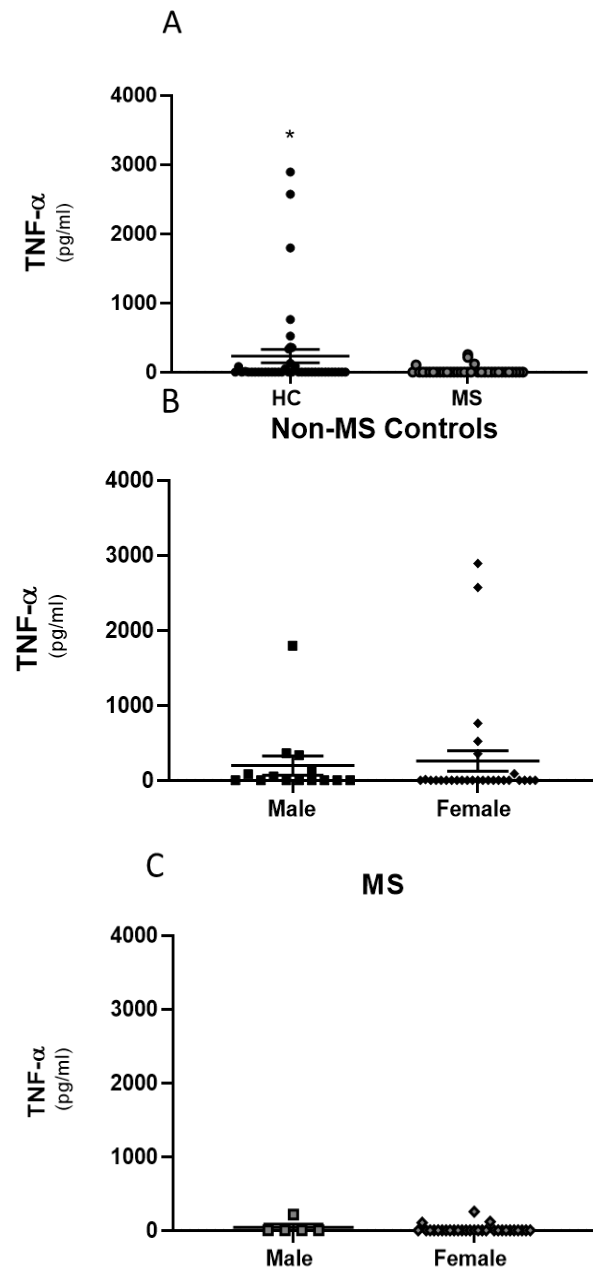


Figure 4.3 Concentration of the cytokine TNF- α in plasma isolated from HCs and pwMS.

Whole blood was collected from healthy volunteers and pwMS, plasma isolated and assessed for cytokine analysis by ELISA. The concentration of **(A)** TNF- α present in plasma from pwMS and HC volunteers was determined by ELISA. **(B)** The plasma concentration of TNF- α in males and females of the HC group. **(C)** Plasma TNF- α concentration in males and females in the MS group. Data are presented as mean $\pm$ SEM. Data were not parametric and the test for significance used was the Mann-Whitney test. * $p < 0.05$ versus MS subjects.

4.6 Concentration of the inflammatory chemokine RANTES in plasma from pwMS and HCs

RANTES is a pro-inflammatory chemokine linked to MS pathology. Indeed, RANTES has been detected in CSF and brain lesions of pwMS (365, 378) and data elsewhere indicates that the expression of RANTES in the CSF of pwMS in relapse is significantly upregulated in comparison with those not in relapse (366). RANTES is produced in large quantities in THP-1 macrophages (Fig 3.1C). Thus, we assessed the concentration of this chemokine in plasma isolated from pwMS and HCs. Whole blood samples were collected from pwMS and control individuals, and plasma isolated for analysis of the RANTES chemokine by ELISA. RANTES was detected in every sample tested (Table 8) and data in Fig. 4.4A indicates that the concentration of RANTES protein was similar between controls and pwMS (Fig. 4.4A). Indeed, the average concentration of RANTES in plasma from HCs was 1345.76 ($\pm$ 61.93) pg/ml, in comparison to 1185.23 ($\pm$ 87.70) pg/ml in pwMS (Table 8). Similarly, both males and females of the HC cohort, as demonstrated in Fig. 4.4B, had equivalent levels of RANTES protein in plasma. The same trend was observed in males and females in the MS group (Fig. 4.4C).

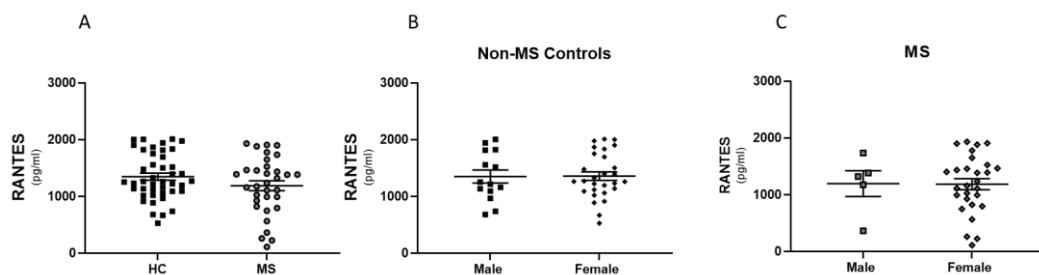


Figure 4.4 Concentration of the chemokine RANTES is unchanged in plasma isolated from HCs and pwMS. Whole blood was collected from healthy volunteers and pwMS, plasma isolated and RANTES concentration was assessed by ELISA. The concentration of **(A)** RANTES present in plasma of pwMS and HC volunteers, **(B)** the plasma concentration of RANTES in males and females of the HC group and **(C)** plasma RANTES concentration in males and females in the MS group, as determined by ELISA. Data are presented as mean $\pm$ SEM. Data were analysed using the Mann-Whitney test.

4.7 Assessment of IFN- β protein expression in plasma from MS and HC cohorts

IFN- β is protective in the context of MS (191, 356) and is an important DMT used in its treatment (190). It was first proved an effective therapy in RRMS in 1993 (379), and since then a multitude of IFN- β -based therapies have been developed (380). IFN- β is an anti-viral protein, and is linked to endosomal viral signalling via TLR7/8 (Fig. 3.3D). Previous work done in our laboratory has shown a desensitisation of the TLR3 pathway with respect to induction of IFN- β following TLR3 stimulation, in PBMCs isolated from MS individuals (317). IFN- β was not expressed at protein level in THP-1 macrophages (Fig. 3.2D). Thus, in this series of experiments, an assessment of human plasma offers a valuable insight into differences in IFN- β production between pwMS and HCs, as well as the difference between *in vitro* cell culture and *in vivo* experiments. Whole blood samples were collected from pwMS and HCs, and plasma was isolated for analysis of IFN- β protein expression by ELISA. The IFN was detected in plasma from 15/43 HCs and 10/35 pwMS (Table 8). As indicated in Fig. 4.5A, minimal amounts of this cytokine was expressed in plasma, with no significant alterations in IFN- β expression observed between HCs and pwMS (Fig. 4.5A), which is in accordance with literature elsewhere (381). Similarly, data presented in Fig. 4.5B indicate that there was no difference in expression of IFN- β in plasma between male and female HCs (Fig. 4.5B). Interestingly, males within the MS cohort had significantly elevated concentrations of IFN- β protein in plasma, in comparison to their female counterparts ($p < 0.05$; Fig. 4.5C).

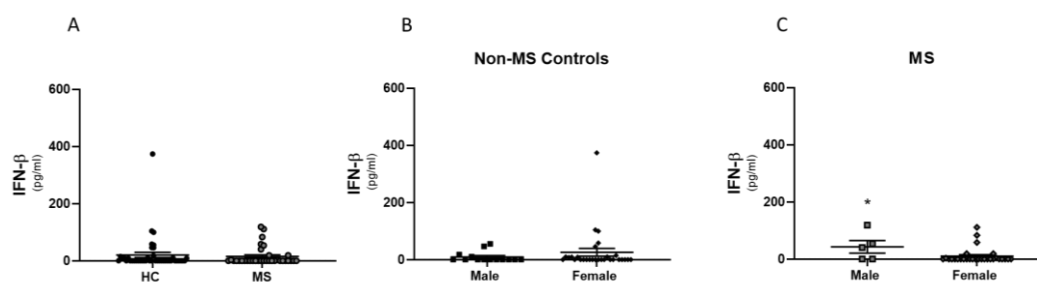


Figure 4.5 Concentration of IFN- β in plasma isolated from HCs and pwMS. Whole blood was collected from healthy volunteers and pwMS, plasma isolated and assessed for IFN- β protein expression by ELISA. The concentration of **(A)** IFN- β protein present in plasma of pwMS and HC volunteers, **(B)** plasma concentrations of IFN- β in males and females of the HC group and **(C)** plasma IFN- β concentrations in males and females in the MS group, as determined by ELISA. Data are presented as mean $\pm$ SEM. Data was not parametric and the test for significance used was the Mann-Whitney test. * $p < 0.05$ versus female pwMS.

4.8 Plasma IL-1 β protein expression in plasma samples from MS and HC cohorts

Central and peripheral inflammation is linked to MS pathogenesis (382). IL-1 β is a prototypical inflammatory cytokine produced via activation of the inflammasome (357), in addition to inflammasome-independent mechanisms (361). IL-1 β has also been shown to play a key role in the pathogenesis of EAE (383, 384), as well as promoting excitotoxic neurodegeneration in the context of MS (385). Given these findings we next set out to determine the expression profile of IL-1 β in plasma samples from pwMS and HCs. Whole blood was collected from pwMS and HCs, the plasma was isolated and ELISAs were run to determine the concentration of IL-1 β . The data presented in Fig. 4.7 demonstrates that a trend towards increased IL-1 β concentration was determined in plasma from HCs, compared to pwMS, albeit insignificant (Fig. 4.6A). The cytokine was only detected in 14/27 plasma samples from controls (450.49 ± 141.32 pg/ml), compared to 12/19 samples from pwMS (180.04 ± 86.73 pg/ml) (Table 8). Among HCs, the expression profile of IL-1 β in plasma samples from males and females was subsequently assessed (Fig. 4.6B). As indicated in Fig. 4.6B, plasma from female cases demonstrated a trend towards higher concentrations of IL-1 β in plasma, when compared to plasma from males, but this was not significant (Fig. 4.6B). A similar pattern was determined in pwMS, where plasma from females demonstrated a trend towards elevated levels of IL-1 β protein, compared to plasma from males (Fig. 4.6C). Overall, these results suggest that pwMS tend to have an equivalent expression of IL-1 β in circulating plasma compared to HCs, and that there is a trend towards elevated levels of plasma IL-1 β in blood samples from females, compared to males. Plasma IL-1 β concentration in the HC cohort is higher than reported elsewhere (362). However, there was a high degree of variability in plasma IL-1 β concentrations in HC individuals, the majority of HC individuals had a very low concentration of IL-1 β in plasma samples (Fig. 4.6 A). Again, this may reflect an underlying infection in certain individuals at the time of blood draw. Several individuals with MS were prescribed DMTs at the time of the study, which act to reduce inflammation and thus may account for the low IL-1 β plasma concentrations in individuals with MS (Fig. 4.6 A).

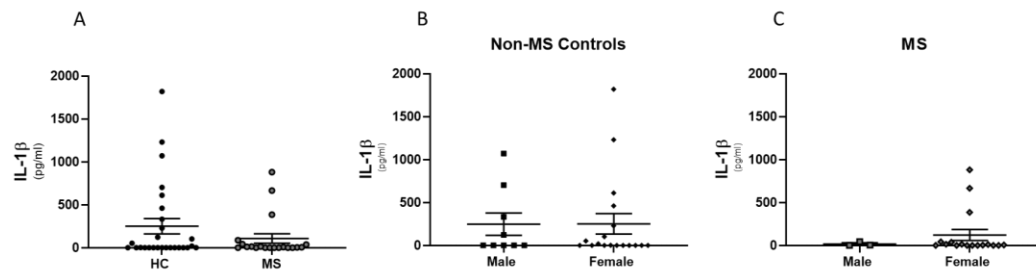


Figure 4.6 Protein concentration of the cytokine IL-1 β in plasma isolated from HCs and pwMS. Whole blood was collected from healthy volunteers and pwMS, plasma isolated and assessed for IL-1 β protein expression by ELISA. The concentration of **(A)** IL-1 β present in plasma of pwMS and HC volunteers, **(B)** the plasma concentration of IL-1 β in males and females of the HC group and **(C)** plasma IL-1 β concentration in males and females in the MS group, as determined by ELISA. Data are presented as mean $\pm$ SEM. Data were analysed using the Mann-Whitney test.

4.9 CRP expression in whole blood plasma samples isolated from healthy volunteers and pwMS

CRP is an acute phase protein implicated in MS disease pathology (386), and is considered a biomarker of the disease (386), although the evidence to support this is inconclusive (387). First discovered in 1930 (388), CRP is produced by the liver in the acute phase response to combat inflammation, activating complement cascades and the adaptive immune system (389). Data elsewhere indicates that CRP is expressed in equal concentrations in serum isolated from both MS and control cohorts, although CRP is elevated in MS relapses (364). Thus, we assessed the levels of this inflammatory protein in plasma from both MS and HC groups. As shown in Fig. 4.7, CRP protein was expressed at similar levels in plasma from MS and control cohorts (Fig. 4.7A), which is consistent with the literature (364). Control subjects had an average plasma CRP protein concentration of $3259.48 (\pm 142.03)$ pg/ml, compared to $3323.28 (\pm 116.01)$ pg/ml in plasma from pwMS, and CRP was detected in all samples tested. Following this, any dichotomy between male and female expression of peripheral circulating CRP was assessed (Fig. 4.7B). The expression of the acute phase protein was comparable in plasma from both males and females in the control (Fig. 4.7B) and MS (Fig. 4.7C) groups.

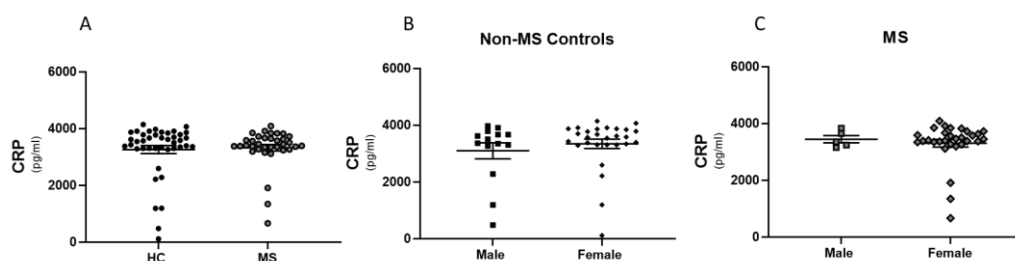


Figure 4.7 Concentration of CRP is comparable in peripheral plasma isolated from HCs and pwMS. Whole blood was collected from healthy volunteers and pwMS, plasma isolated and assessed for CRP protein expression by ELISA. The concentration of **(A)** CRP present in plasma of pwMS and HC volunteers, **(B)** the plasma concentration of CRP in males and females of the HC group and **(C)** plasma CRP concentration in males and females in the MS group was determined by ELISA. Data are presented as mean $\pm$ SEM. Data were not parametric and the test for significance used was the Mann-Whitney test.

4.10 TLR7/8 mRNA expression in PBMCs isolated from pwMS and HCs

PBMCs consist of B cells, T lymphocytes and NK cells and these cells have a prominent role in MS disease progression and pathogenesis (150, 390-393). In addition, Gjelstrup *et al.*, (2018) noted that pwMS have significantly increased numbers of non-classical monocytes (CD14⁺16⁺⁺/DC14^{dim}CD16^{bright}), and reduced classical monocyte populations, in comparison to HCs when assessing PBMC populations (394). Such non-classical monocytes have inflammatory activity and high sensitivity to viral ligands (395, 396) with links to autoimmune diseases such as rheumatoid arthritis (396). Non-classical monocytes preferentially respond to viral RNA through the TLR7/8 signalling axis via release of pro-inflammatory cytokines such as IL-1 β and TNF- α , as well as macrophage inflammatory protein-1 alpha (MIP-1 α) (396). TLR7 signalling has also been implicated in MS pathogenesis. Indeed, O'Brien *et al.*, (2010) determined that TLR7 stimulation resulted in reduced disease severity in EAE and increased levels of protective IFN- β (177). In addition, TLR7^{-/-} mice have reduced EAE severity resulting from reduced cellular infiltration of the spinal cord, as well as an observed reduction in APCs such as pDCs and B cells which play roles in propagation of autoimmunity in EAE (176). There was also an increased number of protective Tregs in TLR7^{-/-} EAE mice, thought to stem from reduced pDC numbers (176). TLR8 signalling is also altered in MS, with the expression profile of TLR8 reduced in PBMCs from pwMS, compared to HCs (183). TLR3 and TLR4 expression in PBMCs was previously investigated in our laboratory and the results concluded that the expression of both receptors was comparable between HCs and pwMS (manuscript in preparation). Given these findings, in the next set of experiments we set out to determine if discrepancies exist between the TLR7/8 signalling axis in PBMCs from pwMS and HCs by investigating the relative expression of both *TLR8* and *TLR7* mRNA in PBMCs isolated from whole blood of pwMS and HCs. PBMCs were isolated from 19 HCs and 12 pwMS, RNA was extracted and converted to cDNA for analysis of *TLR8* and *TLR7* mRNA by RT-qPCR. Data in Fig. 4.8 indicates that *TLR8* mRNA expression was comparable between pwMS and HCs (Fig. 4.8A). Indeed, the average delta CT value for *TLR8* in PBMCs from HCs was 18.14 ($\pm$ 0.36), compared to 17.33 ($\pm$ 0.58) in pwMS. Similarly, *TLR7* mRNA expression was comparable between pwMS and HCs (Fig. 4.8B). The average delta CT value for *TLR7* in PBMCs from HCs was 18.22 ($\pm$ 0.43), compared to 18.55 ($\pm$ 0.87) in pwMS (Fig. 4.8B). This suggests that the relative expression of both TLR7 and TLR8 are not altered in peripheral immune cells from pwMS, when compared to PBMCs from a cohort of HC volunteers.

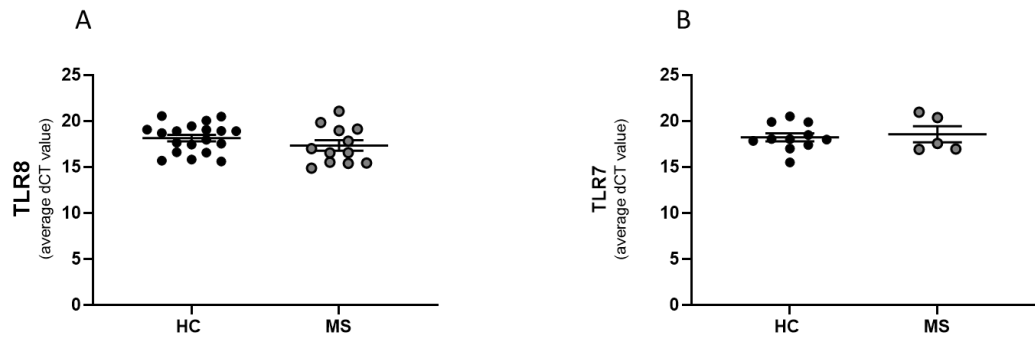


Figure 4.8 *TLR7* and *TLR8* mRNA expression in PBMCs from HCs and pwMS. PBMCs were isolated from whole blood of pwMS and HCs. RNA was extracted and cDNA synthesis performed. The basal expression of *TLR7/8* mRNA was assessed via RT-qPCR and the average delta CT values are presented. **(A)** Basal expression of *TLR8* in PBMCs isolated from pwMS and HCs. **(B)** Basal expression of viral *TLR7* in PBMCs isolated from whole blood of control and MS subjects. Data was presented as mean delta CT value $\pm$ SEM. Data was parametric and analysed using an unpaired student's *t*-test.

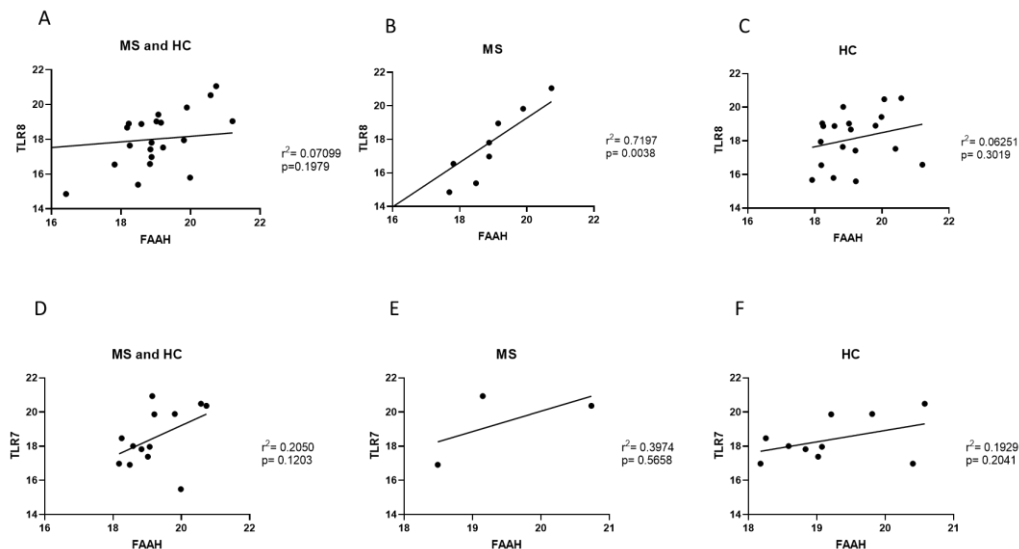
4.11 Expression of components of the eCS in PBMCs isolated from pwMS and HCs

A key aim of this project was to better understand the eCS and its role in the context of MS. Data elsewhere indicates that the eCS is altered in MS, with the expression levels of the eCB AEA elevated in the CSF of pwMS, and in peripheral lymphocytes in comparison to HCs (289). Thus, we next set out to determine if the expression profile of components of the eCS are altered in MS. In previous experiments performed in the laboratory (John-Mark Fitzpatrick, PhD student, Dr Downer laboratory) it was confirmed that both *CNR1* and *CNR2* mRNA are expressed in PBMCs from HCs and pwMS, and no alterations in the expression profile of the cannabinoid receptors was determined in the study cohort (Appendix 4, 7.1). This is in general agreement with available literature (397, 398). Data also indicates that *CB₁* and *CB₂* mRNA are expressed at higher levels in whole blood from MS individuals, compared to HCs (399).

In addition to the eCB receptors, other important components of the eCS include the enzymes FAAH and MAGL. These are responsible for the hydrolysis of the eCBs AEA and 2-AG (285, 286), respectively. Data suggest that targeting of FAAH/MAGL expression may act to ameliorate inflammation in CNS disorders (400, 401). Indeed, FAAH deletion is protective in EAE (402). There are few data assessing the expression profiles of FAAH and MAGL hydrolytic enzymes in PBMCs from pwMS and HCs. Centonze *et al.*, (2007), has previously shown that the levels of AEA are elevated in CSF and peripheral lymphocytes isolated from pwMS (289). From the data presented below in Fig. 4.9A, our findings indicate that the mRNA expression of the FAAH enzyme did not differ widely in PBMCs from HCs and pwMS. Indeed, the average delta CT value for *FAAH* in PBMCs from HCs was 19.13 ($\pm$ 0.21), compared to 18.68 ($\pm$ 0.42) in pwMS (Fig. 4.9A). Similarly, MS had no significant impact on *MAGL* mRNA expression in PBMCs (Fig. 4.9B). The average delta CT value for *MGL* in PBMCs isolated from HCs was of 13.39 ($\pm$ 0.26), compared to an average of 13.11 ($\pm$ 0.56) in the MS cohort. This suggests that MS has no significant impact on the relative expression of both FAAH and MAGL in PBMCs.

4.12 Correlations between the expression profiles of components of the eCS and *TLR7/8* mRNA in PBMCs from pwMS and HCs

Following collation of PCR data from PBMCs isolated from the whole blood of pwMS and HCs, we next determined whether correlations existed between the expression of *TLR8* or *TLR7* mRNA and components of the eCS. All correlation data are presented in Fig. 4.10. *TLR8* mRNA expression was correlated with the expression of *FAAH* mRNA (Fig. 4.10A, B, C) and *MAGL* mRNA (Fig. 4.10G, H, I), while *TLR7* mRNA expression was correlated with *FAAH* (Fig. 4.10D, E, F) and *MAGL* mRNA expression (Fig. 4.10J, K, L). Interestingly, *TLR8* mRNA expression significantly correlated with both *FAAH* and *MAGL* mRNA expression in PBMCs from pwMS (Fig. 4.10B, 4.10H; $p < 0.01$). There was no significant correlation between the expression of *TLR7* mRNA and either *MAGL* or *FAAH* mRNA in all samples tested. This suggests that the expression of TLR8, but not TLR7, correlates with the expression profile of the eCS in immune cells from pwMS.



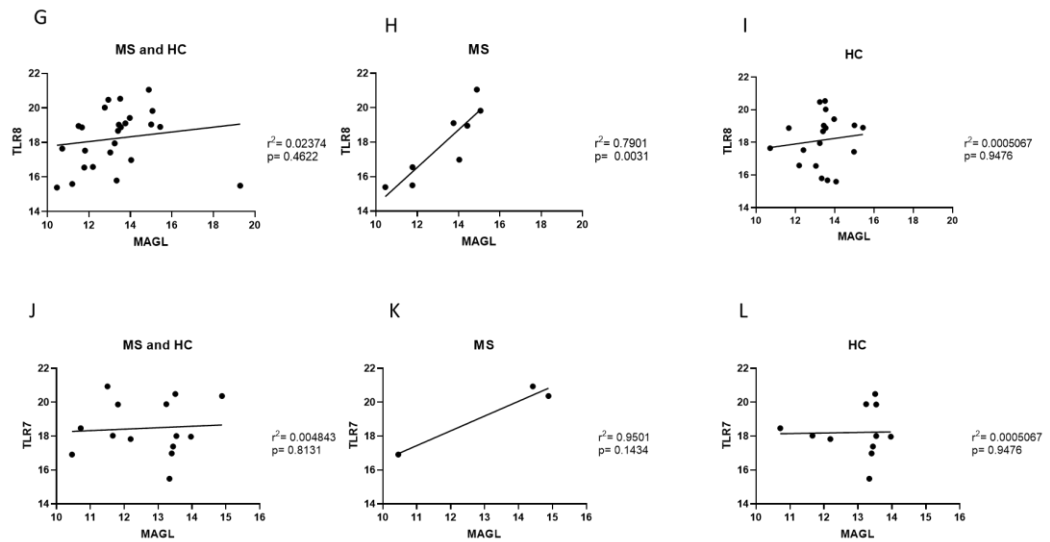


Figure 4.10 Correlations between *TLR7/8* mRNA expression and the expression of components of the eCS in PBMCs isolated from pwMS and HCs. (A) Basal expression of *TLR8* v basal expression of *FAAH* across both cohorts in PBMCs. (B) Basal expression of *TLR8* versus basal expression of *FAAH* in PBMCs in the MS cohort. (C) Basal expression of *TLR8* versus basal expression of *FAAH* in PBMCs in HCs. (D) Basal expression of *TLR7* versus basal expression of *FAAH* in PBMCs across both cohorts. (E) Basal expression of *TLR7* versus basal expression of *FAAH* in PBMCs in the MS cohort. (F) Basal expression of *TLR7* versus basal expression of *FAAH* in PBMCs from HCs. (G) Basal expression of *TLR8* versus basal expression of *MAGL* in PBMCs from both cohorts. (H) Basal expression of *TLR8* versus basal expression of *MAGL* in PBMCs from pwMS. (I) Basal expression of *TLR8* versus basal expression of *MAGL* in PBMCs from HCs. (J) Basal expression of *TLR7* versus basal expression of *MAGL* in PBMCs from both cohorts. (K) Basal expression of *TLR7* versus basal expression of *MAGL* in PBMCs from the MS cohort. (L) Basal expression of *TLR7* versus basal expression of *MAGL* in PBMCs from HCs. Data are presented as mean delta CT values. Linear regression analysis was performed and Pearson's r values are reported. $p < 0.05$ was deemed statistically significant.

4.13 Correlations between the expression profiles of components of the eCS in PBMCs

Following the assessment of links between viral TLR signalling and components of the eCS in PBMCs from both HCs and pwMS, we next determined if a relationship exists between the expression profiles of components of the eCS in PBMCs. In Parkinson's disease (PD), the expression of the *CNR2* gene is significantly upregulated in the substantia nigra, while its expression is significantly downregulated in the putamen of post-mortem brain tissue from Parkinson's disease patients (403). In addition, the expression of *MAGL* is significantly upregulated in the putamen and significantly mitigated in the substantia nigra in PD (403). This may suggest a relationship between the expression of cannabinoid receptors and *MAGL* in neuroinflammation. In addition, increased endogenous levels of *FAAH* substrates such as AEA, mediated by *FAAH* inhibition, was shown to mitigate neuroinflammation induced by TLR3 (404). This suggests a link between viral signalling and the eCS. Here, we determined that the expression of *CNR1* mRNA demonstrated a significant negative correlation with *MAGL* mRNA expression in PBMCs from pwMS (Fig. 4.11B; $p < 0.05$), this was not seen in PBMCs from HCs (Fig. 4.11C). Similarly, *CNR2* mRNA expression in PBMCs significantly correlated with *MAGL* mRNA expression in PBMCs from pwMS (Fig. 4.11E; $p < 0.05$), once again this was not observed in PBMCs from HCs (Fig. 4.11F). Overall, no significant correlation was determined between *CNR1/CNR2* and *MAGL* mRNA expression in PBMCs from the full sample cohort (Fig. 4.11A, Fig. 4.11D). It is also noteworthy that an inverse relationship was not noted between the expression of *CNR1/CNR2* and the *FAAH* mRNA in PBMCs (Appendix 4, 7.8). This indicates that the relationship between *MAGL* mRNA expression and *CNR1/CNR2* mRNA expression may be skewed in peripheral immune cells in pwMS. An increase in sample size would increase the power and is required to make these finding conclusive.

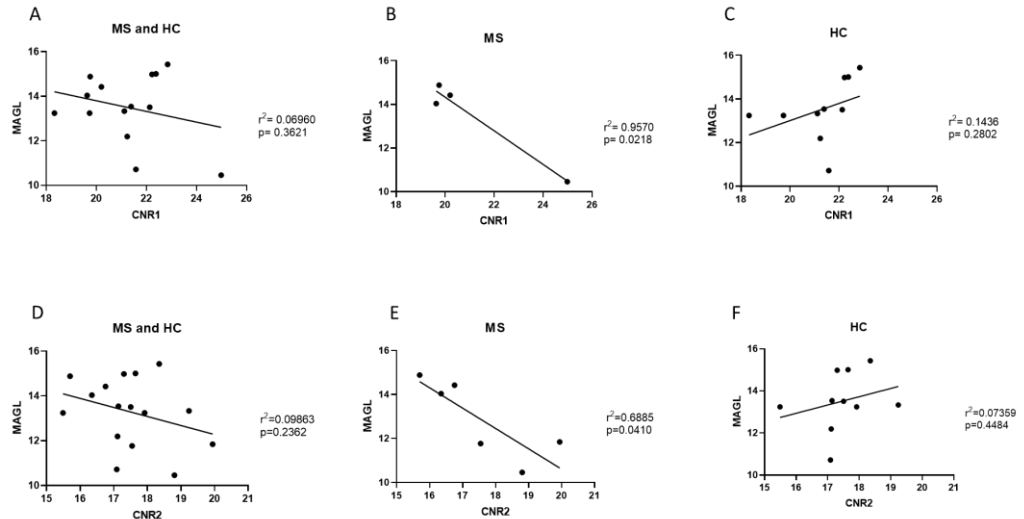


Figure 4.11 Correlations between the mRNA expression of *CNR1/CNR2* and *MAGL* in PBMCs. PBMCs were isolated from whole blood of pwMS and HCs. RNA was extracted, cDNA synthesis was undertaken and the basal expression of *CNR1* and *CNR2* mRNA, in addition to *MAGL* mRNA, was assessed via RT-qPCR. Delta CT values are presented. Linear regression was performed and Pearson's r value reported. **(A)** Basal expression of *CNR1* versus basal expression of *MAGL* in PBMCs across both cohorts. **(B)** Basal expression of *CNR1* versus basal expression of *MAGL* in PBMCs in the MS cohort. **(C)** Basal expression of *CNR1* versus basal expression of *MAGL* in PBMCs from HCs. **(D)** Basal expression of *CNR2* versus basal expression of *MAGL* across both cohorts. **(E)** Basal expression of *CNR2* versus basal expression of *MAGL* in PBMCs in the MS cohort. **(F)** Basal expression of *CNR2* versus basal expression of *MAGL* in PBMCs from HC cases. Data are presented as delta CT values. $p < 0.05$ was deemed statistically significant.

**Chapter 5 Neuropathological characterisation
of TLR7/8 expression, and the expression of
components of the eCS, in post-mortem brain tissue
with relevance to MS**

5.1 Demographics of non-MS control and MS cases in post-mortem tissue analysis

MS is a chronic autoimmune condition associated with damage to neurons in the WM and GM of the brain and the spinal cord (405). TLR signalling has been shown to be altered in the CNS of both murine EAE and in MS in humans. Indeed, there is evidence to suggest that the expression of both TLR3 and TLR4 are increased in the CNS tissue of pwMS (406). Data elsewhere indicates that *TLR2* mRNA expression is increased in cells of the CSF in MS, while the expression of *TLR2* and *TLR4* mRNA is increased in central lesions in EAE mice (407). TLR signalling has been implicated in MS pathology (189) and it has been demonstrated that TLR inhibition can ameliorate disease severity. For example, in a study carried out in EAE mice, an increase in the expression of several TLRs, including the endosomal TLR7 and TLR8 proteins, was observed in the spinal cord (171). TLR7 deficient mice have reduced disease severity in EAE, and demonstrate significantly reduced demyelination in the spinal cord (176). TLR inhibition also promotes protection of the BBB in EAE (187). In addition, the eCS is dysregulated in the context of MS. Indeed, the levels of AEA are increased in the CSF of individuals with RRMS, as well as in peripheral lymphocytes (289). Thus, assessing viral TLR expression, along with the expression of inflammatory cytokines/chemokines and eCS components, provides insight into the cellular inflammatory signalling events occurring in MS individuals at the time of death, particularly when compared to non-MS controls.

Post-mortem brain samples were provided by the UK MS Society Tissue Bank and were collected following informed consent by the donors via a prospective donor scheme according to Ethics committee approval (Appendix 2). The data provided in post-mortem reports was compiled in Table 8, and the tissue provided included snap frozen cortical blocks and formalin-fixed paraffin-embedded brain sections from 32 individuals (8 non-MS control, 12 PPMS, 12 SPMS). In both PPMS and SPMS cohorts, six sections of NAWM containing a CAL were also assessed. The average age of the non-MS control cohort was 71.00 ($\pm$ 4.81) years, the average age of individuals with PPMS was 53.83 ($\pm$ 3.71) years, and 57.67 ($\pm$ 2.28) years in the SPMS cohort. Individuals with PPMS had an average disease duration of 15.83 ($\pm$ 2.41) years, compared to SPMS individuals where the disease duration was 30.44 ($\pm$ 2.13) years. Individuals with SPMS were confined to a wheelchair for 11.40 ($\pm$ 3.01) years on average, compared to 7.00 ($\pm$ 1.11) years in the PPMS cohort. The average pH of the CSF from non-MS controls was 7.08 ($\pm$ 0.62) at the

time of neuropathological assessment, compared to 6.66 (± 0.14) and 6.57 (± 0.10) in PPMS and SPMS cases, respectively.

Table 9. Demographics of MS Neuropathology Cohort

Clinical Category	Non-MS Control	PPMS	SPMS
<i>n</i>	8	12	12
Sex (Female/Male)	2/6	6/6	9/3
Age (years)	71.00 (± 4.81)	53.83 (± 3.71)	57.67 (± 2.28)
MS duration (years)	n/a	15.83 (± 2.41)	30.44 (± 2.13)
Wheelchair time (years)	n/a	7.00 (± 1.11)	11.40 (± 3.01)
Post-mortem delay (hours)	23.50 (± 2.84)	20.39 (± 4.03)	18.00 (± 1.59)
CSF (pH)	7.08 (± 0.62)	6.66 (± 0.14)	6.57 (± 0.10)

Data expressed as Mean ($\pm$ SEM); MS: Multiple sclerosis; PPMS: Primary Progressive MS; RRMS: Relapsing Remitting MS; SPMS: Secondary Progressive MS; n/a: not applicable

5.2 Assessment of demyelination in the NAWM of post-mortem brain samples from individuals with PPMS and SPMS

Demyelination within the CNS is a hallmark of MS pathogenesis (405). Demyelination occurs when autoreactive immune cells become activated by a myelin antigen, and act to destroy myelin within the CNS by release of inflammatory mediators such as IFN- γ (408). Myelin insulates neurons within the CNS and its loss leads to functional disability such as visual and mobility impairment (409-411). It can also lead to a variety of cognitive changes (412-414) and previous data from our laboratory indicates that MS is associated with a decline in cognitive function (345). Formalin-fixed paraffin-embedded sections of post-mortem brain tissue were stained with LFB by Mr. Richard McGee and Dr. Siew Mei Yap (previous members of Downer laboratory). LFB stains myelin protein blue, leaving white unstained areas where demyelination has occurred, as indicated previously (Chapter 2, Fig. 2.1). Thirty images of LFB stained NAWM brain samples were used as part of this project (8 non-MS controls, 12 PPMS (6 NAWM/6 tissue sections containing a CAL) and 12 SPMS (6 NAWM/6 tissue sections containing a CAL)) to determine the percentage demyelination in each section. Interestingly, it has been shown that the number of CALs in pwMS is linked to disability, and individuals with numerous CALs present with more aggressive forms of the disease (411). Using the ImageJ software, a scale was created using the scale provided in each image, and the area of the NAWM and demyelination measured. To yield the percentage demyelination, the overall measure for demyelination was divided by the total area of the brain section and multiplied by 100. The Inkscape programme was then used to sketch a map of the tissue for reference purposes. Having assessed the percentage demyelination in each stained sample, we determined whether there was any difference between the percentage demyelination in sections from non-MS controls, PPMS cases and SPMS cases (Fig. 5.1A). The data below indicate that demyelination was significantly upregulated in sections of tissue from PPMS cases containing a CAL, compared to NAWM in non-MS control sections (Fig. 5.1A; $p < 0.001$). Similarly, demyelination was significantly increased in the SPMS tissue containing a CAL, when compared to non-MS controls (Fig. 5.1A; $p < 0.05$). This fits with wider literature, where CALs are indicative of higher lesion loads (411). Images presented in Fig. 5.1B are representative examples from each category of LFB-stained brain tissue (left) alongside the Inkscape tracing of these images (right) (Fig. 5.1B). The remaining images were analysed using ImageJ and Inkscape, and are presented in the Appendices (Appendix 4, Fig. 7.9, 7.10, 7.11).

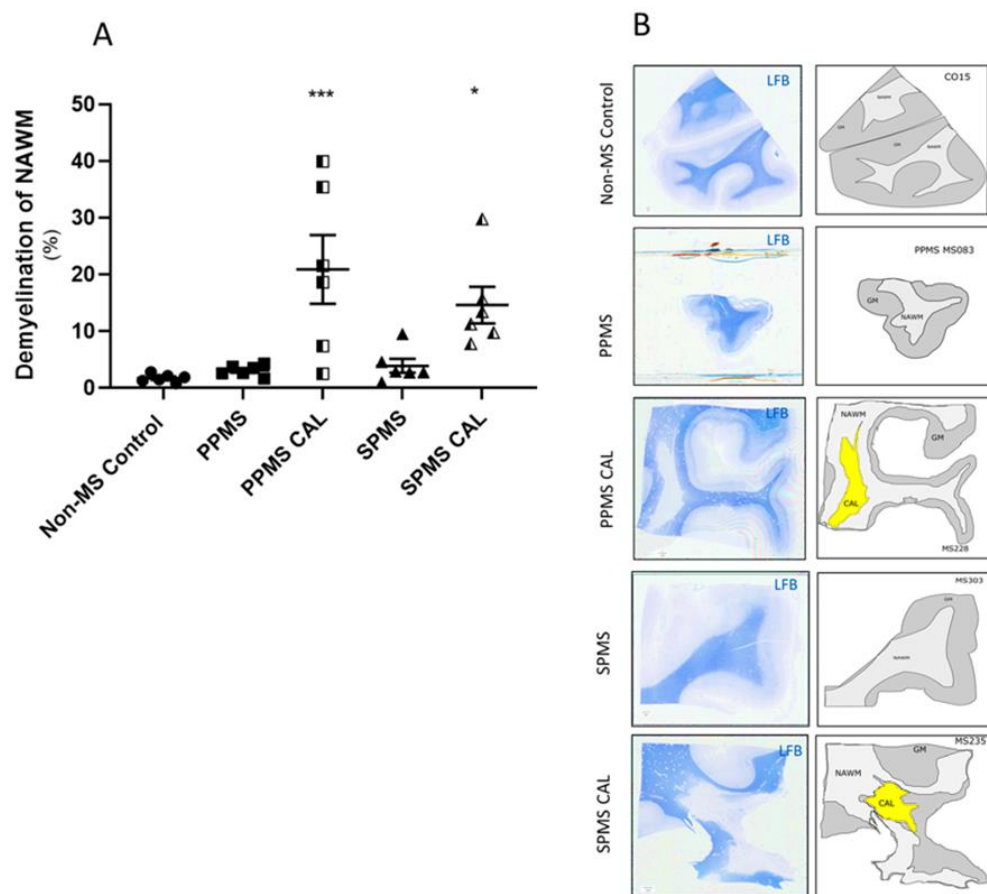


Figure 5.1 Demyelination is increased in CALs in PPMS and SPMS cases. Demyelination was determined by measuring areas of demyelination on LFB-stained images of cortical brain sections. **(A)** Percentage demyelination in LFB-stained images from non-MS control, SPMS and PPMS cases. **(B)** LEFT: LFB-stained images of NAWM of non-MS controls, PPMS cases and SPMS cases, in addition to CALs in SPMS and PPMS cases. RIGHT: Map of the brain section assessed for demyelination created in Inkscape, with NAWM denoted by an off-white, GM denoted by grey and CALs denoted in yellow. Data was presented as mean $\pm$ SEM, symbols represent individual samples. Data was analysed using a one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$ versus non-MS controls.

5.3 Post-mortem brain weight differs between non-MS controls and SPMS cases

Demyelination within the CNS and brain atrophy are hallmarks of pathology in MS (405, 415, 416). Thus, we set out to determine whether there was a difference between the brain weight of non-MS control, PPMS and SPMS cases. As the data in Fig. 5.2A indicates, SPMS cases had significantly lower average brain weights, when compared to non-MS control ($p<0.01$) and PPMS cases ($p<0.05$) (Fig. 5.2A). This is not surprising as individuals with SPMS experience increased periods of demyelination over a longer life span, along with reduced remyelination, when compared to PPMS cohorts (169). This may ultimately impact on brain weight. Both PPMS cases and non-MS control cases had comparable brain weights at the time of death. Following from this, we assessed whether brain weight in females differed from brain weight in males across all cohorts. It has recently been shown that a distinct dichotomy exists between the immune responses of males and females (369), and there is currently much interest in how males and females may differentially combat inflammation (417, 418). Such research may be key to developing effective therapies for inflammatory diseases in the future. Data presented in Fig. 5.2B indicates that males had significantly higher brain weights than females in the full study cohort (Fig. 5.2B; $p<0.01$). This is in agreement with the literature elsewhere (419). Intriguingly, differences in brain weights between males and females in the non-MS cohort was not significantly different, although there was a trend towards decreased brain weight in females in non-MS cases (Fig. 5.2C). We next assessed brain weight in male and females in the MS cohort; the data showed that males had significantly heavier brains than females in the context of MS (Fig. 5.2D; $p<0.05$).

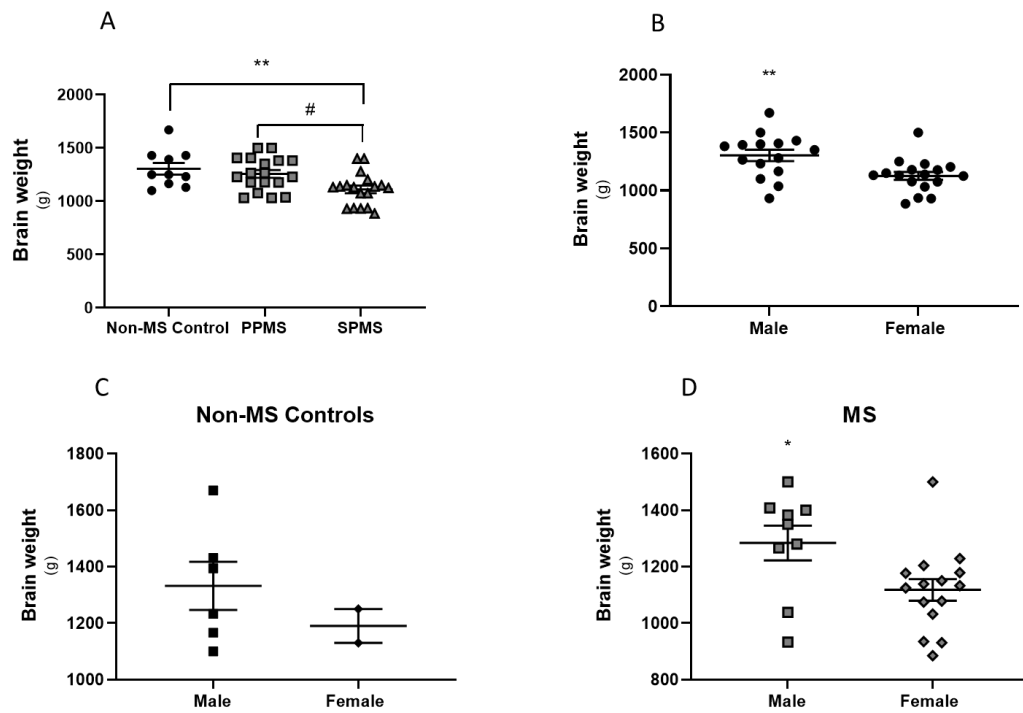


Figure 5.2 Brain weight in MS and non-MS control cases. Shortly after death, an autopsy was performed and a post-mortem report compiled for all cases. Post-mortem brain weight was included in all reports. The data was compiled to determine differences in brain weight between **(A)** non-MS controls, PPMS and SPMS cases, **(B)** males and females in both MS and non-MS control cohorts, **(C)** males and females in the non-MS cohort, and **(D)** between males and females in the MS cohort. Data were presented as mean $\pm$ SEM. Symbols represent individual samples. Data were analysed using **(A)** one-way ANOVA and Dunnett's multiple comparisons test, **(B,D)** unpaired student's *t*-tests, or **(C)** using the Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$ versus non-MS control or female cases. # $p < 0.05$ versus PPMS cases.

5.4 Correlations between disability, disease duration, age and brain weight in MS and non-MS control cases

Having assessed the differences in brain weight in MS and non-MS cases, we sought to determine if there were any significant correlations between aspects of MS pathology and several important time-sensitive aspects of the disease and its progression. Unsurprisingly, we determined that there was a significant negative correlation between brain weight of individuals with MS and disease duration (Fig. 5.3A; $p=0.043$). This was expected as pwMS have been shown to have accelerated brain atrophy, when compared to HCs (420). Hence, we next assessed whether brain weight correlated with age in both MS and non-MS control cases. There was a strong negative correlation ($p=0.059$) between both variables (Fig. 5.3B), suggesting that brain weight in both MS and non-MS controls reduces with age. This is consistent with available literature, as demyelination can occur to a degree with ageing in individuals with no history of neurodegenerative disease which contributes to brain atrophy (421, 422). Thus it is unsurprising that loss of brain mass is prevalent in both MS and non-MS cases. We next determined whether there was a link between disease duration in MS cases and the amount of time spent in a wheelchair. Data presented in Fig. 5.3C indicates that there was a significant positive correlation between these two variables, suggesting that there is a causal relationship between the span of the disease and the subsequent time spent in a wheelchair (Fig. 5.3C; $p<0.001$).

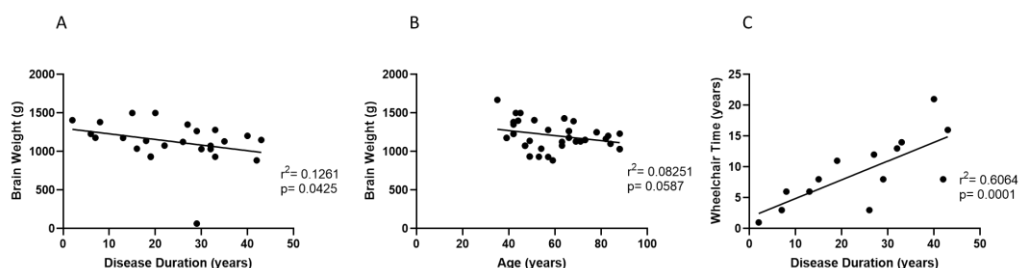


Figure 5.3 Correlations between disability, disease duration, ageing and brain weight in progressive MS cases and non-MS control cases. Data from post-mortem reports of PPMS, SPMS and non-MS control cases were gathered and compiled. **(A)** Correlation between brain weight (g) of MS cases and disease duration (years). **(B)** Correlation between age (years) and brain weight (g) in SPMS and PPMS individuals, as well as non-MS controls. **(C)** Correlation between wheelchair time (years) in MS cases and the disease duration (years). Data was presented as individual values. Linear regression was performed and Pearson's r value reported. $p<0.05$ was deemed statistically significant.

5.5 Relative expression of *TLR8* mRNA in post-mortem brain tissue

Although initially believed to affect only the WM of the brain, MS has also been shown to affect GM, although to a lesser extent (115). Lesions involving GM are associated with clinical disability, such as cognitive and motor impairment in pwMS (117, 118, 423). TLR expression, as previously stated, is altered in the CNS of pwMS, including endosomal TLR3 expression in MS lesions, which is largely undetectable in healthy donor brains (406). Interestingly, endosomal *TLR8* mRNA expression is altered in PBMCs from pwMS, along with the proclivity of this receptor to induce the expression of several cytokines associated with MS pathology (183). Thus, the central expression of *TLR8* mRNA, along with other relevant proteins, was assessed both in NAWM and NAGM of MS and non-MS controls, to determine the impact of disease pathology on viral and eCB signalling in MS. Having determined that the *TLR8* gene was expressed in PBMCs from pwMS and HCs (chapter 4, Fig. 4.8A), we set out to assess the level of central *TLR8* mRNA expression in NAWM and NAGM in both non-MS controls and MS cases. To our knowledge, *TLR8* mRNA expression has not been investigated in MS brain tissue to date, although it was found to be present in primary cell cultures of human glial cells, confirming its presence in cells of the CNS (406). CALs were only found in NAWM and thus were not included in NAGM analysis. Punches of brain tissue were taken from each specimen, RNA was extracted and converted to cDNA for analysis of *TLR8* mRNA by RT-qPCR. Data presented in Fig. 5.4 indicates that *TLR8* mRNA was expressed in the CNS, and that there was no significant difference in relative expression of *TLR8* mRNA in the NAWM of non-MS controls and both cohorts of MS assessed in the study, including tissue blocks containing CALs (Fig. 5.4A). This suggests that the expression profile of TLR8 in WM of the CNS is not altered in disease, and that TLR8 signalling does not have a functional role to play within CALs in MS. *TLR8* mRNA was not abundantly expressed in NAGM, and a significant increase in the expression of *TLR8* mRNA was determined in NAGM of PPMS cases, compared to both non-MS controls cases and SPMS cases (Fig. 5.4B; $p < 0.01$). To our knowledge, the central expression of *TLR8* mRNA in post-mortem CNS samples from MS cases is a novel finding.

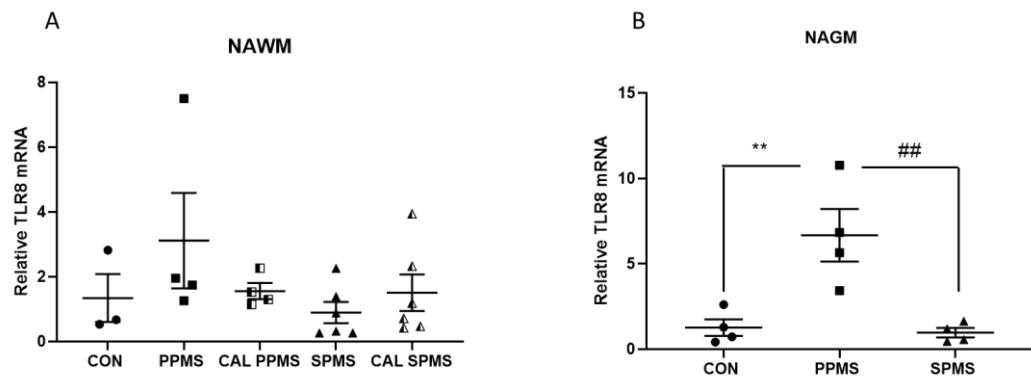


Figure 5.4 Expression of *TLR8* mRNA is increased in NAGM of PPMS cases. Brain tissue was taken in punches from non-MS controls, PPMS and SPMS cases, RNA was extracted and cDNA synthesis performed. The basal expression of *TLR8* mRNA was assessed via RT-qPCR and relative mRNA expression presented. **(A)** Relative expression of *TLR8* mRNA in NAWM from non-MS control cases, NAWM from PPMS cases, CALs from PPMS cases, NAWM from SPMS cases and CALs from SPMS cases. **(B)** Relative expression of *TLR8* mRNA in NAGM from non-MS controls, NAGM from PPMS and SPMS cases. Data was presented as mean $\pm$ SEM, with symbols representing individual samples. Data were analysed using **(A)** the Kruskal-Wallis test followed by Dunn's multiple comparisons test or **(B)** one-way ANOVA followed by Dunnett's multiple comparisons test. ** $p < 0.01$ versus non-MS controls. ## $p < 0.01$ versus SPMS cases.

5.6 Relative expression of *TLR7* mRNA in post-mortem brain tissue

TLR7 has been implicated in the pathogenesis of MS and has been detected at elevated levels in the spinal cord during the early stages of EAE (171). In addition, *TLR7* deficient mice have reduced disease severity in murine EAE (176). *TLR7* is also thought to be protective in the context of MS (179), and stimulation of *TLR7* with a synthetic agonist is protective in the context of EAE, acting to reduce disease severity (177). We previously showed that *TLR7* is basally expressed in PBMCs isolated from HCs and pwMS (Chapter 4, Fig. 4.8B). Hence, in this set of experiments, we assessed the expression of *TLR7* mRNA centrally in post-mortem brain tissue from non-MS controls, PPMS cases and SPMS cases. Punches of post-mortem brain tissue were taken, RNA extracted and converted to cDNA for analysis of *TLR7* mRNA by RT-qPCR. Data presented in Fig. 5.5A indicates there was no significant difference in *TLR7* mRNA expression in NAWM between cohorts, although PPMS cases demonstrated a trend towards increased *TLR7* mRNA expression within NAWM (Fig. 5.5A). Interestingly, similar to data presented in Fig. 5.4 B, our findings indicate that *TLR7* mRNA expression was significantly increased in NAGM from PPMS cases, when compared to NAGM from SPMS cases (Fig. 5.5B; $p < 0.05$).

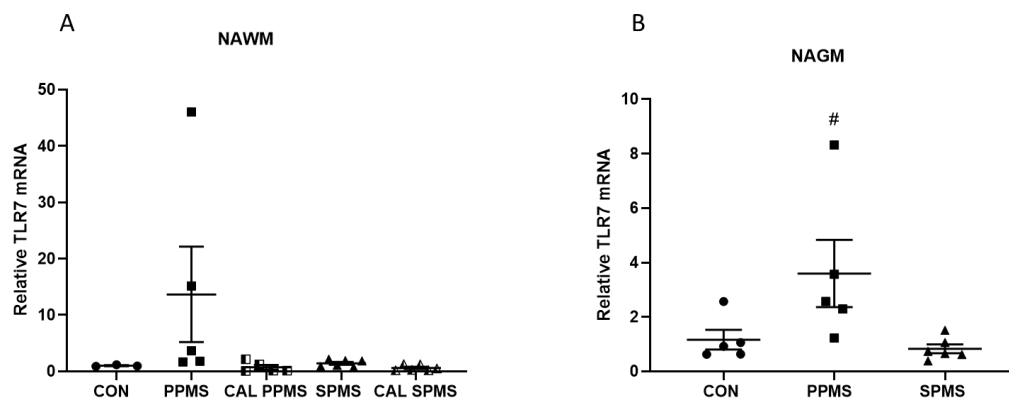


Figure 5.5 *TLR7* mRNA expression is increased in NAGM from PPMS cases, when compared to NAGM from SPMS cases. Post-mortem brain tissue samples were taken in punches from non-MS controls, PPMS cases and SPMS cases. RNA was extracted and converted to cDNA. The basal expression of *TLR7* mRNA was assessed via RT-qPCR and the relative mRNA expression presented. **(A)** Relative expression of *TLR7* mRNA in NAWM from non-MS controls, NAWM from PPMS cases, CALs from PPMS cases, NAWM from SPMS cases and CALs from SPMS cases. **(B)** Relative expression of *TLR7* mRNA in NAGM from non-MS controls, NAGM from PPMS cases and NAGM from SPMS cases. Data was presented as mean $\pm$ SEM, symbols represent individual samples. Data were not parametric and were analysed using the Kruskal Wallis test and Dunn's multiple comparisons test. # $p < 0.05$ versus SPMS cases.

5.7 Relative expression of *CNR1/CNR2* mRNA in post-mortem brain tissue

eCB signalling has been implicated in MS disease pathology and evidence indicates that this system is dysregulated in the context of neuroinflammation (424), as well as having neuroprotective propensity in MS (120, 425). It was previously determined in the laboratory that both CB₁ and CB₂ receptors (encoded by *CNR1* and *CNR2* genes) were expressed peripherally in PBMCs from HCs and pwMS (Appendix 4, 7.1) (John-Mark Fitzpatrick, PhD student, Dr Downer laboratory). Thus, we set out to determine the central expression profiles of these receptors by assessing *CNR1/CNR2* mRNA expression in NAWM and NAGM from non-MS controls, PPMS cases and SPMS cases. Punches of brain tissue were taken, RNA was extracted and converted to cDNA for analysis of *CNR1/CNR2* mRNA by RT-qPCR. As Figure 5.6A shows, *CNR1* expression was identified in NAWM of MS cases and non-MS controls (Fig. 5.6A). *CNR2* mRNA expression in NAWM was expressed by very few samples tested (Appendix 4, 7.2). Data presented in Fig. 5.6B indicates that there was no significant difference in *CNR1* mRNA expression in NAGM between non-MS control and MS cases (Fig. 5.6B). In addition, *CNR2* mRNA was detected in a small number of NAGM samples included in this study (Appendix 4, 7.2).

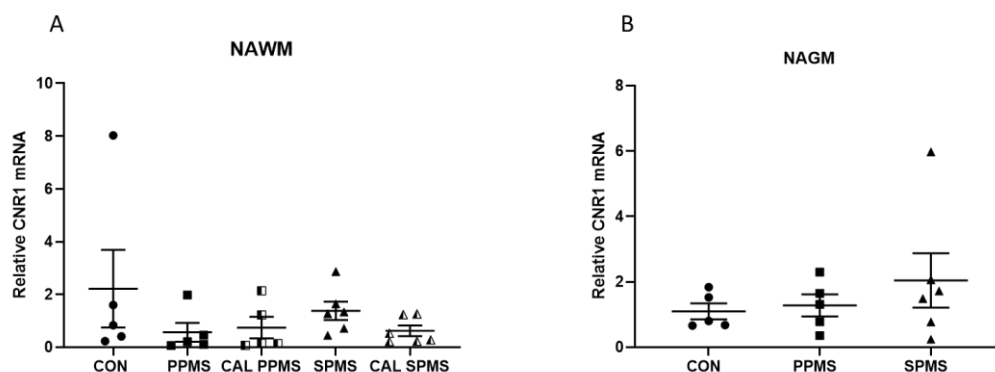


Figure 5.6 Expression of *CNR1* mRNA in NAWM and NAGM from non-MS controls and MS cases. Post-mortem brain tissue punches were taken from non-MS controls, PPMS cases and SPMS cases. RNA was extracted and cDNA synthesis performed. The basal expression of *CNR1* mRNA was assessed via RT-qPCR and the relative mRNA expression presented. **(A)** Basal expression of *CNR1* mRNA in NAWM from non-MS controls, NAWM from PPMS cases, CALs from PPMS cases, NAWM from SPMS cases and CALs from SPMS cases. **(B)** Basal expression of *CNR1* mRNA in NAGM from non-MS controls, NAGM from PPMS and NAGM from SPMS cases. Data was presented as mean $\pm$ SEM, symbols represent individual samples. Data were not parametric and were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test.

5.8 Expression of *FAAH* mRNA in post-mortem brain tissue

FAAH is an important hydrolytic enzyme involved in breakdown of the eCB AEA (285). *FAAH* has been suggested as a therapeutic target in MS and previous studies have shown that depletion of *FAAH* is protective in EAE (292, 426). *FAAH* is also expressed on cortical neurons of both HCs and pwMS (427). In the previous chapter, we determined that *FAAH* mRNA was expressed in PBMCs isolated from individuals with MS and healthy volunteers (Chapter 4, Fig. 4.9A). We sought to determine whether central expression of this enzyme was altered in MS cases when compared to non-MS controls. Punches of post-mortem brain tissue were taken from each study cohort, RNA was extracted and converted to cDNA for analysis of *FAAH* mRNA by RT-qPCR. Data presented in Fig. 5.7A indicates that *FAAH* mRNA was expressed in NAWM and no significant difference in its expression profile was observed between non-MS controls and MS cases (Fig. 5.7A). Similarly, *FAAH* mRNA was expressed in NAGM from non-MS control and MS cases, and no significant change in the relative expression of *FAAH* mRNA was determined in NAGM specimens from all cohorts (Fig. 5.7B).

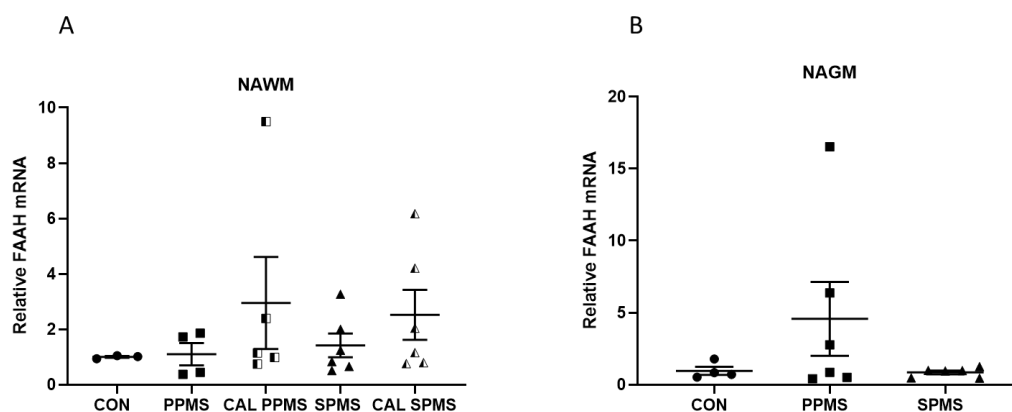


Figure 5.7 Expression of *FAAH* mRNA in NAWM and NAGM from MS cases and non-MS control cases. Tissue punches were taken from NAWM brain samples from non-MS controls, NAWM samples from PPMS cases, PPMS cases containing CALs, NAWM samples from SPMS cases and SPMS cases with CALs. RNA was extracted and converted to cDNA. The basal expression of *FAAH* mRNA was assessed via RT-qPCR and the relative mRNA expression was presented. **(A)** Expression of *FAAH* mRNA in NAWM from non-MS controls, NAWM from PPMS cases, CALs from PPMS, NAWM from SPMS cases and CALs from SPMS cases. **(B)** Expression of *FAAH* mRNA in NAGM from non-MS controls, NAGM from PPMS and NAGM from SPMS cases. Data was presented as mean $\pm$ SEM, with symbols representing individual samples. Data were not parametric and were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test.

5.9 Expression of *MAGL* mRNA in post-mortem brain tissue

MAGL is another important hydrolytic enzyme of the eCS that is mainly involved in the breakdown of 2-AG (286). A reduction in *MAGL* mRNA expression has been noted in several locations in the CNS during neuroinflammation in MS, and other inflammatory diseases (401, 403). Selective *MAGL* inhibitors have been shown to ameliorate the clinical progression of disease in murine EAE, acting to increase 2-AG levels in the spinal cord, thereby reducing axonal damage and leukocyte infiltration (401). We previously determined that *MAGL* mRNA was expressed in PBMCs isolated from whole blood samples from both HCs and pwMS (Chapter 4, Fig. 4.9B). Hence, we next assessed the expression of the enzyme within the NAWM and NAGM of MS cases and non-MS controls. Intriguingly, the expression of *MAGL* mRNA was significantly attenuated in NAWM from PPMS cases, CALs from PPMS cases, and CALs from SPMS cases, when compared to non-MS controls (Fig. 5.8A; $p < 0.05$). In NAGM, the data below indicates that there was no significant difference in *MAGL* mRNA expression between non-MS controls, PPMS cases or SPMS cases (Fig. 5.8B).

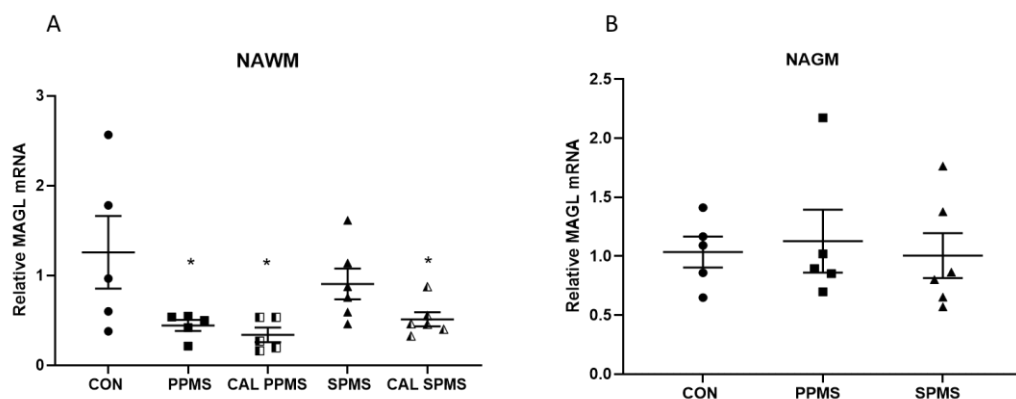


Figure 5.8 *MAGL* mRNA expression in NAWM and NAGM from MS cases and non-MS control cases. Post-mortem brain tissue punches were taken from non-MS control cases, PPMS cases and SPMS cases. RNA was extracted and converted to cDNA. The basal expression of *MAGL* mRNA was assessed via RT-qPCR and the relative expression of mRNA presented. **(A)** Basal expression of *MAGL* mRNA in NAWM from non-MS controls, NAWM from PPMS cases, CALs from PPMS cases, NAWM from SPMS cases and CALs from SPMS cases. **(B)** Basal expression of *MAGL* mRNA in NAGM from non-MS controls, NAGM from PPMS cases and NAGM from SPMS cases. Data was presented as mean ± SEM, symbols represent individual samples. Data were not parametric and were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test. * $p < 0.05$ versus non-MS control cases.

Chapter 6 Discussion

Discussion

This project set out to determine whether TLR7 and TLR8 signalling, in addition to eCB signalling, are altered both centrally and peripherally in the context of MS. In addition, we assessed whether the pCB, CBD, affected both endosomal TLR and eCB signalling in human-derived macrophages in cell culture. The novel insight gained here is that *TLR7/8* mRNA expression is altered centrally in NAGM of PPMS cases, as evident by a significant increase in expression in the cortex. This was not seen in cortical samples from SPMS cases. No change in the expression profile of *TLR7/8* mRNA was determined peripherally, in terms of experiments assessing the receptor expression in PBMCs isolated from pwMS (RRMS). In addition, the expression of components of the eCS remained unaltered in MS, both in terms of the expression of CB₁, CB₂, FAAH and MAGL in PBMCs, and in cortical brain samples, when compared to non-MS cases. This suggests that the endosomal TLR7/8 signalling axis plays a role in MS pathology in the CNS. In culture, we found that exogenous administration of the pCB, CBD, did not impact TLR7/8 signalling in THP-1 macrophages, and furthermore did not impact the expression of components of the eCS following activation through TLR7/8. Of note, however, both *CNR1* and *MAGL* mRNA expression are altered following incubation of THP-1 macrophages with a TLR7/8 agonist. This suggests that signalling via endosomal TLR7/8 may impact the eCS in macrophages, and that this may have far reaching implications *in vivo* in the context of MS.

6.1 TLR7 and TLR8 expression in THP-1-derived macrophages

This study first set out to identify whether endosomal TLR7/8 signalling was operative in THP-1 macrophages. This would enable us to assess the role of endosomal TLRs in inflammation, and assess such signalling in the context of MS. Here we determined that both TLR7 and TLR8 are expressed at similar levels in THP-1 macrophages (Table 2), suggesting that THP-1 cells are a good *in vitro* model to assess endosomal TLR signalling. In support of this, data elsewhere indicate that endosomal signalling activates NF-κB (78), and while signalling via TLR8 induced the production of type I IFNs in THP-1 monocytes, signalling through TLR7 did not (78). In addition, PMA-differentiated THP-1 macrophages showed no proclivity for the induction of IFN stimulated genes (ISGs) through TLR7 or TLR8 (78). PMA-differentiated THP-1 macrophages have also been noted to have significantly reduced levels of molecules involved in downstream TLR

signalling, including IRF7, IRAK1, IRAK4 and MyD88 (78), which are involved in the induction of type I IFNs via TLR7/8. The transcription factor IRF3 is a common signalling intermediate involved in IFN induction through the TRIF adaptor (5) which is utilised by endosomal TLR3 as well as TLR4. Previous experiments from our laboratory have confirmed that TLR3/4 signalling is operative via IRF3 and the induction of type I IFNs in THP-1 macrophages (79). Our findings suggest that TLR7/8 signalling does not promote type I IFN expression *in vitro*, and this should be taken into account when interpreting results gathered from THP1-macrophages assessing IFN production in response to TLR agonists. The reason for a lack of TLR7/8-induced IFN- β expression in THP-1 macrophages will be the target of future studies.

6.2 Dose-dependent effect of CL075 on the expression of CXCL10, TNF- α and RANTES in THP-1 macrophages

The proclivity of the TLR7/8 agonist CL075 to induce TLR activation *in vitro* has been assessed in THP-1 monocytes, as well as THP-1 macrophages (81, 428). These studies indicate that incubation of cells with TLR7/8 agonists at doses from 2.5 $\mu\text{g/ml}$ to 5 $\mu\text{g/ml}$, induced TLR7/8 activation and subsequent downstream signalling (81, 428). Data presented herein support this, indicating that incubation of THP-1 macrophages with the TLR7/8 agonist CL075 in a range of concentrations promoted the dose-dependent expression of CXCL10 and TNF- α , with maximal induction of both determined at [2.5 $\mu\text{g/ml}$]. CL075 did not significantly promote RANTES expression in macrophages with any of the concentrations of CL075 tested, but the baseline production of RANTES was elevated prior to CL075 incubation in this cell type. This may suggest that PMA-driven differentiation of THP-1 macrophages results in a significant upregulation in the endogenous expression of this chemokine. Such an observation has also been made elsewhere, and it has been suggested to result from increased activity at the CCL5 promoter following PMA stimulation (429). This was reported to result from Y-box protein-1 (YB-1) protein degradation following PMA stimulation, whereby YB-1 acts as a potential regulator of RANTES activity (429). The concentrations of CL075 employed in the present study for stimulation of pro-inflammatory protein production was also in line with several other studies in THP-1 cells (80, 81).

6.3 CL075 time-dependently promotes the expression of CXCL10 and TNF- α protein in THP-1 macrophages

TNF- α is an important cytokine in MS pathogenesis, having been identified in the CSF from pwMS (377), as well as in chronic and acute lesions within the CNS (430). Interestingly, remission in MS has been characterised by reduced TNF- α expression in serum and CSF (377). Data presented in this study indicate that incubation of THP-1 macrophages with CL075 at [2.5 μ g/ml] significantly upregulated the expression of TNF- α at the 24 h time point. CXCL10 is an important inflammatory chemokine that plays a major role in chemotaxis, and is involved in the migration of cells into the CNS (431). This migratory effect has been exhibited in THP-1 monocytes (432). In addition, expression of CXCL10 is upregulated in the CSF of individuals with RRMS and SPMS, but not PPMS (433). Thus far, there is limited evidence to suggest that TLR7/8 activation promotes CXCL10 expression in THP-1 macrophages. Our findings indicate that TLR7/8 activation promoted an increase in CXCL10 protein expression in macrophages, and indicate that the signalling machinery governing TLR7/8-induced CXCL10 expression is operative in THP-1 macrophages. Although CXCL10 is a known ISG, it has been determined that CXCL10 induction can occur in the absence of type I IFNs, through TLR7 in a p38 MAPK-dependent manner (434).

Data elsewhere indicates that RANTES release into the supernatant by PMA-differentiated THP-1 macrophages was significantly upregulated following incubation with 2.5 μ g/ml CL075 for 24 h, when compared to control cells (81). However, it was also noted that PMA differentiated THP-1 macrophages had significantly reduced ability to produce RANTES when compared to THP-1 monocytes (81). Data presented in this project indicate that TLR7/8 activation did not significantly promote RANTES protein expression in macrophages, although there was a trend towards increased production of RANTES following incubation with CL075 for 24 h. Future experiments will assess the effect of longer CL075 incubations (+ 24 h) on the expression profile of such inflammatory intermediates.

Interestingly, our findings indicate that IFN- β protein was not detected in supernatants collected from THP-1 macrophages following incubation with CL075 at all timepoints tested. These findings are supported by Eng and colleagues (2018), indicating that THP-1 macrophages do not promote type I IFNs via TLR7/8 signalling (78). In a separate study using human airway epithelial cells, it was noted that CL075 did not induce the

production of IFN- β at any time point between 8 h and 24 h (435). In human PBMCs, CL075 promotes the induction of TNF- α , with negligible effect on IFN- α expression (436). Thus, THP-1 macrophages, although having a marked reduction in their ability to stimulate ISGs through endosomal TLR7/8, still mirror, to an extent, TLR7/8 activation in primary immune cells. This suggests that although THP-1 macrophages are a suitable *in vitro* model for assessment of inflammatory TLR signalling, the lack of IFN induction should be addressed in future experiments. This may be due to alterations in the expression profile of IRFs following differentiation of THP-1 monocytes to macrophages. Interestingly, this lack of IFN- β induction was not observed in previous experiments from the laboratory, with focus on TLR3 and TLR4 signalling in response to poly(I:C) and LPS treatment, respectively. Indeed, published data from our laboratory indicates that stimulation of THP-1 macrophages with either LPS or poly(I:C) promoted type I IFN expression in THP-1 macrophages (79). This indicates that the ability of TLR3/4 to induce type I IFNs in macrophages is most likely due to MyD88-independent signalling via the TRIF and TRAM adaptors (79). In addition, differentiation of THP-1 monocytes to macrophages using PMA was previously shown to reduce IRAK1, IRAK4, MyD88 and IRF7 expression, thus potentially impacting MyD88-dependent induction of type I IFNs (78). This supports the data presented in this study and suggests that PMA-differentiation of THP-1 macrophages alters endosomal MyD88-dependent TLR signalling (summarised in Figure 6.1 and 6.2).

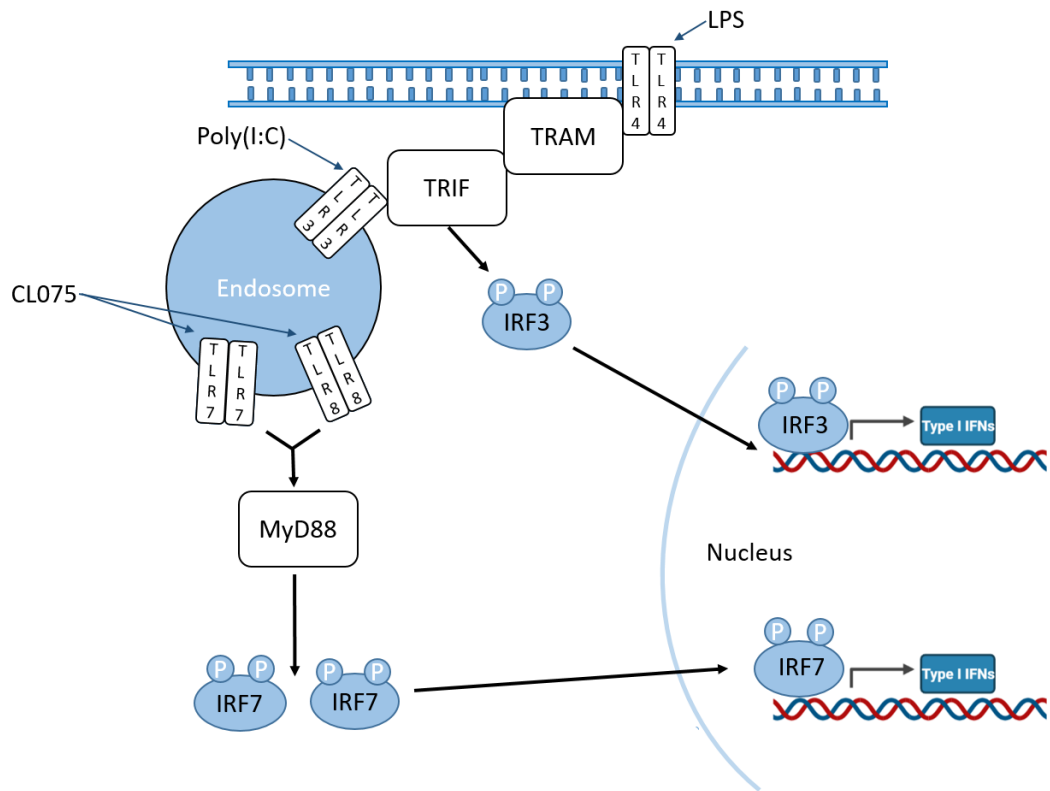


Figure 6.1 MyD88-dependent TLR signalling and subsequent induction of type I IFNs in THP-1 monocytes. To induce type I IFNs, endosomal TLR7 and TLR8 employ the use of a MyD88-dependent pathway, whereby cytoplasmic IRF7 is phosphorylated, sequesters to the nucleus, and stimulates the production of type I IFNs. TLR3 and TLR4 act via the TRIF/TRAM pathway to induce production of type I IFNs through phosphorylation/activation of IRF3. Following activation, IRF3 translocates to the nucleus and induces the production of type I IFNs.

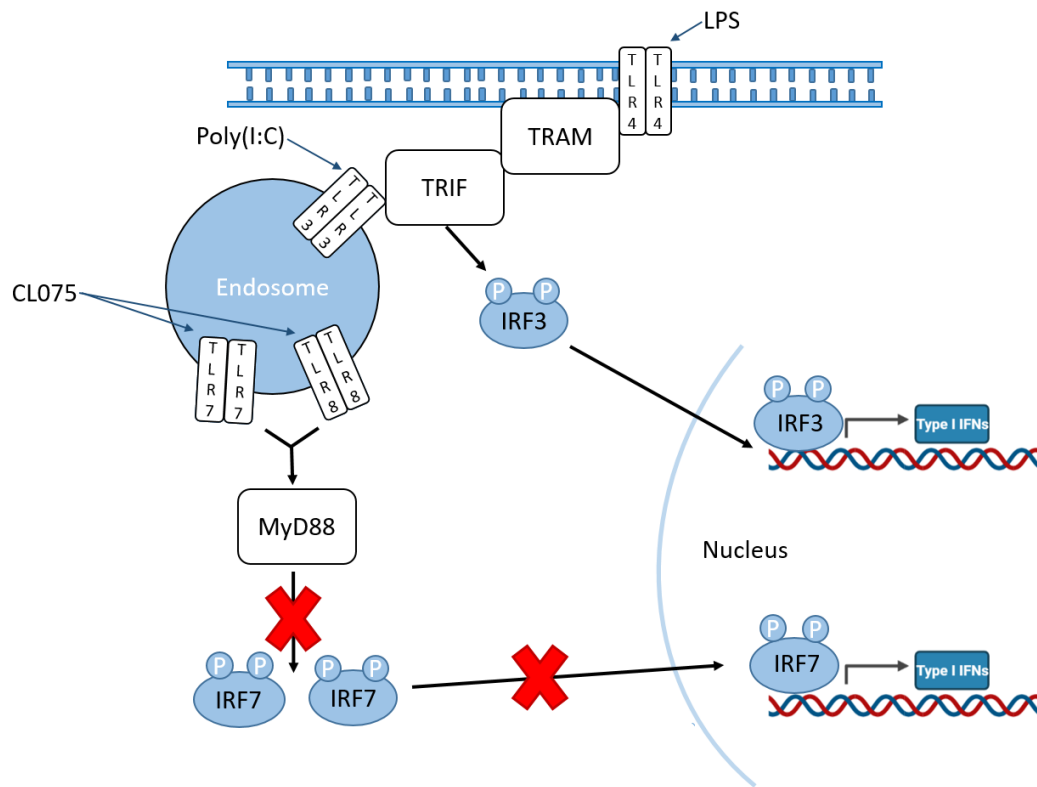


Figure 6.2 The potential impact of PMA differentiation of THP-1 monocytes to macrophages on endosomal MyD88-dependent TLR signalling and subsequent induction of type I IFNs. The data herein suggest that TLR7/8 signalling to promote type I IFN production in THP-1 macrophages was not operative, as indicated by the red 'X'. We speculate that this may be a result of PMA-induced differentiation of monocytes to macrophages which may impact the expression profile of cellular factors required for induction of IFNs.

Having assessed CL075 induction of the cytokines and chemokines at protein level in THP-1 macrophages, we next assessed the effects of TLR7/8 activation on the gene expression of inflammatory mediators. Our findings indicate that TLR7/8 activation promoted *CXCL10* mRNA expression at 6 h, and this returned to baseline at 24 h. Interestingly, both *TNF-α* and *RANTES* mRNA expression were significantly upregulated at both 6 h and 24 h, when compared to controls. This adheres to convention, whereby an increase in mRNA expression precedes an induction at the protein level. Indeed, our findings indicate that CL075 induction of *TNF-α* at the protein level was noted at 24 h following CL075 exposure. This is in agreement with published work suggesting that *TNF-α* protein expression is upregulated following incubation with CL075 at the 24 h timepoint in THP-1 macrophages (81). Interestingly, *RANTES* mRNA expression was significantly upregulated at both 6 h and 24 h post-CL075 stimulation of macrophages, but no significant increase in *RANTES* expression was noted at the protein level. It is reasonable to suggest that a later time point of 36 h or 48 h stimulation with CL075 may promote an induction in the protein expression of this chemokine in THP-1 macrophages. In addition, this induction of *RANTES* at the mRNA level may not translate to heightened induction of the chemokine at the protein level due to the impact of PMA differentiation on post-translational modification/maturation of the *RANTES* protein. Indeed, *RANTES* promoter activity is increased following THP-1 macrophage differentiation using PMA (429). Data presented herein also indicate that *IFN-β* mRNA expression was increased (albeit insignificantly) in THP-1 macrophages following CL075 treatment, and this did not translate to the level of protein. The peak of *IFN-β* mRNA induction was observed at 6 h, thus it would be expected that induction of the *IFN-β* protein would have occurred by 24 h. This did not occur and indicates that CL075 at a concentration of [2.5 µg/ml] does not have the proclivity to induce *IFN-β* protein expression in THP-1 macrophages at the 24 h timepoint.

6.4 CL075 is not toxic to THP-1 macrophages

We assessed whether treatment with the TLR7/8 ligand was toxic in THP-1 macrophages by assessing the impact of CL075 treatment for 6 h and 24 h at [2.5 µg/ml] on cell viability using MTT assays, as a measure of cellular metabolic activity. CL075 did not impact the viability of cells at a concentration of 2.5 µg/ml at either 6 h or 24 h, with a viability of almost 100% determined at both time points. This is consistent with data elsewhere

indicating that CL075 was not toxic to THP-1 macrophages or monocytes (78, 81). The use of a positive control, 10% DMSO, showed a significant reduction in cell viability. In addition to assessing the impact of CL075 on viability, we assessed whether CL075 promoted cell death in THP-1 macrophages as a result of the overproduction of NO. NO is a ROS and can promote cellular apoptosis and necrosis if its production is not tightly regulated (330). Using the Griess assay, we examined the effect of CL075 on nitrite production in THP-1 macrophages at timepoints ranging from 10 min to 24 h. Data presented herein indicate that there was no change in nitrite production at any timepoint tested, when compared to control cells. Overall, our findings indicate that CL075 does not promote cell death in THP-1 macrophages at a concentration of [2.5 µg/ml], and that the effect of CL075 on cellular cytokine/chemokine expression in macrophages are not associated with cytotoxicity.

6.5 Components of the eCS (CNR1, CNR2, FAAH and MAGL) are expressed in THP-1-derived macrophages

Interestingly, differentiation of THP-1 macrophages from monocytes has been shown to promote an increase in expression of CB₁ at the level of mRNA and protein, accompanied by a decrease in CB₂ expression (437). The expression of CB₁ and CB₂ mRNA in THP-1 macrophages has also been confirmed in separate studies (79). In terms of the eCB metabolising enzymes, data elsewhere has shown that *FAAH* and *MAGL* mRNA were not detected in THP-1 monocytes (438), while other studies demonstrate *MAGL* (439) and *FAAH* (440) expression in THP-1 cells. We sought to determine the presence of components of the eCS in THP-1 macrophages. Our findings indicate that all four components of the eCS were expressed at the level of mRNA in THP-1 macrophages, thus providing an adequate cell line for assessment of the impact of cannabinoid signalling on cellular responses.

6.6 The impact of CBD on induction of cytokines/chemokines in THP-1 macrophages stimulated with CL075

CBD has been shown to be neuroprotective in the context of neurodegenerative diseases (258) and has the ability to exert significant anti-inflammatory effects *in vivo* (441). Understanding the impact of pCBs on the inflammatory profile of endosomal TLR

signalling may prove useful in future development of therapeutics. CBD has been shown to impact TLR signalling both *in vivo* and *in vitro*. Indeed, in the U937 monocyte cell line, CBD attenuates LPS-induced RANTES expression (442). This is similar to a study carried out previously by our laboratory, whereby CBD was shown to attenuate TLR4-dependent induction of IRF3 activation and CXCL10/IFN- β expression, in addition to inhibiting TLR3-induced IFN- β and CXCL10 expression (79). Interestingly, data herein is in contrast to these findings, where CBD incubation, at both 6 h and 24 h, had no impact on TLR7/8-induced CXCL10 and TNF- α expression. Similarly, CBD, at all concentrations tested, had no impact on basal RANTES protein expression in THP-1 macrophages. This dichotomy between the impact of CBD on TLR3/4- and TLR7/8-induced signalling may be due to the impact of CBD on MyD88-independent pathways utilised by TLR3/4, as opposed to the classical MyD88-dependent pathway utilised by all other TLRs (79). The study carried out previously in our laboratory also showed that induction of TNF- α in THP-1 macrophages by LPS acting through TLR4 was not negated by pre-treatment with CBD (10 μ M) (79). Similar to these findings, no alteration in the expression of TNF- α induced by LPS in THP-1 macrophages were observed following pre-treatment with CBD at concentrations of 0.1, 1 and 10 μ M (443). Again, this is likely due to the inability of CBD to target TLR4 signalling through the classical MyD88-dependent pathway. The proclivity of CBD to inhibit the MyD88-independent pathway, and its potential impact on cytokine/chemokine/IFN induction, will be assessed closely in future experiments to determine where exactly in the TRIF/TRAM pathway CBD interacts.

Experiments performed previously in the laboratory have also assessed the impact of both THC and CBD, when delivered alone and in combination, on TLR3/4-induced expression of cytokines, chemokines and IFNs in THP-1 macrophages at the mRNA level. Indeed, previous data from the laboratory indicate that both THC and CBD attenuate both TLR3 and TLR4-induced expression of *IFN- β* and *CXCL10* mRNA (79). This is likely due to the ability of TLR4 to signal in a MyD88-independent manner through TRIF/TRAM. We assessed the proclivity of CBD to inhibit CL075-dependent induction of *CXCL10*, *TNF- α* , *RANTES* and *IFN- β* mRNA at 24 h. Interestingly, pre-treatment of CBD potentiated CL075-induced *CXCL10* mRNA expression, albeit insignificantly. Interestingly, in THP-1 macrophages stimulated by a TLR3 agonist, poly(I:C), pre-treatment with CBD (10 μ M) showed a significant inhibition of *CXCL10* mRNA induction (79). Similarly in LPS-stimulated THP-1 macrophages, pre-treatment with CBD (10 μ M) attenuated CXCL10 production at the mRNA level, but this did not reach statistical significance (79). This

indicates that signalling through TLR7/8 in macrophages may be more resilient to the anti-inflammatory properties of CBD, than signalling via TLR3/4.

While CL075 incubation for 24 h resulted in a significant induction of *TNF- α* mRNA in macrophages, our findings indicate that pre-treatment with CBD (10 μ M) did not significantly attenuate TLR7/8-induced *TNF- α* mRNA expression. Elsewhere, it was shown that autoimmune T cells isolated from EAE mice and stimulated with MOG 35-55 showed no significant attenuation of the *TNF- α* gene transcript by CBD, in concurrence with our results (444). Similarly, treatment of THP-1 macrophages with CBD (10 μ M) did not impact LPS-induced *TNF- α* expression (79). Another study in support of this confirmed that CD4⁺ splenocytes, when isolated from both vehicle-treated EAE mice and EAE mice treated with CBD which are subsequently treated with the MOG 35-55 peptide *in vitro*, exhibited increased levels of *TNF- α* secretion *in vitro* when compared to CD4⁺ splenocytes isolated from naïve mice (445). Indeed, the expression levels of *TNF- α* in both vehicle and CBD-treated EAE mice was comparable (445). CBD was shown to attenuate disease severity overall, however (445).

Our findings also indicate that CL075 treatment for 24 h resulted in a significant induction of *RANTES* mRNA expression in THP-1 macrophages, and this was not altered by pre-treatment with CBD. This is in contrast to findings in human U937 monocytes, where it was observed that *RANTES* induction using LPS was attenuated by treatment of CBD (442). In addition, CBD (5 mg/kg) treatment of TMEV-IDD mice significantly reduced *RANTES* mRNA expression in the pre-frontal cortex which was increased following onset of disease (235). Again, it is likely that the increased activity at the *RANTES* promoter following differentiation to THP-1 macrophages (429) may contribute to our findings.

Data presented in this thesis also demonstrate that incubation with CL075 did not significantly promote *IFN- β* mRNA expression in THP-1 macrophages. Minimal expression of *IFN- β* was evident following stimulation with CL075 at [2.5 μ g/ml]. This is again likely due to THP-1 macrophages having lost the ability to induce type I IFN signalling in response to TLR7/8 stimulation following differentiation (78). In addition, treatment with CBD alone, or pre-treatment with CBD prior to CL075 treatment, did not impact *IFN- β* mRNA expression in THP-1 macrophages. This suggests that CBD does not have the proclivity to restore the activity of downstream molecules involved in TLR7/8 signalling, including MyD88, IRFs, IRAK1 and IRAK4, which are potentially altered following THP-1 macrophage differentiation (78). TLR3/4 signalling in THP-1

macrophages is capable of inducing type I IFN production through the MyD88-independent pathway (78), and previous data from our laboratory indicate that this is inhibited by CBD (79). Thus, TLR3/4 signalling is intact in THP-1 macrophages following PMA differentiation, and CBD appears to only target the TRIF-dependent signalling pathway, and not TLR7/8 signalling mechanisms, in THP-1 macrophages. In support of this, data elsewhere indicates that pre-treatment of BV-2 microglia with CBD (10 μ M) prior to stimulation with LPS resulted in significant attenuation of IFN- β production from microglia (323).

Our findings also demonstrate that incubation with CBD alone did not increase CXCL10, TNF- α , RANTES or IFN- β expression in macrophages, indicating that the pCB does not promote inflammatory signalling in macrophages. Table 10 and 11 below summarise our findings regarding the impact of CBD on the expression of CXCL10, TNF- α , RANTES and IFN- β mRNA and protein in THP-1 macrophages stimulated with CL075 at [2.5 μ g/ml] for 24 h.

Table 10. Impact of CBD on CL075 induction of cytokines and chemokines at the level of mRNA in THP-1 macrophages at 24h

Cytokine/chemokine	Impact of CL075 at [2.5 μ g/ml] on mRNA expression	Impact of CBD (10 μ M) on CL075-dependent mRNA expression
CXCL10	–	↑
TNF- α	↑	–
RANTES	↑	–
IFN- β	–	–
↑: significantly increased mRNA expression; ↓: significantly reduced mRNA expression; –: no significant effect on mRNA expression		

Table 11. Impact of CBD on CL075 induction of cytokines and chemokines at the protein level in THP-1 macrophages at 24 h

Cytokine/chemokine	Impact of CL075 at [2.5 µg/ml] on protein expression	Impact of CBD (10 µM) on CL075-dependent protein expression
CXCL10	–	–
TNF-α	↑	–
RANTES	↑	–
IFN-β	n/a	n/a
↑: significantly increased protein expression; –: no significant effect on protein expression; n/a: not applicable		

6.7 Impact of CL075 and CBD treatment on *TLR8*, *TLR7*, *CNR1*, *CNR2*, *FAAH* and *MAGL* mRNA expression in macrophages

Previous studies performed by our laboratory have shown that treatment of THP-1 macrophages with TLR agonists such as poly(I:C) and LPS promote the expression of various inflammatory cytokines/chemokines in THP-1 macrophages (79). Here our findings indicate that exposure of macrophages to CL075 significantly increased the expression of *TLR8* mRNA in THP-1 macrophages at the 24 h timepoint. Pre-treatment with CBD (10 µM) did not significantly attenuate CL075-induced *TLR8* mRNA expression. To our knowledge this finding is novel. Treatment of THP-1 macrophages with CBD alone did not impact *TLR8* mRNA expression, suggesting that CBD is not an endogenous stimulator of TLR8 transcription. Furthermore, CL075 did not have the proclivity to enhance the expression of *TLR7* mRNA in macrophages, and no significant alterations in the relative expression of TLR7 were noted following treatment with CBD alone, or following CBD pre-treatment prior to CL075 incubation for 24 h. In addition, data presented in this report indicate that *TLR7* mRNA expression was over ten-fold lower than relative *TLR8* mRNA expression in THP-1 macrophages following stimulation with CL075. This indicates that stimulation of THP-1 macrophages with CL075 results in significantly increased expression of the TLR8 receptor at the mRNA level. This suggests

that the activity at the TLR8 promoter may be upregulated following stimulation with CL075 in THP-1 macrophages. Functional assessment of TLR8 in THP-1 macrophages will be important in future research in order to determine the impact of this increase in receptor expression *in vitro*.

Data presented herein indicate that THP-1 macrophages express both *CNR1* and *CNR2* mRNA, as well as *FAAH* and *MAGL* mRNA. In support of this, data elsewhere indicate that treatment of macrophages with oxidised low density lipoproteins (oxLDLs) (which are endogenous TLR ligands (446)) promoted THP-1 macrophage differentiation to foamy macrophages (447). As a result of this incubation with oxLDLs however, the relative expression of *CNR1*, *FAAH* and *MAGL* mRNA were significantly upregulated in the cells (447). This indicates that TLR stimulation can alter expression of eCS components. Data presented herein indicate that CL075 had an intriguing effect on the expression profile of components of the eCS in THP-1 macrophages. Indeed, treatment with CL075 for 24 h resulted in a significant reduction in *CNR1* mRNA expression, and pre-treatment with CBD (10 μ M) did not significantly alter *CNR1* mRNA expression. Interestingly, the expression of *CNR2* mRNA in THP-1 macrophages was not altered by incubation with CL075 for 24 h, and furthermore *CNR2* mRNA expression was not affected by pre-treatment with CBD. These results are unsurprising due to the poor affinity of CBD for both CB₁ and CB₂ (256). In terms of *FAAH*, CL075 and CBD did not significantly alter *FAAH* mRNA expression in THP-1 macrophages. This is in contrast to other studies that have observed an inhibition of *FAAH* following treatment with CBD, leading to an increase in the levels of AEA (448). Interestingly, our findings indicate that *MAGL* mRNA expression was significantly increased in macrophages following incubation with CL075 for 24 h. This increase was not significantly altered following pre-treatment with CBD. To date, there is no evidence to suggest that CL075 impacts the expression of *MAGL* mRNA. Overall, these data suggest that CL075 promotes TLR8 transcription in THP-1 macrophages and that this ligand may impact the expression of components of the eCS in THP-1 macrophages, in particular the expression of *CNR1* and *MAGL* mRNA. The mechanisms governing such transcriptional regulation warrant full investigation. Table 12 below summarises the impact of CBD on induction of *TLR7* and *TLR8* mRNA, as well as *CNR1*, *CNR2*, *FAAH* and *MAGL* mRNA following treatment of THP-1 macrophages with CL075 at [2.5 μ g/ml] for 24 h. In addition, a summary scheme outlining the impact of CL075 and CBD on THP-1 macrophages in this project is outlined in Fig. 6.3.

Table 12. Impact of CBD and CL075 on induction of TLR7/8 and components of the eCS at the level of mRNA in THP-1 macrophages

Gene	Impact of CL075 at [2.5 µg/ml] on mRNA expression	Impact of CBD (10 µM) on CL075-dependent mRNA expression
TLR8	↑	–
TLR7	–	–
CNR1	↓	–
CNR2	–	–
FAAH	–	–
MAGL	↑	–
↑: significantly increased mRNA expression; ↓: significantly reduced mRNA expression; –: no significant effect on mRNA expression		

6.8 Clinical characteristics and QoL in pwMS and HCs in the cohort of study participants

The majority of individuals affected by MS are female (98, 449), and the demographics of the MS cohort recruited to this project reflect this. Indeed, in the MS cohort recruited to this study, 85% of participants were female, this number is representative of the higher female:male ratio of MS, which affects between 2-3 times more women than men (83). In comparison, 65% of HCs in the study were female. HCs were age-matched with pwMS (35.50 ± 2.64 years compared to 38.72 ± 1.55 years). The average EDSS of 2.16 (± 0.40) indicated that overall in the MS cohort the majority of individuals had minimal neurological disability. In addition, the average disease duration in pwMS recruited to this study was over 5 years, allowing us to gain insight into the peripheral cellular pathophysiology of established, but generally well-managed (as indicated by EDSS scores), MS.

Individuals affected by MS are treated with a variety of medications, and a wide range of DMTs exist for pwMS, including IFN- β therapy, humanised monoclonal antibodies, chemotherapy drugs and immunosuppressants. IFN- β therapy was first licensed as an effective therapy in RRMS in 1993 (379) and since then a multitude of IFN- β -based therapies have been developed, including intramuscular IFN- β -1a (Avonex from Biogen)

and PEGylated type I IFNs, such as Plegridy (Biogen) (380). The majority of pwMS in this study were treated with tysabri at the time of recruitment. This humanised monoclonal antibody has proven efficacious in pwMS, although there are heightened risks of developing PML associated with the duration of treatment (450), particularly in individuals receiving treatment for more than two years (451). Participants in this study also reported the use of the immunomodulatory therapies including gilenya, rituximab, tefidera and copaxone. The most common DMT in use worldwide for the management of RRMS is IFN- β therapy (380); however just five individuals reported the use of IFN- β therapy (avonex and plegridy) in the current study. One individual reported cannabis use at the time of recruitment, and three individuals were taking vitamin D supplements. Vitamin D use is linked to reducing the risk of developing MS (452), as well as reducing clinical activity of the disease (453, 454). Three individuals in the study were smokers, and one of these participants had an EDSS above 5 (data not shown). Smoking can influence disease progression in MS, and smokers tend to progress more quickly from RRMS to SPMS, than individuals who had never smoked (455). In addition, smokers have an increased T2 lesion volume compared to individuals who have never smoked (455). Folic acid was taken by two individuals in the study, which has been shown to improve QoL in pwMS (456).

Following collation of all self-reporting QoL questionnaires and depressive symptomology questionnaire data, the data highlight the significant impact of MS on QoL. Indeed, pwMS were noted to have significantly reduced scores in terms of physical function, physical health, energy, sexual function, social function, mental health, cognitive function, reduced health distress, emotional wellbeing, and overall QoL. pwMS were also noted to have significantly increased depressive symptomology as defined by the QIDS-SR₁₆, when compared to HCs. This highlights the pleiotropic nature of MS and its impact on QoL. Interestingly, depressive symptomatology does not have a causal relationship with increased disability (as defined by EDSS) in MS, rather depressive symptomology may be an emotional side effect of MS pathology and CNS atrophy (127). However, the incidence of major depression in pwMS is up to 10 times higher than the general population, as well as being upregulated in comparison to other neurologic disorders (457). These results are supported by literature elsewhere (458), indicating that MS significantly impacts QoL.

6.9 Cytokine, chemokine and CRP assessment in plasma from whole blood samples isolated from healthy volunteers and pwMS

Sysmex data was obtained at the time of venepuncture via the use of a Sysmex haematology analyser, providing a readout of the cellular profiles of each participant blood sample. We compiled the data obtained from both the MS and HC cohorts and examined the data for any differences in cellular read-outs (Table 6). No significant differences were found between the profile of whole blood isolated from pwMS and HCs, apart for MCH, which was significantly increased in pwMS. MCH represents the average amount of haemoglobin present within each red blood cell. Interestingly, high levels of free haemoglobin within blood serum may be linked to MS pathology and may be attributed to atrophy of the brain (459), acting to enhance neurodegenerative processes by the release of haem and iron (459).

Enhanced expression of inflammatory cytokines is common in MS, occurring both centrally and within the periphery (460). Indeed, peripheral inflammation has been linked to the triggering and exacerbation of MS relapses, causing further damage within the CNS and axonal loss (461). The data collected as part of this study revealed that the inflammatory chemokine CXCL10 was detected in plasma from over half of the individuals assessed in both cohorts, with plasma isolated from HCs demonstrating a higher concentration of the inflammatory chemokine, when compared to plasma from pwMS. CXCL10 has been linked to MS pathology and its expression is upregulated in blood serum and CSF from pwMS (433, 462). Interestingly, in a cohort of individuals with MS treated previously with IFN- β , those who had failed to develop neutralising antibodies for IFN- β had significantly increased levels of various chemokines in peripheral blood, including CXCL10, while those pwMS who had developed neutralising antibodies for IFN- β had no observable difference to HCs. This was found at both the level of mRNA and protein (463). CSF concentration of CXCL10 was also found to correlate with the number of leukocytes present within the CSF (462). The reduced detection and concentration of this chemokine in plasma from pwMS assessed in this project may be related to DMT use that may negate the expression of circulating CXCL10.

TNF- α is an important cytokine in MS pathogenesis. Indeed, the expression of TNF- α in the CSF has been linked to disease progression in MS (350). In this study, the TNF- α protein was not detected in the majority of plasma samples obtained from the participants in this study, both in HC and MS cohorts (detected in thirteen and four cases,

respectively). The average TNF- α level was 765.97 ($\pm$ 275.50) pg/ml in plasma from HCs, compared to 73.75 ($\pm$ 38.88) pg/ml in plasma from pwMS. This reduction may be linked to DMT use in pwMS. The chemokine RANTES has previously been identified as a genetic risk marker for MS (464), and this chemokine has roles to play in synaptic excitability and inflammation in MS (465). RANTES protein was detected in plasma samples from all individuals in the study, and the expression pattern was similar in HCs (1345.76 $\pm$ 61.93 pg/ml) and in pwMS (1185.23 $\pm$ 87.70 pg/ml). As mentioned previously, the type I IFN, IFN- β , has been shown to be protective in the context of MS, particularly in early RRMS, where it is believed to exert its effects through anti-inflammatory processes (192-194). Here we determined that IFN- β protein was not expressed in plasma samples isolated from the majority of individuals who took part in the project (detected in 29% of pwMS and 35% of HCs). In addition, it was evident from our assessment that IFN- β protein expression was comparable in plasma from both cohorts.

Much evidence suggests that IL-1 β has a role in the pathophysiology of MS via activation of the inflammasome. Indeed, data suggests that activation of the inflammasome may be responsible for MS relapse and inflammation in MS (466). Indeed, inhibitors of the NLRP3 inflammasome alleviate MS severity by reducing IL-1 β expression (467-469). In addition, high levels of NLRP3-associated proteins, such as IL-1 β , caspase-1 and IL-18, have been noted in CSF and blood samples from pwMS (359, 470, 471). Indeed, IL-1 β in CSF has been shown to correlate with the progression from RRMS to progressive MS (472). Given these findings, we assessed the impact of MS on plasma IL-1 β expression. Data presented in this study indicate that 52% of HCs recruited to this study had detectable levels of IL-1 β protein in their plasma, compared with 63% of pwMS. Unexpectedly, a non-significant elevation in plasma IL-1 β protein expression was determined in samples from HCs, compared to samples from pwMS. Indeed, individuals with MS had an average plasma IL-1 β concentration of 180.04 ($\pm$ 86.73) pg/ml, compared to 450.49 ($\pm$ 141.32) pg/ml in plasma samples from the HC cohort. Again, the reduced concentration of IL-1 β in plasma from pwMS assessed in this project may be related to DMT use that may negate the expression of peripheral IL-1 β .

The inflammatory protein CRP has been implicated in the pathophysiology of MS and is indicative of an adverse outcome in the context of disease (473). CRP was detected in plasma from all individuals who took part in this project. Indeed, CRP was also the most highly expressed protein we assessed by ELISA in plasma, with an average concentration of 3259.48 pg/ml in plasma from HCs, and plasma CRP concentrations of 3323.38 pg/ml

detected in plasma from individuals with MS. This is interesting as CRP is generally produced in the context of inflammation (389). As CRP is a common marker of infection, these results suggest that the cellular read-outs recorded in both pwMS and HCs are not linked to infection.

6.10 Effect of gender on cytokine/chemokine expression in plasma isolated from HCs and pwMS

Various cytokines and chemokines are differentially expressed in males and females due to gender specific immune responses (369). Thus we assessed cytokine and chemokine expression in plasma samples from males and females in both HC and MS cohorts. Our data indicate that females in the MS cohort may demonstrate a trend towards increased CXCL10 production in plasma, but this was not statistically significant. This was expected as there has been little evidence from human studies to suggest that, under normal conditions, males and females have altered peripheral levels of CXCL10. However, data from TLR4 macrophage studies in murine models demonstrate that macrophages from males produce more CXCL10 than macrophages from females (372).

TNF- α is linked to disease progression (474), and in this project we demonstrate that TNF- α expression is significantly upregulated in plasma from HCs, when compared to plasma from pwMS. This is in contrast to studies elsewhere that have shown a significant increase in *TNF- α* mRNA in PBMCs from pwMS, when compared to PBMCs from healthy donors (475). This may be due to the action of DMTs in the current MS cohort, acting to reduce peripheral inflammation. We also assessed plasma TNF- α expression in male and female participants; no significant difference was determined between males and females in terms of TNF- α production in plasma, in both HC and MS cohorts. This is in contrast to studies elsewhere that have identified an increase in TNF- α -producing CD3⁺ immune cells in males with RRMS, when compared to females with RRMS (370). This suggests that peripheral TNF- α production/function is dysregulated in the context of MS and may be important in disease progression.

RANTES expression is elevated in CSF of individuals with active MS (365, 465), as well as being expressed in cells within demyelinating lesions of the CNS (365). Here, we found no significant difference between levels of RANTES in plasma of individuals with MS and

HCs. In addition, the expression pattern of RANTES in plasma was not altered by gender in either HC cases, or in individuals with MS.

IFN- β therapy acts to combat inflammation, particularly in the relapsing-remitting phases of disease (195). We confirmed that HCs and pwMS demonstrate similar expression levels of IFN- β in plasma, indicating that MS does not affect type I IFN signalling in the periphery. This is consistent with data elsewhere (381). In addition, no significant alterations in plasma levels of IFN- β were recorded between males and females among HCs. Intriguingly, males in the MS cohort demonstrated significantly increased levels of IFN- β protein in plasma, compared to females, suggesting that gender impacts endogenous plasma IFN signature in MS.

As part of this project, we assessed IL-1 β expression in plasma from control individuals and pwMS, while also assessing the impact of gender on endogenous plasma IL-1 β expression. IL-1 β expression is upregulated in CSF and serum from pwMS, when compared to CSF/serum from HCs (476). Here we observed that although there appeared to be a trend towards increased IL-1 β levels in plasma from control individuals compared to pwMS, this was not significant. Again, this may reflect the impact of DMT use on peripheral IL-1 β expression in individuals with RRMS. In addition, it may also stem from an increased presence of soluble IL-1 receptor (sIL-1R) in plasma isolated from pwMS, compared to HCs, which has been documented in other neurodegenerative diseases such as Alzheimer's disease (477). Although there was no significant difference observed between male and female plasma levels of IL-1 β in both HCs and pwMS, there was a trend for females to have slightly elevated levels of IL-1 β in plasma. This is of interest as elevated or poorly regulated IL-1 β expression is evident in a large number of autoimmune diseases (478), to which women are at a greater risk of developing than men (369). Thus this data may be indicative of a heightened risk of females to develop autoimmunity than males.

The acute phase protein CRP is generally upregulated in the periphery following inflammation, where it acts to initiate the complement cascade (389). A study by Soilu-Hänninen *et al.*, (2005), suggests that there are comparable levels of CRP measured in serum from individuals with RRMS and healthy donors, although CRP was shown to increase significantly following MS relapse (364). We show here that CRP plasma levels are comparable between pwMS and HCs. In addition, there was no difference determined in the plasma levels of CRP in both males and females in both the HC group

and MS cohort. This suggest that underlying infection was not responsible for the cellular responses observed in either the HC or MS cohorts assessed in this study.

6.11 TLR7/8 expression in PBMCs isolated from pwMS and HCs

IFN- β therapy has been shown to upregulate endosomal receptors including TLR3, TLR7 and TLR9 in PBMCs from both HCs and pwMS, and pDCs are the main cell type in which TLR7 is upregulated in pwMS (479). In terms of TLR7, stimulation of TLR7 may be protective in MS. Indeed, in EAE, repeated stimulation with a TLR7 ligand was shown to reduce antigenic T cell responses by affecting DC activation, accompanied by lower levels of IFN- γ and Th1/Th2 cytokines (480). However, TLR7 deficient mice have been shown to have reduced severity of EAE (176). There may be a disparity between the data gathered in murine models of EAE and the functions of TLR7 in MS. Indeed, some data indicate that *TLR7* mRNA expression was significantly reduced in PBMCs from pwMS compared to HCs (180). In contrast, our findings indicate that *TLR7* mRNA was expressed at similar levels in PBMCs isolated from blood of individuals with RRMS and HCs. The role of TLR8 in MS pathogenesis has not been studied extensively, and data presented herein indicate that *TLR8* mRNA expression was comparable in PBMCs from both individuals with RRMS and HCs. Data elsewhere suggests that endosomal TLR8 expression is downregulated, and its function is dysregulated, in PBMCs isolated from pwMS (183).

6.12 Expression of components of the eCS in PBMCs isolated from pwMS and HCs

In previous experiments conducted by the laboratory, it was determined that both *CNR1* and *CNR2* mRNA are expressed in PBMCs from pwMS and HCs (John-Mark Fitzpatrick, PhD student, Dr. Downer laboratory) (Appendix 4, 7.1). This is in agreement with studies elsewhere (397). In this study we assessed the expression of the hydrolytic enzymes FAAH and MAGL in PBMCs from pwMS and HCs. FAAH and MAGL are involved in the degradation of the eCBs, AEA and 2-AG, respectively (285, 286). The expression of AEA is elevated in peripheral lymphocytes from pwMS, and this in turn is linked to reduced expression and dysregulated function of FAAH (289). In a separate study it was found that *FAAH* mRNA expression was not altered in B, NK or T cells in pwMS, when compared to immune cells from controls (291). To our knowledge, research into the expression of MAGL has not been undertaken extensively in MS. In this project, we present evidence

that the expression of *MAGL* mRNA in PBMCs was comparable between individuals with MS and HCs. Similarly, the expression of *FAAH* mRNA was also comparable in PBMCs from both study cohorts. We suggest that this assessment of *MAGL* and *FAAH* mRNA expression in PBMCs from individuals with MS and HCs is novel, and warrants further investigation to ascertain whether the functionality, as well as expression of these metabolising enzymes, is intact in MS. Here we also ascertained that there was a significant negative correlation between *MAGL* mRNA expression, and the expression of *CNR1* and *CNR2* mRNA in PBMCs from pwMS. This negative correlation was not observed in the HC cohort.

6.13 Demographics of non-MS control and MS cases in post-mortem tissue analysis

The pathophysiology associated with MS is evident in both the WM and GM of the brain, as well as in the spinal cord, by the formation of demyelinating lesions and subsequent loss of neurons (85). Having received ethical approval to assess post-mortem cortical human brain (Appendix 2), we first examined the demographics of a cohort of MS and non-MS cases obtained from the UK MS Brain Bank. We compiled the data to assess any baseline alterations between PPMS cases, SPMS cases and non-MS control cases. The average age of non-MS cases in this part of the study was 71.00 years at time of death. This was compared to an average of 53.83 years in PPMS cases, and 57.67 years in SPMS cases. This was expected, as individuals with progressive MS have a shorter life expectancy, on average, than control individuals (481). The average age of disease duration among PPMS cases was 15.83 years, compared to 30.44 years in SPMS cases. Again, this was expected given the natural course of the disease (85). Wheelchair time in SPMS cases averaged over 7 years, whereas PPMS cases had spent approximately 11 years in a wheelchair prior to death. The post-mortem delay was highest in non-MS cases, with an average delay of 23.50 h, followed by PPMS cases with a post-mortem delay of 20.39 h, and an average post-mortem delay of 18.00 h in SPMS cases. This is important to note in terms of the impact of such delay on the integrity of the tissue for biochemical analysis. The pH of CSF at the time of death was also measured. In non-MS cases, an average CSF pH of 7.08 was recorded, and PPMS and SPMS cases recorded an average CSF pH of 6.66 and 6.57, respectively. CSF pH has been linked to both respiratory and non-respiratory processes (482), and may have an impact on cerebral function (482). Further research regarding the impact of MS on CSF pH is warranted.

6.14 Demyelination in NAWM in cortical brain samples

Demyelination is a hallmark of MS pathology, contributing to the formation of lesions and subsequent axonal loss, and resulting in disability (85). Demyelination was assessed in non-MS cases, PPMS and SPMS cases, in both NAWM and CALs. CALs are areas of active demyelination at the time of death, and are often associated with failure of full remyelination due to their expanding nature, particularly in SPMS (169). Interestingly, the tight junction protein, zonula occludens 1 (ZO-1) is abnormally expressed in CALs in SPMS and PPMS cases (483). This expression may be linked to leakage across the BBB, inflammation and axonal loss. It has been reported that full remyelination is less frequent in SPMS, when compared to PPMS (169), although here we focused on the activity of demyelination rather than the process of remyelination.

Having assessed the percentage demyelination in non-MS cases, PPMS (NAWM and CAL) cases and SPMS (NAWM and CAL) cases, data presented herein indicate that PPMS samples containing a CAL demonstrated significantly increased demyelination, when compared to non-MS cases. In some PPMS cases, demyelination was as high as 40% in cortical brain samples. SPMS CALs also demonstrated significantly elevated demyelination, compared to non-MS control NAWM, although not to the same extent as PPMS. No significant difference in the percentage demyelination was determined between CALs in either PPMS or SPMS cases. Overall, in NAWM from PPMS and SPMS cases, minimal evidence of demyelination was determined. Overall, this analysis indicates that chronic inflammation is a prominent feature at each phase of disease. The pathophysiology of progressive MS is unlikely to rely heavily on the formation of new lesions within the CNS, but rather the gradual expansion of pre-existing lesions and subsequent axonal loss.

6.15 Post-mortem brain weight in non-MS controls and SPMS cases

Having assessed demyelination in cortical brain samples, we next assessed brain weights across the MS subtypes, as well as between males and females in each cohort. Our findings indicate a stepwise reduction in brain weight in non-MS cases, PPMS cases and SPMS cases. In fact, SPMS cortical brain weight was significantly reduced in comparison to the brain weight of both non-MS cases and PPMS cases. This suggests reduced

remyelination capacity in the SPMS brain, compared to the PPMS brain, in agreement with the literature available (169). This reduced ability to remyelinate is a likely contributor to higher brain atrophy in the CNS in SPMS.

Several studies have determined that males and females differ in terms of brain weight (484). This is not surprising due to body mass differences between males and females. Data presented in this study indicate that brain weights are significantly lower in females across all cases. This is in agreement with research elsewhere on the dichotomy of male and female brain mass (484). Interestingly, in non-MS cases there was no difference in brain weight between males and females. This may be due to the inclusion of only two female cases in the non-MS cohort. Finally, we found that males of the MS cohort had significantly heavier brains than female MS cases. This is likely due to the influence of sex hormones. Indeed, women with MS have lower levels of testosterone in serum, when compared to HCs (485). Testosterone has been linked to neurogenesis in the adult brain (486), thus it may prove protective in the context of MS, and could contribute to increased brain weight in males, when compared to females. In rodents it has been shown that high doses of testosterone enhances adult neurogenesis via enhanced cell survival (487). This increase in cell survival was not noted with the use of estradiol (487). These results suggest a role for testosterone in increasing cell viability in the male brain compared to the female brain. Interestingly, studies elsewhere have indicated that males with MS have more pronounced atrophy in regions of GM, when compared to females with MS (488). This suggests that distinct cellular pathologies occur in both the GM and WM areas of the brain in MS, and that the degree of atrophy observed in each may be related to gender. This increase in localised GM atrophy in men may be linked to a poorer prognosis in disease, as pathology of the GM atrophy has been associated with both neurological and physical disability in MS (489). In addition, studies indicate that 43% of pwMS display apathy, with a higher incidence in males, which may be linked to GM atrophy (129).

Data presented in this project also demonstrate a negative correlation between brain weight and disease duration in both SPMS and PPMS cases. This suggests that progressive brain atrophy occurs in the cortex with prolonged disease in SPMS and PPMS cases. This is unsurprising as CNS atrophy occurs with age (421, 422), and remyelination becomes less frequent in progressive MS (490). This is represented by 'smoldering' plaques in individuals with a protracted disease course for over 10 years (491). Thus the failure to remyelinate may contribute to brain atrophy in MS. Lastly, we determined a

positive correlation between wheelchair time and disease duration in MS. This was expected given that physical and neurological disability progress over time in MS.

6.16 TLR7/8 expression in post-mortem brain tissue

Altered expression of TLRs have been confirmed in animal models of MS (492). Indeed, high expression levels of both TLR3 and TLR4 have been identified in MS lesions (406), however the expression of TLR3/TLR4 was not noted in the majority of leukocytes that had infiltrated the CNS (406). In terms of TLR adaptors, it is clear that MyD88 is essential for development of EAE (173), with data indicating that MyD88^{-/-} are resistant to the development of the disease (173). MyD88 is involved in both the induction of pro-inflammatory cytokines such as IL-23 and IL-6, as well as the induction of Th17 cells in EAE (173). Data elsewhere also indicates that both TLR4^{-/-} and TLR9^{-/-} mice develop more severe forms of EAE (173). In addition, significantly increased expression of both TLR2 and TLR4 is evident in CSF mononuclear cells from pwMS (407). Interestingly, activation of the endosomal TLRs in neurons, in particular TLR3, TLR7 and TLR8, alters neuronal outgrowth and synapse formation (493). Data presented herein indicate that there was no significant difference in relative *TLR7* and *TLR8* mRNA expression in NAWM and CALs in MS cases, when compared to non-MS cases. However, *TLR7* mRNA expression was significantly increased in NAGM from PPMS cases, when compared to SPMS and control cases. Similarly, in NAGM, *TLR8* mRNA expression was significantly upregulated in PPMS cases, when compared to both non-MS cases and SPMS cases. Endogenous TLR7 and TLR8 expression is low in primary cultures of microglia isolated from WM MS samples (406), and data elsewhere suggest that *TLR8* mRNA expression is reduced in PBMCs from pwMS (183). In murine EAE, TLR8 expression is elevated in the spinal cord (181), and treatment of mice with 1,25-Dihydroxyvitamin D3 reduces the expression of TLR8 in the spinal cord in EAE (182). In addition, the EAE disease score in 1,25-Dihydroxyvitamin D3 treated mice was significantly reduced (182), suggesting a role for TLR8 in disease pathology. Overall, these findings suggest that TLR7 and TLR8 expression are upregulated in the GM in human tissue in MS, and suggests that TLR7/8 have an inflammatory role to play centrally in MS.

6.17 *CNR1/CNR2 expression in post-mortem brain tissue*

Both CB₁ and CB₂ are expressed in the brain, with CB₁ being one of the most widely expressed GPCRs in the CNS (261) with many central functions in appetite, memory and learning, addiction and anxiety (494). CB₂ is considered the peripheral cannabinoid receptor, and is abundantly expressed in peripheral immune cells and tissues (233) where it has been shown to play a role in neuroinflammation (495). In our assessment of central expression of the cannabinoid receptor genes, we determined that *CNR1* mRNA was expressed at similar levels in both NAWM and CALs in PPMS and SPMS cases, when compared to non-MS controls. Similarly, comparable levels of *CNR1* mRNA were expressed in NAGM in non-MS cases, SPMS cases and PPMS cases. This is interesting as CB₁ has been shown to be neuroprotective in neurodegenerative diseases (496). In one study carried out by Benito *et al.*, (2007), CB₁ was identified on cortical neurons and in pyramidal cells of both HCs and individuals with SPMS, with immunoreactivity of CB₁ noted in neurons in the WM (427), which is in agreement with our data. Interestingly, CB₁ expression was also confirmed in cells within lesions in SPMS (427), indicating a potential role of the receptor in MS pathophysiology. Elsewhere, CB₁ has been identified in a portion of infiltrating T lymphocytes in SPMS (427). Although our data suggest that *CNR1* mRNA expression does not change in progressive MS, we cannot infer that CB₁ function is normal in these cases, or that the local expression of eCBs that act via the receptor are dysregulated. Indeed, in a separate study it was identified that the binding properties of CB₁ are significantly reduced in the striatum in EAE mice (289). Interestingly, the binding properties of CB₁ in the cortex of EAE mice displayed no alteration compared to HCs (289). This may indicate that there are no functional alterations or changes in the expression profile of CB₁ in the MS cortex.

Importantly, in our analysis, *CNR2* mRNA was not detected in the majority of cortical samples assessed (Appendix 4, 7.2). This is consistent with a separate study (427). Interestingly, CB₂ expression in SPMS brain is confined to active lesions, as well as in peripheral zones of chronic lesions (427), suggesting that CB₂ has a role to play in neuroinflammation of MS. Data from Benito and colleagues (2007) indicate that the majority of cells expressing CB₂ had a microglial phenotype, although CB₂ was also expressed in macrophages of lesions, and in a portion of WM astrocytes (427). Our data is in contrast to data elsewhere that indicate elevated levels of CB₂ in CNS lesions in MS (497, 498). CB₂ is expressed to a lesser extent centrally than CB₁, and is predominantly restricted to activated microglia and neurons of the brainstem (269, 270). Our results

suggest that there is no significant alteration in *CNR2* mRNA expression in the cortex of individuals with progressive MS. Overall, conclusions relating to MS pathophysiology from such findings should be made with caution, given that our assessment of the *CNR1* and *CNR2* mRNA in CNS tissue was limited to a single method of analysis. However, it is unlikely that rearrangement of CB₁ and CB₂ has taken place in cortical samples investigated herein. Future experiments will profile *CNR1* and *CNR2* expression in CNS tissue using protein analysis alongside mRNA assessment.

6.18 Expression of FAAH and MAGL in post-mortem brain tissue

FAAH is a hydrolytic enzyme responsible for the degradation of the eCB, AEA (285). Inhibition of FAAH has been linked to controlling spasticity in EAE (426). Furthermore, the concentration of AEA is upregulated in the CSF of individuals with RRMS (289); this may be indicative of FAAH reduction and (or) dysfunction in the disease. FAAH has been identified in cortical neurons of pwMS and HCs, and has been detected in active lesions in SPMS (427). In addition, both the expression of FAAH, and its activity, are significantly reduced in peripheral lymphocytes isolated from pwMS (289). We assessed central FAAH expression in the MS cortex and found that the expression of *FAAH* mRNA in NAWM and CALs was comparable to non-MS control expression levels. Furthermore, there was no significant alteration in the expression of *FAAH* mRNA in NAGM of PPMS and SPMS cases, when compared to non-MS cases. Thus we conclude that expression of FAAH does not change centrally in progressive MS, although its ability to regulate levels of AEA was not assessed in the current study. This will be the focus of future studies.

We also assessed the CNS expression profile of MAGL, an enzyme involved in the hydrolytic breakdown of 2-AG (286). To date, to our knowledge, the expression profile of MAGL has not been characterised in the CNS in human MS studies. However MAGL inhibitors have been shown to control spasticity in EAE (426), suggesting that the enzyme is a central player in MS pathogenesis. Our findings indicate that the expression of *MAGL* mRNA is altered in progressive MS, particularly PPMS where reduced *MAGL* expression was noted in both NAWM and CALs of PPMS cases when compared to non-MS control cases. CALs found in SPMS cases also had reduced expression of *MAGL* mRNA when compared to non-MS control cases. These findings suggest that MAGL expression in the CNS is impacted by MS, particularly in the chronic stages of disease in NAWM. Again, caution should be applied when forming conclusions relating to MS pathophysiology

from such findings, given that our assessment of the eCB metabolising enzymes in the CNS was limited to a single method of analysis. Future experiments will profile FAAH and MAGL expression in CNS tissue using a combination of immunohistochemical and western blot analysis.

Limitations of the study

A limitation of the study was the limited number of plasma samples in which cytokines such as TNF- α , IFN- β and IL-1 β were undetected. This may be reflective of inflammatory conditions occurring in the periphery of these individuals, and also may be a result of DMT usage. In addition, we were confined to low n numbers for neuropathological assessment, in particular. It would be of benefit to increase the number of cortical brain samples for analysis to improve the power of the study. During the course of this study there was limited ability to assess IFN expression in THP-1 macrophages following activation of TLR7/8 signalling, thus suggesting that differentiation of THP-1 monocytes to THP-1 macrophages affected the ability of the TLR7/8 signalling axis to induce type I IFNs. Although THP-1 macrophages have been shown to be an adequate model for investigating TLR and eCB signalling *in vitro* they are not the optimal model for assessing cellular signalling of individuals with MS. Ideally, moving forward in future studies, monocytes isolated from the blood of HC individuals, as well as pwMS, subsequently differentiated to macrophages *in vitro*, would be the ideal cell model to use in future experiments. The use of such primary macrophages would more accurately depict TLR and eCB signalling *in vivo*. It would also be of interest to investigate differences in cellular signalling in monocyte-derived macrophages isolated from individuals with RRMS, SPMS and PPMS. A further limitation of the study is the lack of RRMS cases included in post-mortem analysis. Inclusion of such a sample cohort would enable an assessment of neuropathological features across all MS subtypes. From a neuropathology perspective, SPMS and PPMS have parallels in terms of pathology and disease progression. Hence, inclusion of RRMS cases would highlight immunopathological differences between acute and chronic MS. In addition, it would also have been preferable to include age-matched controls in the non-MS cases cohort supplied by the UK MS Brain Bank. Inclusion of more female cases in neuropathological samples would reflect MS incidence and improve statistical power in the analysis of gender differences. Indeed, MS predominantly affects women at a ratio of 2-3:1 (83, 98). Overall, this limits the conclusions that can be made

in terms of neuropathological gender differences. Finally, it should be recognised that post-mortem delay may result in protein degradation and thus may skew some of the neuropathological read-outs reported in this study.

Future studies

Our findings indicate that TLR7/8 activation did not induce IFN- β protein expression in THP-1 macrophages. Data elsewhere indicate that the expression of MyD88, IRAK1, IRAK4 and IRF7 are reduced in PMA-differentiated THP-1 macrophages (78), which may account for this. Future studies will employ the use of an alternative cell type to assess TLR7/8-induced IFN signalling. In addition, an alternative differentiation strategy may be utilised in THP-1 cells that may not impact the expression profile of TLR7/8 signalling intermediates. These include the use of macrophage colony-stimulating factor (M-CSF) and compounds such as byrostatin (499), an activator of the protein kinase C family, initially utilised for its anti-tumour activity. TLR7 activation has been shown to induce high amounts of IFN- α in pDCs (500), hence it would be of interest to assess the production of IFN- α production by THP-1 macrophages. IFN- α is a type I IFN produced mainly by pDCs, especially following stimulation of TLR7 or TLR9 by viral RNA or DNA (501). IFN- α is preferentially used in the treatment of both viral infections such as hepatitis B and C, as well as its use in cancer patients (381) with both solid tumours and haematological malignancies (502). Interestingly, IFN- α is an ISG, thus early type I IFN production can potentiate further production of IFN- α through IRF7 (503). Future experiments will assess the proclivity of CL075 to induce IFN- α expression in THP-1 cells, and also determine the impact of CBD on such signalling.

Our findings indicate that CBD had no impact on CL075-induced signalling in THP-1 macrophages. This is contrast to the impact of CBD on MyD88-independent signalling induced by LPS and poly(I:C) in THP-1 macrophages (79). Future experiments will assess the impact of CBD on TRIF and TRAM adaptors of the TLR3/4 pathway in THP-1 macrophages, in order to determine where exactly in the pathway CBD exerts its inhibitory potential. In addition, future experiments will assess additional cannabinoids including CBG and CBDV, as well as a range of terpenes such as limonene, to determine their potential impact on TLR signalling. The potential impact of pCBs/terpenes on TLR7/8 signalling in cells isolated from pwMS would also be of interest, to determine whether any compounds derived from *Cannabis L. sativa* modulate the TLR7/8 signalling

pathway. Shedding light on the potential role of the inflammasome, both centrally and peripherally, in different subtypes of MS would be of significant value in further experiments.

In future research, it would also be beneficial to assess the impact of CL075 on the induction of TLR7/8, along with components of the eCS described herein, in primary human cells isolated from pwMS, in particular PBMCs. Subsequently, the impact of CL075 on immune subtypes, including monocyte-derived macrophages, DCs, Th1 and Th17 cells isolated from pwMS and HCs, would improve the clinical relevance of the project. The impact of CBD on CL075-dependent signalling in these cells will add therapeutic relevance.

Conclusion

TLRs play a key role in MS pathogenesis. The importance of innate immunity in combatting neurodegeneration and immune cell infiltration of the CNS is clear, with dysregulated auto-antigenic immune cells and altered signalling pathways heavily implicated in neurodegenerative diseases such as MS. Here we have shown that both TLR7 and TLR8 may play important functional roles in the development and disease propagation within MS. In this study we identified that both *TLR7* and *TLR8* mRNA expression are significantly enhanced in NAGM of PPMS, suggesting that endosomal TLR signalling may have roles to play in chronic stages of the disease, contributing to neurodegeneration and axonal loss. Indeed, GM involvement in MS pathogenesis has been established, having links to disability (119), cognitive impairment and motor dysfunction, although the cause of GM damage in MS has yet to be fully elucidated (504).

We identified that *TLR8* mRNA was significantly upregulated in THP-1 macrophages following incubation with CL075, but no increase was noted in terms of *TLR7* mRNA. In addition, *CNR1* mRNA expression was significantly downregulated following incubation with CL075, while *MAGL* mRNA expression was significantly elevated in THP-1 macrophages. This suggests that crosstalk may exist between TLR7/8 signalling and the eCS in macrophages. In addition, in primary PBMCs, the expression of *TLR8* mRNA in PBMCs from pwMS significantly correlated with both *MAGL* and *FAAH* mRNA expression. Overall, this suggests that dysregulation of TLR8 signalling, which has been identified

previously in PBMCs of pwMS (183), may have a prominent role in altering the eCS in the context of MS.

The expression of TLR7/8, and components of the eCS (CNR1, CNR2, FAAH, MAGL), was not altered in PBMCs from pwMS, when compared to PBMCs from HCs. This may reflect the impact of DMTs on the relative expression of such markers in circulating immune cells. Indeed, IFN- β therapy has been shown to upregulate the expression of *TLR7* mRNA in PBMCs from pwMS (479). In addition, IFN- β therapy reduces AEA levels in immune cells from pwMS (291), thus identifying the proclivity of some DMTs to impact on expression of components of the eCS.

pCBs, such as CBD, have neuroprotective propensity (258), and previous work from our laboratory has identified anti-inflammatory activity of CBD in terms of inhibiting the ability of both LPS and poly(I:C) to induce a variety of chemokines and IFNs (CXCL10 and IFN- β) via TLR3/4 activation in THP-1 macrophages (79). Intriguingly, data herein demonstrates that CBD did not have the proclivity to inhibit CL075-dependent induction of cytokines and chemokines (CXCL10, TNF- α and RANTES) through activation of the TLR7/8 signalling axis. Thus, CBD may not impact on the classical MyD88-dependent TLR signalling pathways. This may have implications in the future in terms of the cellular effects of cannabinoid therapeutics in MS.

Overall, our findings indicate that treatment with the pCB CBD did not impact TLR7/8-induced inflammatory signalling in macrophages. A summary of our findings on the impact of CL075 and CBD on TLR7/8 signalling, as well as *TLR7/8* and *eCS* mRNA expression in THP-1 macrophages has been outlined in Fig. 6.3. We also characterised the expression profile of TLR7, TLR8 and the eCS (CB₁, CB₂, FAAH and MAGL) in PBMCs isolated from healthy volunteers and pwMS, and in post-mortem human cortical brain samples from PPMS, SPMS, and non-MS control cases. Our findings indicate that TLR7/8, and the eCS, are expressed in immune and CNS tissue, and that CL075 can alter the expression of TLR8, and components of the eCS, in macrophages. We also provide evidence of alterations in TLR7/8 expression, and the eCS, in the cortex of MS cases. A final summary of the findings of this thesis has been included below in Figure 6.4.

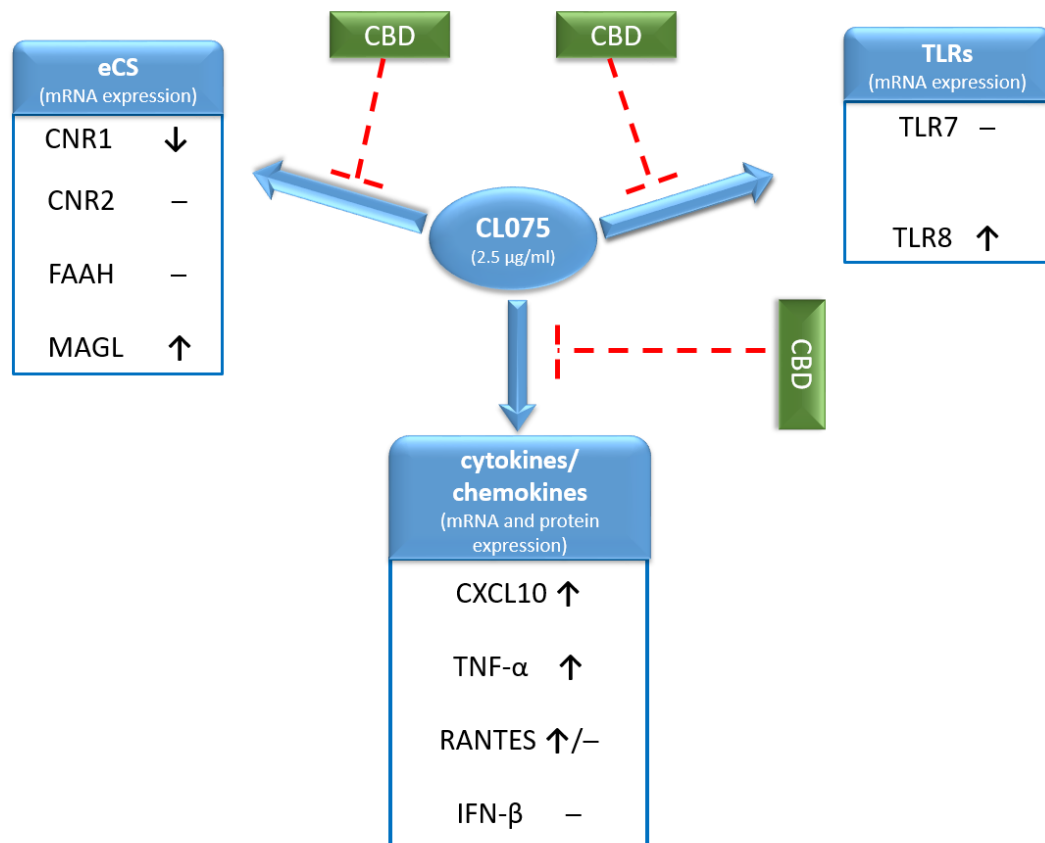


Figure 6.3 Impact of CL075 and CBD on the expression of cytokines/chemokines, the eCS and TLR7/8 in THP-1 macrophages. This scheme summarises the main *in vitro* findings of this project. Incubation of THP-1 macrophages with CL075 at [2.5 µg/ml] for 24 h resulted in a significant upregulation of CXCL10 and TNF-α at the protein level, while significantly upregulating *CXCL10*, *TNF-α* and *RANTES* expression at the mRNA level. IFN-β expression was not upregulated by incubation with CL075. Incubation with CL075 also resulted in increased expression of *TLR8*, but not *TLR7*, mRNA in THP-1 macrophages. In terms of the eCS, CL075 treatment did not alter the expression of *CNR2* or *FAAH* mRNA in THP-1 macrophages, although it did increase the expression of *MAGL* mRNA, and reduce expression of *CNR1* mRNA. Pre-treatment of THP-1 macrophages with CBD (10 µM) for 30-45 mins did not impact CL075 signalling in terms of the production of CXCL10,

TNF- α or RANTES. In addition, pre-treatment with CBD did not alter the effect of CL075 on *CNR1*, *MAGL* and *TLR8* mRNA expression.

No inhibition:

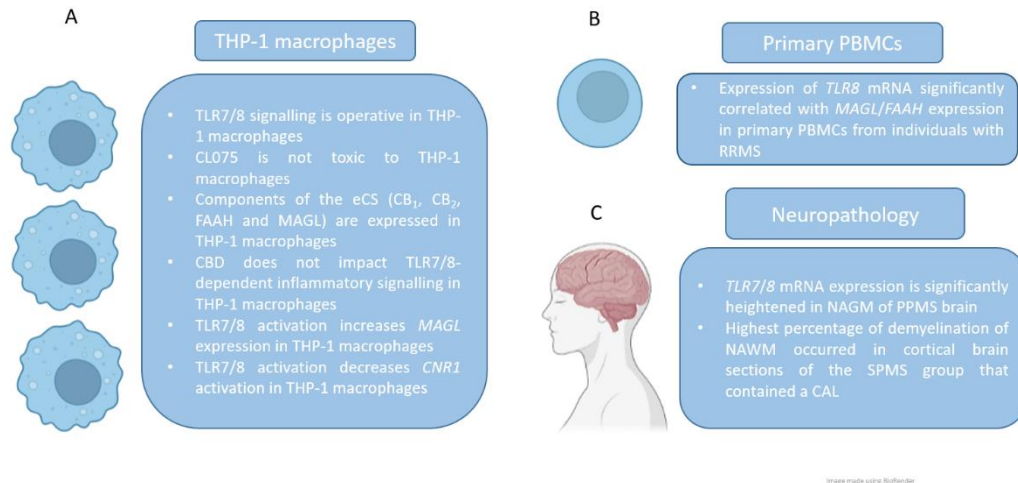


Figure 6.4 Summary of Findings. This diagram summarises the main findings of this thesis, both *in vitro* and in neuropathological tissue isolated from individuals with MS and HCs. **(A)** Describes the results of experiments carried out in THP-1 monocyte-derived macrophages, **(B)** presents the data gathered from experiments run on mRNA derived from primary PBMCs isolated from HCs and pwMS. In **(C)** the most relevant findings from assessment of post-mortem cortical brain tissue of HCs and pwMS are described.

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Appendices

Appendix 1 Peer-reviewed publications

Review Article

Cannabidiol modulation of oxidative stress and signalling

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Cannabidiol (CBD), one of the primary non-euphoric components in the *Cannabis sativa* L. plant, has undergone clinical development over the last number of years as a therapeutic for patients with Lennox-Gastaut syndrome and Dravet syndromes. This phytocannabinoid demonstrates functional and pharmacological diversity, and research data indicate that CBD is a comparable antioxidant to common antioxidants. This review gathers the latest knowledge regarding the impact of CBD on oxidative signalling, with focus on the proclivity of CBD to regulate antioxidants and control the production of reactive oxygen species. CBD is considered an attractive therapeutic agent for neuroimmune disorders, and a body of literature indicates that CBD can regulate redox function at multiple levels, with a range of downstream effects on cells and tissues. However, pro-oxidant capacity of CBD has also been reported, and hence caution must be applied when considering CBD from a therapeutic standpoint. Such pro- and antioxidant functions of CBD may be cell- and model-dependent and may also be influenced by CBD dose, the duration of CBD treatment and the underlying pathology.

Introduction

Production of reactive oxygen species (ROS) is commonly associated with oxidative stress and its pathological role in inflammatory diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), atherosclerosis and inflammatory bowel disease (IBD) [1–5]. During infection, immune cells produce ROS via the NADPH oxidase 2 (NOX2) complex as a mechanism to eradicate pathogens [6]. When NOX2-generated ROS production is dysregulated due to mutations in NOX2 complex proteins, this can result in defective phagocyte function characterized by severe and recurrent infections defined as chronic granulomatous disease (CGD) [7]. Moreover, ROS produced in the phagosome to activate proteolytic enzymes can escape the immune cell, thus damaging the surrounding tissue [8]. Despite this, it is clear that a deficiency in ROS production has the proclivity to aggravate disease processes [9]. In addition, leukocytes isolated from individuals with chronic MS produce less superoxide than those with a milder disease [10], and a similar scenario has been identified in Guillain-Barré syndrome (a subtype of acute inflammatory demyelinating polyneuropathy), where evidence suggests that leukocytes produce lower levels of oxygen radicals in the most severe cases of disease [11]. Taken together, these findings support a complex role of ROS in regulating inflammation in disease.

In recent years, cannabinoid molecules, such as cannabidiol (CBD) and Δ^9 -tetrahydrocannabinol (Δ^9 -THC), have drawn attention due to their anti-inflammatory, antioxidant and neuroprotective properties [12,13]. The most well-described targets for cannabinoids are their specific receptors, the cannabinoid receptors CB₁ and CB₂ [14,15], but their pharmacological actions are not solely limited to these receptors. Indeed, cannabinoids are lipophilic and certain cannabinoids have also been shown to target a wide range of receptors, including the peroxisome proliferator-activated receptors (PPARs), the transient receptor potential cation channel subfamily V member 1 (TRPV1), G-protein-coupled receptor 55 (GPR55), the 5-hydroxytryptamine receptor subtype 1A (5-HT1A), glycine α 1 and α 1 β receptors, in addition to ion

Received: 25 May 2021

Revised: 27 July 2021

Accepted: 30 July 2021

Accepted Manuscript Online:

09 August 2021

Version of Record published:

24 August 2021

channels (Ca^{2+}) and enzymes such as the adenosine membrane transporter phospholipase A2, lipoxygenase (LO) and cyclooxygenase-2 (COX-2) [16–22]. Depending both on the cannabinoid structure and cell/tissue targeted, the pharmacological effects of cannabinoids may vary.

Overall, cannabinoids have been shown to possess therapeutic efficacy in several inflammatory and neuronal diseases [23]. Given that the production of ROS is an intrinsic feature of neuroinflammation and peripheral immune responses, this review aims to gather the latest knowledge on the action of cannabinoids on oxidative signalling, with focus on the phytocannabinoid CBD. CBD is selected for review given recent advances in its therapeutic development [24–26].

Oxidative signalling and stress

The production and maintenance of controlled levels of intracellular ROS has a key role in several physiological functions, including the maintenance of redox homeostasis, cell cycle signalling and hormone production [27,28]. When present, ROS can regulate several signalling pathways by reacting with transcription factors and genes, modifying their structure and thus their function. Hence, ROS can modulate gene expression patterns and signalling proteins related to the stress response and cell survival mechanisms [29]. It is when this homeostasis is impaired, by either an overproduction of ROS or inefficient ROS scavenging mechanisms, that oxidative stress ensues, promoting cellular damage, lipid peroxidation, DNA modifications and enzyme inactivation, and when persistent, can ultimately lead to cell death and tissue destruction [30–32]. This rationale has been the basis for the development of several anticancer drugs [33–35].

ROS

ROS represent a group of unstable oxygen radicals and molecules with strong oxidizing properties. Once formed, ROS are converted to other oxidative species or eliminated by the antioxidant mechanisms of the cell [29,36]. The most common ROS are the superoxide anion ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\text{HO}^\bullet$) [29]. There are three major cellular mechanisms of ROS production as described in Figure 1: (A) ROS are produced via the mitochondrial electron transport chain, in complex I and II; (B) production via the enzymatic reaction catalysed by the enzyme xanthine oxidase (XO); and (C) by NOX as a defence against pathogens [37–39]. To maintain the levels of ROS under control, the enzyme superoxide dismutase (SOD) converts $\text{O}_2^{\bullet-}$ into H_2O_2 (D), which is then converted into water, a reaction catalysed by catalase (CAT) and/or glutathione peroxidase (GPx) (E) [40,41]. However, during periods of high ROS generation, H_2O_2 may cross cell membranes and react with $\text{O}_2^{\bullet-}$ and metal cations (Fe^{2+} or Cu^+) to form $\text{HO}^\bullet$ (Fenton and Haber–Weiss reactions) (F, G) [42]. Furthermore, oxygen radicals can also react with nitrogen species, namely nitric oxide (NO) (H). The reaction of $\text{O}_2^{\bullet-}$ with NO can generate the production of peroxynitrite (ONOO^-) (H) [43], a particularly reactive radical that may promote generalized oxidative/nitrosative damage, including DNA fragmentation and lipid degradation.

NOX2 production of ROS

One of the main cellular sources of ROS is the NOX2 complex, which belongs to a family of NADPH oxidases. NOX are transmembrane enzymes involved in the electron transport across biological membranes by oxidizing intracellular NADPH = NADH, while reducing molecular oxygen into $\text{O}_2^{\bullet-}$ anions [37]. Among the seven NOX members identified to date, NOX2 has the ability to produce an oxidative burst to eliminate pathogens [44]. The NOX2 complex consists in a transmembrane catalytic core, a heterodimer containing gp91phox and the protein p22phox. On the cytosolic side of the membrane, the proteins p47phox, p67phox, p40phox and Rac (a small GTP-binding protein) regulate the complex activity [37,45].

Antigen-presenting cells can produce ROS upon activation via the NOX2 complex [6,46]. Interestingly, gp91phox is also expressed in bone marrow and thymus, contributing to the role of NOX2-derived ROS in B-/T-cell development and maturation [47,48]. Although phagocytes express high levels of the NOX2 complex, components of this complex are also found in nonphagocytic cells, such as cardiomyocytes, endothelial cells and muscle cells [49–51]. In resting cells, the NOX2 complex remains inactive, and upon exposure to certain stimuli, the complex becomes primed or activated [52]. NOX2 priming prepares the complex to produce ROS, initiating a weak oxidative response without eliciting an oxidative burst. This can occur upon stimulation by pro-inflammatory cytokines (e.g. tumour necrosis factor- α [TNF- α] and interleukin [IL]-1 β), toll-like receptor [TLR] agonists, e.g. lipopolysaccharide [LPS], flagellin), OONO^- and proteases. The priming induces a partial phosphorylation of p47phox that alters its conformation, thus decreasing its autoinhibition and facilitating its translocation and interaction with the NOX2 enzymatic core. This mechanism exerts tight control over NOX2 activation to prevent unintended $\text{O}_2^{\bullet-}$ release [53,54]. Full activation of

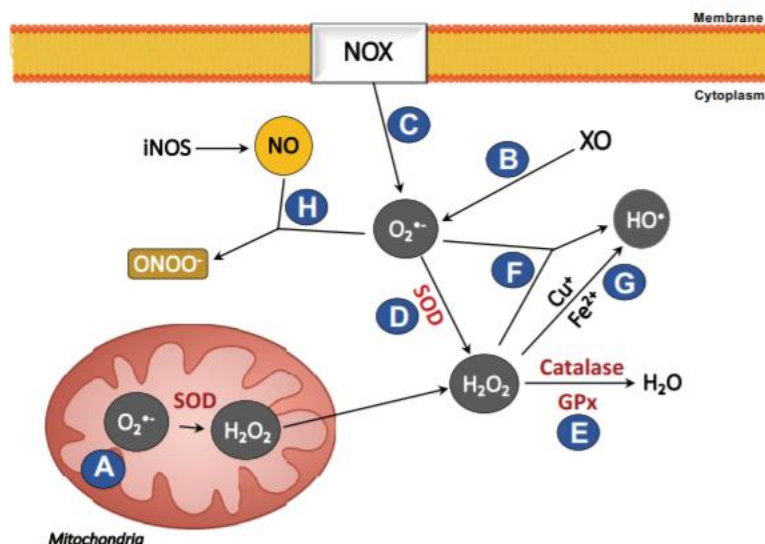


Figure 1. Modulation of cellular ROS

The majority of ROS originate from various sources within the cell. Production is via (A) the mitochondrial electron transport chain, (B) the enzymatic reaction catalysed by XO and (C) by NOX. Endogenous antioxidant mechanisms are exerted via (D) SOD conversion of O₂^{•-} to H₂O₂, followed by conversion to water by (E) CAT and/or GPx. When ROS production exceeds the endogenous antioxidant mechanisms capacity, (F and G) H₂O₂ may react with O₂^{•-} to form HO[•] (Haber-Weiss and Fenton reactions). (H) Oxygen radicals can also react with NO to generate ONOO⁻.

the NOX2 complex requires additional stimuli, such as phorbol 12-myristate 13-acetate, a protein kinase C activator and formyl-methionyl-leucyl phenylalanine, which acts via the formylpeptide receptor. Upon activation, NOX2 increases the production of superoxide [55], and therefore, mutations in any of the components of NOX2 dysregulate its activity and thus ROS signalling [7,56].

Antioxidant mechanisms

Cellular antioxidant mechanisms regulate ROS signalling and furthermore prevent/reduce the oxidation of unintended molecules. Antioxidant mechanisms include enzymes such as CAT, SOD, GPx, thioredoxin and peroxiredoxin, in addition to the radical scavengers such as reduced glutathione (GSH) [40,41,57,58]. As mentioned previously, CAT catalyses the conversion of H₂O₂ to water (Figure 1), and although this enzyme is highly expressed during inflammation, the GPx enzyme has even higher affinity to the H₂O₂ radical. GPx activity depends on the GSH ability to be oxidized to glutathione disulfide (GSSG) in the presence of NADPH, thus functioning as a proton donor. The GSH pool is afterwards replenished by both its regeneration from GSSG mediated by GSH reductase and *de novo* synthesis [59,60]. Additionally, dietary antioxidant molecules are also useful at maintaining the level of ROS by directly scavenging ROS, namely tocopherols (vitamin E) and ascorbic acid (vitamin C) [61,62].

More research is still needed to achieve a deeper understanding on the specific roles for each particular oxygen radical. Current methodologies are limited in their ability to measure isolated radicals but rather a group of radicals, making it difficult to differentiate amongst them. What is known today in the field is based on the use and development of various ROS scavengers, enzyme inhibitors and antioxidant enzymes. Moreover, many *in vitro* studies

employ the use of high levels of ROS and thus do not represent the physiological fine-tune modulatory effects of ROS *in vivo*.

In consideration of antioxidant mechanisms, much data indicate that nuclear factor erythroid 2-related factor-2 (Nrf2), a ubiquitously expressed redox-sensitive transcription factor, is important in initiating the transcription of antioxidant and cytoprotective genes [63]. In the cytoplasm, Nrf2 is bound to its inhibitor Kelch-like ECH-associated protein1 (Keap1), and in response to pro-oxidant stress, oxidative modification of Keap1 dissociates Keap1 from Nrf2, facilitating the nuclear translocation of Nrf2 [64]. Nrf2 binds to antioxidant response elements (AREs) to orchestrate the expression of antioxidant enzymes, including SOD [65], to promote a reduction in ROS. Antioxidants can act by promoting Nrf2 activation [66,67], and deficiencies in Nrf2 factor have been associated with increased inflammation and carcinogenesis [68]. The enzyme heme oxygenase-1 (HO-1) is a key Nrf2 gene target, and a large body of data indicate that HO-1 possesses diverse antioxidant and anti-inflammatory capacity [69]. HO-1 is an enzyme that catalyses the degradation of heme and is induced under conditions of oxidative stress [70]. Importantly, HO-1 has the proclivity to negate the production of ROS [71] and inflammatory mediators [72], making HO-1 inducers potential therapeutic targets in disease [73].

ROS and oxidative signalling

While some studies suggest that oxidative stress plays a critical role in the progression of diseases including cancer, diabetes and CNS disorders [1–5,74], many studies have failed to demonstrate that the therapeutic effects of antioxidants translate to a clinical setting [75]. Indeed, although antioxidants are believed to wield anti-inflammatory effects, their assessment as therapeutics in both human and rodent studies has provided contradictory findings [76–79]. On the contrary, the use of pro-oxidant molecules, such as phytol (NOX2 activator and vitamin E precursor), can improve, or even prevent, ongoing inflammation in animal models [80]. Considering this, the controlled production of ROS is recognized to exert crucial effects in regulating biological functions and cell signalling. This occurs via the rapid generation and removal of ROS within defined cell compartments, thus avoiding sustained signalling and/or oxidative stress [28]. Contrary to previous interpretation, ROS signalling may regulate the inflammatory response.

As previously described, to eliminate invading pathogens, phagocytic cells produce ROS into phagolysosomes following NOX2 activation, and this results in a pH increase within the vesicles, triggering the activation of proteolytic enzymes which in turn destroys the engulfed pathogens/debris [8,81]. Radicals that escape the vesicle membrane into the cytoplasm are rapidly eliminated by the cell antioxidant mechanisms. It is important to note that there are some indications that ROS production can regulate the activation of the NLRP3 inflammasome during inflammation [82,83]. The inflammasome consists in a protein complex that can activate caspase-1 to control the production of IL-1 β , a classic pro-inflammatory cytokine [84]. Although ROS may regulate NLRP3 inflammasome activation, it appears to be independent of NOX2 activation. Other known NLRP3 inflammasome activators include innate stimuli (e.g. pathogens and environmental insults) and particulate adjuvants (e.g. alum, silica and urate crystals) [85].

As previously discussed, NO plays a role on oxidative signalling mechanisms [43], in addition to its important role in modulating physiological functions such as neurotransmission, vasodilation and immunomodulation [86]. NO is synthesized on demand and is produced by one of the three known isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). The nNOS and eNOS isoforms are constitutively present in neuronal and endothelial cells, respectively [86], while iNOS expression is inducible and regulated at the transcriptional level, particularly in inflamed tissues [87]. As mentioned previously, the reaction of $O_2^{\bullet-}$ with NO can generate ONOO $^-$ [43] (Figure 1), which can contribute to generalized oxidative/nitrosative damage, including DNA fragmentation and lipid degradation.

Oxidative stress ensues when ROS are produced in high quantities without control, and ROS production plays a key role in neuroimmune disorders, resulting in several harmful nonspecific events such as DNA laddering and lipid peroxidation. While some radicals react rapidly and rarely escape the cell membrane (e.g. $O_2^{\bullet-}$), other more stable radicals can cross the cell membrane and diffuse to adjacent tissues (e.g. H_2O_2) [88]. Moreover, $O_2^{\bullet-}$ gives rise to HO $^\bullet$ and perhydroxyl (HOO $^\bullet$) radicals [29,89], with data indicating that the HO $^\bullet$ radical can inactivate the mitochondrial enzyme pyruvate dehydrogenase [90], depolymerize gastrointestinal mucin [91] and inflict oxidative DNA damage [92]. The HOO $^\bullet$ is also highly deleterious, initiating lipid peroxidation [93], disturbing membrane permeability [94] and demonstrating toxicity [95]. Such events may be associated with mutagenesis in chronic intestinal inflammation [96] and in addition to intestinal inflammation, much data link the production of ROS to the pathology of a range of inflammatory diseases such as RA, atherosclerosis, MS and IBD [1–5]. The key role of ROS in physiological processes adds to the complexity of targeting ROS production therapeutically, and the balance between ROS elimination and generation may be key in terms of the management of disease.

Cannabinoids

The introduction of medicinal cannabis, and cannabis-based therapeutics, to mainstream medicine has proved a controversial topic, although medicinal benefits of the *Cannabis sativa* L. (*C. sativa*) plant have been recorded for centuries. Indeed, evidence exists for medicinal use of cannabis dating back to the fourth century when used in the treatment of a broad range of ailments [97]. Much public reticence with the introduction of cannabinoids to a medical setting are associated with the recreational use of cannabis, the euphoric effects of the drug and the evidence linking cannabis abuse with psychosis [98]. With the discovery and characterization of many compounds in *C. sativa*, in addition to an increase in our understanding of cannabinoid pharmacology and toxicology, selective cannabinoid-based therapeutics continue to make advancement in pre-clinical and clinical studies.

To date, a large body of data suggest that cannabinoids have therapeutic properties, alleviating symptoms of several CNS disorders. Indeed, cannabinoids can mitigate inflammation, reduce CNS spasticity, alleviate neuropathic pain, and a body of evidence indicates that cannabinoids provide neuroprotection following injury or inflammation in the CNS [99,100]. The most well-known cannabinoids are the phytocannabinoids synthesized by *C. sativa*, including THC, a euphoric component of the plant, in addition to CBD, cannabiol (CBN), cannabichromene (CBC) and cannabigerol (CBG), which are considered non-euphoric cannabinoids [101]. Cannabinoids also include a class of lipid messengers known as endogenous cannabinoids (eCBs), with *N*-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoyl-glycerol (2-AG) representing the most actively studied eCBs [102]. Both AEA and 2-AG are synthesized on demand in the body to mediate diverse physiological functions via interaction with a range of receptors, particularly the G-protein-coupled receptors (GPCRs), CB₁ and CB₂ [103]. The system of eCBs and cannabinoid receptors constitutes the eCB system (ECS). In addition, many synthetic agonists, antagonists and inverse agonists for the cannabinoid receptors have been developed and such classes of molecules are under investigation.

Phytocannabinoids

C. sativa is an annual dioecious plant containing a diverse repertoire of botanical cannabinoids commonly known as phytocannabinoids. To date approximately 150 phytocannabinoids have been characterized in the plant [104]. The primary phytocannabinoids are THC and CBD, which represent the most commonly studied phytocannabinoids in experimental and clinical settings, demonstrating a wide range of effects on physiological processes. Other phytocannabinoids currently under investigation include the less well-characterized phytocannabinoids, CBG and CBC. The biosynthetic pathways involved in the generation of the main classes of phytocannabinoids involve the formation of acidic cannabinoids including cannabigerolic acid (CBGA), the precursor of tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) and cannabichromenic acid (CBCA), which have poor oxidative stability. Following oxidation or decarboxylation, neutral forms of cannabinoids are generated by heating or naturally as the plant ages [105]. Other compounds isolated from *C. sativa* include a variety of terpenes [106]. Indeed, over 200 terpenes have been identified, including the monoterpenes (limonene, α -pinene and linalool) and sesquiterpenes (β -caryophyllene), which share the same biological precursor with phytocannabinoids. Terpenes produced by the plant are responsible for the plant aroma [107], acting as botanical insecticides and attracting predatory mites [108].

Phytocannabinoids and terpenes accumulate in the secretory cavity of the glandular trichomes in *C. sativa* [109,110] and are present in the highest quantity on the female flower of the plant. Male plants produce lower levels of phytocannabinoids [111]. Glandular trichomes consist of a sac-like cavity packed with secretory vesicles known as glandular hairs. Glandular trichomes of *C. sativa* alter morphology and metabolite content during flower maturation, and phytocannabinoids/terpenes are found on the calyx and the underside of anthers of flowers, leaves and bracts [109]. Trichomes rupture due to environmental stress or damage (due to high temperatures and herbivorous consumption), resulting in the release of phytocannabinoids and terpenes as a noxious, sticky liquid on the plant surface.

The ECS

The activity of phytocannabinoids was initially considered to result from their proclivity to fluidise membranes. However, in the early 1990s the cannabinoid receptors CB₁ [14] and CB₂ [15], the receptors of the ECS, were identified. Both receptors are GPCRs and are responsible for many of the effects of cannabinoids on physiological systems. CB₂ is abundantly expressed in immune tissues [112] and is responsible for many immunomodulatory effects, while CB₁ expression is predominantly confined to the CNS, with expression also identified in peripheral tissues including the heart, reproductive organs and thymus [112]. Indeed, CB₁ is considered one of the most highly expressed GPCRs in the brain [113]. Under normal physiological conditions CB₂ exhibits low basal expression in the CNS, with evidence

indicating that this receptor is expressed in the brain stem [114] and on hippocampal pyramidal neurons [115]. Importantly, in pathological conditions, data suggest that the expression of the CB₂ receptor is up-regulated, particularly on microglial cells [116]. Cannabinoids vary in their affinity for the cannabinoid receptors. Indeed, THC is a partial agonist for both CB₁ and CB₂ [117], whereas CBD has low affinity for both CB₁ and CB₂ [117,118].

The discovery of receptors that mediate the cellular action of components of *C. sativa* was followed by the subsequent identification of the eCBs, AEA [119] and 2-AG [120,121], again in the early 1990s. eCBs vary in their affinities for the cannabinoid receptors; AEA has relatively high affinity for CB₁ but little to no affinity for CB₂, while 2-AG is an agonist at both CB₁ and CB₂ [122]. AEA and 2-AG are non-charged, hydrophobic lipid molecules that can act to control neurotransmission [123] and are produced on demand in response to an increase in intracellular Ca²⁺ [124–126]. Basal concentrations of 2-AG in brain tissue are 170 times greater than AEA [127]. Furthermore, AEA is a member of the family of *N*-acylethanolamines, other members of which include the eCB-like compounds, palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), both of which possess diverse physiological functions [128–130].

Although CB₁ and CB₂ are considered classic cannabinoid receptors, much evidence suggests the existence of further receptor targets for cannabinoids, including nuclear PPARs, GPR55 and the TRPV1. Although GPR55 shares a low amino acid sequence homology with both CB₁ and CB₂, data indicate that GPR55 is a receptor for cannabinoid ligands including AEA, PEA, THC and CBD [16,17,131–135]. TRPV1 is a cationic channel receptor dependent on intrinsic and extrinsic calcium concentrations, and evidence indicates that TRPV1 is a target for CBD, 2-AG and AEA [18,136,137]. This receptor exists largely on sensory neurons and cannabinoid activity through this receptor has shown significant analgesic potential [138]. Several studies have also shown that CBD targets PPAR- γ [13,19,139,140], and it should also be noted that PEA and OEA can exert their effects via PPARs [141–143]. In terms of CBD, further CB_{1/2}-independent cellular targets include serotonin (5-HT) receptors [19,20,144,145] and both μ - and δ -opioid receptors [146].

Synthetic cannabinoids

Multiple synthetic cannabinoid compounds have been developed with the aim to pharmacologically target specific aspects of the ECS and to facilitate the development of cannabinoid therapeutics [147]. Firstly, the compound WIN55,212-2, an aminoalkylindole derivative, with affinity for both cannabinoid receptors, but binding to CB₂ with higher affinity, represents one of the most actively studied synthetic cannabinoids [148,149]. Indeed, research on this compound has shown that WIN55,212-2 is an analgesic in rodent models of neuropathic pain [150] and mechanical allodynia [151], while topical administration of WIN55,212-2 was shown to reduce intraocular pressure in patients with glaucoma [152]. HU-210 is another synthetic cannabinoid agonist that has been extensively researched. HU-210 is a high affinity CB₁/CB₂ receptor agonist [153,154] with agonist activity also at GPR55 [16]. This cannabinoid has been shown to be protective in models of experimental colitis [155] and photoreceptor degeneration [156]. The cannabinoid agonist CP55,940 shares biochemical properties with THC, demonstrates high affinity for CB₁ and CB₂ [157,158] and is also a GPR55 agonist [16]. CP55,940 has been shown to mitigate tumour-evoked hyperalgesia in murine models in a dose-dependent manner [159] and also possesses antioxidant capacity in neutrophils [160]. The JWH family of synthetic cannabinoids have also been shown to be effective agonists of the cannabinoid receptors. Indeed, evidence indicates that JWH-133, a selective CB₂ agonist [161], ameliorates spasticity in murine MS [162] and prevents microglial cell activation and inflammation following exposure to β -amyloid [163]. Finally, ACEA is a well described synthetic CB₁ agonist and analogue of AEA [164]. ACEA has the proclivity to protect ischemic neurons from oxygen-glucose deprivation/reoxygenation and middle cerebral artery occlusion [165].

Overall, there is a large array of synthetic cannabinoid ligands currently employed in cannabinoid pharmacological research. Such synthetic cannabinoid ligands are useful pharmacological tools that may be used to identify therapeutic avenues in the ECS.

Antioxidant and anti-inflammatory effects of cannabinoids: focus on CBD

The antioxidative and anti-inflammatory properties of cannabinoids across a variety of tissue types and cellular models have been studied for several decades and are well described [166]. A summary of the antioxidant capacity of CBD is presented in Figure 2. In particular, much evidence has highlighted that CBD, the major non-euphoric phytocannabinoid in *C. sativa*, has an array of anti-inflammatory effects, with proclivity to modulate oxidative processes in neuropathic and inflammatory models [167]. As discussed previously, CBD can act through cannabinoid

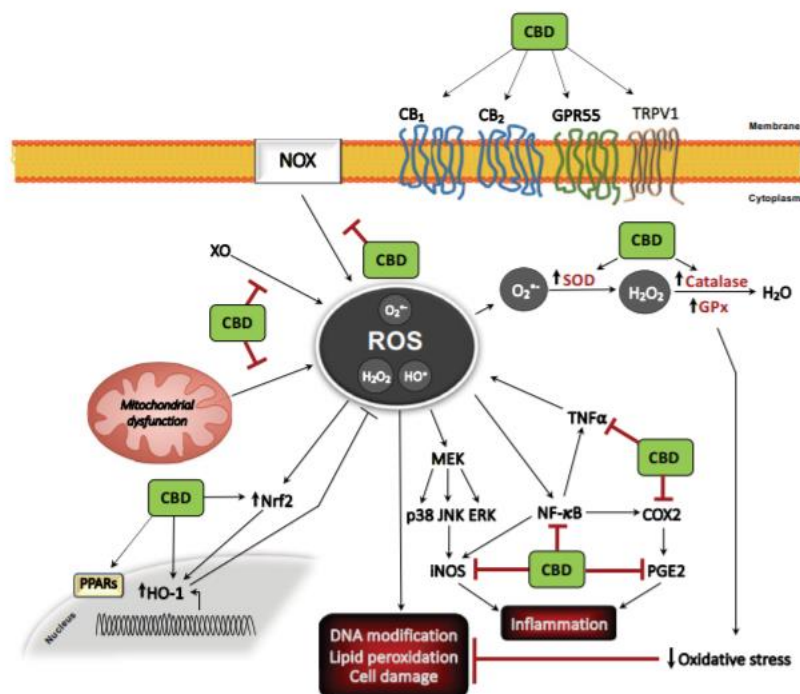


Figure 2. Overview of the antioxidant propensity of CBD

receptor-dependent and -independent mechanisms, and demonstrates minimal agonist activity (and very low affinity) for both CB₁ and CB₂ [117,118]. Furthermore, CB_{1/2}-independent mechanisms of action for CBD have also been identified, including PPAR-γ [13,19,139,140], TRPV1 receptor [18], GPR55 [16], 5-HT receptors [19,20,144,145] and μ-/δ-opioid receptors [146]. Much data suggest that CBD has antioxidant capacity, and CBD has been shown to reduce oxidative metabolism in polymorphonuclear leukocytes [168] and H₂O₂-treated nucleus pulposus cells [169], and furthermore reduces oxidative stress parameters in aged pancreatic cells [170]. In addition, CBD has the proclivity to improve cell viability following H₂O₂ treatment [169]. In addition, data from Barichello and colleagues (2012) indicate that CBD reduces host immune responses and exerts an anti-inflammatory effect (reduced cortical TNF-α) in the CNS of rats exposed to intrathecal *Streptococcus pneumoniae* [171]. This is significant given the key role played by TNF-α in the CNS, particularly in events associated with neuroinflammation, neurodegeneration and neurogenesis [172–174]. Broadly, CBD has an inhibitory effect on TNF-α expression in an array of inflammatory models, which has been recently reviewed by Nicols and Kaplan (2020) [175]. Interestingly, CBD attenuates neural production of ROS following cadmium chloride treatment, in a manner similar to vitamin E (α-tocopheryl acetate) [176], and evidence also suggests that it is more neuroprotective than ascorbate and α-tocopherol against glutamate toxicity [177]. Owing to its functional and pharmacological diversity, and evidence of its comparable antioxidant capacity to known antioxidants, CBD is an attractive agent for therapeutic immunomodulation and has been studied in conditions including intractable epilepsy [26], Huntington's disease [178] and schizophrenia [179], amongst others.

Antioxidant mechanisms of action of CBD

Regulation of antioxidants

A body of research evidence indicates that CBD modifies redox balance by altering the level and activity of antioxidant molecules (Figure 2). Indeed, CBD has been shown to target the regulation of redox-sensitive transcription factors such as Nrf2 in microglia [180], keratinocytes [181] and endothelia [182], which is important given the key role of Nrf2 in initiating the transcription of antioxidant and cytoprotective genes [63]. Indeed, recent data have shown that CBD can target the expression of Keap1 and Nrf2 in a pulmonary artery smooth muscle cells, which may contribute to its antioxidant effect in a model of pulmonary arterial hypertension [183]. CBD has also been shown to regulate the expression of the inducible antioxidant enzyme HO-1 in keratinocytes [184], adipose tissue-derived mesenchymal stem cells [185], neuroblastoma cells [186] and smooth muscle [187], which may impact the proclivity of this phytocannabinoid to regulate cellular ROS levels. Indeed, CBD significantly increases HO-1 mRNA and protein expression in human umbilical artery smooth muscle cells in a time- and concentration-dependent manner independent of CB receptors, and this effect was reversed via glutathione precursor N-acetyl cysteine (NAC), indicating the participation of ROS signalling in this process [187]. Similar effects of CBD were determined in human umbilical vein endothelial cells [182]. Data elsewhere indicate that CBD can also regulate the activity of SOD and the enzymatic activity of Cu-, Zn- and Mn-SOD, which metabolize superoxide radicals [181,188]. The vasorelaxant effects of CBD are reduced by a SOD inhibitor, suggesting that CBD acts via SOD to promote its vascular actions [189]. CBD also attenuates hippocampal oxidative damage post-oxygen-glucose-deprivation/reperfusion injury, up-regulating GSH levels and simultaneously increasing SOD1 and GPx activity following injury [190]. In support of this, *in vivo* administration of CBD attenuates the reduction in reduced/oxidized glutathione ratio (GSH/GSSG) in myocardial tissue of diabetic mice [188], and furthermore prevents GSH depletion in cardiac tissue following doxorubicin cardiotoxicity [191].

Free radical scavenging capacity

A body of data indicates that CBD demonstrates intrinsic free radical scavenging capacity (Figure 2). Indeed, CBD has been shown to ameliorate LPS-induced ROS production in microglia [192]. Furthermore, CBD inhibits mitochondrial superoxide generation in high glucose-stimulated human coronary endothelial cells [193] and reduces mitochondrial ROS generation following hippocampal oxidative damage post-oxygen-glucose-deprivation/reperfusion injury [190]. Neuroprotective effects of CBD have been shown in models of retinal neurotoxicity whereby CBD directly mitigates N-methyl-D-aspartate (NMDA)-mediated oxidative stress by potentially targeting the production of nitrotyrosine, a product of tyrosine nitration [194]. Similarly, oligodendrocyte progenitor cells and keratinocytes are protected from H₂O₂-induced cell death by CBD, with this phytocannabinoid demonstrating ROS scavenging properties against H₂O₂-induced ROS in the progenitor cells and keratinocytes [195,196]. CBD was recently shown to exert a similar effect on H₂O₂-induced ROS in intestinal cell monolayers [197]. Furthermore, data from Branca and colleagues (2019) indicate that CBD attenuates neural ROS production following cadmium chloride exposure in a manner similar to α -tocopheryl acetate [176], and CBD also dose-dependently reduces β -amyloid-induced ROS production in neurons [198]. In parallel, CBD has been shown to ameliorate cisplatin-induced production of renal nitrotyrosine in a model of nephrotoxicity [199] and has also been shown to dose-dependently reduce tert-butyl hydroperoxide-induced ROS production in keratinocytes [184]. In support of this, CBD reduces chemotactic peptide-induced ROS production in polymorphonuclear leukocytes [168] and furthermore *in vivo* administration of CBD ameliorates the production of ROS, in addition to lipid peroxides, in myocardial tissue of diabetic mice [188]. Finally, recent data from Baeeri and colleagues (2020) indicate that CBD attenuates age-related increases in ROS production in pancreatic islets [170], overall indicating the proclivity of CBD to exert free radical scavenging capacity in response to a multitude of stimuli.

Redox balance

CBD principally affects redox balance via intrinsic mechanisms. Indeed, evidence indicates that CBD interrupts free radical chain reactions, transforming free radicals into more inert molecules via the electrophilic aromatic molecular region and hydroxyl groups of its phenol ring [200]. Using cyclic voltammetry, Hampson et al. (1998) demonstrated that CBD donated electrons at a similar potential to known antioxidants, and using the iron-catalysed ROS production system (Fenton reaction), CBD was shown to prevent hydroperoxide-induced oxidative damage in neurons [177]. Again, using cyclic voltammetry, Hamelink and colleagues (2005) demonstrated that CBD is a comparable antioxidant to the common antioxidants tocopherol and butylated hydroxytoluene [201], with data indicating that CBD can reduce ROS production by chelating transition metal ions involved in the Fenton reaction [202]. Neuroprotective effects

of CBD have also been shown following anti-Yo-associated paraneoplastic cerebellar degeneration, where data indicate that CBD minimizes the downgrading of the mitochondrial membrane potential induced by anti-Yo in a manner similar to the ROS scavenger butylated hydroxytoluene, although simultaneously potentiated Yo-induced ROS production [203]. CBD has also been shown to protect hippocampal neurons from energy stress via modulation of glucose consumption by activation of the pentose-phosphate pathway in an oxygen-glucose-deprivation/reperfusion injury model [190].

Indirect antioxidant actions on XO and NOX

In addition to its intrinsic antioxidant function, CBD alters oxidative metabolism through more indirect mechanisms to modulate downstream mediators of oxidative stress (Figure 2). Indeed, Atalay and colleagues (2020) recently demonstrated that CBD attenuates XO activity in keratinocytes exposed to UVB irradiation and H_2O_2 [204]. CBD has been shown to reduce the expression of the superoxide generators RENOX (NOX4) and NOX1 in a mouse model of cisplatin-induced nephrotoxicity [199], and in a comprehensive study, Rajesh et al. (2010) identified the proclivity of CBD to attenuate diabetes-induced mRNA expression of NADPH oxidase subunits (p22phox, p67phox and gp91phox) in myocardial tissue using a model of diabetic cardiomyopathy [188]. Elsewhere, CBD has been shown to attenuate LPS-induced intracellular NADPH synthesis in microglia [192], and similarly, *in vivo* administration of CBD reduces the hepatic expression of the NADPH NOX2 isoforms p67phox/gp91phox and nitrosative stress in ethanol-fed mice, while reducing oxidative burst in neutrophils isolated from the livers of ethanol-fed mice [205]. CBD has also been shown to reduce nitrosative stress in cardiac tissue following doxorubicin-induced cardiac injury in rats, promoting a reduction in nuclear factor- κ B (NF- κ B), Fas ligand, caspase-3 and TNF- α in cardiac tissue following doxorubicin administration [191]. Similarly, CBD attenuates H_2O_2 -induced COX2 and iNOS in nucleus pulposus cells [169], attenuates iNOS expression in the myocardium of diabetic mice [188] and in rats following doxorubicin cardiotoxicity [191], and furthermore attenuates NO expression in paw tissue following sciatic nerve injury and intraplantar injection of complete Freund's adjuvant [167]. In parallel, Rajesh and colleagues (2007) demonstrated that CBD blunts iNOS expression in high glucose-stimulated human coronary endothelial cells [193], and data elsewhere indicate that CBD inhibits cisplatin-induced renal iNOS in a mouse model of nephrotoxicity [199]. CBD has also been shown to ameliorate plasma levels of prostaglandin E_2 (PGE $_2$) in animal models of neuropathic and inflammatory pain, using sciatic nerve constriction and intraplantar injection of complete Freund's adjuvant, respectively [167]. By lowering ROS levels, CBD therefore protects non-enzymatic antioxidants, preventing their oxidation. Recent evidence also suggests that CBD can ameliorate H_2O_2 -induced IL-1 β expression and the expression of other NLRP3 inflammasome-related genes, and this area warrants full investigation [196].

Pro-oxidant effects of CBD

Despite data indicating that CBD promotes antioxidative metabolism in various cells/systems, conflicting data exist regarding the influence of CBD on redox status. The effects of CBD on cell viability [170,181,195,206–210] and proliferation [187,211] are dose-dependent, and this may also be the case with respect to its antioxidant function. Indeed, in terms of ROS production, CBD has been shown to disrupt mitochondrial integrity and also induce ROS production and apoptosis in human CD14 $^{+}$ monocytes in a time-dependent manner [206]. Studies also indicate that CBD elevates intracellular ROS production in several cell systems, including human THP-1 monocytes [207], mouse macrophages [212], breast cancer cells [213] and human glioma cells [214]. Similarly, CBD promotes ROS production to promote apoptosis in leukaemia cells [208], thymocytes [211] and splenocytes [209], and data from Panja and colleagues (2019) indicate that CBD potentiates Yo-induced ROS production in a model of postparaneoplastic cerebellar degeneration treatment [203]. CBD has also been shown to overcome oxaliplatin resistance in human colorectal cancer cells by inducing mitochondrial dysfunction by increasing intracellular ROS and decreasing SOD2 [215]. Data from Gonzalez-Garcia *et al.* (2017) indicate that CBD induces apoptosis of encephalitogenic cells through oxidative stress induction (increase in ROS) in murine MS [210]. CBD has also been shown to increase the oxygen consumption rate and enhance mitochondrial bioenergetics [190], and at high concentrations can promote COX-2 expression in LPS-treated macrophages [216]. In further support of the pro-oxidant capacity of CBD, it is also important to note that CBD has been shown to increase the expression of the NOX4 and p22phox NAP(P)H oxidases [208], and also enhances the production of the p47 subunit of pro-oxidative NADPH oxidase in endotoxin-treated neutrophils from healthy subjects [217]. Finally, CBD promotes a reduction in GSH in splenocytes [209] and has been shown to deplete GSH in human gliomas [214].

Overall, CBD modulates redox function at multiple levels and a variety of downstream effects are presented in the literature. These pro- and antioxidant functions may be cell- and model-dependent, and may also be influenced by the dose of CBD delivered, the duration of CBD treatment and the underlying pathology.

Cannabis-based therapeutics

Cannabinoid-based therapeutics are currently approved for a range of symptoms in various disorders; anorexia associated with loss of appetite in acquired immunodeficiency syndrome (AIDS), chemotherapy-associated nausea, spasticity in people with (pw)MS and the treatment of seizures in Lennox-Gastaut and Dravet syndromes. The therapeutics include Epidiolex[®], Sativex[®], Marinol[®] and Cesamet[®], and such cannabinoid-based therapeutics are used as treatment strategies in those patients where conventional treatments are ineffective.

Of particular relevance to this review, the CBD-based therapeutic in the form of Epidiolex[®] has recently entered the clinic in the management of epileptic seizures. Epidiolex[®], a purified solution of CBD oil formulated for oral administration [24], is a medication approved by the US Food and Drug administration (FDA) and the European Medicines Agency (EMA) for patients with Lennox-Gastaut Syndrome and Dravet syndromes. CBD demonstrates efficacy in reducing convulsive seizure frequency in double-blind placebo-controlled trials [218], and in a prospective open-label study assessing the efficacy of Epidiolex[®] in patients with treatment resistant epilepsy, Epidiolex[®] improved the severity and frequency of seizures, and this was sustained for up to 48 weeks of treatment [219]. Devinsky and colleagues (2018) also demonstrated that 10 and 20 mg/kg doses of Epidiolex[®] (administered as two doses per day over a 14-week period) ameliorated drop seizures in patients with Lennox-Gastaut syndrome, when compared with placebo control cases [25].

In a second cannabis-based therapeutic, CBD is combined with THC as an oromucosal spray (Sativex[®]), developed to manage spasticity in pwMS [220]. Sativex[®] can be used as an add-on therapy to ameliorate MS symptoms, with each 100 µl Sativex[®] actuation delivering 2.5 mg CBD and 2.7 mg THC, in addition to other constituents [221,222]. In terms of effectiveness in ameliorating spasticity, data indicate that the majority of pwMS that administer Sativex[®] experience an improvement in spasticity score within a 4-week period [223].

Finally, both Marinol[®] (a pharmaceutical formulation of synthetic THC) and Cesamet[®] (a synthetic analogue of THC) are FDA approved cannabinoid therapeutics that can be administered orally in tablet form for the management of chemotherapy-induced nausea/emesis in patients who are refractory to other antiemetics [224,225]. Marinol[®] is also indicated for anorexia associated with loss of appetite in AIDS patients [225], and data suggest that Cesamet[®] has efficacy as an analgesic in chronic non-cancer pain [226].

It should be noted that given that adverse effects may be associated with cannabinoids, certain cannabis-based therapeutics are contraindicated in patients with a history of psychotic illness, schizophrenia, substance abuse and also in pregnancy and patients who are breast feeding. Certain cannabis-based therapeutics are also not recommended for use in certain age groups.

Conclusions

It is clear that cannabinoid use in the clinical setting has a wide variety of uses in terms of ameliorating symptoms of neurological disease, such as seizure disorders and MS, and further cannabis-based therapeutics are in the pipeline for a range of disorders. Epidiolex[®], a purified solution of CBD, is in the clinic for the management of epileptic seizures. Given the recent advancements in the clinical development of CBD, this review has focussed on the signalling targets for this phytocannabinoid, and in doing so, aimed to outline both the complex role played by ROS in disease processes, in addition to the key role of ROS in regulating biological functions. It is clear that ROS are integral players in neuroinflammation, peripheral immune responses and physiological processes, and herein we have summarized evidence that CBD readily targets oxidative signalling and ROS production. Further research is required to fully understand the interplay of this phytocannabinoid with oxidative signalling. The balance between the ability of CBD to regulate both the generation and elimination of ROS may govern its effectiveness in impacting the symptoms of disease.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This work is supported by Enterprise Ireland [grant number IP/2018/0740 (to E.J.D.)] and the Provosts PhD Project Awards at Trinity College Dublin (to E.J.D.).

Abbreviations

2-AG, 2-arachidonoyl-glycerol; 5-HT1A, 5-hydroxytryptamine receptor subtype 1A; AEA, anandamide; AIDS, acquired immunodeficiency syndrome; ARE, antioxidant response element; CAT, catalase; CBC, cannabichromene; CBCA, cannabichromenic acid; CBD, cannabidiol; CBDA, cannabidiolic acid; CBG, cannabigerol; CBGA, cannabigerolic acid; CBN, cannabinol; CGD, chronic granulomatous disease; COX-2, cyclooxygenase-2; eCB, endocannabinoid; ECS, eCB system; EMA, European Medicines Agency; eNOS, endothelial NOS; FDA, US Food and Drug administration; GPCR, G-protein-coupled receptor; GPR55, G-protein-coupled receptor 55; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; HO-1, heme oxygenase-1; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; iNOS, inducible NOS; Keap1, kelch-like ECH-associated protein1; LO, lipoxygenase; LPS, lipopolysaccharide; MS, multiple sclerosis; NAC, N-acetyl cysteine; NF- κ B, nuclear factor- κ B; NMDA, N-methyl-D-aspartate; nNOS, neuronal NOS; NO, nitric oxide; NOS, nitric oxide synthase; NOX2, NADPH oxidase 2; Nrf2, nuclear factor erythroid 2-related factor-2; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PGE₂, prostaglandin E₂; PPAR, peroxisome proliferator-activated receptor; pwMS, people with MS; RA, rheumatoid arthritis; ROS, reactive oxygen species; SOD, superoxide dismutase; THC, tetrahydrocannabinol; THCA, tetrahydrocannabinolic acid; TLR, toll-like receptor; TNF- α , tumour necrosis factor- α ; TRPV1, transient receptor potential cation channel subfamily V member 1; XO, xanthine oxidase.

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Appendix 2 Ethical Approval Letters



Tel: + 353-21-490 1901
Fax: + 353-21-490 1919

UCC

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee

Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

Our ref: ECM 4 (r) 12/03/13

20th February 2013

Dr Eric Downer
Lecturer
Department of Anatomy & Neuroscience
Room 4.11
Western Gateway Building
University College Cork
College Road
Cork

Re: Analysis of inflammatory signalling molecules in post-mortem human multiple sclerosis tissue.

Dear Dr Downer

Expedited approval is granted to carry out the above study in:

- University College Cork.

The following document has been approved:

- Signed Application Form.

We note that the co-investigator involved in this study will be:

- Dr Yvonne Nolan.

Yours sincerely


Dr Michael Hyland
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

The Clinical Research Ethics Committee of the Cork Teaching Hospitals, UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised by the Department of Health and Children to carry out the ethical review of clinical trials of investigational medicinal products. The Committee is fully compliant with the Regulations as they relate to Ethics Committees and the conditions and principles of Good Clinical Practice.

Ollscoil na hÉireann, Corcaigh - National University of Ireland, Cork.



Coláiste na Tríonóide, Baile Átha Cliath
Trinity College Dublin

Ollscoil Átha Cliath | The University of Dublin

Dr Eric Downer
Room 2.11
Discipline of Physiology
Trinity Biomedical Sciences Institute
Trinity College Dublin
Dublin 2

07th January 2016

Ref: 20151001

Title of Study: Assessment of inflammatory signalling intermediates in human
post mortem human multiple sclerosis tissue

Dear Dr Downer,

Further to a meeting of the School of Medicine Research Ethics Committee held in
October 2015, we are pleased to inform you that the above project has been
approved.

Yours sincerely,

Professor Thomas Rogers
Chairperson
School of Medicine Research Ethics Committee

An tOllamh Paul Browne MB, FRCP, FRCPath
Ceann Scoil an Leighis

Ms. Orla Bannon
Riarthóir na Scoile

Scoil an Leighis
An Institiúid Eolaíochtaí Bithleighis
Coláiste na Tríonóide, Baile Átha Cliath
Ollscoil Átha Cliath
152-160 Sráid an Phiarsaigh
Baile Átha Cliath 2, Éire.

Professor Paul Browne MB, FRCP, FRCPath
Head of School of Medicine

Ms. Orla Bannon
School Administrator

School of Medicine
Trinity Biomedical Sciences Institute
Trinity College Dublin
The University of Dublin
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Coláiste na Tríonóide, Baile Átha Cliath
Trinity College Dublin
Ollscoil Átha Cliath | The University of Dublin

Dr. Eric J. Downer
Dept. Physiology,
Room 2.07, Level 2,
School of Medicine
Trinity Biomedical Sciences Institute
Trinity College, 152-160 Pearse Street,
Dublin 2

22nd July 2016

Ref: 160502

Title of Study: "The impact of cannabinoids on endogenous interferon beta
in multiple sclerosis (MS) - an in vitro study"

Dear Dr. Downer,

Further to a meeting of the Faculty of Health Sciences Ethics Committee held in
July 2016, we are pleased to inform you that the above project has been
approved without further audit.

Yours sincerely,

Prof. Brian O'Connell
Chairperson
Faculty Research Ethics Committee

Dámh na nEolaíochtaí Sláinte
Foirgneamh na Ceimice,
Coláiste na Tríonóide,
Ollscoil Átha Cliath,
Baile Átha Cliath 2, Éire.

Faculty of Health Sciences
Chemistry Building,
Trinity College Dublin,
The University of Dublin,
Dublin 2, Ireland.

www.healthsciences.tcd.ie

Beaumont Hospital Ethics (Medical Research) Committee

Chairperson: Professor Gerry McElvaney
Convenor: Dr. Peter Branagan

Administrator: Gillian Vale

19th December 2018

REC reference: 16/47

Professor Eric Downer
Assistant Professor
Director Human health and Disease
Dept of Physiology
Trinity College, Dublin 2

To: edowner@tcd.ie

Dear Professor Downer,

RE: 16.47 – Dr. Eric Downer (TCD) - The impact of cannabinoids on endogenous interferon beta in multiple sclerosis (MS) – an in vitro study
Consultant co-investigators: Dr. Lisa Costelloe, Consultant Neurologist, Beaumont Hospital

Background:

Application Received April 2016, Reviewed 20th May 2016, Approved 22nd June 2016
Details: 4 year study – due to finish 2020* = data collection + blood sample collection + questionnaires
Participants: MS patients, adults with capacity
Funding: Irish Research Council & GW Pharmaceuticals
PhD student project
Amendment: #1, 28/10/16 – add questionnaire
Amendment: #2, 2/12/18 – extend study to 2023*

Further to correspondence dated 2nd December 2018 (received 4th December 2018), I confirm that this amendment to extend this study has been approved.

Receipt of research ethics committee approval is not, and should be regarded as evidence of compliance with GDPR 2016 or the Health Research Regulations 2018. Please note it is the responsibility of the Data Controller/Joint Data Controllers/PI to ensure and monitor compliance with relevant data protection legislation and regulation

If you have not already done so, please submit your draft Data Protection Impact Assessment (DPIA) to the Beaumont Hospital Data Protection Officer as per submission instructions, available here https://beaumontethics.ie/home/t_dpia.htm You may need a Beaumont Hospital employee to do this on your behalf.

Yours sincerely,



Dr. Peter Branagan
Convenor
Beaumont Ethics (Medical Research) Committee

Ethics (Medical Research) Committee Beaumont Hospital Dublin 9
Tel: 353-1-809 2680 Email: beaumontethics@rcsi.com <https://beaumontethics.ie>

Beaumont Hospital Ethics (Medical Research) Committee

Chairperson: Professor Gerry McElvaney
Convenor: Dr. Peter Branagan

ERIC Downer
(with Lisa O'Shea - Beaumont)

16/47

October - December 2018

To Whom It May Concern

RE. Health Research Regulations 2018

I am contacting you in your capacity as the principal investigator of a research study in the context of the Health Research Regulations.

Please begin to complete Section E2 of the most recent ethics application form, dated 31.8.18, with a view to ensuring that suitable and specific measures are taken to safeguard the fundamental rights and freedoms of the data subject as per Section 3 of the Health Research Regulations 2018

Secondly, please liaise with the relevant Data Protection Officer in relation to the need for a Data Protection Impact Assessment.

If necessary, please submit a Data Protection Impact Assessment to the Data Protection Officer.

Thirdly, please review the participant information leaflets and consent forms for your study with a view to ensuring they meet the requirements for explicit consent as per GDPR 2016.

If necessary, please submit an amendment to the relevant Research Ethics Committee(s) to revise the participant information leaflets and consent forms for your study to bring them in line with the requirements for explicit consent as per GDPR 2016.

This committee has placed adjusted template Patient Information Leaflets & Consent Forms on its website with a view to assisting researchers in meeting the requirements for explicit consent.

Should you decide to submit an amendment to the relevant Research Ethics Committee(s), please ensure you obtain expert legal / data protection advice from your legal department / data protection officer as appropriate.

Kind regards

Yours sincerely

Administrator
Ethics (Medical Research) Committee

Ethics (Medical Research) Committee, Beaumont Hospital, Dublin 9
Tel: 353-1-809 2680 Email: beaumontethics@rcsi.com <https://beaumontethics.ie>

Beaumont Hospital Ethics (Medical Research) Committee

Chairperson: Professor Gerry McElvaney
Convenor: Dr. Peter Branagan

Administrator: Phil Oglesby

22nd November 2016

REC reference: 16.47

Professor Eric Downer
Assistant Professor
Director Human health and Disease
Dept of Physiology
Trinity College
Dublin 2

To: edowner@tcd.ie

Dear Professor Downer,

RE: 16.47 STUDY TITLE: "The impact of cannabinoids on endogenous interferon beta in multiple sclerosis (MS) – an in vitro study".

The Beaumont Ethics Committee has reviewed the revised documentation in relation to the requested amendments to the above study and is happy to provide Ethical Approval.

With best regards,

Yours sincerely,



Dr. Peter Branagan
Convenor
Beaumont Ethics (Medical Research) Committee

"The REC must be satisfied with the scientific quality of the research proposal"
- Council of Europe (2011) Guid for Research Ethics Committee Members.

Ethics (Medical Research) Committee Beaumont Hospital Dublin 9
Tel: 353-1-809 2680 Email: gvale@rcsi.ie www.beaumontethics.ie

Beaumont Hospital Ethics (Medical Research) Committee

Chairperson: Professor Gerry McElvane
Convenor: Dr. Peter Branagan

Administrator: Phil Oglesby

27th July 2016

REC reference: 16.47

Prof Eric Downer,
Assistant Professor in Human Health and Disease,
Department of Physiology,
Level 2,
Trinity Biomedical Sciences Institute (TBSI),
Trinity College,
152-160 Pearse Street,
Dublin 2.

To: edowner@tcd.ie

Dear Prof Downer,

RE: The impact of cannabinoids on endogenous interferon beta in multiple sclerosis (MS)- an in vitro study

The Committee has reviewed the changes made and are happy to approve this study. Please note the original copy of approval letter was sent on 22nd June 2016 and we apologise for any confusion with the address.

With best regards

Yours sincerely,


Dr. Peter Branagan
Convenor
Ethics (Medical Research) Committee

c.c.

"The REC must be satisfied with the scientific quality of the research proposal"
- Council of Europe (2011) Guide for Research Ethics Committee Members

Ethics (Medical Research) Committee Beaumont Hospital Dublin 9
Tel: 353-1-809 2680 Email: beaumontethics@rcsi.ie www.beaumontethics.ie

Beaumont Hospital Ethics (Medical Research) Committee

Chairperson: Professor Gerry McElvaney
Convenor: Dr. Peter Branagan

Administrator: Phil Oglesby

REC reference: 16.47

27th July 2016

Prof Eric Downer,
Assistant Professor in Human Health and Disease,
Department of Physiology,
Level 2,
Trinity Biomedical Sciences Institute (TBSI),
Trinity College,
152-160 Pearse Street,
Dublin 2.

To: edowner@tcd.ie

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With best regards

Yours sincerely

Dr. Peter Branagan
Convenor
Ethics (Medical Research) Committee

c.c.

"The REC must be satisfied with the scientific quality of the research proposal"
- Council of Europe (2011) Guide for Research Ethics Committee Members

Ethics (Medical Research) Committee Beaumont Hospital Dublin 9
Tel: 353-1-809 2680 Email: beaumontethics@rcsi.ie www.beaumontethics.ie

Beaumont Hospital Ethics (Medical Research) Committee

Chairperson: Professor Gerry McElvaney
Convenor: Dr. Peter Branagan

Administrator: Phil Oglesby

3rd May 2016

REC reference: 16/47

Dr. Eric Downer
Assistant Professor in Human Health and Disease
Physiology Dept,
Level 2, Trinity Biomedical Science Institute
Trinity College
152-160 Pearse Street
Dublin 2

To: edowner@tcd.ie
c.c. lisacostello@beaumont.ie hardimao@tcd.ie

Dear Dr. Downer

RE: 16/47 – Study: “The impact of cannabinoids on endogenous interferon beta in multiple sclerosis (MS) – an in-vitro study”

Co-Investigator: Dr. Lisa Costello/Professor Orla Hardiman

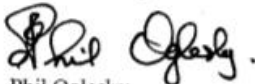
I acknowledge receipt of the following documentation:

1. Ethics Application Form 5.6, V.1, dated 27.4.16
2. Research Protocol Study (5 copies)
3. Patient Information Leaflet
4. Patient Consent Form
5. Multiple Sclerosis Quality of Life (MSQOL)-54 Instrument
6. The Quick Inventory of Depressive Symptomatology (16-Item) Self-Report (QODS-SR₁₆)
7. Checklist and signed Signature Page

I can confirm that these documents have been scheduled for review at the next committee meeting due to take place on the 20th May 2016.

Kind regards

Yours sincerely



Phil Oglesby
Acting Administrator
Ethics (Medical Research) Committee

**Appendix 3 Study Questionnaires (MSQOL-54, QIDS-SR₁₆,
background information), study participant information
leaflet, participant consent form**

Study questionnaire

Study title: The impact of cannabinoids on endogenous interferon beta in multiple sclerosis (MS) - an in vitro study"

This questionnaire is entirely confidential and only the investigators mentioned below will have access to the information you provide. Please fill out all sections in detail for us. If you have any questions or do not understand a particular question please do not hesitate for clarification.

Regards, Dr. Lisa Costelloe (Co-investigator), Mr. John-Mark Fitzpatrick (Principal Researcher), Dr. Eric Downer (Principal Investigator), Researchers (Dr. Downer lab), Dr. Margaret O'Brien (Co-investigator)

NAME (PRINT): _____ DATE: _____

What is your age? _____

Please circle: Male / Female

How long have you had MS? _____ months

EDSS (completed by Neurologist) _____

General health concerns – please answer the following questions	YES	NO	If yes, please provide further details
Do you have a blood disorder?			
Do you have thyroid disease?			
Do you have diabetes?			
Do you have an autoimmune disease other than MS?			
Do you have any allergies?			
Do you have a bacterial, viral or fungal infection?			
Do you smoke?			
Do you smoke cannabis?			

Do you have any other medical condition?			
--	--	--	--

Please list below any medications you take:	Medication:	Dose:	Reason for medication:

Multiple Sclerosis Quality of Life (MSQOL)-54 Instrument

For Further Information, Contact:

Barbara G. Vickrey, MD, MPH
UCLA Department of Neurology
C-128 RNRC; Box 951769
Los Angeles, CA 90095-1769
Voice: 310.206.7671
Fax: 310.794.7716

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INSTRUCTIONS:

This survey asks about your health and daily activities. Answer every question by circling the appropriate number (1, 2, 3, ...).

If you are unsure about how to answer a question, please give the best answer you can and write a comment or explanation in the margin.

Please feel free to ask someone to assist you if you need help reading or marking the form.

1. In general, would you say your health is:

(circle one number)

Excellent.....1

Very good.....2

Good.....3

Fair.....4

Poor.....5

2. Compared to one year ago, how would you rate your health in general now?

(circle one number)

Much better now than one year ago 1

Somewhat better now than one year ago.....2

About the same..... 3

Somewhat worse now than one year ago..... 4

Much worse now than one year ago..... 5

- 3-12. The following questions are about activities you might do during a typical day. Does **your health** limit you in these activities? If so, how much?
(Circle 1, 2, or 3 on each line)

	Yes, Limited a Lot	Yes, Limited a Little	No, Not Limited at All
3. <u>Vigorous</u> activities, such as running, lifting heavy objects, participating in strenuous sports	1	2	3
4. <u>Moderate</u> activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	1	2	3
5. Lifting or carrying groceries	1	2	3
6. Climbing <u>several</u> flights of stairs	1	2	3
7. Climbing <u>one</u> flight of stairs	1	2	3
8. Bending, kneeling, or stooping	1	2	3
9. Walking <u>more than a mile</u>	1	2	3
10. Walking <u>several blocks</u>	1	2	3
11. Walking <u>one block</u>	1	2	3
12. Bathing and dressing yourself	1	2	3

- 13-16. During the **past 4 weeks**, have you had any of the following problems with your work or other regular daily activities **as a result of your physical health?**

(Circle one number on each line)

	YES	NO
13. Cut down on the <u>amount of time</u> you could spend on work or other activities	1	2
14. <u>Accomplished less</u> than you would like	1	2
15. Were limited in the <u>kind</u> of work or other activities	1	2
16. Had <u>difficulty</u> performing the work or other activities (for example, it took extra effort)	1	2

- 17-19. During the **past 4 weeks**, have you had any of the following problems with your work or other regular daily activities **as a result of any emotional problems** (such as feeling depressed or anxious).

(Circle one number on each line)

	YES	NO
17. Cut down on the <u>amount of time</u> you could spend on work or other activities	1	2
18. <u>Accomplished less</u> than you would like	1	2
19. Didn't do work or other activities as <u>carefully</u> as usual	1	2

20. During the **past 4 weeks**, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

(circle one number)

Not at all 1
Slightly 2
Moderately 3
Quite a bit 4
Extremely 5

Pain

21. How much **bodily** pain have you had during the **past 4 weeks**?

(circle one number)

None 1
Very mild 2
Mild 3
Moderate 4
Severe 5
Very severe 6

22. During the **past 4 weeks**, how much did **pain** interfere with your normal work (including both work outside the home and housework)?

(circle one number)

Not at all 1
A little bit 2
Moderately 3
Quite a bit 4
Extremely 5

23-32. These questions are about how you feel and how things have been with you **during the past 4 weeks**. For each question, please give the one answer that comes closest to the way you have been feeling.

How much of the time during the **past 4 weeks**...

(Circle one number on each line)

	All of the Time	Most Of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	None of the Time
23. Did you feel full of pep?	1	2	3	4	5	6
24. Have you been a very nervous person?	1	2	3	4	5	6
25. Have you felt so down in the dumps that nothing could cheer you up?	1	2	3	4	5	6
26. Have you felt calm and peaceful?	1	2	3	4	5	6
27. Did you have a lot of energy?	1	2	3	4	5	6
28. Have you felt downhearted and blue?	1	2	3	4	5	6
29. Did you feel worn out?	1	2	3	4	5	6
30. Have you been a happy person?	1	2	3	4	5	6
31. Did you feel tired?	1	2	3	4	5	6
32. Did you feel rested on waking in the morning?	1	2	3	4	5	6

33. During the **past 4 weeks**, how much of the time has your **physical health or emotional problems** interfered with your social activities (like visiting with friends, relatives, etc.)?

(circle one number)

All of the time 1

Most of the time 2

Some of the time 3

A little of the time 4

None of the time 5

Health in General

- 34-37. How TRUE or FALSE is each of the following statements for you.

(Circle one number on each line)

	Definitely True	Mostly True	Not Sure	Mostly False	Definitely False
34. I seem to get sick a little easier than other people	1	2	3	4	5
35. I am as healthy as anybody I know	1	2	3	4	5
36. I expect my health to get worse	1	2	3	4	5
37. My health is excellent	1	2	3	4	5

Health Distress

How much of the time during the **past 4 weeks...**

(Circle one number on each line)

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	None of the Time
38. Were you discouraged by your health problems?	1	2	3	4	5	6
39. Were you frustrated about your health?	1	2	3	4	5	6
40. Was your health a worry in your life?	1	2	3	4	5	6
41. Did you feel weighed down by your health problems?	1	2	3	4	5	6

Cognitive Function

How much of the time during the **past 4 weeks...**

(Circle one number on each line)

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	None of the Time
42. Have you had difficulty concentrating and thinking?	1	2	3	4	5	6
43. Did you have trouble keeping your attention on an activity for long?	1	2	3	4	5	6
44. Have you had trouble with your memory?	1	2	3	4	5	6
45. Have others, such as family members or friends, noticed that you have trouble with your memory or problems with your concentration?	1	2	3	4	5	6

Sexual Function

46-50. The next set of questions are about your sexual function and your satisfaction with your sexual function. Please answer as accurately as possible about your function during the last 4 weeks only.

How much of a problem was each of the following for you during the past 4 weeks?

(Circle one number on each line)

MEN	Not a problem	A Little of a Problem	Somewhat of a Problem	Very Much a Problem
46. Lack of sexual interest	1	2	3	4
47. Difficulty getting or keeping an erection	1	2	3	4
48. Difficulty having orgasm	1	2	3	4
49. Ability to satisfy sexual partner	1	2	3	4

(Circle one number on each line)

WOMEN	Not a problem	A Little of a Problem	Somewhat of a Problem	Very Much a Problem
46. Lack of sexual interest	1	2	3	4
47. Inadequate lubrication	1	2	3	4
48. Difficulty having orgasm	1	2	3	4
49. Ability to satisfy sexual partner	1	2	3	4

50. Overall, how satisfied were you with your sexual function during the past 4 weeks?

(circle one number)

- Very satisfied 1
- Somewhat satisfied 2
- Neither satisfied nor
dissatisfied 3
- Somewhat dissatisfied 4
- Very dissatisfied 5

51. During the past 4 weeks, to what extent have problems with your bowel or bladder function interfered with your normal social activities with family, friends, neighbors, or groups?

(circle one number)

- Not at all 1
- Slightly 2
- Moderately 3
- Quite a bit 4
- Extremely 5

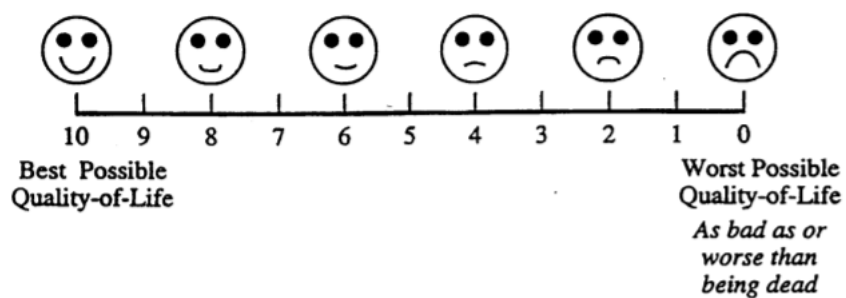
52. During the past 4 weeks, how much did *pain* interfere with your enjoyment of life?

(circle one number)

- Not at all 1
- Slightly 2
- Moderately 3
- Quite a bit 4
- Extremely 5

53. Overall, how would you rate your own quality-of-life?

Circle one number on the scale below:



54. Which best describes how you feel about your life as a whole?

(circle one number)

- Terrible 1
- Unhappy.....2
- Mostly dissatisfied 3
- Mixed - about equally
satisfied and dissatisfied 4
- Mostly satisfied.....5
- Pleased 6
- Delighted.....7

The Quick Inventory of Depressive Symptomatology (16-Item) (Self-Report) (QIDS-SR16)

Name or ID: _____ Date: _____

CHECK THE ONE RESPONSE TO EACH ITEM THAT BEST DESCRIBES YOU FOR THE PAST SEVEN DAYS.

During the past seven days...

1. Falling Asleep:

- ☐ 0 I never take longer than 30 minutes to fall asleep.
- ☐ 1 I take at least 30 minutes to fall asleep, less than half the time.
- ☐ 2 I take at least 30 minutes to fall asleep, more than half the time.
- ☐ 3 I take more than 60 minutes to fall asleep, more than half the time.

2. Sleep During the Night

- ☐ 0 I do not wake up at night.
- ☐ 1 I have a restless, light sleep with a few brief awakenings each night.
- ☐ 2 I wake up at least once a night, but I go back to sleep easily.
- ☐ 3 I awaken more than once a night and stay awake for 20 minutes or more, more than half the time.

3. Waking Up Too Early:

- ☐ 0 Most of the time, I awaken no more than 30 minutes before I need to get up.
- ☐ 1 More than half the time, I awaken more than 30 minutes before I need to get up.
- ☐ 2 I almost always awaken at least one hour or so before I need to, but I go back to sleep eventually.
- ☐ 3 I awaken at least one hour before I need to, and can't go back to sleep.

4. Sleeping Too Much:

- ☐ 0 I sleep no longer than 7-8 hours/night, without napping during the day.
- ☐ 1 I sleep no longer than 10 hours in a 24-hour period including naps.
- ☐ 2 I sleep no longer than 12 hours in a 24-hour period including naps.
- ☐ 3 I sleep longer than 12 hours in a 24-hour period including naps.

During the past seven days...

5. Feeling Sad:

- ☐ 0 I do not feel sad.
- ☐ 1 I feel sad less than half the time.
- ☐ 2 I feel sad more than half the time.
- ☐ 3 I feel sad nearly all of the time.

Please complete either 6 or 7 (not both)

6. Decreased Appetite:

- ☐ 0 There is no change in my usual appetite.
- ☒ 1 I eat somewhat less often or lesser amounts of food than usual.
- ☐ 2 I eat much less than usual and only with personal effort.
- ☐ 3 I rarely eat within a 24-hour period, and only with extreme personal effort or when others persuade me to eat.

- OR -

7. Increased Appetite:

- ☐ 0 There is no change from my usual appetite.
- ☐ 1 I feel a need to eat more frequently than usual.
- ☐ 2 I regularly eat more often and/or greater amounts of food than usual.
- ☐ 3 I feel driven to overeat both at mealtime and between meals.

Please complete either 8 or 9 (not both)

8. Decreased Weight (Within the Last Two Weeks):

- ☐ 0 I have not had a change in my weight.
- ☐ 1 I feel as if I have had a slight weight loss.
- ☐ 2 I have lost 2 pounds or more.
- ☐ 3 I have lost 5 pounds or more.

- OR -

9. Increased Weight (Within the Last Two Weeks):

- ☐ 0 I have not had a change in my weight.
- ☐ 1 I feel as if I have had a slight weight gain.
- ☐ 2 I have gained 2 pounds or more.
- ☐ 3 I have gained 5 pounds or more.

The Quick Inventory of Depressive Symptomatology (16-Item) (Self-Report) (QIDS-SR₁₆)

During the past seven days...

10. Concentration / Decision Making:

- ☐ 0 There is no change in my usual capacity to concentrate or make decisions.
- ☐ 1 I occasionally feel indecisive or find that my attention wanders.
- ☐ 2 Most of the time, I struggle to focus my attention or to make decisions.
- ☐ 3 I cannot concentrate well enough to read or cannot make even minor decisions.

11. View of Myself:

- ☐ 0 I see myself as equally worthwhile and deserving as other people.
- ☐ 1 I am more self-blaming than usual.
- ☐ 2 I largely believe that I cause problems for others.
- ☐ 3 I think almost constantly about major and minor defects in myself.

12. Thoughts of Death or Suicide:

- ☐ 0 I do not think of suicide or death.
- ☐ 1 I feel that life is empty or wonder if it's worth living.
- ☐ 2 I think of suicide or death several times a week for several minutes.
- ☐ 3 I think of suicide or death several times a day in some detail, or I have made specific plans for suicide or have actually tried to take my life.

13. General Interest

- ☐ 0 There is no change from usual in how interested I am in other people or activities.
- ☐ 1 I notice that I am less interested in people or activities.
- ☐ 2 I find I have interest in only one or two of my formerly pursued activities.
- ☐ 3 I have virtually no interest in formerly pursued activities.

During the past seven days...

14. Energy Level:

- ☐ 0 There is no change in my usual level of energy.
- ☐ 1 I get tired more easily than usual.
- ☐ 2 I have to make a big effort to start or finish my usual daily activities (for example, shopping, homework, cooking, or going to work).
- ☐ 3 I really cannot carry out most of my usual daily activities because I just don't have the energy.

15. Feeling Slowed Down:

- ☐ 0 I think, speak, and move at my usual rate of speed.
- ☐ 1 I find that my thinking is slowed down or my voice sounds dull or flat.
- ☐ 2 It takes me several seconds to respond to most questions and I'm sure my thinking is slowed.
- ☐ 3 I am often unable to respond to questions without extreme effort.

16. Feeling Restless:

- ☐ 0 I do not feel restless.
- ☐ 1 I'm often fidgety, wringing my hands, or need to shift how I am sitting.
- ☐ 2 I have impulses to move about and am quite restless.
- ☐ 3 At times, I am unable to stay seated and need to pace around.

Participant Information Leaflet

Study title: "The impact of cannabinoids on endogenous interferon beta in multiple sclerosis (MS) - an in vitro study"

Principal investigator's name: Dr. Eric J. Downer

Principal investigator's title: Assistant Professor, Trinity College Dublin

Telephone number of principal investigator: 01 896 2076

Consultant co-investigator's name: Dr. Lisa Costelloe/Prof. Orla Hardiman

Consultant co-investigator's title: Consultant Neurologist

Data Controller's/joint Controller's Identity: Trinity College Dublin

Data Controller's/joint Controller's Contact Details: Trinity College, Dublin 2.

Data Protection Officer's Identity: John Eustace

Data Protection Officer's Contact Details: Secretaries Office, Trinity College, Dublin 2.

You are being invited to take part in a clinical research study being carried out at Beaumont Hospital and Trinity College Dublin by Dr. Eric Downer's research laboratory at Trinity College Dublin. Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP (doctor). Take time to ask questions – don't feel rushed and don't feel under pressure to make a quick decision. You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. If you decide not to take part it won't affect your future medical care. You can change your mind about taking part in the study any time you like. Even if the study has started, you can still opt out. You don't have to give us a reason. If you do opt out, rest assured it won't affect the quality of treatment you get in the future.

Why is this study being done?

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system (brain and spinal cord). Its cause is unknown but it is believed to be associated with an overactive immune system (which normally takes part in the body's defense against infections). The immune system produces many inflammatory mediators (substances that fight infections but in some situations attack the body's own structure). Many such inflammatory substances are produced in the brain in MS and may be found in the fluid surrounding the brain and also in the blood. The purpose of this study is to identify new inflammatory mediators in blood cells isolated from MS patients. The number of blood cell types and inflammatory innate immune signalling mechanisms will be assessed in isolated blood cells, and the ability of novel drugs to target innate immune signalling in blood cells, will be determined. These inflammatory mediators will be correlated with quality of life and depression scores. This may aid in the development of new MS therapies.

Who is organising and funding this study?

Researchers: Dr. Eric Downer (TCD), Dr. Lisa Costelloe (Beaumont), Prof. Orla Hardiman (Beaumont), Dr. Margaret O'Brien (Beaumont), Mr. John Mark Fitzpatrick (TCD), Dr. Noreen Boyle (TCD), PhD students/researchers (Dr. Downer's lab; TCD)

Funding body: Irish Research Council, GW Pharmaceuticals, Trinity College Dublin

Why am I being asked to take part?

The purpose of this study is to identify new inflammatory mediators in blood cells.

How will the study be carried out?

The study will involve the collection of a single blood sample and completion of study questionnaires related to health.

What will happen to me if I agree to take part?

You are being invited to take part in a clinical research study carried out at Beaumont Hospital and Trinity College Dublin. At the time of consent you will be asked to complete a number of questionnaires to assess quality of life, depression and mood. A standard neurological examination may be performed by your doctor. You will be asked for permission to obtain a small amount of blood (maximum of 50ml; 3 tablespoons) on one occasion for this study. You may be asked for permission to look at your medical records. This information will be kept private and confidential. Involvement in this study should take approximately 20-30 minutes.

What other treatments are available to me?

This study does not involve you taking any additional treatments.

What are the benefits?

MS is one of the most prevalent diseases of the nervous system in the Western world and currently there is no cure. Therapies are centered on relapse reduction and symptom management. However many patients respond sub-optimally to currently available therapies and side effects of medication are common. This study aims to examine anti-inflammatory changes in blood cells from MS patients. This may lead to the development of novel therapies for MS in the future. By participating in this study you would be contributing to this goal.

What are the risks?

Blood taking may be associated with some short-lasting discomfort of a needle stick. To minimize this appropriate tourniquet, alcohol wipes, gauze sponges and adhesive bandages and tape will be used. There will be no risks to you aside from that associated with routine venipuncture by a qualified phlebotomist. These include the possibility that you will experience discomfort as the needle is inserted and you may have a small bruise afterwards. It is also possible that you may feel faint, in which case the chair can be reclined until you recover. Emergency procedures are in place to deal with any unexpected adverse events, which although statistically possible are extremely rare. These could include thrombosis of the vein due to trauma and infection which results in thrombophlebitis.

What if something goes wrong when I'm taking part in this study?

If you decide not to participate, or if you quit, you will not give up any benefits which you had before entering the study. Your decision not to participate or to withdraw from the study will not restrict your access to health care services normally available to you. You understand that your Principal Investigator or the sponsoring company/university may stop your participation in the study at any time without your consent.

Will it cost me anything to take part?

No. Furthermore, you will not receive any expenses for being part of this study.

Is the study confidential?

Your identity will remain confidential over the duration of the study. Your name will not be published and will not be disclosed to anyone outside the hospital or outside the Trinity College research team. Your identity will only be available to researchers directly involved in the study. Your GP will not be contacted as part of this study. All medical information will be kept private and confidential. Blood samples and questionnaire data will be assessed for inflammatory changes associated with MS in a research laboratory in Trinity College Dublin. The research findings may potentially be published in research articles and/or presented at research conferences. Your identity will remain confidential in any publications and/or presentations.

Data Protection

You have the right to withdraw consent to your personal data being used in this research project. You will be able to do this by contacting Dr. Eric Downer, Dept. Physiology, Trinity Biomedical Sciences Institute, Trinity College, Dublin 2. Phone No: 01 896 2076

1. We will be using your personal data information in our research to carry out our research study which is in the public interest.
2. We are processing your data for scientific research purposes.
3. The recipient of your data is Dr. Eric Downers laboratory, Trinity College Dublin, GW Research Ltd., Royal Holloway Hospital and academic collaborators.
4. The data will be stored for approximately 7 years after completion of the study. This is to allow correlation of the data with further studies. At present funding is in place to conduct this study until 2023.
5. No risks and/or implications may arise for you as a result of the data processing.
6. You have a right to withdraw from the study at any time by contacting Dr. Eric Downer.
7. You have a right to request access to your data and a copy of it, unless your request would make it impossible or make it very difficult to conduct the research.
8. You have a right to have any inaccurate information about you corrected/deleted, unless your request would make it impossible (or very difficult) to conduct the research.
9. You have a right to data portability, meaning you have a right to move your data from one controller to another in a readable format.
10. There will be no automated decision making, including profiling as part of this study. You have a right to object to automated processing including profiling if you wish.
11. You will be informed if we intend to further process your personal data and you will be provided with information on that other purpose.
12. You will be informed if we wish to transfer your data to a country outside of the EU or an international organisation and you will be advised of the safeguards we have in place to protect your data.

Consent to Future Uses

By giving consent to take part in this study you have only given permission for your data/biological material to be used for the current research. We are seeking permission to store the data/biological material for possible future uses in our research. This may entail the assessment of new inflammatory proteins involved in the disease process. For example, by consenting to **future research related to the current study** you are giving consent to processing

for an MS-related research study and other future unnamed research studies in the same area of inflammation research. Your consent could not go beyond inflammation to other areas. The data/blood samples will be stored in Dr. Downers laboratory at Trinity College Dublin, a laboratory dedicated to understanding the inflammatory processes associated with human disease and aims to develop new therapeutic avenues. Biological samples may be shared with academic/industry collaborator's (inside and outside EU) for assessment of inflammatory read-outs. This laboratory is supported by grants (government-funded, company funded, society funded, university funded) with no potential conflicts of interest. You are being invited to participate to provide invaluable blood samples and questionnaire data that are central to understanding the processing of inflammation in the human body. Questionnaire data related to Quality of Life and depression, alongside biological material (blood immune cells/plasma) will be stored for future use. Participation is voluntary and consent given is an unambiguous indication of your wishes. You can change your mind at any time and withdraw from the study by contacting dr. Eric Downer. The research is approved by the School of Medicine Research Ethics Committee, Trinity College Dublin, and the Beaumont Hospital Research Ethics committee.

Where can I get further information?

You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Dr. Eric Downer who can be emailed at edowner@tcd.ie or telephoned at 01 896 2076. If your Principal Investigator learns of important new information that might affect your desire to remain in the study, he will tell you.

If you need any further information now or at any time in the future, please contact:

Name: Dr. Eric Downer

Address: Dept. Physiology, Trinity Biomedical Sciences Institute, Trinity College, Dublin 2.

Phone No: 01 896 2076 (this will be answered only during office hours)

Participant Consent Form

Study title: "The impact of cannabinoids on endogenous interferon beta in multiple sclerosis (MS) - an in vitro study"

Name of researchers: Dr. Eric Downer (TCD), Prof. Orla Hardiman (Beaumont), Dr. Lisa Costelloe (Beaumont), Dr. Margaret O'Brien (Beaumont), Mr. John-Mark Fitzpatrick (TCD), Dr. Noreen Boyle (TCD), PhD students/researchers (Dr. Downer lab; TCD)

I have read and understood the Information Leaflet about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	Yes ☐	No ☐
I understand that I don't have to take part in this study and that I can opt out at any time. I understand that I don't have to give a reason for opting out and I understand that opting out won't affect my future medical care.	Yes ☐	No ☐
I understand that I can withdraw my biological material at any time without any negative repercussions.	Yes ☐	No ☐
I understand that my biological material will be disposed of in a lawful and respectful way.	Yes ☐	No ☐
I am aware of the potential risks, benefits and alternatives of this research study.	Yes ☐	No ☐
I give permission for researchers to look at my medical records. I have been assured that information about me will be kept private and confidential.	Yes ☐	No ☐
I have been given a copy of the Information Leaflet and this completed consent form for my records.	Yes ☐	No ☐
I give my permission for information collected about me to be stored or electronically processed for the purpose of academic scientific research and to be used in <u>related studies or other studies in the future</u> .	Yes ☐	No ☐
I consent to take part in this research study having been fully informed of the risks, benefits and alternatives.	Yes ☐	No ☐
I give explicit informed consent to have my data processed as part of this research study.	Yes ☐	No ☐
I consent to give a blood sample or samples for this research project. I understand that giving a blood sample or samples for this research is my own decision.	Yes ☐	No ☐
I consent to be contacted by researchers as part of this research study	Yes ☐	No ☐

FUTURE CONTACT		
I consent to be re-contacted by researchers about possible future research related to the current study for which I may be eligible.	Yes ☐	No ☐

STORAGE AND FUTURE USE OF INFORMATION		
RETENTION OF RESEARCH MATERIAL IN THE FUTURE		
I give permission for my biological material/data to be stored for <u>future academic research related</u> to the current study in, and outside, the EU, but only if the research is approved by a Research Ethics Committee.	Yes ☐	No ☐
I agree that some future research projects may be carried out by researchers working for <u>commercial/pharmaceutical companies</u> .	Yes ☐	No ☐
I understand I will not be entitled to a share of any profits that may arise from the future use of my material/data or products derived from it.	Yes ☐	No ☐
DESTRUCTION OF RESEARCH MATERIAL		

I request that my <i>biological material be destroyed but</i> I give permission for my <i>data</i> derived from my biological material to be <i>stored</i> for <i>possible future research related</i> to the current study <i>without further consent</i> being required but only if the research is approved by a Research Ethics Committee.	Yes ☐	No ☐
I request that all biological material/data previously collected can no longer be used by researchers and is destroyed.	Yes ☐	No ☐

To be completed by the PARTICIPANTS

Patient Name (Block Capitals)	Patient Signature	Date

Translator Name (Block Capitals)	Translator Signature	Date

Legal Representative/Guardian Name	Legal Representative/Guardian Signature	Date

To be completed by the Principal Investigator or nominee.

I, the undersigned, have taken the time to fully explain to the above participant the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them.

Name (Block Capitals)	Qualifications	Signature	Date

3 copies to be made: 1 for participant, 1 for PI and 1 for hospital records.

Appendix 4 Supplementary Data

7.1 *CNR1/CNR2* expression in PBMCs isolated from pwMS and HCs

As mentioned previously in the results section (4.11), it was previously determined that both *CNR1* and *CNR2* mRNA are expressed in PBMCs isolated from whole blood of both pwMS and HCs. PBMCs were isolated from HCs and pwMS, RNA was extracted and converted to cDNA for analysis of *CNR1* and *CNR2* mRNA by RT-qPCR. This experiment was conducted previously by Dr John-Mark Fitzpatrick (previous PhD student, Dr Downer laboratory). As indicated in Fig. 7.1A, the expression of *CNR1* mRNA is comparable in pwMS and HCs, although individuals with MS have a trend towards increased *CNR1* mRNA expression, albeit insignificant (Fig. 7.1A). Similarly, comparable expression levels of *CNR2* mRNA were determined in PBMCs from both HCs and pwMS (Fig. 7.1B).

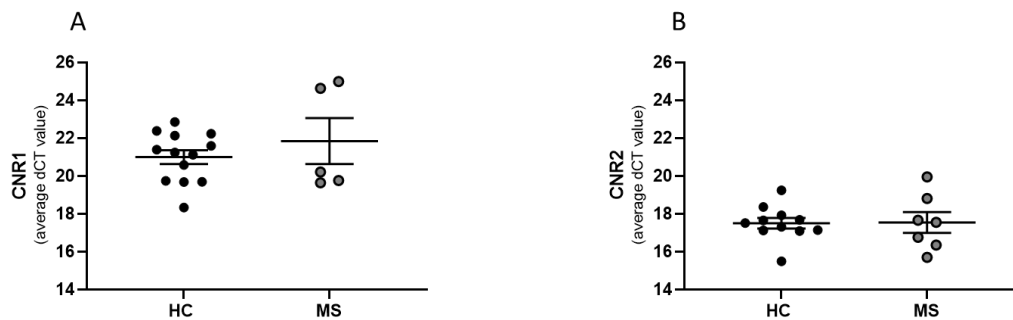


Figure 7.1 *CNR1* and *CNR2* mRNA expression is comparable in PBMCs isolated from pwMS and HCs. PBMCs were isolated from the whole blood of HCs and pwMS, RNA was extracted and converted to cDNA. The basal expression of *CNR1* and *CNR2* mRNA was assessed via RT-qPCR and the delta CT values presented. **(A)** Basal expression of *CNR1* mRNA in PBMCs from pwMS and HC subjects. **(B)** Basal expression of *CNR2* mRNA in PBMCs isolated from pwMS and HCs. Data was presented as mean delta CT value $\pm$ SEM. Data was analysed using Mann-Whitney or an unpaired *t*-test.

7.2 Expression of *CNR2* mRNA in NAWM and NAGM from non-MS controls and MS cases

As mentioned in chapter 5 (5.7) the expression of both *CNR1* and *CNR2* mRNA was assessed in post-mortem cortical tissue from non-MS cases, PPMS cases and SPMS cases, both in NAWM and NAGM. The endogenous expression of *CNR2* mRNA in post-mortem cortical tissue was low. Indeed, most samples assessed did not express *CNR2* mRNA, and there was no *CNR2* mRNA expressed in control cases, either in NAWM or NAGM. This is unsurprising as *CNR2* is expressed to a greater extent in the periphery as opposed to centrally in the brain. Indeed, *CNR2* mRNA has been identified, to some extent, within areas of the brain, including neurons of the brainstem and in microglia. Having assessed the expression of *CNR2* mRNA in PBMCs, we sought to assess its expression centrally and determine whether the expression differed in NAWM and NAGM from non-MS cases, SPMS cases and PPMS cases. Cortical punches of NAWM and NAGM brain tissue were taken, RNA was extracted and converted to cDNA for analysis of *CNR2* mRNA by RT-qPCR. As shown in Fig. 7.2, few cases had detectable levels of *CNR2* either or NAWM or NAGM. Indeed, none of the non-MS cases expressed *CNR2* mRNA in the cortex (Fig. 7.2). It is difficult to draw conclusions from these underpowered data.

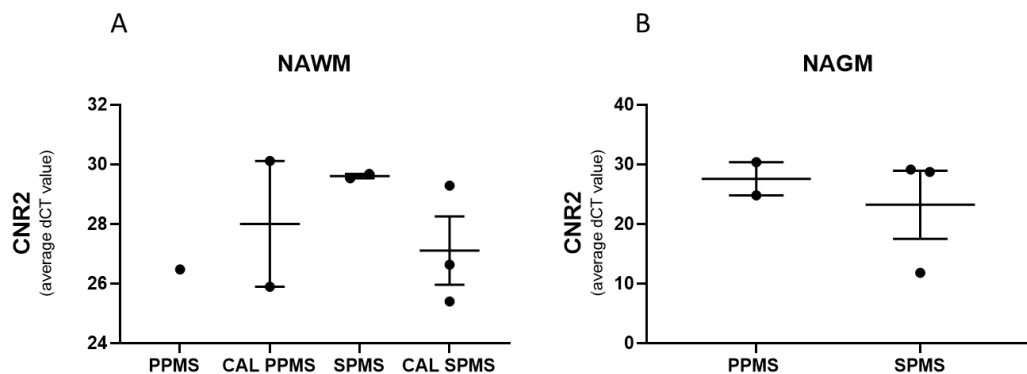


Figure 7.2 *CNR2* mRNA expression in NAWM and NAGM. Post-mortem brain tissue punches were taken from non-MS control cases, PPMS cases and SPMS cases. RNA was extracted and converted to cDNA. The basal expression of *CNR2* mRNA was assessed by RT-qPCR and the dCT values presented. **(A)** Basal expression of *CNR2* mRNA in NAWM from PPMS cases, CALs from PPMS cases, NAWM from SPMS cases and CALs from SPMS cases. **(B)** Basal expression of *CNR2* mRNA in NAGM from PPMS and SPMS cases. Data was presented as mean $\pm$ SEM, symbols represent individual samples. Data were not parametric and were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test, or an unpaired *t*-test (NAGM).

7.3 LPS time-dependently induces the production of the inflammatory chemokine CXCL10, and cytokines IL-1 β and TNF- α , in THP-1-derived macrophages. The impact of the TLR4 agonist, LPS, on the induction of CXCL10, IL-1 β and TNF- α were assessed during the course of this project. THP-1-derived macrophages were treated with LPS at final concentrations of 100 ng/ml at timepoints ranging from 10 min to 24 h. The supernatants were harvested and analysed by ELISA to determine the protein concentration of TLR4-associated chemokines/cytokines (Fig. 7.3A). Data presented in Fig. 7.3A shows that LPS induces significantly heightened levels of CXCL10 in THP-1 macrophages following stimulation for 6 h (Fig. 7.3A). Interestingly, Fig. 7.3B shows that there is no significant induction of IL-1 β protein in THP-1 macrophages following treatment with LPS at any timepoint ranging from 10 min to 24 h. The most notable induction of IL-1 β appears to be at 3 h post-stimulation with LPS, although this was not significant (Fig. 7.3B). In addition, no significant induction of TNF- α was noted in THP-1 macrophages at the level of protein following stimulation with LPS (Fig. 7.3C). Optimal induction of TNF- α was similarly noted after 3 h post-LPS stimulation, although this did not reach significance.

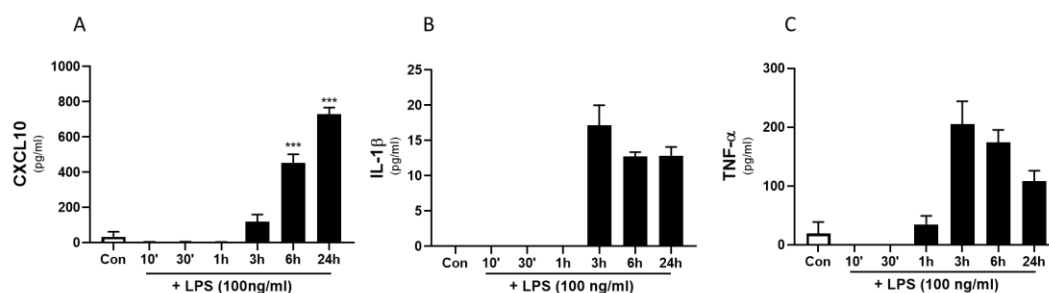


Figure 7.3 LPS significantly enhances production of CXCL10 in THP-1 macrophages. THP-1-derived macrophages were treated with LPS (100 ng/ml) for 10 min, 30 min, 1 h, 3 h, 6 h and 24 h, and the concentrations of (A) CXCL10 (B) IL-1 β and (C) TNF α determined in supernatants by ELISA. Data are presented as the mean $\pm$ S.E.M of duplicate determinations from 4 independent passages. Data were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test. * $p < 0.001$ versus untreated control cells.

7.4 LPS is not toxic to macrophages

We determined if over-production of nitrite, as a result of stimulation with LPS, was occurring in THP-1 macrophages. Overproduction of nitrite is linked to dysregulated production of cellular NO, associated with cell damage and cell death. The Griess assay was performed on THP-1 macrophages incubated with LPS (100 ng/ml) at time points ranging from 10 min-24 h (Fig. 7.4). As can be seen below in Fig. 7.4, stimulation of THP-1 macrophages with LPS did not promote nitrite production in THP-1 derived macrophages. The average nitrite concentration recorded in this was 0.12 μ M. This indicates that LPS is not toxic to THP-1 macrophages and does not induce production of nitrite at the timepoints tested.

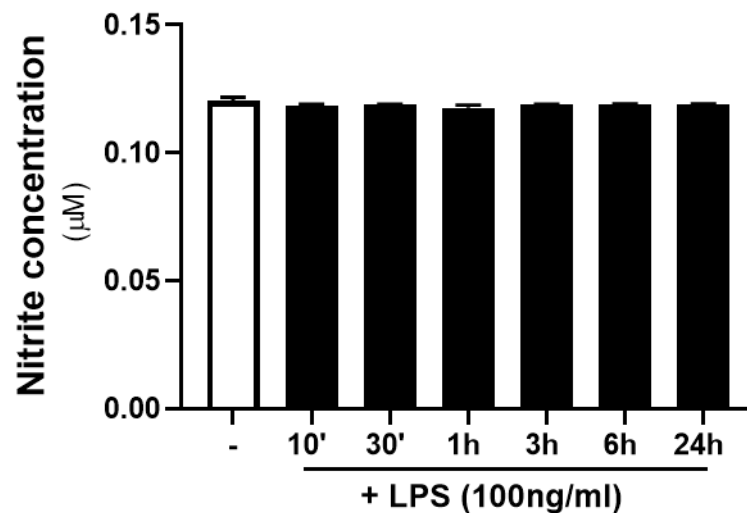


Figure 7.4 Effect of LPS on nitrite production. THP-1-derived macrophages were treated with LPS (100 ng/ml) for 10 min, 30 min, 1 h, 3 h, 6 h and 24 h, and nitrite concentrations determined at each time point via the Griess reaction. Data are presented as the mean $\pm$ S.E.M of triplicate determinations from 4 independent passages. Data were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test.

7.5 Effect of CBD on LPS-induced IL-1 β expression in THP-1 macrophages

Having determined that LPS stimulation of THP-1 macrophages induced expression of IL-1 β , although insignificantly, we sought to determine the impact of CBD on TLR4-dependent induction of the inflammatory cytokine. IL-1 β is a potent inflammatory cytokine, classically induced through activation of the inflammasome, whereby it is cleaved into its mature form from pro-IL-1 β . THP-1 monocytes were differentiated to macrophages following 48 h incubation with PMA (10 ng/ml). The cells were then incubated with CBD at [10 μ M] for 6 h. THP-1-derived macrophages were pre-treated for 30-45 mins with CBD, followed by addition of LPS at a working concentration of 100 ng/ml for 6 h. At time zero, supernatants were harvested and analysed for cytokine/chemokine expression by ELISA (Fig. 7.5). The *in vitro* portion of this experiment, whereby the cells were differentiated and stimulated with LPS/CBD was performed previously by Dr. John-Mark Fitzpatrick and Eleanor Minogue. The stored supernatants were then tested later by ELISA. It is evident in Figure 7.5 below that LPS induces heightened production of IL-1 β in THP-1 macrophages, albeit insignificant. Pre-treatment with CBD did not impact this induction. Treatment with CBD at [10 μ M] alone did not promote IL-1 β expression. This indicates that CBD does not have the proclivity to attenuate IL-1 β production in response to TLR4 activation.

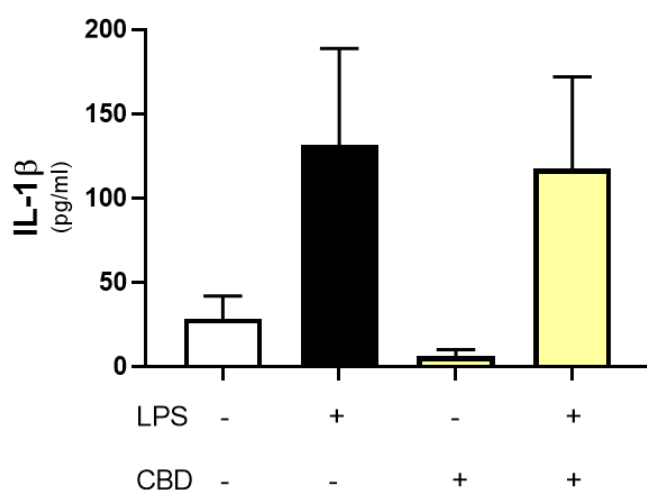


Figure 7.5 Effect of CBD on LPS-induced IL-1 β expression in macrophages. THP1-derived macrophages were pre-treated (for 45 min) with CBD (10 μ M) prior to incubation with LPS (100 ng/ml; 6 h), and the concentration of IL-1 β determined in supernatants by ELISA. Data are presented as the mean $\pm$ S.E.M of duplicate determinations from 4 independent passages. Data were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test.

7.6 Effect of CBD on LPS-induced IL-10 expression in THP-1 macrophages

IL-10 is an important anti-inflammatory cytokine and may be protective in the context of MS. Indeed, it was shown by Wei and colleagues (2019), that IL-10 may be an important biomarker in individuals with clinically isolated syndrome (CIS) whereby individuals who experienced relapses had significantly low serum levels of IL-10 than those who did not (505). THP-1 monocytes were differentiated to macrophages following 48 h incubation with PMA (10 ng/ml). THP-1-derived macrophages were pre-treated for 30-45 mins with CBD at [10 μ M], followed by addition of LPS at a working concentration of 100 ng/ml for 6 h. At time zero, supernatants were harvested and analysed for cytokine/chemokine expression by ELISA (Fig. 7.6). The *in vitro* part of this experiment, where cells were differentiated and then stimulated with LPS/CBD was carried out previously in the laboratory by Dr. John-Mark Fitzpatrick and Eleanor Minogue. The stored supernatants were tested later by ELISA. LPS and CBD did not significantly impact the expression of anti-inflammatory IL-10 in THP-1 macrophages.

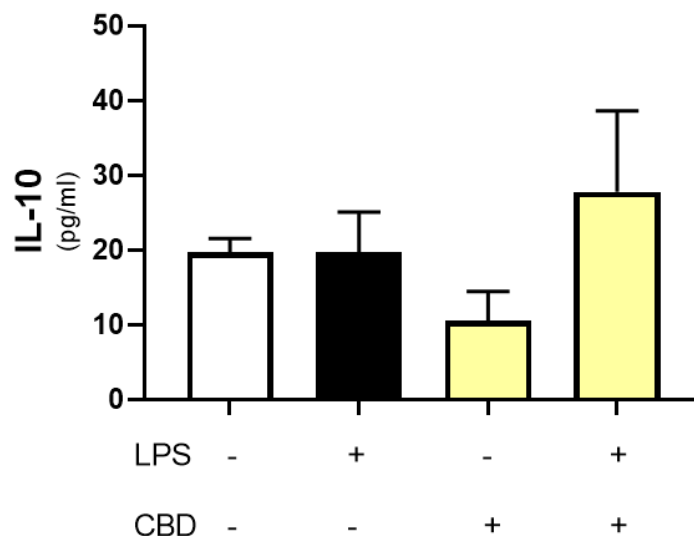


Figure 7.6 Effect of CBD and LPS on IL-10 expression in macrophages. THP1-derived macrophages were treated (for 45 min) with CBD (10 μ M) prior to incubation with LPS (100 ng/ml; 6 h). The concentration of IL-10 was determined in supernatants by ELISA. Data are presented as the mean $\pm$ S.E.M of duplicate determinations from 4 independent passages. Data were analysed using the Kruskal-Wallis test and Dunn's multiple comparisons test.

7.7 Correlations between IL-1 β , TNF- α and IFN- β expression in plasma in whole blood samples isolated from pwMS and sysmex data

To determine whether there was any correlation between the concentrations of IL-1 β , TNF- α and IFN- β measured in plasma isolated from pwMS and cellular components in whole blood, we conducted correlations between the concentrations of IL-1 β , TNF- α and IFN- β in plasma and the WBC number, MXD number, LYM number and the NEUT numbers in whole blood (Fig. 7.7). As can be seen below in Figure 7.5, a positive correlation between the concentration of both IL-1 β and TNF- α in plasma from pwMS, and the MXD number was identified ($p=0.0027$, $p=0.0008$, respectively) (Fig. 7.7B, F). This suggests that the presence of such pro-inflammatory cytokines in the periphery of individuals with MS is related to the number of lymphocytes, neutrophils and other immune cells in whole blood. There was a trend towards a negative correlation between the concentration of IFN- β in plasma and the number of neutrophils in whole blood isolated from pwMS. This suggests that increased neutrophil numbers in whole blood isolated from pwMS may correlate with reduced expression of IFN- β in plasma.

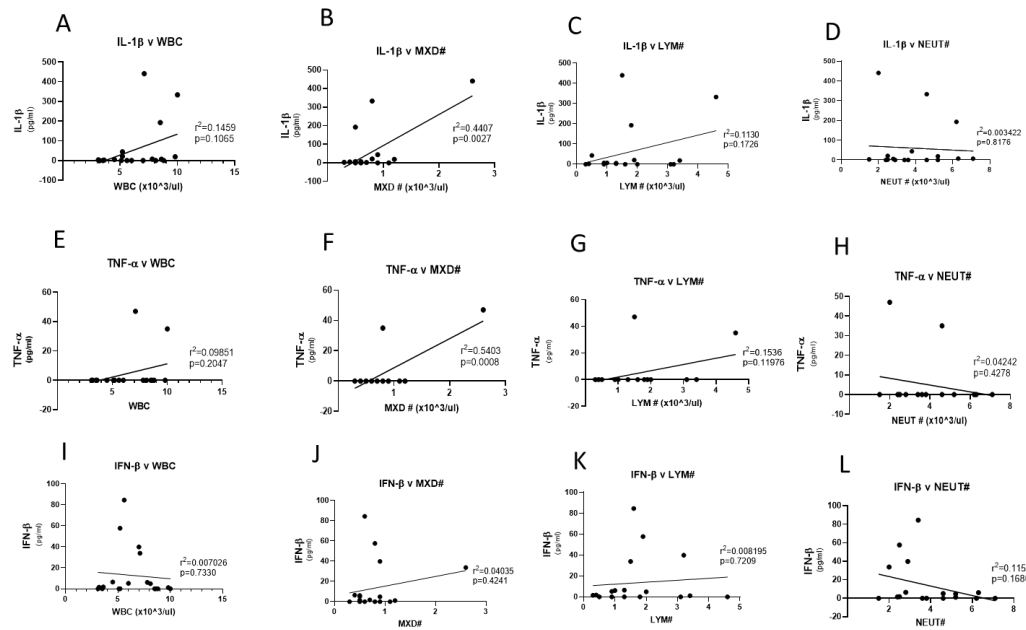


Figure 7.7 Correlations between sysmex data and the concentrations of IL-1 β , TNF- α and IFN- β in plasma isolated from pwMS . (A) Concentration of IL-1 β in plasma from pwMS v WBC in whole blood isolated from pwMS. **(B)** Concentration of IL-1 β in plasma from pwMS v MXD# in whole blood isolated from pwMS. **(C)** Concentration of IL-1 β in plasma from pwMS v LYM# in whole blood isolated from pwMS. **(D)** Concentration of IL-1 β in plasma from pwMS v NEUT# in whole blood isolated from pwMS. **(E)** TNF- α concentration in plasma from pwMS v WBC in whole blood isolated from pwMS. **(F)** Concentration of TNF- α in plasma from pwMS v MXD# in whole blood isolated from pwMS. **(G)** Concentration of TNF- α in MS plasma v LYM# in whole blood isolated from pwMS. **(H)** Concentration of TNF- α in plasma from pwMS v NEUT# in whole blood isolated from pwMS. **(I)** IFN- β concentration in plasma from pwMS v WBC in whole blood isolated from pwMS. **(J)** Concentration of IFN- β in plasma from pwMS v MXD# in whole blood isolated from pwMS. **(K)** Concentration of IFN- β in MS plasma v LYM# in whole blood isolated from pwMS. **(L)** Concentration of IFN- β in plasma from pwMS v NEUT# in whole blood isolated from pwMS. Data was presented as mean concentration (pg/ml). Linear regression was performed and Pearson's r value reported. $p < 0.05$ was deemed statistically significant.

7.8 Correlations between *CNR1*, *CNR2*, and *FAAH* mRNA expression in PBMCs isolated from pwMS and HCs

Data presented in this thesis indicate that the expression of both *CNR1* and *CNR2* mRNA in PBMCs from pwMS significantly correlated with the expression of *MAGL* mRNA. We sought to determine if any significant correlations were present between the mRNA expression of the cannabinoid receptors *CNR1/CNR2* and *FAAH* mRNA in PBMCs from pwMS and HCs. Interestingly, as can be seen in Fig. 7.8, there was no significant correlation between *FAAH* mRNA expression and *CNR1/CNR2* mRNA in both cohorts. This suggests that the expression of *FAAH* mRNA in peripheral immune cells may not bear any relationship to the expression of cannabinoid receptors (Fig. 7.8).

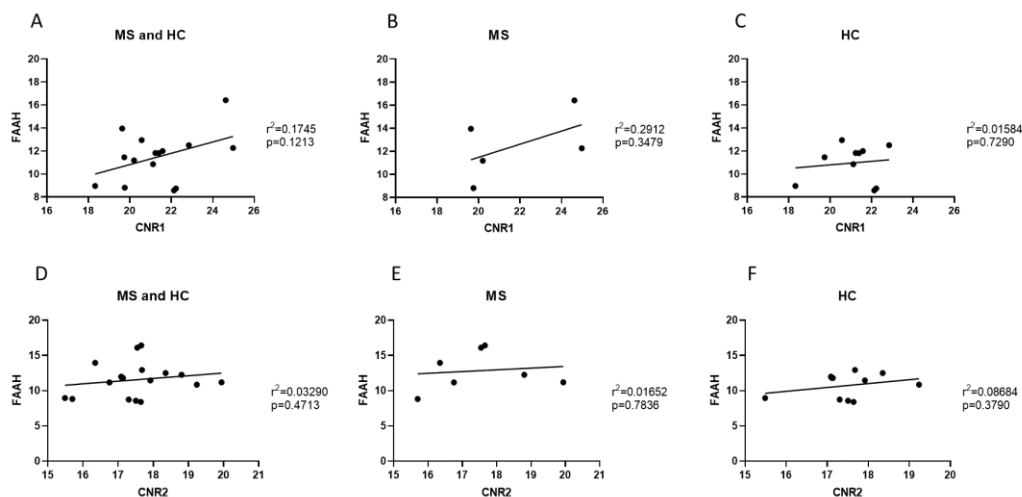


Figure 7.8 *FAAH* mRNA expression does not correlate with expression of *CNR1* or *CNR2* in PBMCs isolated from HCs and pwMS. PBMCs were isolated from whole blood of HCs and pwMS. RNA was extracted, cDNA synthesis was performed and the basal expression of *CNR1* and *CNR2* mRNA, as well as *FAAH* mRNA, was assessed via RT-qPCR. Delta CT values are presented. Linear regression was performed and Pearson's *r* value is reported. **(A)** Basal expression of *CNR1* v basal expression of *FAAH* in PBMCs across both HC and MS cohorts. **(B)** Basal expression of *CNR1* v basal expression of *FAAH* in PBMCs in the MS cohort. **(C)** Basal expression of *CNR1* v basal expression of *FAAH* in PBMCs from HCs. **(D)** Basal expression of *CNR2* v basal expression of *FAAH* across both MS and HC cohorts. **(E)** Basal expression of *CNR2* v basal expression of *FAAH* in PBMCs in the MS cohort. **(F)** Basal expression of *CNR2* v basal expression of *FAAH* in PBMCs in HCs. Data was presented as mean delta CT value.

7.9 ImageJ assessment of demyelination and Inkscape tracing of NAWM and CALs in post-mortem tissue from non-MS control cases

Demyelination is important in MS pathophysiology due to its role in axonal damage. Demyelinating lesions in the CNS of pwMS can also impact clinical symptoms of disease such as visual, sensory and motor dysfunction. In addition to the five representative images shown in chapter 5 (Figure 5.1B), 25 further formalin-fixed paraffin-embedded sections of post-mortem brain tissue from non-MS control cases, PPMS and SPMS cases were provided by the UK MS Brain Bank. These sections were stained with LFB by Mr Richard Magee and Dr Siew Mei Yap previously in the laboratory. Overall, thirty images were stained with LFB, 8 of these were non-MS control cases, 12 were PPMS cases (6 NAWM/6 NAWM containing CALs) and 12 were SPMS cases (6 NAWM/6 NAWM containing CALs). We sought to determine the percentage of demyelination occurring within each sample. Images of demyelination mapping in non-MS control cases, as determined via staining with LFB, are included in Fig. 7.9. Using ImageJ, a scale was set using the scale provided in each image, areas of demyelination were mapped, and the total area of demyelination calculated (Fig. 7.9). The percentage demyelination in each sample is outlined in Table 12. The Inkscape programme was then used to map both the NAWM and NAGM in each sample, and is displayed alongside the representative LFB-stained image showing demyelination below.

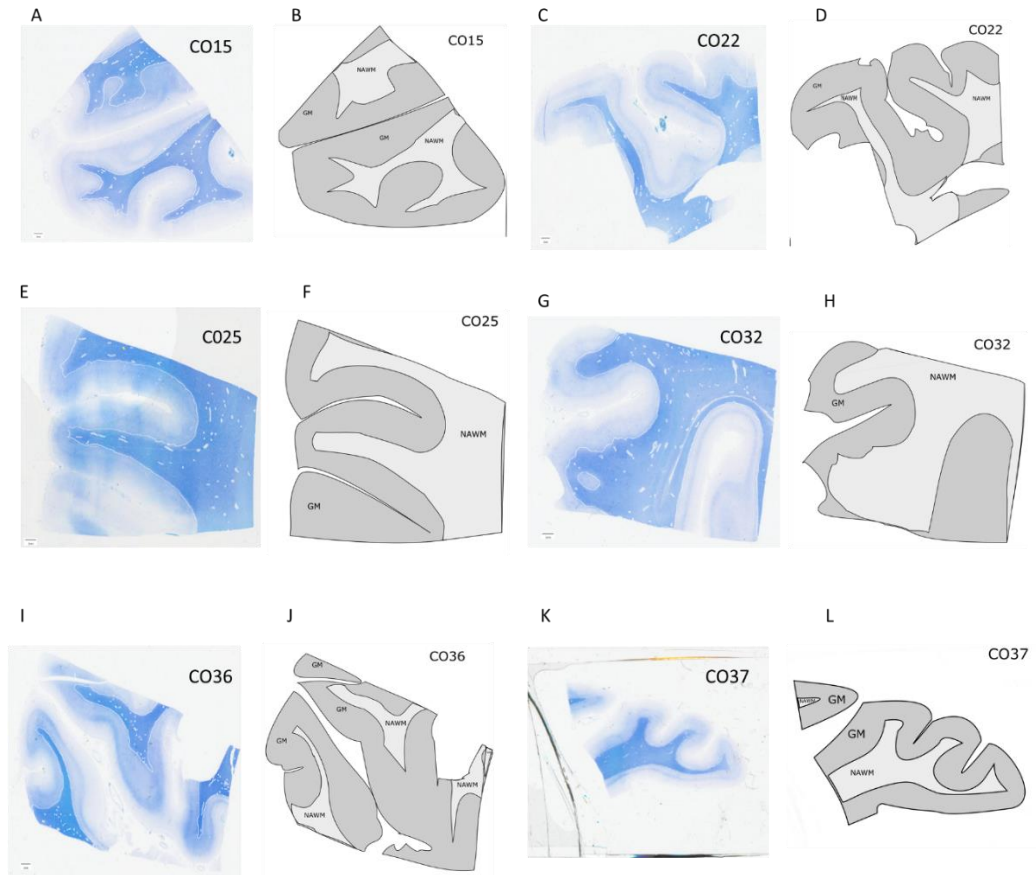
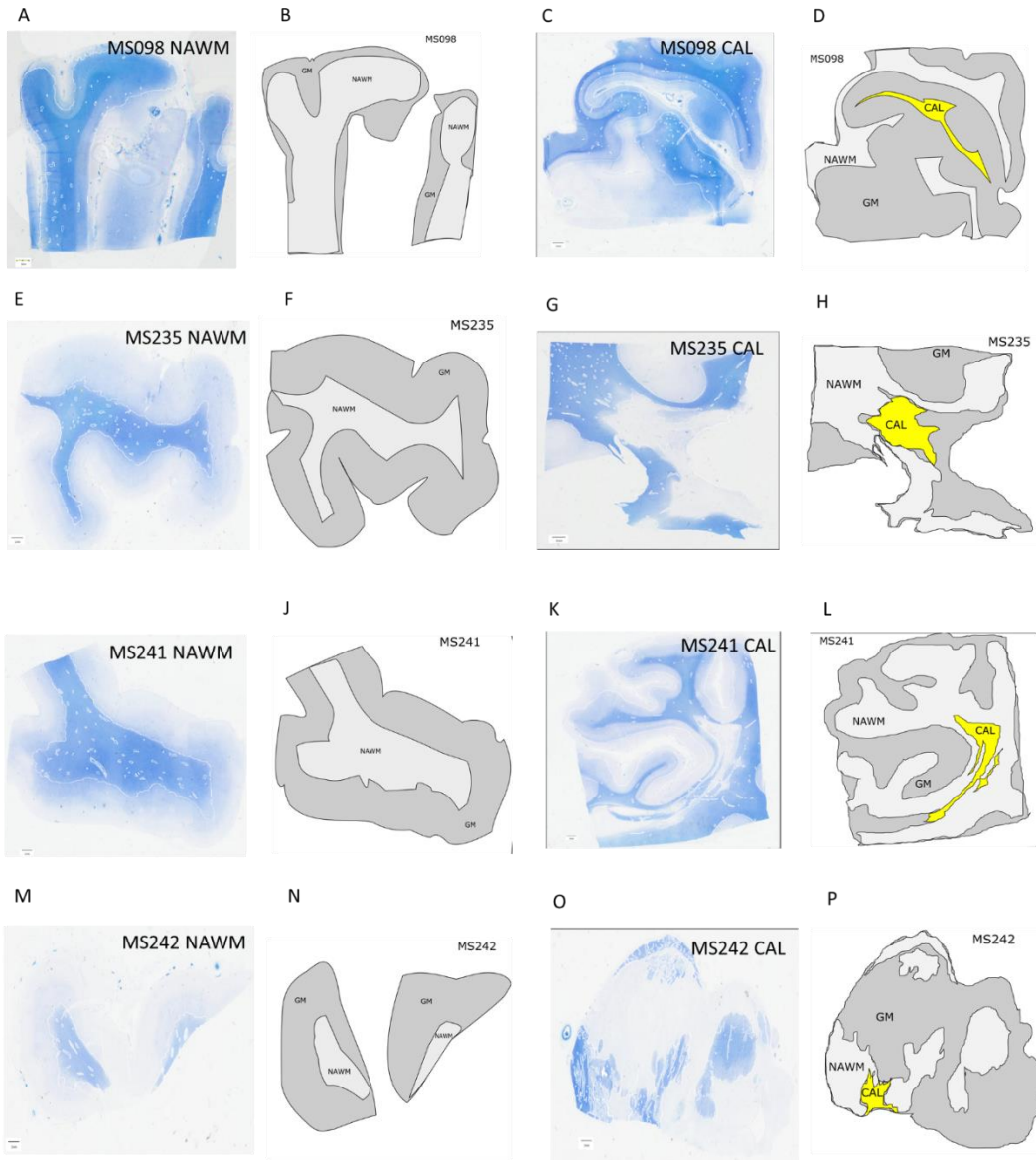


Figure 7.9 Demyelination as assessed using ImageJ and Inkscape mapping of NAWM/NAGM in post-mortem cortex of non-MS control cases. Demyelination in non-MS control cases was determined using the ImageJ programme, as is shown in **(A, C, E, G, I, and K)**. Demyelination is marked and outlined in white in each of these images. In addition, mapping of the NAWM and NAGM in the same tissue was performed using the Inkscape programme. These maps are shown in **(B, D, F, H, J, L)**. NAWM is denoted by an off-white colour and GM is denoted in grey.

7.10 ImageJ assessment of demyelination and Inkscape tracing of NAWM and CALs in post-mortem tissue from SPMS cases

In addition to non-MS control cases we received 12 cortical sections from the brain of SPMS cases (6 NAWM containing a CAL, and 6 NAWM). These sections were stained using LFB by Mr Richard Magee and Dr Mei Yap previously in the laboratory. Similar to the non-MS control cases, an assessment and mapping of demyelination within each section of stained NAWM was assessed using the ImageJ programme (Fig. 7.10). Inkscape was used to map the section of NAWM, including any CALs which are denoted in yellow (Fig. 7.10). The total demyelination occurring within both SPMS NAWM and within NAWM containing a CAL, are outlined below in Table 12/13.



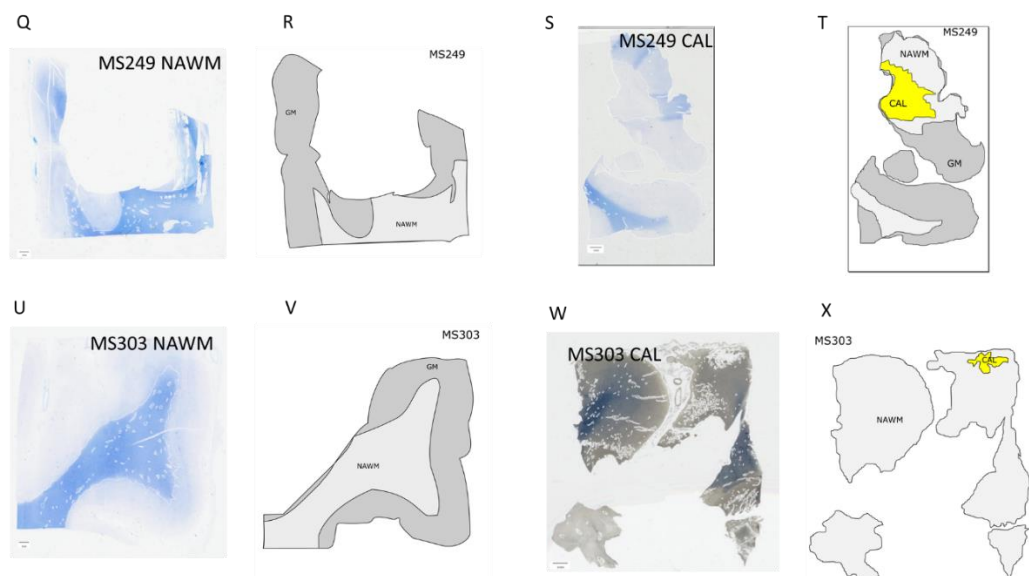
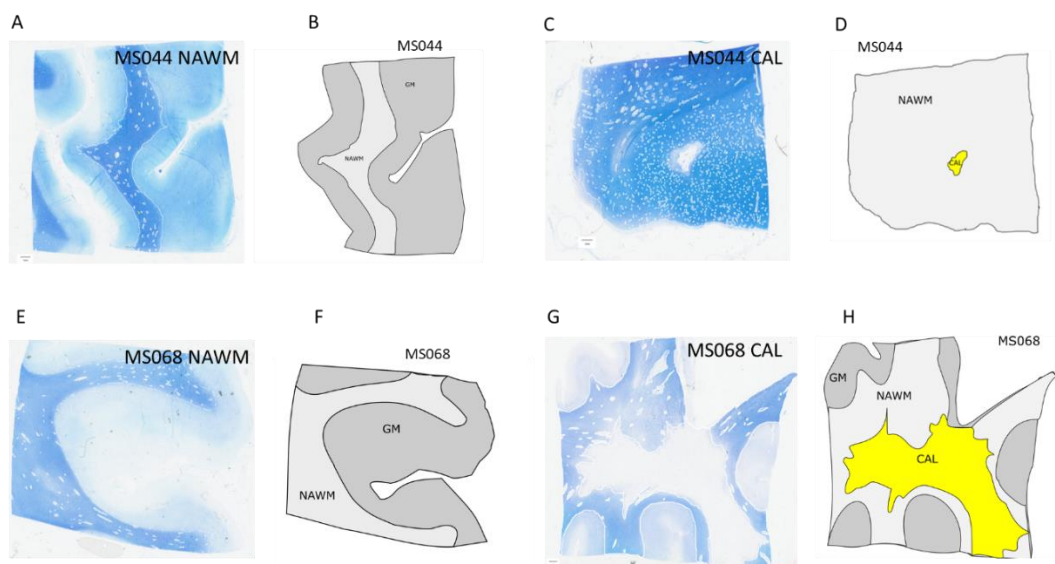


Figure 7.10 Demyelination in sections of NAWM and CALs from SPMS cases.

Demyelination was determined by measuring areas of demyelination in LFB-stained images of cortical brain sections. Images denoting demyelination in SPMS cases are shown in (A, C, E, G, I, K, M, O, Q, S, U, W) while mapping of the NAWM, NAGM and CALs were performed using the Inkscape programme, as can be seen in (B, D, F, H, J, L, N, P, R, T, V, X) . NAWM is denoted by an off-white colour, CALs are denoted in yellow and GM is denoted by grey.

7.11 ImageJ assessment of demyelination and Inkscape tracing of NAWM and CALs in post-mortem tissue from PPMS cases

Twelve sections of stained NAWM were also provided by the UK MS Brain Bank (6 NAWM, 6 NAWM containing a CAL). Each PPMS section sample had an accompanying sample of NAWM containing a CAL. Each section provided was stained with LFB by Mr Richard Magee and Dr Siew Mei Yap, to allow the quantification of demyelination. The percentage demyelination in each section was determined using the ImageJ programme (Table 12, 13), and the LFB-stained images which have been mapped for demyelination (Fig. 7.11). Subsequently, the Inkscape programme was employed to map out the tissue, indicating the NAWM, GM and CALs (Fig. 7.11). NAWM is denoted as off-white, GM is shown as grey and CALs are denoted in yellow. The total percentage demyelination in all PPMS cases, those containing a CAL and those not containing a CAL, are outlined in Table 12/13 below.



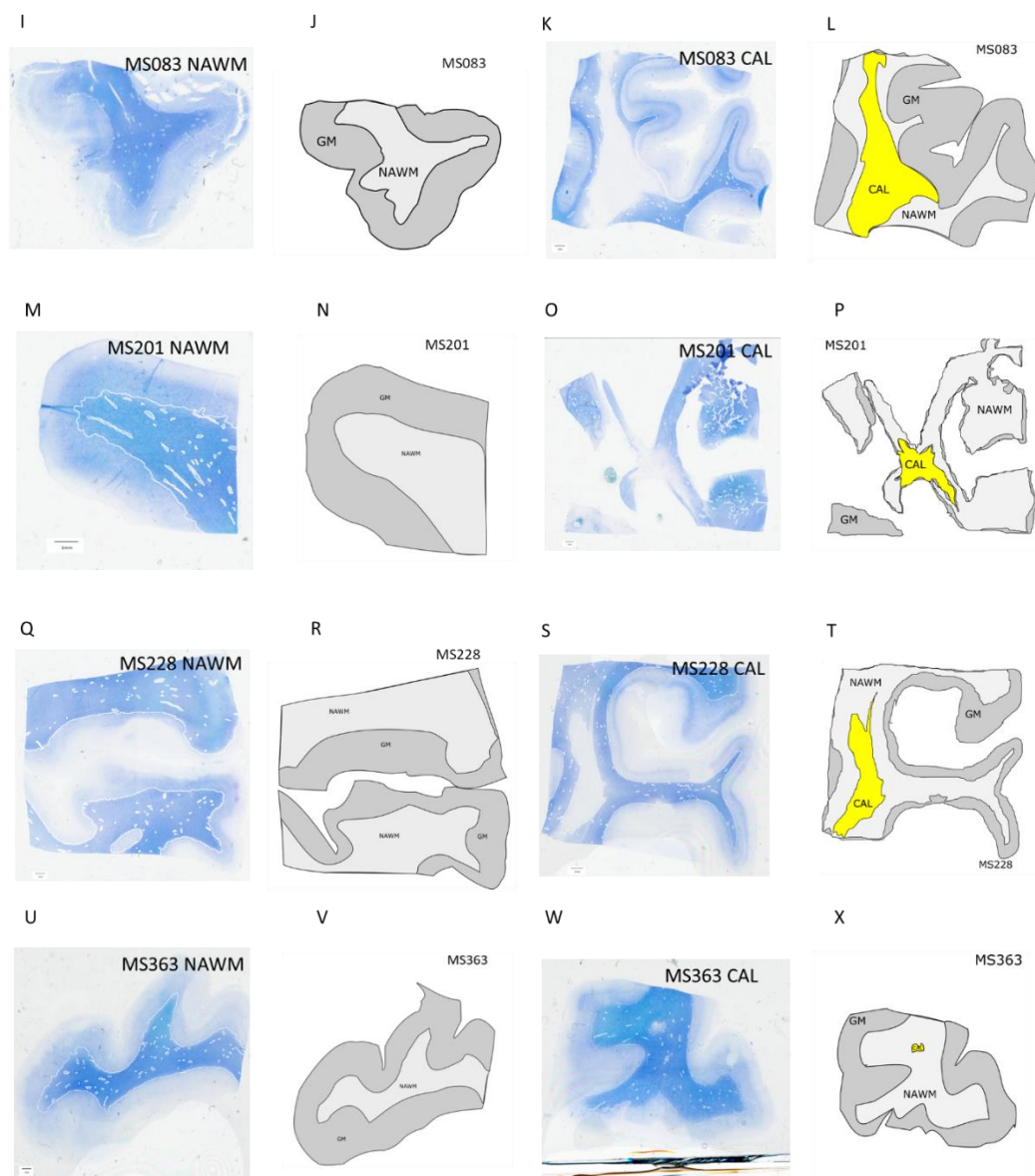


Figure 7.11 Demyelination assessment in sections of NAWM and CALs from PPMS cases. Demyelination was determined in LFB-stained images of cortical brain sections. LFB-stained images of PPMS cases assessed for percentage demyelination in ImageJ are shown in (A, C, E, G, I, K, M, O, Q, S, U, W). Inkscape was used to map the NAWM, GM and CALs in samples. These mappings are shown in (B, D, F, H, J, L, N, P, R, T, V, X). As above, NAWM is denoted by an off-white colour, CALs are yellow and GM is denoted by grey.

7.11 Demyelination assessment in non-MS control, PPMS and SPMS cases

Demyelination is a hallmark of MS pathogenesis and contributes to the damage and loss of neurons and subsequent disability associated with MS. As previously stated, we sought to determine the percentage of demyelination that had occurred within both SPMS (NAWM and CAL) and PPMS (NAWM and CAL) subcortical tissue, and compare the degree of demyelination determined to non-MS cases. Overall, 30 sections of post-mortem brain tissue was obtained from the UK MS Brain Bank: 8 non-MS controls, 12 SPMS (6 NAWM/ 6 containing a CAL) and 12 PPMS (6 NAWM, 6 CAL). Ethical approval was given to work on these samples (Appendix 2). These post-mortem tissue sections were stained previously with LFB in the laboratory by Mr Richard Magee and Dr Siew Mei Yap. Images of these stained samples were analysed using the ImageJ programme. The scale provided with each sample was used to set a scale. Following this, each area of demyelination, as denoted in white, was outlined and its area determined. The total area of demyelination was thus determined. This figure was subsequently divided the total area of NAWM tissue in the sample, this was multiplied by 100 to yield the percentage demyelination value observed in each sample of NAWM. Table 12 below shows the percentage demyelination observed in all non-MS control cases and all NAWM tissue from both PPMS and SPMS cases. NAWM tissue with a CAL (6 PPMS/ 6 SPMS) was also provided from each SPMS case and PPMS case. Although the CAL in NAWM of these cases was generally responsible for the largest degree of demyelination determined in the NAWM sample, there were separate areas of demyelination observed in sections containing a CAL. Thus, total demyelination within the section was assessed, as well as the demyelination observed within the CAL alone. We display these values in Table 13.

Table 13. Percentage demyelination in subcortical post-mortem brain tissue in non-MS control cases, SPMS cases and PPMS cases

Code	Sample Type	Percentage demyelination (%)
CO15	Control	1.48%
CO22	Control	2.15%
CO25	Control	1.27 %
CO32	Control	2.83%
CO36	Control	1.91%
CO37	Control	0.83%
MS098	SPMS	0.99%
MS235	SPMS	2.89%
MS241	SPMS	2.76%
MS242	SPMS	4.60%
MS249	SPMS	9.54%
MS303	SPMS	2.72%
MS044	PPMS	3.79%
MS068	PPMS	2.62%
MS083	PPMS	1.65%
MS201	PPMS	4.32%
MS228	PPMS	3.52%
MS363	PPMS	2.64%
MS: multiple sclerosis; SPMS: secondary progressive MS; PPMS: primary progressive MS		

Table 14. Percentage demyelination in NAWM tissue containing CALs in SPMS and PPMS cases

Code	Sample type	Percentage demyelination in CAL alone (%)	Percentage demyelination in overall sample (%)
MS098	SPMS	8.69%	9.78%
MS235	SPMS	13.97%	15.63%
MS241	SPMS	6.94%	7.83%
MS242	SPMS	6.88%	29.83%
MS249	SPMS	12.43%	13.44%
MS303	SPMS	1.39%	11.25%
MS044	PPMS	0.84%	7.38%
MS068	PPMS	37.94%	39.98%
MS083	PPMS	34.32%	35.44%
MS201	PPMS	18.75%	21.58%
MS228	PPMS	17.20%	18.63%
MS363	PPMS	1.27%	2.51%
MS: multiple sclerosis; SPMS: secondary progressive MS; PPMS: primary progressive MS; CAL: chronic active lesion			