Assessing Toll-like receptor 3/4 signalling as a phytocannabinoid target in immune cells

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By

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Declaration of authorship

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Ms. Lucy Curham contributed to experiments in Fig. 3.4, 3.11, and 4.5
Ms. Eleanor Minogue contributed to experiments in Fig. 4.3, 4.4, 4.5
Mr. Phillip Gavigan contributed to experiments in Fig. 6.2.
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Abstract

Toll-like receptors (TLRs) are the sensors of pathogen associated molecules that trigger tailored innate immune intracellular signalling responses. TLRs are expressed on cells of the immune system and play an important role in immune cell activation and inflammatory responses. Indeed, TLRs have been implicated in many diseases, with data from human and animal studies identifying TLRs as players in the pathogenesis of multiple sclerosis (MS). Uncontrolled and atypical activation of TLR signalling can result in neuroinflammation, and in macrophages, innate immune responses to bacterial (via TLR4) and viral (via TLR3) signalling is key in mediating cellular inflammation. Targeting TLR3/4 signalling with novel therapeutics may represent an important avenue on the road to developing improved therapies for MS, and possibly other neuroinflammatory conditions.

Cannabinoids are biologically active compounds extracted from the hemp plant *Cannabis sativa* L. (*C. sativa*), commonly known as phytocannabinoids, synthesised in our bodies (endogenous cannabinoids) or are artificially created (synthetic cannabinoids). Cannabinoids can reduce the symptoms associated with experimental autoimmune encephalomyelitis (EAE), the murine model of MS, and clinical studies have demonstrated the therapeutic potential of phytocannabinoids in people with (pw)MS. Indeed, Sativex is an oromucosal spray containing cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) as its most abundant phytocannabinoid components, in addition to other phytocannabinoids and non-phytocannabinoid components, and has been shown to palliate symptoms associated with MS. The full cellular mechanism of action of the components of Sativex in human cells is unclear. A growing body of literature indicates that cannabinoids can modulate TLR-induced inflammatory signalling events. Indeed, evidence suggests that TLR3 signalling via TIR-domain-containing adaptor-inducing IFN-β (TRIF)-dependent activation of interferon regulatory factor 3 (IRF3), in addition to TLR4 signalling via myeloid differentiation primary response 88 (MyD88) to nuclear factor (NF)-κB, may be targeted by cannabinoids to regulate cellular inflammation.

The data presented herein characterised TLR3 and TLR4 signalling in the human THP-1 monocyte cell line, THP-1-derived macrophages and primary human peripheral blood mononuclear cells (PBMCs) in terms of inflammatory cytokine (TNFα) chemokine (CCL5, CXCL10) and type I interferon (IFN)-β expression. This study demonstrates that THP-1 monocytes are a poor model for investigating TLR3-induced signalling mechanisms and that TLR3 and TLR4 signalling is operative in THP-1-derived macrophages. Importantly, CBD and THC differentially targeted MyD88-dependent and independent signalling mechanisms via TLR3 and TLR4 in THP-1-derived macrophages, in terms of IFN-β and CXCL10 expression. Data also suggests that THC and CBD do not target TLR signalling via the cannabinoid receptors (CB_1 and CB_2) and the PPARγ receptor in THP-1 macrophages. In addition, the effects of THC and CBD were examined in PBMCs isolated from pwMS and healthy control (HC) donors. PBMC data identify THC and CBD as potential novel regulators of TLR3/4 signalling in primary immune cells, and highlights possible mechanisms to be targeted in the development of new cannabinoid therapeutics for the treatment of disorders such as MS. Finally, the effect of a range of novel highly purified botanically-derived cannabinoids on the viability of THP-1 monocytes, THP-1 macrophages, and PBMCs from HC donors and pwMS was determined. Findings here suggest that phytocannabinoids are generally well tolerated by immune cells, however the effect was dependent on the cannabinoid used, the concentration administered, and the immune cell examined. Overall this study offers mechanistic insight on the role of phytocannabinoids in modulating cellular inflammatory signalling events controlled by TLR3/4.

Publications

Peer-reviewed published papers:

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4. **Fitzpatrick J-M**, Anderson RC, McDermott KW. MicroRNA: Key regulators of oligodendrocyte development and pathobiology. *Int J Biochem Cell Biol*. 2015;65:134-138. DOI: 10.1016/j.biocel.2015.05.021

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Courses

- Postgraduate Certificate in Innovation and Entrepreneurship completed as part of the structured aspect of my PhD (30 ECTs).
- Venepuncture training course completed. Registered phlebotomist since July 2017.

List of abbreviations

2-AG - 2-arachidonyl glycerol

$5\text{-}HT_{1A}$ - serotonin receptors

AC - adenylate cyclase

AD - Alzheimer's disease

AEA - anandamide

AMPK - adenosine monophosphate-activated protein kinase

AP-1 - activator protein-1

APCs - antigen presenting cells

Aβ - amyloid-beta

BBB - blood brain barrier

BG-12 - Dimethyl fumarate

C. sativa - *Cannabis sativa* L.

cAMP - cyclic AMP

CB_1 and CB_2 - cannabinoid receptors

CBC - cannabichromene

CBCA - cannabichromenic acid

CBD - cannabidiol

CBDA - cannabidiolic acid

CBDV - cannabidivarin

CBG - cannabigerol

CBGA - cannabigerolic acid

CBGV - cannabigevarin

CD14 - cluster of differentiation 14

cDNA - Complementary DNA

CIS - clinically isolated syndrome

CNS - central nervous system

CSF - cerebrospinal fluid

Ct – cycle threshold

CTCF - corrected total cell fluorescence

DAMPs - danger-associated molecular patterns

DCs - dendritic cells

DMTs - disease-modifying therapies

dsRNA - double stranded RNA

EAE - experimental allergic encephalomyelitis

EBV - Epstein-Barr virus

ECS - endogenous cannabinoid system

EDSS - expanded disability status scale

ELISA - Enzyme-linked Immunosorbent Assay

EMA - European medicines agency

ERK - extracellular signal-regulated kinase

FAAH - fatty acid amide hydrolase

FDA - United States food and drug administration

GA - Glatiramer acetate

GPP - geranyl diphosphate

GPR55 - G protein coupled receptor 55

HC - healthy control

HCT - haematocrit

HGB - haemoglobin

HPRA - Health Products Regulatory Authority

HSCT - Hematopoietic stem cell transplantation

IFNAR - IFN-α receptor

IFNs - interferons

IKK - inhibitor of kappaB kinase

IL - Interleukin

IRAK - IL-1R-associated kinase

IRF3 - IFN regulatory factor 3

ISG - IFN-stimulated gene

ISGF3 - IFN-stimulated gene factor 3

ISREs - IFN-stimulated response elements

IκB - inhibitor of κB

JAK1 - Janus kinase 1

JNK - c-Jun-N-terminal kinase

LPS - lipopolysaccharide

LRR - leucine-rich repeat

MAGL monoacylglycerol lipase

MAP - mitogen-activated protein

MBP - myelin basic protein

MCH - mean corpuscular haemoglobin

MCHC - mean corpuscular haemoglobin concentration

MCP-1 - monocyte chemoattractant protein 1

MCV - mean corpuscular volume

MEP - plastidal 2-C-methyl-D-erythritol 4-phosphate

MHC - major histocompatibility complex

miRNAs - micro-RNAs

MMD – major depressive disorder

MRI - magnetic resonance imaging

MS - Multiple Sclerosis

MSQOL-54 – Multiple Sclerosis Quality of Life-54

mTOR - mechanistic target of rapamycin

MxA - myxovirus resistance A

MyD88 - myeloid differentiation primary response 88

NADA - N-arachidonoyl dopamine

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

NGS - normal goat serum

NK - natural killer cells

NLRs – NOD-like receptors

NO - nitric oxide

NOD - nucleotide-binding and oligomerization domain

NOS - nitric oxide synthase

NPA - nitropropionic acid

Nrf2 - nuclear factor erythroid 2-related factor

OLA - olivetolic acid

OPCs - oligodendrocyte precursor cells

OXPHOS - oxidative phosphorylation

PAMPs - pathogen-associated molecular patters

PBMCs - peripheral blood mononuclear cells

PD - Parkinson's disease

PKA - protein kinase A

PLGA - polymeric biodegradable lactic-glycolic acid

PLTs - platelets

PMA - Phorbol myristate acetate

polyI:C - Polyinosine-polycytidylic

PPAR - peroxisome proliferator-activated receptor

PPMS - primary-progressive MS

PRDs - positive regulatory domains

PRMS - progressive-relapsing MS

PRRs - pathogen-recognition receptors

pwMS - People with MS

QIDS-SR_{16} - Quick Inventory of Depressive Symptomatology (16-item) (self-report)

RA - rheumatoid arthritis

RBC - red blood cell

RDW - red blood cell distribution width

RIG-I - retinoic acid inducible gene-I

RIP-1 - receptor interacting protein-1

RLRs – RIG-1-like receptors

ROS - reactive oxygen species

RRMS - relapsing-remitting MS

rRNA - ribosomal RNA

RT-qPCR - real-time quantitative polymerase chain reaction

S1P - sphingosine 1-phosphate

SARS-CoV-2 - severe acute respiratory syndrome coronavirus 2

sCB - synthetic cannabinoids

SEM - standard errors of the mean

SLE - systemic lupus erythematosus

SPMS - secondary-progressive MS

SR141716A - CB_1 antagonist

ssRNA - single stranded RNA

STAT - signal transducer and activator of transcription

TAK1 - transforming growth factor beta-activated kinase 1

THC - Δ^9-Tetrahydrocannabinol

THCA - Tetrahydrocannabinolic acid

THCV - tetrahydrocannabivarinic

TIR - Toll/IL-1 receptor

TLRs - Toll-like receptors

TMEV - Theiler’s murine encephalomyelitis virus

TNF - tumour necrosis factor

TOLLIP - Toll-interacting protein

TRAF6 - TNF-receptor-associated factor 6

Tregs - regulatory T cells

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

TRPA1 - TRP ankyrin-1

TRPV1 - transient receptor potential cation channel subfamily V member 1

TYK2 - tyrosine kinase 2

URB597 - FAAH inhibitor

WBC - white blood cell

Contents

Declaration of authorship i

Acknowledgements ii

Abstract iv

Publications vi

Awards vii

Courses vii

List of abbreviations viii

List of Figures xvi

List of Tables xviii

Chapter 1: Introduction 1

Introduction 2

- 1.1 Introduction to Innate Immunity 2
- 1.2 TLRs 2
- 1.3 TLR signalling mechanisms 3
- 1.4 TLR3 5
- 1.5 TLR4 7
- 1.6 THP-1 cells: a cell culture model to investigate TLR signalling 8
- 1.7 Multiple Sclerosis 9
- 1.8 Pathology and Types of MS 10
- 1.9 Pathogenesis and Aetiology of MS 12
- 1.10 Current treatments of MS 14
- 1.11 New and emerging treatments in MS 18
- 1.12 The role of TLR3 and TLR4 signalling in disease: focus in MS pathogenesis 19
- 1.13 Cannabis and Cannabinoids 23
- 1.14 Phytocannabinoids 23
- 1.15 The endogenous cannabinoid system (ECS) 26
- 1.16 sCB 28
- 1.17 Cannabinoids and neuroinflammation: focus on MS 28
- 1.18 The role of cannabinoids in innate immunity 30
- 1.19 The effect of cannabinoids on cellular metabolism and viability 34
- 1.20 Cannabis based medicines 36
- Hypothesis 38
- Overall aims 38

Chapter 2: Materials and methods 39

- 2.1 Culture of cell lines 40
- 2.1.1 Culture of the THP-1 monocyte cell line 40
- 2.1.2 Phorbol myristate acetate (PMA)-induced THP-1 monocyte differentiation 40
- 2.2 Isolation of PBMCs 40
- 2.3 Drug treatments 41
- 2.3.1 LPS stimulation 41
- 2.3.2 Poly(I:C) stimulation 41
- 2.3.3 Cannabinoid treatment 42
- 2.4 Thiazoyl blue (MTT) cytotoxity assay 42
- 2.5 Immunocytochemistry 43

2.5.1 Preparation of sterile coverslips 43
2.5.2 IRF-3/NF-κB immunocytochemistry 43
2.6 Enzyme-linked Immunosorbent Assay (ELISA) 45
Day One 45
Day Two 45
2.7 Quantitative real-time polymerase chain reaction 46
2.8 Western immunoblotting 47
2.9 Quick Inventory of Depressive Symptomatology (16-item) (self-report) (QIDS-SR_{16}) questionnaire, Quality of Life-54 (MSQOL-54) questionnaire and blood count profiling 48
2.10 Statistical Analysis 49
Chapter 3 51
Characterisation of TLR3 and TLR4 signalling events in THP-1 monocytes, THP-1-derived macrophages, and primary human peripheral blood mononuclear cells 51
3.1 Introduction 52
Aims 53
3.2 TLR3 is expressed in THP-1 monocytes and poly(I:C) does not regulate *TLR3* or *TLR4* mRNA expression 54
3.3 TLR3 activation does not modulate TNFα, RANTES or IFN-β expression in THP-1 monocytes 56
3.4 TLR3 is expressed on THP-1-derived macrophages and treatment with poly(I:C) increases *TLR3*, but not *TLR4*, mRNA 58
3.5 TLR3 activation enhances nuclear IRF3 expression and downstream induction of IFN-β and CXCL10 mRNA/protein in THP-1-derived macrophages 60
3.6 TLR3 is expressed on primary human PBMCs, and treatment with poly(I:C) does not increase *TLR3* or *TLR4* mRNA in PBMCs 62
3.7 TLR3 activation does not increase RANTES or TNFα protein, in addition to *IFN-β* mRNA, in PBMCs seeded at a low density 64
3.8 Poly(I:C) treatment increases IFN-β and CXCL10 (mRNA and protein) expression, in addition to *TLR3* mRNA, in PBMCs seeded at a high density 65
3.9 TLR4 is expressed on THP-1 monocytes 68
3.10 TLR4 activation induces RANTES, TNFα and IFN-β expression in THP-1 monocytes 69
3.11 TLR4 is expressed in THP-1-derived macrophages 71
3.12 TLR4 activation induces NF-κB and IRF3 activation, IκB-α degradation, while promoting the downstream expression of TNFα, IFN-β and CXCL10 in THP-1-derived macrophages 73
3.13 TLR4 is expressed in primary human PBMCs and the effect of LPS treatment on TLR4 receptor expression in PBMCs 76
3.14 Effect of LPS on TNFα, IFN-β and CXCL10 expression in primary PBMCs 78
3.15 Discussion 80
Kinetics of response to TLR3 activation in human immune cells 80
Kinetics of response in human immune cells to TLR4 activation 83
Results Chapter 4 86
THC and CBD differentially target TLR3 and TLR4 signalling events in THP-1 monocytes and THP-1-derived macrophages 86
4.1 Introduction 87
Aims 88

4.2 CB_1 and CB_2 cannabinoid receptors are expressed in THP-1 monocytes and THP-1-derived macrophages 89
4.3 THC and CBD do not attenuate TLR4-induced TNFα or RANTES expression in THP-1 monocytes 90
4.4 The impact of THC, CBD, and a THC:CBD combination on THP-1 monocyte viability 92
4.5 CBD, THC and THC:CBD (1:1) inhibit TLR3-induced IRF3 activation and induction of CXCL10/IFN-β in THP-1 macrophages 93
4.6 Effect of CBD and THC on TLR4-induced IκB-α degradation, NF-κB nuclear sequestration and TNFα/CXCL8 protein production in THP-1-derived macrophages 95
4.7 CBD and THC regulate TLR4-induced IRF3, CXCL10 and IFN-β expression in THP-1-derived macrophages 97
4.8 The role of CB_1 and CB_2 cannabinoid receptors, and the nuclear PPARγ receptor, in mediating the effects of CBD and THC on TLR4-induced CXCL10 and IFN-β, and TLR3-induced CXCL10 expression 99
4.9 Discussion 101

Chapter 5 106

Examining the proclivity of THC and CBD to modulate TLR3/4 signalling in primary human PBMCs from healthy control subjects and pwMS 106
5.1 Introduction 107
Aims 108
5.2 Whole blood cell composition profiles of HC subjects 109
5.3 Demographics of healthy donors and pwMS 111
5.4 Comparison of the cellular composition of whole blood from HC and pwMS participants 114
5.5 PBMCs from pwMS are desensitised in terms of TLR3-induced CXCL10 expression, when compared to cells from healthy subjects 116
5.6 THC and CBD target TLR3-induced CXCL10 and IFN-β expression in primary PBMCs from HC subjects and pwMS 118
5.7 A 1:1 combination of THC:CBD inhibits basal CXCL10 expression in PBMCs 120
5.8 Stratifying the responses of PBMCs from pwMS to poly(I:C), THC and CBD treatment in terms of DMT use at the time of analysis 122
5.9 THC and CBD potentiate LPS-induced TNFα expression in PBMCs 125
5.10 THC and CBD differentially target MyD88-independent signalling events regulated by TLR4 in PBMCs from HC and MS subjects 128
5.11 Stratifying the response of PBMCs to LPS, THC and CBD treatment in cells from pwMS in terms of current DMT use 131
5.12 Discussion 133

Chapter 6 139

Examining the effect of highly purified botanically-derived phytocannabinoids on immune cell viability 139
6.1 Introduction 140
Aims 141
6.2 The effect of DMSO, ethanol and a panel of eight highly purified phytocannabinoid extracts on the viability of THP-1 monocytes 142
6.3 The effect of a panel of eight highly purified phytocannabinoid extracts on the viability of THP-1-derived macrophages 145

6.4 Examining the proclivity of TLR3/4 agonists, and eight purified phytocannabinoids, to alter the viability of primary human PBMCs isolated from healthy volunteers **147**
6.5 Examining the proclivity of TLR3/4 agonists and eight botanically-derived cannabinoid extracts to alter the viability of primary PBMCs from pwMS **150**
6.6 Discussion **153**

Chapter 7: Discussion **160**

7.1 General Discussion **161**
7.2 Limitations of the studies **173**
7.3 Future studies **174**

References **176**

Appendices **204**

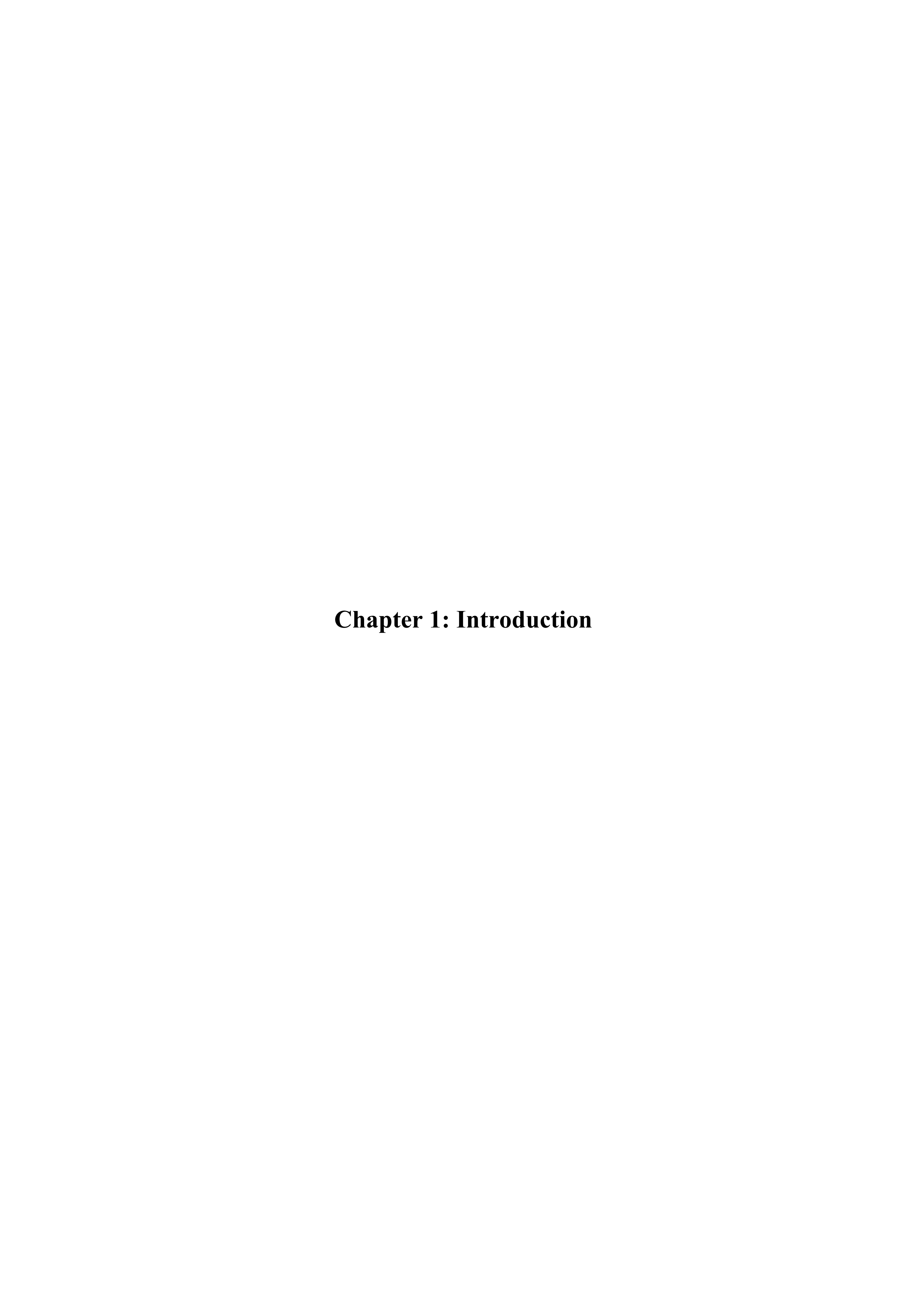
List of Figures

Figure 1: Overview of TLR-induced MyD88-dependent and -independent intracellular signalling mechanisms.......... 4
Figure 2. Overview of TLR3 and IFNAR signalling pathways.......... 7
Figure 3. Biosynthesis of the major phytocannabinoids.......... 24
Figure 4. Potential cellular therapeutic targets for cannabinoids in MS pathogenesis 34
Figure 3.1. Poly(I:C) does not alter TLR3 or TLR4 mRNA expression in THP-1 monocytes. 55
Figure 3.2. Poly(I:C) does not alter RANTES, TNFα and IFN-β expression in THP-1 monocytes. 57
Figure 3.3. Effect of poly(I:C) on relative TLR3 and TLR4 mRNA expression in THP-1-derived macrophages. 59
Figure 3.4. Poly(I:C) activates IRF3 and induces IFN-β/CXCL10 expression in THP-1-derived macrophages. 61
Figure 3.5. Effect of poly(I:C) on TLR3 and TLR4 mRNA expression in PBMCs plated at low cell densities. 63
Figure 3.6. TLR3 activation does not promote TNFα, RANTES and IFN-β expression in PBMCs from healthy volunteers. 64
Figure 3.7. Poly(I:C) increases expression of TLR3, IFN-β and CXCL10 in primary human PBMCs. 67
Figure 3.8. LPS does not affect THP-1 monocyte viability.......... 68
Figure 3.9. LPS promotes TNFα, RANTES and IFN-β expression in THP-1 monocytes. 70
Figure 3.10. LPS does not alter THP-1-derived macrophage viability. 72
Figure 3.11. LPS promotes MyD88-dependent and independent signalling in THP-1-derived macrophages. 75
Figure 3.12. Effect of TLR4 activation on TLR3 and TLR4 mRNA expression in PBMCs from HC subjects.......... 77
Figure 3.13. Effect of LPS on TNFα, IFN-β and CXCL10 expression in primary human PBMCs. 79

Figure 4.1. The effect of THC and CBD on LPS-induced TNFα and RANTES protein expression in THP-1 monocytes..91
Figure 4.2. THC, CBD and a THC:CBD combination are not toxic to THP-1 monocytes. 92
Figure 4.3. THC, CBD, and THC:CBD (1:1 combination) inhibit MyD88-independent signalling via TLR3 in THP-1-derived macrophages. 94
Figure 4.4. The effect of THC, CBD and THC:CBD on TLR4 signalling in THP-1-derived macrophages 96
Figure 4.5. CBD, THC and THC:CBD inhibit TLR4-induced IRF3, CXCL10 and IFN-β expression in THP-1-derived macrophages. 98
Figure 4.6. THC and CBD do not act via CB1, CB2 or the PPARγ receptor to modulate TLR signalling. 100
Figure 5.1. Blood cell profiles in samples collected from healthy volunteer's post-venepuncture. 109
Figure 5.2. MSQOL-54 and QIDS-SR16 data in HC and MS cohorts................113
Figure 5.3. Whole blood cellular profiles in samples from HC cases and pwMS. 115
Figure 5.4. Effect of TLR3 activation on CXCL10 and IFN-β expression in PBMCs from HC and MS subjects...117
Figure 5.5. Effect of THC, CBD, and a 1:1 combination on TLR3-induced CXCL10 and IFN-β expression in PBMCs from HC cases and pwMS.............................119
Figure 5.6. A THC:CBD (1:1) combination inhibit CXCL10 expression in primary PBMCs..121
Figure 5.7. Analysis of the effect of DMTs on the cellular responses of PBMCs from pwMS to poly(I:C), THC and CBD treatment. 124
Figure 5.8. THC, CBD, and THC:CBD differentially target TNFα production in PBMCs from HC and MS cohorts. 127
Figure 5.9. The effect of THC and CBD on TLR4 signalling independent of MyD88 in PBMCs from HC cases and pwMS. 130
Figure 5.10. Effect of DMTs on cellular responses to LPS, THC and CBD. 132
Figure 6.1. The effect of DMSO, ethanol, THC, CBD, CBDA, CBDV, THCA, THCV, CBG and CBC on THP-1 monocyte cell viability. 144
Figure 6.2. The effect of THC, CBD, CBDA, CBDV, THCA, THCV, CBG and CBC on THP-1-derived macrophage cell viability...146
Figure 6.3. The effect of poly(I:C), LPS, THC, CBD, CBDA, CBDV, THCA, THCV, CBG and CBC on the viability of primary human PBMCs isolated from healthy volunteers. 149
Figure 6.4. The effect of poly(I:C), LPS, THC, CBD, CBDA, CBDV, THCA, THCV, CBG and CBC on the viability of primary PBMCs from pwMS. 152
Figure 7.1. Proposed mechanism by which THC and CBD target TLR3/4 signalling in THP-1 macrophages. 165
Figure 7.2. Schematic outlining the primary findings in Chapter 5. 169
Figure 7.3. Schematic outlining the effect of THC, CBD, CBDV, CBDA. THCV, THCA, CBC and CBG (dose range 0.1, 1, 10 μM) on the viability of immune cells using MTT assays. 172

List of Tables

Table 1: TLR3 is expressed on THP-1 monocytes 55
Table 2: *TLR3* is expressed on THP-1-derived macrophages 59
Table 3. *TLR3* is expressed in human PBMCs 63
Table 4. *TLR4* expression in THP-1 monocytes. 68
Table 5. *TLR4* expression in THP-1-derived macrophages. 72
Table 6. TLR4 is expressed on healthy human PBMCs 77
Table 7. CB_1 and CB_2 receptor expression in THP-1 monocytes and THP-1-derived macrophages. 89
Table 8. Constitutive expression of PPARγ in THP-1-derived macrophages 99
Table 9. Blood profiles in samples from healthy subjects maintained at RT post-venepuncture. 110
Table 10. Blood profiles in samples from control subjects maintained on ice post-venepuncture. 110
Table 11. Demographic data from HC subjects and pwMS included in the study. 112



Chapter 1: Introduction

Introduction

1.1 Introduction to Innate Immunity

The human immune system is a complex arrangement of tissues and cells that work together to fight against invading pathogens and prevent disease and infection. This system consists of two branches, innate and adaptive, which are not separate, but synergistically cooperate to eradicate host infection. The cells of the immune system originate in bone marrow from hematopoietic stem cells. These cells can differentiate to a common lymphoid progenitor which can differentiate further to adaptive immune cells (B cells, T cells, natural killer (NK) cells, and NK-T cells) or into a common myeloid precursor which can differentiate to innate immune cells (monocytes, macrophages, neutrophils, dendritic cells (DCs), eosinophils, basophils and mast cells) [1]. Originally, the innate immune system was considered an inelegant precursor to the more sophisticated adaptive immune system, and Immunologists regarded the innate immune system as the initiation event that took place to enable the mature adaptive immune response to confer its protective effect on the organism. The production of innate immune cytokines such as interleukin-1 (IL-1), tumour necrosis factor (TNF), and IL-6, was unknown, alongside the signalling events that governed the production of interferons (IFNs) [2]. As research advanced, the importance of innate immunity became clear. The innate immune system is now recognised as the first line of host defence against pathogens. This system detects the presence of infection, and regulates the initiation of the adaptive immune response [3]. The discovery of DCs, complement and Toll-like receptors (TLRs) has further expanded research interest in this field.

1.2 TLRs

TLRs are pathogen-recognition receptors (PRRs) that recognise specific conserved pathogen patterns from microorganisms, termed pathogen-associated molecular patters (PAMPs), or danger-associated molecular patterns (DAMPs) from damaged tissue. TLRs are expressed on/in immune cells and cells of the central nervous system (CNS), however TLR expression is ubiquitous and has been detected on many organs and cell types [4]. To date 10 functional TLRs have been characterised in humans and 12 in mice [5]. TLR3, 7, 8 and 9 are expressed on endosomal compartments, while TLR1, 2, 4, 5, 6, and 10 are expressed on cellular membranes.

TLRs are categorised as a family of type I transmembrane receptors, and these receptors contain an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain responsible for downstream signal transduction [6]. TLR ligands differ greatly in structure and origin, nevertheless, common motifs do exist. The mechanism(s) by which TLRs recognise ligands is still not fully characterised, however, some data suggest that ligands are recognised by direct binding [3]. The cellular location of TLRs determines their recognition of specific biological ligands. Indeed, TLRs expressed on the cell surface recognise bacterial PAMPs, while TLRs expressed intracellularly (predominately on endosomes) recognise viral single stranded RNA (ssRNA), double stranded RNA (dsRNA) and unmethylated CpG DNA [7]. Furthermore, other PRRs exist, including nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs). In humans currently there are 22 known NLRs [8] and 3 members of the RLR family [5], and both PRRs reside in the cytoplasm.

1.3 TLR signalling mechanisms

TLR-induced signal transduction pathways promote the induction of various genes that function in host defence, such as controlling the expression of inflammatory cytokines and chemokines [9]. Ligand binding to TLRs induces conformational changes and dimerization, which promotes the recruitment of adaptor proteins to bind and recruit further downstream signalling molecules. The TLR family are characterised by the presence of type I transmembrane proteins consisting of LRRs, and activate nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) and mitogen-activated protein (MAP) kinases via a TIR domain to induce target genes [10]. TLR recruitment of MAP kinases results in the activation of the activator protein-1 (AP-1) family of transcription factors, resulting in inflammatory cytokine production and the regulation of the inflammatory response [11]. However, some differences exist in the signalling pathways employed by TLRs. Broadly, TLR signalling pathways are characterised as ‘shared’ and ‘specific’ pathways. A shared signalling pathway is utilized by all TLRs, while specific pathways are only activated by certain TLRs, or an individual TLR [3]. The shared signalling pathway, employed by all TLRs, includes several essential components:

(i) the adaptor proteins, myeloid differentiation primary response 88 (MyD88) [12] and Toll-interacting protein (TOLLIP) [13], (ii) a protein kinase, IL-1R-associated kinase (IRAK) [14], and (iii) another adaptor, TNF-receptor-associated factor 6 (TRAF6) [14]. MyD88 is an essential adaptor protein that binds to the TIR domain of the TLR and recruits other proteins to the receptor domain, which in turn induces phosphorylation and the activation of NF-κB, c-Jun-N-terminal kinase (JNK) and p38 MAP kinase [15]. This is known as MyD88-dependent signalling. However, MyD88-independent signalling is also utilized by TLR3 and TLR4. Indeed, TLR3 and TLR4 employ the use of TIR-domain-containing adapter-inducing IFN-β (TRIF), instead of MyD88, to recruit adaptor proteins, which promotes the nuclear sequestration of IFN regulatory factor 3 (IRF3) and promotes the induction of inflammatory cytokines and type I IFNs [16]. Cellular anti-viral signalling also utilises MyD88-dependent signalling via TLR7 and TLR9 to activate the transcription factor IRF7, promoting the downstream production of IFN-α [17]. The mechanisms by which TLRs signal via MyD88-dependent (TLR1, TLR2, TLR4, TLR5, TLR6, TLR11) and -independent (TLR3, TL7, TLR8, TLR9) mechanisms to activate a suite of transcription factors including AP-1, NF-κB, and IRFs, are outlined in Figure 1.

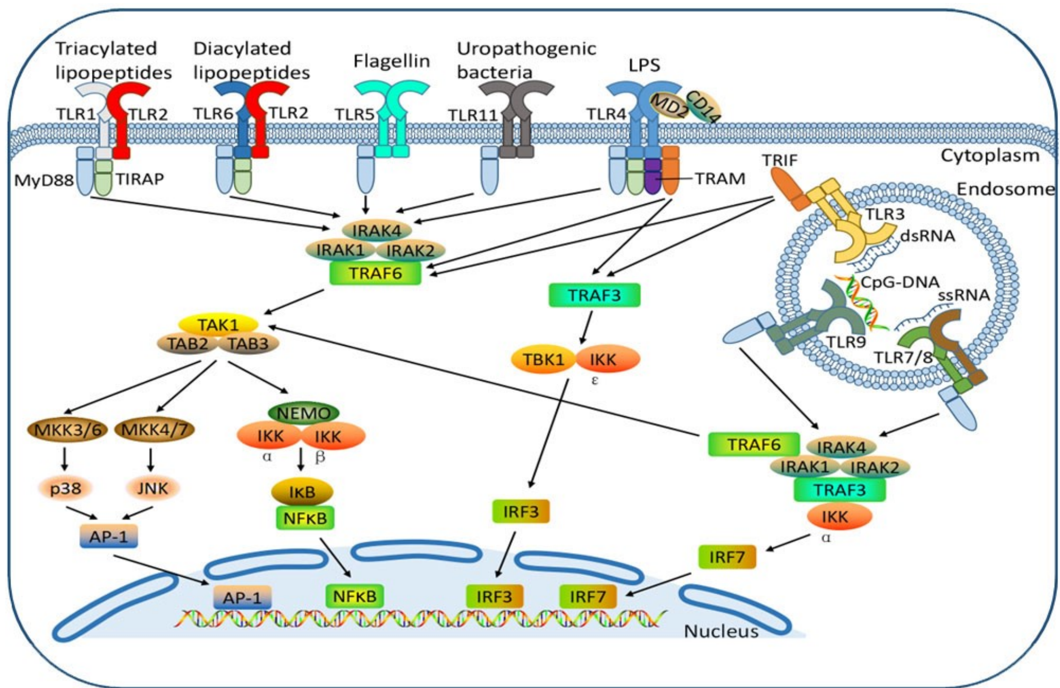


Figure 1. Overview of TLR-induced MyD88-dependent and -independent intracellular signalling mechanisms. From [18].

1.4 TLR3

External nucleic acids, such as dsRNA from viruses, are potent activators of TLR3 to mount a host response to viral infection. TLR3 is a highly conserved TLR among vertebrates [19] and is expressed in the population of peripheral blood mononuclear cells (PBMCs) [20]. Among this population of cells, evidence indicates that TLR3 is expressed at low levels in NK cells, T cells, monocytes and B cells [21]; however, studies elsewhere have shown that TLR3 expression is specific to DCs [22]. TLR3 is a receptor for dsRNA [23] produced by most viruses, and TLR3 has an essential role in combatting viral infection. Polyinosine-polycytidylic (polyI:C) is a synthetic analogue of dsRNA commonly used for research purposes. Indeed, dsRNA and poly(I:C) induce antiviral immune responses through a signalling cascade which promotes the production of both type I IFNs and inflammatory cytokines [24]. TLR3 is a unique TLR in that it does not contain a specific conserved proline residue. Substitution of this residue in other TLRs can render the TLR unresponsive to a known ligand and can abolish signalling activities [25]. This interesting data suggests that TLR3 utilises a different signalling mechanism, with a large body of research evidence indicating that TLR3 utilizes the MyD88-independent signalling pathway by recruiting TRIF, promoting the downstream activation of both IRF3 and NF-κB [26].

NF-κB activation via TLR3 requires the recruitment of TRAF6 via TRIF and the activation of transforming growth factor beta-activated kinase 1 (TAK1) and TAB2. TRAF6-TAK1-TAB2 are translocated to the cytosol where TAK1 is phosphorylated with subsequent activation of NF-κB [27]. Additionally, the kinase receptor interacting protein-1 (RIP-1) has been identified as an essential component of NF-κB activation via TLR3, which is dependent upon the TRIF adaptor [28]. NF-κB is located in the cytosol in an inactive form bound to and inhibited by inhibitor of κB (IκB) proteins. Upstream NF-κB activation is associated with the activation of an inhibitor of kappaB kinase (IKK) complex, which when activated promotes the phosphorylation of IκB proteins. Hence, once IκB is phosphorylated, it is tagged with ubiquitin and degraded, freeing NF-κB. The active protein can then translocate to the nucleus where, alone or in combination with other transcription factors, it can induce target inflammatory gene expression [29].

The IRF family consists of 9 members in mammals and are key regulators of type I IFN expression and the expression of IFN-inducible genes [30]. Of the family of IRFs, IRF3 and IRF7 are the primary inducers of type I IFNs. TLR3 activation by dsRNA promotes IRF3 activation, while ssRNA can activate intracellular endosomal TLR7/8, which leads to the production of IFN-α via recruitment of the adaptor protein TRAF6 and subsequent IRF7 activation [10]. In terms of IRF3 activation, the TRIF adaptor recruits a signalling complex (IKKs, TBK1) that catalyses the phosphorylation of IRF3. The signalling complex is then activated by TRAF3, which has been shown to be crucial in IFN-β induction through TRAF3 deficiency studies [31]. Once IRF3 is phosphorylated, this transcription factor translocates to the nucleus to promote IFN-β expression.

TLR3 activation has also been shown to induce the expression of the pro-inflammatory chemokine RANTES (also known as CCL5) [32] and the pro-inflammatory cytokine TNFα, which are differentially induced in different cell types [33]. Furthermore, TLR3 activation of IRF3, NF-κB and AP-1 can promote the induction of other pro-inflammatory chemokines such as CXCL10, which is an IFN-stimulated gene (ISG) [34, 35]. Indeed, secreted IFN-α and IFN-β, produced following TLR3 activation, can promote autocrine and paracrine signalling through binding to the heterodimeric IFN receptor. IFN cell surface receptor is composed of two subunits, IFN-α receptor 1 (IFNAR1) and receptor 2 (IFNAR2) [36]. IFNAR engagement activates receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which in turn activate the transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2 [37]. Upon phosphorylation of STAT1 and STAT2, dimerization of the transcription factors take place, facilitating the translocation of the heterodimer to the nucleus where it forms a complex with IRF9 (known as IFN-stimulated gene factor 3; ISGF3). This then binds IFN-stimulated response elements (ISREs) to promote the production of ISGs such as CXCL10 [36].

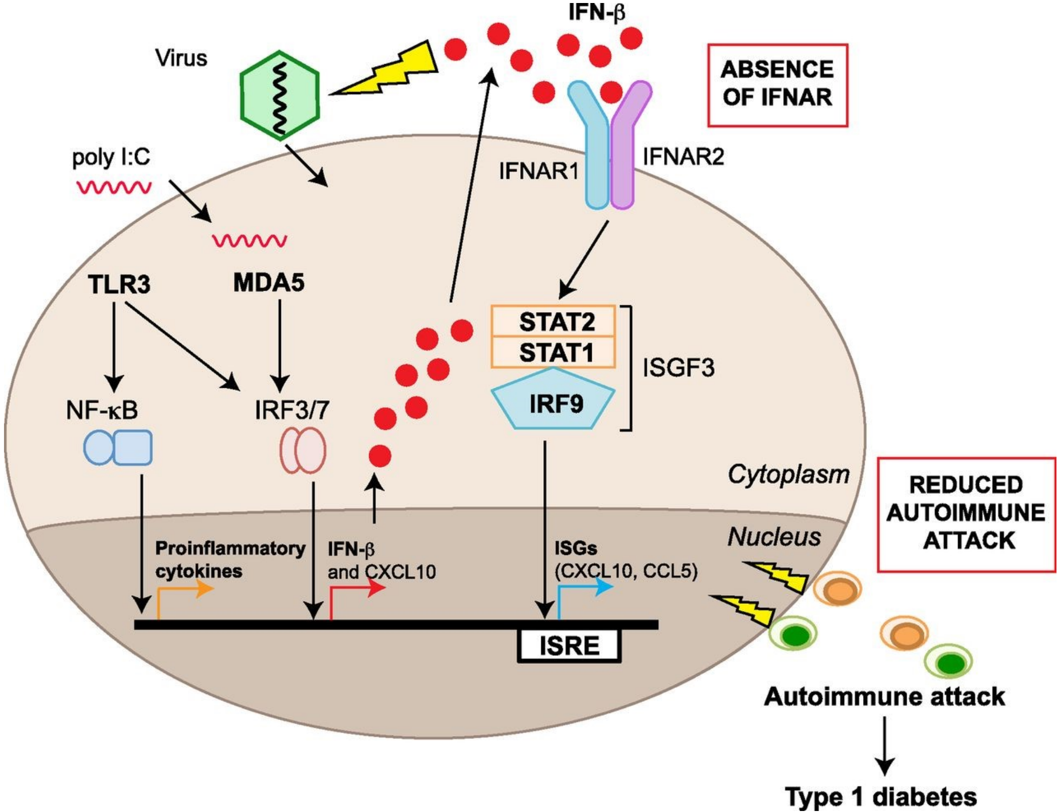


Figure 2. Overview of TLR3 and IFNAR signalling pathways. TLR3 activation induces IFN-β (and CXCL10) expression which can activate IFNAR in an autocrine manner to promote the production of further ISGs. From [38].

1.5 TLR4

The TLR4 signal transduction pathway plays an important role in the host response to bacterial infection and provides the initial immune response to invading bacterial endotoxins in the body. TLR4 is expressed on human immune cells including monocytes, macrophages, granulocytes and mature DCs [21]. TLR4 recognises bacterial infection and therefore can be activated via the use of lipopolysaccharide (LPS), a component of the wall of Gram-negative bacteria, which leads to the induction of pro-inflammatory cytokines and chemokines such as TNFα and RANTES [39]. LPS does not bind to TLR4 directly, instead it incorporates the adaptor protein MD-2 (also known as lymphocyte antigen 96). MD-2 binds to the lipophilic domain of LPS and forms a complex, which then associates with TLR4 to form the activated heterodimer LPS/MD-2/TLR4 [40, 41]. LPS is chaperoned to MD-2 by the cluster of differentiation 14 (CD14) protein, which aids in the formation of the LPS/MD-2/TLR4 complex. Upon activation of the TLR4/MD-2 heterodimer on the surface of the cell, intracellular signalling is induced via two

mechanisms. Indeed, TLR4 signals via the MyD88-dependent pathway which regulates NF-κB activation, and the MyD88-independent pathway (TRIF-TRAM) which activates IRF3 and type I IFNs [42] (Fig. 1). Both of these pathways are competitive and mutually exclusive [43]. TLR4/MD-2 activation on the cellular membrane initiates MyD88-dependent signalling whereas TLR4/TRIF-dependent signalling occurs following internalisation into endosomes which is controlled by CD14 [44].

1.6 THP-1 cells: a cell culture model to investigate TLR signalling

THP-1 cells are a human monocytic cell line adapted from a male with acute monocytic leukaemia which express distinct monocytic markers such as Fc and C3b receptors [45]. THP-1 monocytes are recognised as a valuable tool for investigating the function of monocytes in health and disease [46]. However, it must be noted that caution should be applied when making research comparisons of this cell type to its physiological counterpart, primary human monocytes. Indeed, evidence indicates that primary monocytes are more reactive to LPS when compared to THP-1 cells. This is due to the high expression of CD14 on primary monocytes, which forms a complex with TLR4 and exacerbates the cellular response of primary monocytes to LPS [47, 48]. Conversely, THP-1 cells express low levels of CD14 [49]. However, THP-1 cells are widely employed for modelling a monocytic response and for the study of inflammatory signalling events mediated by TLRs [50, 51].

THP-1 cells are differentiated to a macrophage-like phenotype using phorbol 12-myristate 13-acetate (PMA) [52], 1,25-dihydroyvitamin D3 (VD3) [53], retinoic acid [54], or cytokines (TNFα, IFN-γ) [55]. For the purposes of this study, PMA was used to promote THP-1 monocyte differentiation. However, the use of PMA at high concentrations has been shown to upregulate the expression of certain genes which can result in the activation of intracellular signalling systems associated with inflammation, particularly when other stimuli are used [56]. Therefore, the appropriate concentration of PMA is required to induce cell differentiation, but to limit the upregulation of undesirable genes [56].

THP-1 cells have been widely used to study disease models and to assess TLR3 and TLR4 signalling. Indeed, inhibition of NF-κB attenuates LPS-induced TLR4 activation in THP-1 cells [57], and LPS has been shown to up-regulate TNFα, IL-1β and IL-8 expression in this cell type [58]. Data also indicate that TLR3 is not abundantly expressed in THP-1 cells [59], therefore, there are few studies assessing the impact of poly(I:C) treatment on signalling events in THP-1 cells. However, LPS has been shown to up-regulate TLR3 expression in THP-1 cells [60], and furthermore poly(I:C) can activate the NLRP3 inflammasome in THP-1 cells [61]. Both of these studies employed the use of poly(I:C) transfection to promote endosomal TLR3 activation. In addition, the effects of poly(I:C) on cytokine/chemokine analysis in this cell type are commonly studied using differentiated THP-1-derived macrophages [62]. In contrast, in terms of TLR4 signalling, evidence indicates that THP-1 cells do not require differentiation, or transfection with LPS, to promote TLR4 signalling in THP-1 cells. It is noteworthy however that upon differentiation of THP-1 cells, cytoplasmic NF-κB expression is upregulated, which primes the cells to LPS stimulation [63].

1.7 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory progressive disease of the central nervous system (CNS) and is considered an autoimmune disorder given the involvement of $CD4^+$ T cells in the well-characterised demyelination associated with the disease [64]**.** However, recently there has been a shift in the classic dogma that MS is a T-cell mediated autoimmune disorder due to the emergence of efficacious B-cell targeted therapies [65]. Hallmarks of the disease include demyelination of the myelin sheath, axonal loss, inflammation and gliosis [66]. Both the grey and white matter of the CNS are affected which can lead to neuronal and axonal death [67]. Common symptoms include paraesthesia, diplopia, loss of vision, numbness or weakness of the limbs, bowel or bladder dysfunction, spasticity, ataxia, fatigue and cognitive changes [68]. MS is more common in females than males, with a usual ratio of close to 3:1 reported [69]. MS commonly manifests in patients between the ages of 20 and 40 years, but cases have been reported at all stages of life [70]. The average onset age for MS is 29 years [71].

It is estimated that more than 2 million people worldwide suffer from MS, and currently it is the primary cause of neurological disability in young adults [72]. Worldwide incidence rates and prevalence differs depending on the region and the sub-population specified, and efforts to define a pattern of geographical differences in MS frequency remain difficult. The main problem encountered is the variability in surveyed population sizes, age, ethnic origins, and inadequate recognition of benign and very early cases of the disorder. Moreover, a distinct racial and ethnic pattern does exist for the incidence of MS that has highlighted the rarity of MS among Samis, Turkmen, Uzbeks, Kazakhs, Krygyzis, native Siberians, North and South Amerindians, Chinese, Japanese, African blacks and New Zealand Maoris, as well as the high risk among Sardinians, Parsis and Palestinians. This clearly demonstrates that the different susceptibilities of distinct racial groups are important in understanding the uneven geographical distribution of MS [73].

MS epidemiology is well characterised in Ireland, with a national incidence rate of newly diagnosed MS reported as 6/100,000 (approximately 300 new cases per year) [74]. According to MS Ireland, approximately 8,000 people currently have MS in Ireland. Interestingly, in the North West of Ireland, the prevalence of MS is reported to be as high as 1 in 400. Publications from the UK indicate an incidence rate of 9.64/100,000 [75].

1.8 Pathology and Types of MS

MS is classified into four clinically distinct types: (i) relapsing-remitting MS (RRMS), (ii) secondary-progressive MS (SPMS), (iii) primary-progressive MS (PPMS), and (iv) progressive-relapsing MS (PRMS). RRMS is the most common form of MS, representing approximately 80% of all cases. RRMS begins with a uni- or multi-focal demyelinating attack known as a clinically isolated syndrome (CIS) [76]. Post-initial symptoms, follow on attacks commonly occur, which are classified as relapses. Complete or partial recovery may occur, with or without treatment. Of the four types of clinical MS, in most cases patients are first diagnosed with RRMS [77]. Of the patients diagnosed with RRMS, approximately 50% will develop SPMS after 10-15 years [67], at an average age of 42 years. As time progresses, each relapse can leave the patient with residual disability that over time

can accumulate into permanent disability. SPMS is a chronic phase characterised by attacks without recovery, leading to neurological impairments and consequently progressive physical deterioration of the patient [78]. SPMS, but not PPMS, is preceded by RRMS. PPMS accounts for approximately 10% of new MS cases and is characterised by gradual accrual of disability from the onset [79]. PRMS is the least common subtype of MS, with approximately 5% of all cases of MS categorised as this subtype. PRMS is characterised by progressive neurological deterioration from onset coupled by clear acute relapses, with or without recovery [80].

Clinically when an individual presents with a CIS, a diagnosis of MS is commonly investigated. Patients are commonly mono- or poly-symptomatic depending on the location of inflammatory lesion activity. Commonly reported presentations of the disease are optic neuritis, brainstem and spinal cord syndromes, however there are many less frequent manifestations of the disease [81]. The typical MS relapse progresses over hours or days until reaching a plateau for multiple weeks, followed finally by steady recovery. Individuals recently diagnosed with MS can appear to fully recover from relapses, however, the majority of relapses can result in some permanent damage. Diagnosing MS is based on multiple lines of evidence including medical history, neurological examination using imaging techniques (magnetic resonance imaging; MRI), lumbar puncture for cerebrospinal fluid (CSF) analysis, evoked potentials and blood read-outs [82]. Presently, the McDonald criteria is the mostly widely adopted set of criteria for diagnosing MS by clinicians and researchers. The McDonald criteria focuses on the demonstration of dissemination of lesions in both time and space using MRI [83]. These criteria are constantly re-evaluated and updated using the latest research available. In 2017 the McDonald criteria was updated to facilitate a diagnosis of MS in patients with a CIS, a demonstration of dissemination in space and the presences of CSF-specific oligoclonal bands [84]. Additionally, the progression of disability in pwMS is quantified using the expanded disability status scale (EDSS). The EDSS is the most commonly used scale for measuring disability status in MS, and is also regarded as being an effective method of quantifying disability [85]. The scale functions as a score out of 10, where a score of zero indicates normal neurological examination. Any score up to 5 points reflects fully ambulatory patients, scores over 5 reflect

ambulation status as the primary determinant in the degree of disability, and finally, a score of 10 indicates death due to MS [86].

1.9 Pathogenesis and Aetiology of MS

Much scientific research has elucidated the pathogenesis of MS. Nevertheless, the exact molecular mechanisms of disease progression remain unknown. Generally, it is accepted that clinically observed hallmarks of MS are a consequence of three neural tissue injury mechanisms combining synergistically, inflammation, demyelination, and axonal damage [87]. The inflammatory lesions associated with MS are populated by immune cells such as T cells, B cells, macrophages, and microglia, alongside an extensive repertoire of cytokines, chemokines, antibodies and complement. T-lymphocytes, which are myelin-specific (autoreactive), are thought to underlie nervous system attack and commencement of disease progression [87]. The most common theory of T-lymphocyte activation is through molecular mimicry. Once activated, a cascade of detrimental events occur which ultimately lead to neurodegeneration and microglial scarring. Microglia also contribute to inflammation observed in MS by producing proteolytic enzymes, cytokines, oxidative products and free radicals, all of which display toxicity towards oligodendrocytes and myelin [88]. Consequently, the loss of the protective myelin sheath from axons promotes neuronal degeneration and subsequent neural dysfunction. MS is thought to initiate in the periphery where T cells are primed to CNS autoantigens and cross the BBB where they can then activate microglia and macrophages [89]. Many studies have primarily focused on the effects of $CD4^{+}$ T cells in MS and EAE, however many other lymphocyte subsets have been highlighted to play a role in MS pathogenesis. For instance, $CD8^{+}$ T cells have been found in greater abundance than $CD4^{+}$ T cells in MS lesions [90]. DCs are antigen presenting cells (APCs) which play a role in the activation and differentiation of naïve T cells. The interaction between DCs and T cells determines T cell differentiation into effector T cells (Th1, Th2, and Th17) or Tregs [91]. PRRs such as TLRs are expressed by DCs and activation of these receptors triggers maturation of DCs and increased expression of co-stimulatory molecules which are crucial for activating naïve T cells [92]. Furthermore, co-stimulation occurs through DC-bound co-stimulatory molecules which induces full activation and effector function of the T cell [93]. Understanding the role APCs, and in particular DCs, play in MS

progression is crucial as many approved therapies for MS lead to a significant modulation of DCs [94].

The cause of MS is not attributed to one underlying factor but is considered the combination of an unknown environmental trigger and genetic susceptibility. There is a trend in the global distribution of MS that indicates there is an increased incidence of disease with distance from the equator [95]. Indeed, MS is prevalent in geographical locations populated by northern Europeans. People with MS (pwMS) generally report infection with measles, mumps, rubella, and Epstein-Barr virus (EBV) at later ages than their genetically (HLA-DR2) matching controls [96]. In particular, research indicates that infection with EBV as a young adult increases the risk of developing MS (relative risk 3·0 [95% CI 1·3–6·5]) [97]. Indeed, testing negative for EBV is considered a protective factor for developing MS [98], whereas being symptomatic with EBV increases the risk of developing MS two-fold [99]. In addition other environmental triggers have been linked to MS pathogenesis including low exposure to sunlight, vitamin D deficiency, diet, geomagnetism, air pollutants, radioactive rocks, cigarette use and toxins [100]. The potential role of vitamin D in the development of MS is associated with studies that demonstrate a correlation between latitude and MS prevalence. The latitudinal gradient correlates with exposure to UVB, which can synthesise vitamin D through photolyzing 7-dehydrocholesterol found in the skin to vitamin D3 or cholecalciferol [101]. Low vitamin D levels through reduced exposure to the sun, and low dietary intake in pwMS, coupled with a genetic polymorphism causing low vitamin D expression, have highlighted vitamin D as a potential risk factor in developing MS [102]. Furthermore, a double-blind, randomised, placebo controlled trial of vitamin D_3 in CIS and healthy donors found that there was no alteration in $CD4^+$ T cells between study groups [103]. Additionally, the authors found that vitamin D_3 treatment had no immunological, MRI or clinical evidence of benefit. The environmental component of MS disease aetiology should not be underestimated and may be the leading trigger in disease progression. This is clear from migration studies which demonstrated that migrants moving from what are considered low risk MS countries (such as the West Indies), to high risk MS regions (such as Europe), were not at higher risk of developing MS. However, data indicate that the migrant's children had a higher risk of developing MS due to their geographical location in a high risk

region [104]. This suggests that environmental factors supersede genetic factors during MS aetiology and progression.

Data from familial and population-based studies of MS indicate that there is a genetic component to the disease. Indeed, approximately 1 in 8 pwMS have a family history of the disease, highlighting a genetic susceptibility to MS [105]. In the 1970's the first link between MS and alleles of the major histocompatibility complex (MHC) were identified [106]. More specifically, the markers were refined to DR15 and DQ6. The primary genetic risk associated with MS is in the HLA-DRB1*15 allele. Specifically, homozygotes for HLA-DRB1*15 are more likely than heterozygotes to develop MS, however the mechanism by which this allele influences MS pathogenesis is unknown [107]. The association of these markers are observed in all populations (strongest in northern Europeans), except for the Sardinian population, and some other Mediterranean sub-populations, which have a correlation with DR4 [108]. As increased scientific research of the genome or regions of interest was completed, single nucleotide polymorphic markers for the IL-2 and IL-7 receptor α chains were identified as further susceptibility markers [109]. Indeed, a study using ImmunoChip genotyping array identified 135 potentially associated genetic regions to MS susceptibility, however the odds ratio associated with most of these is low [110]. Interestingly, the majority of such single nucleotide polymorphisms are in close proximity to genes involved in immune function, and are typically found in regulatory, instead of coding, regions. Overall, MS aetiology should be considered as both genetic and environmental.

1.10 Current treatments of MS

Many disease-modifying therapies (DMTs) have been developed for the treatment of MS, however there is currently no cure for the disorder. Several treatment options have been approved by the United States food and drug administration (FDA) and European medicines agency (EMA) and are currently available on prescription to pwMS. MS DMTs show reasonable and varying efficacy and act by several therapeutic mechanisms, including immunomodulation, restoration of the blood brain barrier (BBB) and neuroprotection, and have been shown to reduce the rate of relapse and accrual of disability [71]. Approved medications include beta-interferon (IFN-β), Glatiramer acetate (GA), Tysabri, Gilenya, Mitoxantrone,

Fingolimod and Sativex. Recently, several new DMTs have received regulatory approval for MS, including Aubagio [71] and Dimethyl fumarate (BG-12), both of which are available for the treatment of RRMS. BG-12, like Gilenya and Sativex, is administered orally and has shown efficacy in MS [111]. All immunotherapeutic drugs developed for MS target the RR phenotype of the disease. Importantly, Ocrelizumab is another MS DMT that has been approved by the FDA for treatment of both RRMS and PPMS following a positive phase III clinical trial, with the authors noting that extended observations are required to determine the long-term safety and efficacy profile of the drug [112].

In light of the current severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, there is concern surrounding the use of DMTs as these drugs act as immunosuppressives and may increase the risk of contracting SARS-CoV-2 and severe disease. However, it has been proposed that immunosuppression may not be detrimental in pwMS infected with SARS-CoV-2 [113]. Here the authors state that most DMTs (except for Alemtuzumab) for RRMS do not affect the viral specific $CD8^+$ T cell response, which may assist in eliminating SARS-CoV-2. Additionally, the authors note that DMTs do not generally inhibit immature B cell development, therefore facilitating antibody production and possibly preventing re-infection, as well as allowing antibody development during a vaccine response. Furthermore, a recent case study of a RRMS patient receiving Fingolimod therapy who presented with a severe SARS-CoV-2 infection noted that Fingolimod treatment was associated with lymphopenia, which may be detrimental in recovering from SARS-CoV-2 [114], but was also associated with enhanced lung endothelial cell integrity, which may be beneficial [115]. However, infected SARS-CoV-2 patients with a severe disease course report increased levels of circulating cytokines [116]. Therefore, immunomodulation therapy may be beneficial for infected patients and Fingolimod may be of value in controlling severe SARS-CoV-2 infection. Much work is required to understand how DMTs for RRMS may interact with the SARS-CoV-2 disease course.

IFN-β is one the most commonly used therapies employed for the treatment of MS. Indeed, endogenous IFN-β insufficiency has been shown in MS patients [117]. IFN-β is primarily produced by fibroblasts, however evidence also indicates that IFN-β

is also synthesised by immune cells such as NK cells, B cells, T cells, macrophages and plasmacytoid DCs [118]. Two types of therapy exist, IFN-β1a and IFN-β1b, and administration is either via intramuscular or subcutaneous injection. Avonex, Rebif, Plegridy, Extavia and Betaseron are the current approved IFN-β therapeutics, and they are, along with GA, recognised as first-line disease modifying agents [119]. IFN-β therapy has been shown to reduce relapse rate in RRMS patients, as well as having anti-viral and anti-tumour properties [120]. A comprehensive mechanism(s) of therapeutic actions of IFN-β is incompletely understood, but it is known that IFN-β has anti-inflammatory properties, as well as effects on BBB permeability [121]. Indeed, IFN-β is thought to act by regulating the expression of pro- and anti-inflammatory cytokines in the brain and spinal cord and has been shown to reduce the number of inflammatory cells that can cross the BBB. Specifically, IFN-β can attenuate IL-17 levels and Th17 cell populations in pwMS [122], which are involved in MS progression [123]. Common side-effects of IFN-β treatments include flu-like symptoms, headache, injection site reactions, asthenia, lymphopenia, elevated hepatic enzymes, and pain.

GA, marketed as Copaxone, is a synthetic mimic of myelin basic protein (MBP), containing 4 amino acids (glutamic acid, lysine, alanine, and tyrosine) which acts be competing with endogenous antigenic MBP peptides that complex with MHC class II. GA was initially designed to induce experimental allergic encephalomyelitis (EAE) in mice, a murine model of MS. However, GA was found to suppress EAE and was therefore translated to human MS trials [123]. The mechanism of action of GA involves shifting Th1 cells to a Th2 phenotype, which suppresses inflammation and promotes the activation of regulatory T cells (Tregs) [124]. Clinical trial data indicate that GA reduces relapse rate by approximately 30% in RRMS [125]. GA is administered via injection which can result in injection site reactions, with further reported side effects including fever, cardiovascular, digestive, muscular and respiratory issues.

Dimethyl fumarate, marketed as BG-12 or Tecfidera, is a methyl ester of fumaric acid that has been shown to have immunomodulatory properties. Indeed, Phase III clinical trials in pwMS using BG-12 twice or three times daily resulted in a 53% and 48% reduction in relapses, respectively, when compared to placebo control

[111]. The trial also found that BG-12 increased the time to disability progression in RRMS patients. BG-12 activates the transcription factor nuclear factor erythroid 2-related factor (Nrf2), which is protective against oxidative damage and inflammation, and can also inhibit the migration of inflammatory immune cells across the BBB [126]. BG-12 is administered orally and therefore bypasses the injection site reactions commonly observed during the administration of other DMTs for MS (IFN-β, GA). Side effects of BG-12 treatment include gastrointestinal disturbances such as diarrhoea, nausea, and upper abdominal pain, in addition to decreased lymphocyte counts and elevated liver aminotransferase levels.

Fingolimod, trade name Gilenya, is a sphingosine 1-phosphate (S1P) receptor modulator which upon activation in peripheral immune cells attenuates CNS infiltration of auto-reactive lymphocytes by sequestering lymphocytes in lymph nodes [127]. Phase III clinical trials of fingolimod versus placebo in RRMS patients demonstrated that fingolimod has the proclivity to reduce relapse rate by between 54% and 60%, depending on the dose of drug used [128]. Indeed, trials comparing the effect of IFN-β and fingolimod therapy in RRMS patients demonstrated greater efficacy of fingolimod, when compared to IFN-β, with respect to relapse rate and MRI outcomes; however, there was no difference in progression of disability between the groups tested [129]. Fingolimod is administered orally and common side effects include bradycardia, blurred vision, diarrhoea, back pain, headache, cough, and vomiting.

Teriflunomide (Aubagio) is another orally administered DMT for RRMS. This therapeutic is a more recent addition to the market for treating RRMS and received FDA and EMA approval in 2012 and 2013, respectively. Teriflunomide is an active metabolite of leflunomide, a DMT used for the treatment of rheumatoid arthritis (RA). Teriflunomide acts by inhibiting dihydroorotate dehydrogenase and the proliferation of B and T cells [130]. Indeed, evidence indicates that Teriflunomide inhibits IFN-γ producing T cells, while having no impact on IL-4 and IL-10 producing T cells [131]. Phase III trials in pwMS indicate an association between orally administered Teriflunomide, reduced relapse rate and reduced disability

progression, when compared to placebo [132]. Adverse reactions include elevated alanine aminotransferase, hair thinning and headache.

Finally, several humanised monoclonal antibodies are currently in use as DMTs for RRMS. These include Natalizumab (Tysabri), Alemtuzumab (Lemtrada), Daclizumab (Zinbryta), Ocrelizumab (Ocrevus) and Ofatumumab (Arzerra). Ofatumumab is an anti-CD20 antibody which binds to B cells and reduces the number of autoreactive B cells present in the CNS. Currently, Ofatumumab is under investigation in phase II trials for MS and the results of these trials are highly anticipated. Importantly, Ocrelizumab is the first approved therapy for PPMS, and phase III clinical trials have shown that the use of Ocrelizumab was associated with lower rates of clinical and MRI progression, when compared to placebo control [112]. Alemtuzumab is a further humanised monoclonal antibody against CD52, a marker expressed by B and T cells, and is thought to suppress B and T cell function and autoreactivity [133]. Finally, Natalizumab is a humanised monoclonal antibody against the cellular adhesion molecule α4-integrin. By blocking α4-integrin Natalizumab inhibits immune cell transendothelial migration across the BBB via its interaction with vascular endothelial adhesion molecule-1 [134]. Phase III clinical trials indicate that Natalizumab reduced inflammatory brain lesions, reduced relapse rate and the progression of disability [135].

1.11 New and emerging treatments in MS

There are a number of new approaches currently under investigated for the treatment of MS, with a number of avenues that avoid the use of non-specific immunosuppressive drugs under intense investigation. For example, stem cell transplants are being investigated as a possible therapeutic for MS. Hematopoietic stem cell transplantation (HSCT) was first employed in the 1990's in a leukaemia patient with MS, and in this case a marked improvement in MS brain lesion load was recorded [136]. Furthermore, a meta-analysis of HSCT treatment in pwMS found that at 2 years post-HSCT treatment, 83% of patients demonstrated no evidence of disease activity, and at 5 years this was reduced to 67%. However, the authors suggest that HSCT should only be considered in patients with aggressive RRMS who are refractory to conventional therapies [137]. No phase III clinical

trials of HSCT have been completed to date, with one trial in the USA (BEAT-MS) currently taking place at the time of writing.

DNA vaccines represent another approach currently under investigated for the treatment of MS. To date, one vaccine therapy has progressed to phase II trial evaluation. The DNA vaccine, known as BHT-3009, encodes human MBP, which is the target of autoreactive immune cells in MS. Therefore, the aim of the vaccine is to tolerise pwMS against MBP [138]. Data has shown that BHT-3009 is safe and well tolerated and can reduce inflammatory lesions on brain MRI, in addition to reducing the number of $CD4^+$ T cells in peripheral blood [139], however, more work is needed to determine if vaccine therapy is a viable avenue for MS treatment.

Nanoparticles have also been implicated as an avenue for MS therapy. Indeed, polymeric biodegradable lactic-glycolic acid (PLGA) nanoparticles carrying MOG_{35-55} peptides and recombinant IL-10 have been investigated in a number of studies. In EAE studies, PLGA nanoparticles (MOG_{35-55} peptide and IL-10) have been shown to ameliorate EAE progression and reduce IL-17 and IFN-γ produced by splenic T cells [140]. Therefore, nanoparticles are a promising avenue for treating the symptoms of MS by delivering self-antigens.

1.12 The role of TLR3 and TLR4 signalling in disease: focus in MS pathogenesis

TLRs play a critical role in orchestrating both innate and adaptive immune responses, and over-, or, under-activation of these PRRs can result in the development of inflammatory disorders and autoimmunity. Indeed, overexpression of TLR2 is associated with type 2 diabetes [141] and TLR2, TLR4, and TLR9 polymorphisms are linked with the development of Crohn’s disease [142]. Furthermore, nucleic acid sensing TLRs, which are found on endosomal compartments, including TLR 7, 8, and 9, have been shown to play key roles in numerous autoimmune diseases. For example, sera from patients with systemic lupus erythematosus (SLE) has been shown to contain increased amounts of endogenous ligands for TLR7, 8, and 9, therefore, over-stimulation of such TLRs can contribute to disease pathogenesis in SLE [143]. TLR-targeted therapies are currently being developed given their potential in preventing infectious disease [144], with potential agonists and antagonists under development. Indeed, TLR4

antagonists are regarded as a primary target for treating sepsis, as evidence indicates that viable bacteria and LPS from the gastrointestinal tract may influence the pathophysiology of sepsis [145]. Additionally, high expression levels of TLRs (in particular TLR3 and TLR4), and their endogenous ligands, have been detected in synovial tissue from RA patients, a chronic autoimmune inflammatory disease [146]. Necrotic synovial fluid cells isolated from RA patients have been shown to release RNA, which can then activate TLR3 expressed on synovial fibroblasts [147]. Additionally, TLR4 agonists, such as fibronectin and heat-shock proteins, have been detected in synovial fluid isolated from individuals with RA, further suggesting a role for TLR4 in disease pathogenesis [148]. In the context of MS, the soluble form of TLR2 has been proposed as a potential biomarker for the disease, as data from Hossain and colleagues (2018) indicate significantly elevated levels of soluble TLR2 in serum isolated from pwMS, when compared to healthy control (HC) subjects [149]. Importantly, CNS TLRs are expressed on glial cells (microglia, astrocytes and oligodendrocytes) [150], and are also expressed on activated lymphocytes which can infiltrate the nervous system. Therefore, TLRs and their activation may be critical in the development of neurodegenerative disease.

TLR3 has been implicated to play a role in neurodegenerative disease, particularly MS. Indeed, poly(I:C) stimulation has been shown to suppress demyelination in a murine EAE model via induction of endogenous IFN-β [151]. The transcription factor utilized by TLR3, IRF3, plays a critical role in the development of EAE, as indicated in mice deficient in IRF3 [152]. Additionally, TRIF deficiency improved the severity and neurological scores in EAE [153]. Furthermore, poly(I:C) has been shown to promote myelin repair in oligodendrocyte precursor cells (OPCs) [154]. Interestingly, previous data from our laboratory indicates that PBMCs from pwMS are refractory to poly(I:C) stimulation in terms of TNFα and IL-8 production [155]. These data indicate that the TLR3 signalling axis may be crucial in MS disease progression.

TLR4 has been widely studied in autoimmune disease [156] and has also been implicated in the pathogenesis of MS. Indeed, the Asp299Gly polymorphism on the TLR4 gene has been linked to MS given that PBMCs from pwMS that are

heterozygous for the Asp299Gly mutation demonstrate reduced proliferative capacity, when compared PBMCs from wild-type patients [157]. Data from our laboratory also indicates that PBMCs from newly diagnosed treatment naïve MS cases are hypersensitive to TLR4 stimulation with LPS, showing an increased production of TNFα and IL-8 [155]. Furthermore, TLR4 knockout in $CD4^+$ T cells diminishes disease symptoms in EAE through reduced Th17 and Th1 responses [158]. Additionally, increased levels of *TLR4* mRNA was detected in MOG-induced EAE [159] and Dark Agouti rat EAE models [160]. Elsewhere, there has been conflicting evidence regarding the role of TLR4 in EAE pathogenesis. Indeed, data from Marta *et al*., (2008) indicate that TLR4 knockout mice exhibit an increased severity in EAE symptoms, when compared to wild type mice [161], whereas other laboratories have suggested that TLR4 does not play a part in EAE progression [162]. Interestingly, MyD88 deficient mice are completely resistant to EAE, highlighting a crucial role for this adaptor protein in EAE progression [161]. Recently, it has also been reported that monocytes from pwMS express higher basal levels of *TLR4* mRNA, when compared to monocytes from HC subjects [163]. These data suggest that TLR4 plays a complex role in MS pathogenesis.

IFN-β is an anti-inflammatory cytokine that is a member of the type I IFN family. TLR3 and TLR4 signalling mechanisms promote the induction of IFN-β [16]. Indeed, as discussed previously, TLR3 can act via TRIF and IRF3 to induce the expression of IFN-β. Furthermore TLR4 can signal via a TRIF-dependent mechanism through the kinase RIP1 to activate NF-κB and induce IFN-β expression [164]. Efficient production of IFN-β is well characterised and transcriptional regulation is dependent upon the assembly of a transcription enhancer complex known as the enhanceosome [165]. The enhancer region contains four positive regulatory domains (PRDs I-IV) that are recognised by specific transcription factors. PRD I and III are recognised by IRFs, PRD II is recognised by NF-κB, and PRD IV is recognised by AP-1 (ATF-1/c-Jun) [166]. Therefore, TLR3/4 signalling is critical in the efficient production of type I IFNs. Importantly, IFN-β (Betaseron, Avonex, Rebif) is used as a front-line treatment for RRMS [167], and has been shown to reduce relapse rate in pwMS and also provide protection in EAE [168]. As previously discussed, IFN-β engages IFNAR and initiates JAK-STAT signalling which regulates cytokine/chemokine production. Studies have

shown that IFN-β supresses Th17 immune responses by regulating the expression of specific cytokines including IL-4, IL-10 and IL-27 [169]. Research elsewhere has shown that IFN-β inhibits T-cell activation via down-regulation of the MHC II co-stimulatory molecules and cell adhesion molecules on APCs [170]. Additionally, Arbour and colleagues (2004) have shown that IFN-β can induce T-cell apoptosis in MS [171], and IFN-β also exerts neuroprotective propensity by acting on the novel neurotensin high affinity receptor 1 pathway [172]. Overall, understanding the mechanisms that regulate endogenous IFN-β production may represent an important therapeutic avenue for future drug design.

It has been reported that a subset of pwMS demonstrate an increased endogenous IFN-like activity prior to initiation of IFN-β therapy [173]. Indeed, clinical non-responders to IFN-β therapy have been reported to overexpress ISGs before commencement of therapy, when compared to clinical IFN-β responders. Furthermore, data indicate that there is an upregulation of ISGs in clinical responders following IFN-β treatment, while no change in ISG expression levels were detected in clinical non-responder [173]. The increased IFN signature in clinical responders was attributed to activation of IFNAR1 and JAK-STAT signalling in monocytes, and decreased expression levels of IRAK3, when compared to clinical non-responders [174, 175]. Data elsewhere indicates higher basal expression of IFN-β in serum in a small patient population of non-responders to IFN-β therapy [176]. However, Feng *et al.* [177] reported that active MS is associated with lower expression of ISGs such as 2’,5’-oligoadenylate synthetase and myxovirus resistance A (MxA), and this lower expression is linked to subnormal phosphorylation of STAT1. The authors conclude that IFN signalling may be predictive of MS disease progression and response to therapy. Finally, in a large RRMS patient study, elevated expression of *MX1* mRNA (an ISG) in blood samples was associated with a longer time to first new relapse [178]. Overall, these studies highlight the complex nature of IFN signalling and ISG regulation in MS pathogenesis, and suggest that endogenous IFN signatures may be predictive of disease course.

1.13 Cannabis and Cannabinoids

Cannabis, commonly known as marijuana, is a derivative of an Indian hemp plant *Cannabis sativa* L. (*C. sativa*). Cannabis is the most commonly used illegal substance by adults, with approximately 5 million daily cannabis users worldwide [179]. There is a long history of cannabis use recreationally and medicinally in human populations [180]. The Irish physician William O'Shaugnessy is credited with introducing cannabis as a treatment option in western medicine after researching the medicinal properties of a range of indigenous plants [181]. Various parts of the *C. sativa* plant, including the leaves, flowers, seeds, stalks and resin glands are documented for use as food, fuel and medicine [182]. Structurally, cannabinoids are a set of over 100 oxygen-containing aromatic hydrocarbons [183], and all parts of the male and female plant contain euphoric cannabinoids. Cannabinoids are grouped into three subsets: (a) plant-derived (phyto) cannabinoids are compounds found only in the cannabis plant and comprise all active components of the plant; (b) the endogenous cannabinoids (endocannabinoids) constitute the cannabinoids that naturally occur in the body, (c) and synthetic cannabinoids (sCB) are artificially designed ligands used predominantly for pharmacological scientific research [184].

1.14 Phytocannabinoids

Phytocannabinoids are derived directly from *C. sativa* and are an extensively studied group of cannabinoids. Cannabinoids are classed as terpenophenolic compounds that contain 22 carbons or 21 carbons in neutral form. Neutral cannabinoids are formed by decarboxylation of the acid form of the cannabinoid via increased temperature [185]. Phytocannabinoids are synthesised via two distinct pathways, the polyketide pathway and the plastidal 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [186] (Fig. 3). The polyketide pathway forms olivetolic acid (OLA) from hexanoyl CoA and the MEP pathway produces geranyl diphosphate (GPP). OLA is alkylated with GPP via the enzyme geranylpyrophosphate:olivetolate geranyltransferase, which leads to the production of cannabigerolic acid (CBGA) [187]. Importantly, CBGA is the precursor to the majority of phytocannabinoids, reacting with oxidocyclases to produce an array of cannabinoids. Tetrahydrocannabinolic acid (THCA) synthase converts CBGA to

THCA which can lead to the synthesis of Δ^9-Tetrahydrocannabinol (THC), whereas cannabidiolic acid (CBDA) synthase forms CBDA which can undergo decarboxylation to cannabidiol (CBD), and finally cannabichromenic acid (CBCA) synthase forms CBCA, which results in the production cannabichromene (CBC) following decarboxylation [188-190]. Finally, phytocannabinoids that contain a propyl group, instead of a pentyl group side chain, such as tetrahydrocannabivarinic (THCV) acid and cannabidivarin (CBDV) acid, are instead formed by the combination of GPP with divarinic acid [185]. THCV acid and CBDV acid are decarboxylated to produce THCV and CBDV. Figure 3 below summarises the pathways associated with phytocannabinoid synthesis.

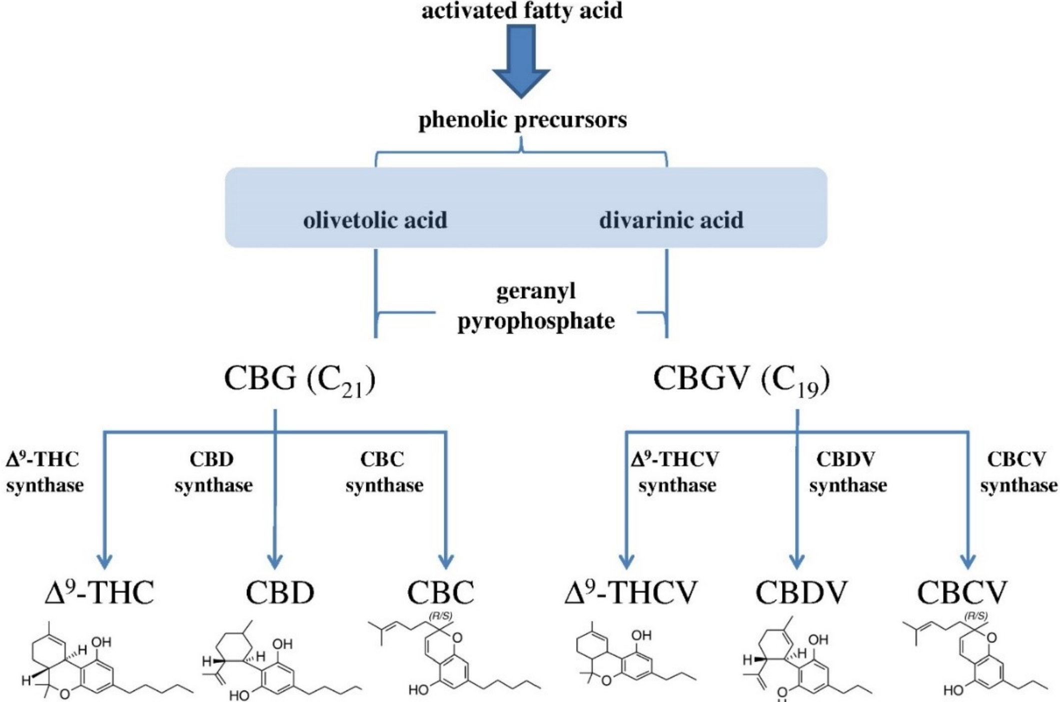


Figure 3. Biosynthesis of the major phytocannabinoids. From [191].

A large group of phytocannabinoids have been isolated and characterised in the plant, the most well-known being THC, the euphoric component of cannabis. CBD is a second major phytocannabinoid of relevance to the present study, which is described as the major non-euphoric ingredient in cannabis [192]. A large body of literature indicates that both THC and CBD have potential as neuroprotective [193], anti-inflammatory [194], antioxidant [195] and anti-excitotoxic compounds [196]. Furthermore, some published data suggest that other phytocannabinoids, including THCV, THCA, CBDV, CBDA, CBC and cannabigerol (CBG), have potential

therapeutic value. All phytocannabinoids indicated above were characterised in terms of their toxicity profile in the present study. Overall, further research is required to delineate the pharmacology of phytocannabinoids, their behaviour physiologically and their therapeutic potential.

Some research, albeit limited, has investigated the propensity of THCA, THCV, CBDA, CBDV, CBG and CBC as neuroprotective, analgesic, anti-nausea, and immunomodulatory compounds. Indeed, THCA, the acid variant of THC, has been shown to be neuroprotective in mice intoxicated with the mitochondrial toxin 3-nitropropionic acid (3-NPA) [197]. THCA can attenuate microgliosis, astrogliosis, and pro-inflammatory markers via engagement of the peroxisome proliferator-activated receptor (PPAR)-γ receptor [197]. In addition, THCA has potential as an anti-emetic and immunomodulatory compound via CB_1-dependent and independent mechanisms [198, 199]. Data elsewhere indicates that THCV decreases inflammation markers in mice injected with carrageenan or formalin [200], and data from Garcia and colleagues (2011) indicate that THCV can alleviate symptoms of Parkinson’s disease (PD) through activation of the cannabinoids receptors (discussed below) CB_2, and antagonizing CB_1 receptors, in rats [201]. In terms of CBDA, there is evidence that CBDA has anti-inflammatory and anti-hyperalgesia effects in a rat model of inflammation [202], and can bind to the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor [203]. CBDV, is the propyl analogue of CBD, acts as an allosteric modulator of CB_1, an antagonist of G protein coupled receptor 55 (GPR55) and can also activate TRPV1 [204]. The potential of CBDV as an anti-convulsant compound has been shown in seizure models in rats [205], while data also indicate that CBDV is an anti-emetic [206]. CBG is a major non-euphoric phytocannabinoid and has been shown to bind to both CB_1 and CB_2 [207], TRPV1 and PPARγ [197] receptors. Data indicate that CBG has potential as an appetite stimulant in pre-satiated rats [208] and as a neuroprotective agent via attenuation of IL-1β, TNFα, IFN-γ and PPARγ expression [209]. Finally, the non-euphoric cannabinoid CBC is a CB_2 receptor agonist [210], activator of TRP ankyrin-1 (TRPA1) channels [211], and can inhibit nitric oxide (NO) production in macrophages [212]. Furthermore, CBC has been shown to be antimicrobial [213], anti-inflammatory [214], an analgesic [215] and to possess anti-depressant properties [216]. Overall, there is

clear evidence that phytocannabinoids have potential as therapies for many inflammatory and neurodegenerative disorders, however, much research is needed to further elucidate their mechanism(s) of action.

1.15 The endogenous cannabinoid system (ECS)

The endocannabinoids, cannabinoid receptors CB_1 and CB_2, and the enzymes that regulate the synthesis and degradation of endocannabinoids, comprise the ECS [217]. To date, two cannabinoid receptors, CB_1 and CB_2, have been cloned and characterised, and both receptors are classic G-protein-coupled receptors [218]. The affinity of endocannabinoids and phytocannabinoids for CB_1 and CB_2 vary greatly between ligands [219]. For example, THC has been reported to bind to CB_1 and CB_2 receptors with Ki values in the low nanomolar range, but has a greater affinity for CB_1 [220]. Furthermore, CBD is reported to have low affinity for both cannabinoid receptors and displaces [^{3}H]CP55940 radioligand binding to the receptors in the micromolar range [219]. In terms of the expression profile of CB_1 and CB_2, the receptor distribution varies quite considerably between both receptors. Indeed, CB_1 is expressed predominately in the CNS, and is the receptor that has a greater affinity for THC and mediates its euphoric effects [221]. Indeed, CB_1 has been detected on many different cell types of the CNS, including several classes of neurons and glial cells [221, 222]. CB_1 expression is not uniform across all types of neurons however, for example GABAergic interneurons express higher levels of CB_1 than glutamatergic principal neurons [223]. Furthermore, in a seminal immunohistochemical analysis of CB_1 expression in the rat CNS performed by Tsou *et al*., (1998), CB_1 expression was determined on axons, cell bodies and dendrites, and was generally well distributed in the forebrain with more restricted distribution in the hindbrain and spinal cord [224]. CB_1 has also been detected, to a lesser extent, on cells of the immune system (B cells, NK cells, neutrophils, T cells and monocytes), testis, vascular endothelium, small intestine and peripheral nerve presynapses [225, 226]. Conversely, the CB_2 receptor is expressed almost exclusively on immune cells (macrophages, mast cells, B and T lymphocytes) and organs of the immune system (spleen, thymus, lymph nodes) [222]. Importantly, some evidence suggests that the CB_2 receptor is expressed on microglia of the CNS [227], and CB_2 receptor mRNA and protein has been localised on brainstem

neurons [228]. In addition, there is evidence that CB_2 is expressed predominately on neuronal somatodendritic areas (postsynaptically) [229].

The most well-known and characterised endocannabinoids are anandamide (AEA) and 2-arachidonyl glycerol (2-AG). 2-AG is a full agonist for CB_1 (EC_{50} = 125 nM) and CB_2 (EC_{50} = 38.9 nM) [230], while AEA has low affinity for CB_1 (EC_{50} = 1349 nM) and higher affinity for CB_2 (EC_{50} = 121 nM) [231]. Additionally, cannabinoids can target non-classical cannabinoid receptors such as PPAR's and TRP channels. Indeed, TRPV1 is activated by AEA (EC_{50} = 5.31 nM) under certain conditions [232] and AEA can also signal via PPARγ (EC_{50} = 8 µM) [233]. Some data indicate that GPR55, another G-protein coupled receptor, is a cannabinoid target [234], and there is some evidence that the serotonin receptors (5-HT_{1A}) are also targets of cannabinoids [235].

Cannabinoid receptor signalling (both CB_1 and CB_2) involves pertussis toxin sensitive G-proteins ($G_{i/o}$) coupling to the inhibition of adenylate cyclase (AC) [236]. AC activation results in increased cyclic AMP (cAMP)/ protein kinase A (PKA) signalling, therefore, $CB_{1/2}$ receptor activation results in the inhibition of cAMP/PKA signalling [222]. CB_1 activation leads to guanosine diphosphate/guanosine triphosphate exchange of the α and βγ subunit proteins, resulting in the regulation of many effector proteins and subsequent biological functions [237]. Cannabinoid receptor signalling has been linked to well characterised signalling events. For example, CB_2 activation promotes MAPK activation, including the activation of extracellular signal-regulated kinase (ERK) signalling [238] and the activation of p38 MAPK pathways [239]. CB_1 activation can also lead to phosphorylation and activation of MAPKs (ERK and p38 MAPK) and JNK [240]. In terms of endocannabinoids, AEA has also been shown to inhibit cAMP and adenylyl cyclase indicating a requirement for $G_{i/o}$ proteins [241]. Additionally, AEA can inhibit neuronal progenitor cell differentiation via attenuation of the ERK pathway [242]. There is also evidence that the endocannabinoid 2-AG can dose-dependently increase cAMP levels in primary adipocytes, which can be reversed through inhibition of CB_1 [243]. Furthermore, ion channels have been implicated in being impacted by cannabinoids. CB_1 receptors can influence activated A-type potassium currents by decreasing cAMP

signalling [244] and potassium channels (K^+) can be inhibited by AEA [245]. Furthermore, 2-AG can increase intracellular free (calcium) Ca^{2+} in a neuroblastoma/glioma hybrid cell model [246] and inhibit sodium (Na) ion channels [247]. Finally, there is also evidence that cannabinoids can modulate a number of neurotransmitter systems, including dopamine, serotonin, noradrenaline, GABA and glutamate (for full review see [248]).

1.16 sCB

sCB represent a large group of artificial compounds which have been developed as pharmacological tools to activate the ECS, and also represent potential avenues for therapeutic development. These compounds interact with CB_1 and CB_2 and elicit cannabimimetic effects similar to THC [249]. However, sCB have been reported to have higher binding affinity at the cannabinoid receptors than THC, in both *in vitro* and *in vivo* studies [250, 251]. Many sCB's have been developed as pharmacological tools, including arachidonyl-2'-chloroethylamide (ACEA), 1-pentyl-3-(1-adamantoyl)indole (AB-001), *R*(+)WIN55,212-2, AM694, CP55,940, HU-210, ADB-PINACA, JWH-018, JWH-175/6, JWH-307, JWH-250, PB-22 and UR-144 [252]. sCBs can also bind and activate non-classical cannabinoid receptors, such as TRP channels and PPARs. Indeed, the synthetic cannabinoid *R*(+)WIN55,212-2 has been shown to modulate intracellular signalling mechanisms controlling IFN-β expression in a PPARα-dependent manner [253]. Additionally, *R*(+)WIN55,212-2 has been found to exert analgesic effects by desensitizing both TRPV1 and TRPA1 [254].

1.17 Cannabinoids and neuroinflammation: focus on MS

Neuroinflammation is a key event in myelin degenerative disorders, particularly MS [255]. A broad range of events are encompassed by inflammation of the CNS, including activation of glial cells, modulation of cytokine and chemokine balance, neuronal dysfunction, and neurodegeneration [256]. Neuroinflammation is a key event in many neurological disorders such as ischaemia, Alzheimer's disease (AD), PD [257], psychiatric disorders (such as schizophrenia and bipolar disorder) [258], and neurodevelopmental disorders such as autism spectrum disorder [259]. There is increasing evidence that cannabinoids have potential to modulate neuroinflammatory events and are therefore under investigation as therapeutic

targets for a range of neuroinflammatory disorders. For example, the endocannabinoid 2-AG has been shown to decrease BBB permeability and inhibit the expression of TNFα, IL-1β, and IL-6 in mice following traumatic brain injury [260]. Additionally, the selective CB_1 agonist ACEA, is protective against inflammatory and endoplasmic reticulum stress in an *in vitro* neuronal model [261]. In terms of MS, large bodies of data indicate that cannabinoids have anti-inflammatory potential in neuroinflammatory events underlying the progression of EAE, the murine model of MS. For example, to study the effect of THC on rodents with EAE, Lyman and co-workers (1989) administered THC to rodents once daily, starting several days prior to inoculation and continuing after inoculation with EAE using MBP. Their findings indicate that the development of EAE was prevented, indicating that THC suppressed the development of EAE [262]. This experiment was also repeated to assess the role of THC on the progression of EAE post-immunisation. In this model, onset of symptoms was delayed and the clinical index lowered [262]. Following histological studies of the spinal cords of rodents, significantly less inflammation was observed in THC-treated rodents, when compared to vehicle-treated rodents. In addition, research has shown that CBD is also an effective phytocannabinoid in terms of ameliorating the clinical signs of EAE. Data from Kozela *et al*., (2011) indicate that CBD reduced the severity of EAE in mice, which was accompanied by diminished axonal damage and inflammation, as well as reduced microglial activation and T-cell recruitment in the spinal cord [263]. Furthermore, the sCB *R*(+)WIN55,212 has potent immunoregulatory properties and can ameliorate the progression of clinical disease symptoms in a viral murine model of MS [264]. Similarly, AEA has been shown to downregulate IL-23 and IL-12 expression in the spinal cord and serum in a viral model of MS [265]. Interestingly, knockout of the enzyme fatty acid amide hydrolase (FAAH), which hydrolyses AEA and acts as a key regulator of AEA [266] and 2-AG [267], results in the clinical remission of EAE [268]. Overall, there is much evidence that indicates that phytocannabinoids, endocannabinoids and sCBs have potential as cannabinoid-based therapies in EAE and MS and can target several mechanisms contributing to the pathogenesis of MS (Figure 4).

Much *in vitro* data also supports the anti-inflammatory nature of cannabinoids in neuroinflammation. Cannabinoids have been shown to supress inflammation in

cultured CNS cells, particularly inflammation induced by IL-1β, IFN-γ, amyloid-beta (Aβ) and hypoxia-ischemia [269]. Cannabinoid receptor signalling has also been linked to neurodegeneration and neuroinflammation in EAE [270]. Indeed, CB_1 knockout mice in EAE show more severe loss of neurofilament and myelin basic protein levels in their spinal cords, when compared to 'normal' EAE mice, highlighting a neuroprotective effect for this receptor [271]. Additionally, CB_2 knockout mice in EAE exhibit an exacerbated clinical score of disease, extended axonal loss, increased $CD4^+$ T cell infiltration and microglial activation, when compared to their wildtype littermates, suggesting a protective role for CB_2 in EAE pathology [270]. Furthermore, evidence indicates that the CSF and peripheral lymphocytes of RRMS patients demonstrate altered levels of endocannabinoids when compared to CSF/lymphocytes from HC subjects, and this has also been demonstrated in the brains of EAE mice [272]. Overall, a large body of research evidence strongly links the cannabinoid system with the pathophysiological mechanisms associated with MS, and this system offers potential for therapeutic interventions in this disease [273].

1.18 The role of cannabinoids in innate immunity

As discussed previously, TLRs are key components of the innate immune system, orchestrating innate immune responses to pathogens and promoting the production of inflammatory mediators. Importantly, there is a growing body of literature which demonstrates that a novel interplay exists between the TLR and cannabinoid systems, both centrally and peripherally [274]. TLRs and cannabinoid receptors share common signalling intermediates (i.e. MAP kinases), with direct cross-talk between these two cascades identified. Indeed, evidence indicates that cannabinoids inhibit TLR-induced cytokine/chemokine expression, in addition to interfering with the activation of TLR-induced transcription factors and signalling proteins [269].

Much research evidence has elucidated the effects of phytocannabinoids (particularly THC and CBD) on immune cell signalling and function. For example, THC inhibits TLR4-induced inflammation [275], induces apoptosis [276], and can inhibit migration [277] in macrophages. THC has also been shown to negatively regulate NK cell activity [278] and induce apoptosis in DCs [279]. Studies on the effects of CBD on the innate immune system have demonstrated that CBD inhibits

neutrophil migration in a periodontitis experimental model in rats which is accompanied by a CBD-induced decrease in the activator of NF-κB ligand RANKL [280]. In the same study, the researchers found that gingival tissues from CBD-treated rats had decreased IL-1β and TNFα expression. Studies elsewhere have highlighted a role for cannabinoids in immune cell signalling and function. Using the BV-2 microglial cell line, researchers found that THC and CBD can regulate the expression of LPS-induced micro-RNAs (miRNAs) which are associated with TLR and NF-κB signalling, including miR-146a and miR-155 [281]. In the same study, CBD treatment alone was sufficient to upregulate miR-34a which is associated with the regulation of cell cycle pathways and Notch-DII1 signalling [281]. Furthermore, microarray and pathway analysis data from the same laboratory indicates that treatment with CBD had a greater impact than THC on LPS-induced gene expression, which may be due to the proclivity of CBD to upregulate genes that encode negative regulators of NF-κB and AP-1 transcriptional activities, which emphasises the immunosuppressant activities of cannabinoids and their ability to target TLR signalling mechanisms [282]. Finally, Kozela *et al.,* (2010) reported that THC and CBD attenuate LPS-induced inflammatory cytokine (IL-1β, IL-6) and IFN-β expression in BV-2 microglial cells and that this effect was independent of the cannabinoid receptors [283]. Here, the researchers also found that CBD, but not THC, reduced NF-κB signalling while upregulating the activation of STAT3 to promote anti-inflammatory signalling. Additionally, both phytocannabinoids decreased LPS-induced STAT1 activation, which is critical in IFN-β-dependent inflammatory signalling processes. There is also evidence that THC and CBD may be protective to methamphetamine-induced neuroinflammation and mitochondrial dysfunction via phytocannabinoid modulation of TLR4-NF-κB signalling (for full review see [284]).

In terms of cannabinoid receptors, CB_1 has been shown to mediate LPS-induced fever responses (including LPS-induced hypothermia, hyperalgesia, and pro-inflammatory cytokine production in macrophages), indicating that CB_1 is pivotal in mediating TLR4-induced febrile responses [285]. Furthermore, cannabinoid-induced modulation of TLR signalling has been reported in adipocytes and endothelia. Indeed, the sCB *R*(+)WIN55,212-2 and endocannabinoid N-arachidonoyl dopamine (NADA), have been shown to ablate both LPS- and FSL-1

(TLR2/6 ligand)-induced proinflammatory cytokine expression in endothelia [286]. TLR4-induced pro-inflammatory cytokine production in adipocytes is blocked by the CB_1 antagonist SR141716A, indicating that CB_1 regulates LPS-induced inflammation in adipocytes [287]. Interestingly, some evidence suggests that cannabinoid-induced effects on TLR signalling may be independent of the cannabinoid receptors [288]. Indeed, using the sCB *R*(+)WIN55,212-2, Downer and colleagues (2011) showed evidence that *R*(+)WIN55,212-2 is a regulator of TLR3 and TLR4 signalling, independent of $CB_{1/2}$. Specifically, *R*(+)WIN55,212-2 inhibited the pro-inflammatory signalling axis activated by TLR3 and TLR4, while amplifying the activation of the IRF3 protein and consequently, IFN-β, with resulting anti-inflammatory effects in EAE [184]. Elsewhere, the CB_2 receptor agonists JWH-133 and JWH-015, in addition to THC, were sufficient to modulate TLR9-induced IFN-α and TNFα expression in primary human pDCs. Indeed, JWH-113, JWH-015 and THC, attenuated the TLR9-induced phosphorylation of IRF7, TBK1, NF-κB and IKKγ in pDCs, which are all key factors in pDC activation [289]. Furthermore, there is evidence linking THC regulation of TLR7-induced IL-1β expression in monocytes through CB_2. Indeed, THC attenuation of TLR7-IL-1β production in a co-culture of monocytes and astrocytes, resulting in decreased astrocyte production of monocyte chemoattractant protein 1 (MCP-1) and IL-6 [290].

In terms of endocannabinoids, studies have shown that AEA can modulate TLR7/8-dependent, but not TLR4/5-dependent, release of cytokines/chemokines in monocytes isolated from pwMS [163]. Similarly, TLR7/8 activation promotes cytokine expression in myeloid DC isolated from healthy individuals and pwMS, and these effects are attenuated by both AEA, in addition to the sCB JWH-015, in a CB_2-dependent manner [291]. Additionally, it has been demonstrated that the novel cannabinoid receptor GPR55 potentiates LPS-induced pro-inflammatory cytokine expression in monocytes [292], suggesting that GPR55 signalling modulates TLR4 signalling in immune cells. Studies such as these demonstrate the potential of cannabinoids to modulate TLR-induced events and highlight their potential as immunosuppressive agents (Figure 4).

Several important findings indicate that cannabinoid and TLR signalling may overlap in the CNS. Cannabinoid receptors are expressed by major glial cells [293] and there is growing evidence that suggests that cannabinoids negatively regulate TLR4-induced inflammation in glial cells. Moreover, cannabinoids, including THC and CBD, can downregulate pro-inflammatory mediator expression induced by the TLR4 agonist LPS in microglia [294]. TLR3 has been shown to promote the expression of neuroinflammatory mediators in the hippocampus of rats, and these effects are regulated by the FAAH inhibitor URB597 [295]. Indeed, administration of a monoacylglycerol lipase (MAGL) inhibitor (MJN110), which results in increased levels of the endocannabinoid 2-AG, does not affect TLR3-induced CXCL10, IRF7, or TNFα expression in the spleen or hypothalamus of rats [296]. Conversely, utilisation of the FAAH inhibitor, URB597, which results in increased levels of AEA, attenuated TLR3-induced inflammatory events in the hypothalamus of rats, indicating a role for endocannabinoid regulation of TLR3-induced neuroinflammatory events [296]. Additionally, data from the same group identified that URB597 treatment was sufficient to attenuate TLR3-induced fever, hypothermia and anxiety-like behaviour in rats. The authors note that URB597 decreased expression of TLR3-induced microglia/macrophage activation [297]. Recent data from Corcoran *et al.,* (2020) [298] have also demonstrated a role for the ECS in pain- and fear-related disorders in rat models. Here, the researchers found that microinjection of MJN110 (which will increase the expression levels of 2-AG) in the anterior cingulate cortex attenuated fear-conditioned analgesia, and that this effect was blocked using the CB_2 antagonist AM630 [298]. Data elsewhere indicates that administration of WIN55,212-2 attenuates the number of LPS-activated microglia in the rat hippocampus *in vivo* [299]. Furthermore, LPS also reduces CB_2 expression on macrophages and microglia, indicating that CB_2 expression undergoes modulatory changes due to cell activation [300]. These studies outline the potential for cannabinoids to regulate TLR-induced mechanisms and further highlight their potential development as therapeutics (see the authors review in appendix 1 for a table summarising cannabinoid overlap with TLRs). For full review of TLR signalling as a cannabinoid target see Fitzpatrick and Downer, 2017 (Appendix 1) [274].

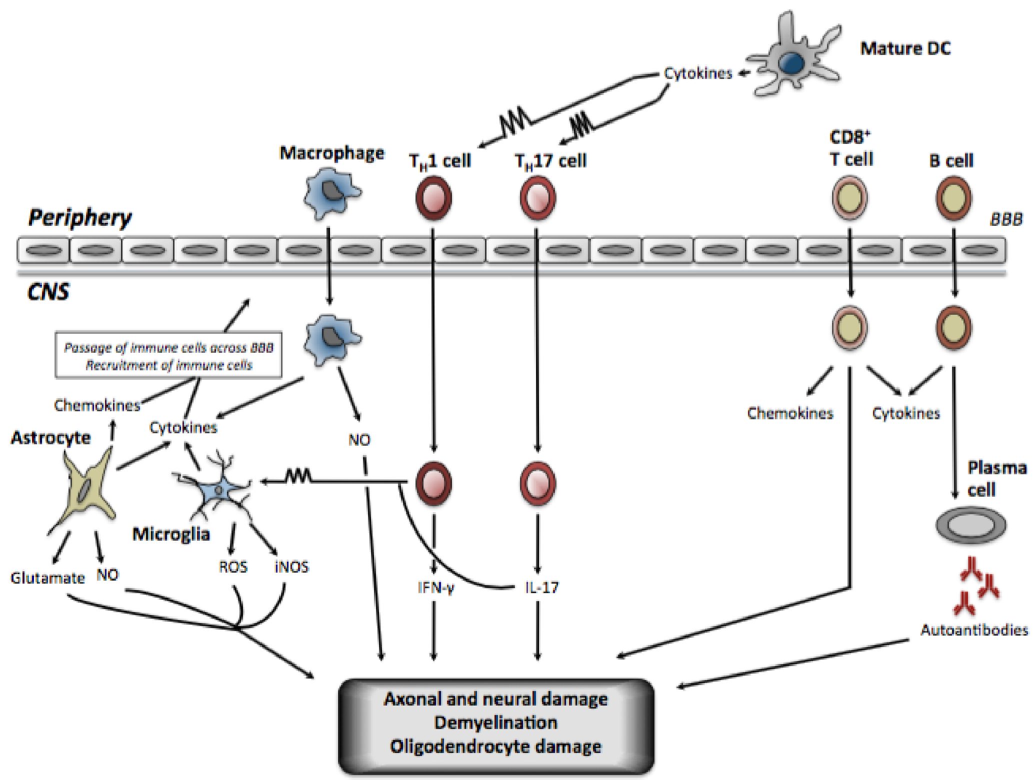


Figure 4. Overview of immune cell activation in MS pathogenesis [274].

1.19 The effect of cannabinoids on cellular metabolism and viability

There is now considerable evidence that cannabinoids can modulate immune cell function by regulating innate immune signalling pathways, altering cytokine/chemokine release, and potentially controlling cellular metabolism. The recently emerging field of immunometabolism has shed light on how metabolic cellular reactions and processes can function as a mechanism to control immunity and inflammation [301]. Indeed, there is evidence that cannabinoids may promote oxidative metabolism by upregulating adenosine monophosphate-activated protein kinase (AMPK), which is the master regulator of cellular energy levels [302]. AMPK is involved in promoting energy production strategies such as mitochondrial biogenesis and autophagy [303]. Indeed, AMPK controls the balance between anabolism and catabolism [304] through the phosphorylation of key proteins including the mechanistic target of rapamycin (mTOR), which is involved in cell growth and metabolism [305], lipid homeostasis [306], glycolysis [307] and mitochondrial homeostasis [308]. Indeed, AMPK activation can increase fatty acid oxidation [309] and mitochondrial biogenesis [310], which can induce an increase

in oxidative phosphorylation (OXPHOS). It has been reported that OXPHOS can skew immune cells towards an anti-inflammatory, tolerogenic phenotype [311]; therefore, AMPK activation can promote a cellular phenotype capable of attenuating inflammation.

Cannabinoids have been linked with the activation of AMPK and may utilize this master regulator of the cell response to energy stress to supress inflammation. For example, CB_2 activation using THC and the sCB JWH-015, has been shown to promote AMPK activation in hepatocellular carcinoma [312]. Findings elsewhere indicate that cannabinoids induce AMPK-dependent autophagy via a reactive oxygen species (ROS)-dependent increase in AMP/ATP ratio in pancreatic cancer cells [313]. However, there is evidence to suggest that cannabinoid activation of AMPK is cell-type specific. Indeed, in a mouse model of obesity, CB_1 receptor stimulation decreased mitochondrial biogenesis through attenuation of AMPK in white adipocytes through extracellular nitric oxide synthase (NOS) downregulation and p38 MAPK activation [314]. Additionally, the metabolic profile of immune cells can be regulated by cannabinoids and is a mechanism by which cannabinoids may control inflammation. One group has shown that knockout of CB_2 can increase glucose uptake and ATP levels in B cells [315]. Indeed, activation of CB_2 with the sCB HU308 has been shown to attenuate LPS-induced NLRP3 inflammasome activation in murine macrophages [316]. The same group also reported that macrophages with CB_2 genetically ablated demonstrate increased inflammasome activity, which the authors mechanistically attribute to the AMPK-mTOR-P70S6K signalling pathway [316]. It is clear that there is evidence implicating a role for cannabinoids in regulating energy metabolism in adaptive and innate immune cells via activation of AMPK. However, there is much work still required to fully elucidate the mechanisms by which cannabinoids alter immune cell metabolism and induce an anti-inflammatory phenotype.

There is also evidence of cannabinoids altering cellular viability, however, these studies are limited. THC treatment in cultured cortical neurons induces apoptosis in a CB_1-dependent manner through activation of JNK and caspase-3 [317]. Indeed, *in vitro* THC treatment of cerebral cortical slices obtained from neonatal rats activates the stress-activated protein kinase, JNK, and caspase-3, however, THC

treatment of adult cortical slices did not impact these pro-apoptotic pathways [318]. These data suggest that neonatal rat brain is more susceptible to the neurotoxic effects of THC than the adult rat brain [318]. Data elsewhere indicate that THC, alongside sCBs (CP55,940 and WIN55212-2), concentration-dependently increase B-cell proliferation, indicating that these tested cannabinoids are not cytotoxic to B cells and increase B cell viability [318]. A toxicity screen (using MTT assays) of a panel of non-psychoactive cannabinoids (CBD, CBC, CBG, THCV and CBGV) in human keratinocyte cells, indicated that all cannabinoids tested were not cytotoxic at the concentrations examined (10-20 μM) [194]. Furthermore, in the THP-1 monocytic cell line, CBD treatment was found to concentration-dependently increase intracellular ROS production and to promote apoptosis [319]. Furthermore, there is evidence that the phytocannabinoid CBG is protective against motor neuron loss after treatment with media from LPS-stimulated macrophages [209]. Indeed, the neuroprotective properties of CBG were associated with reducing nitrotyrosine, superoxide dismutase 1 (SOD1) and iNOS expression, while restoring Nrf-2 levels [209]. Previously reported studies have shown a protective effect of phyto- and synthetic-cannabinoids in B cells, however, there is evidence that the endocannabinoid AEA can inhibit primary human T and B lymphocyte proliferation by promoting DNA fragmentation [320]. Elsewhere, data from Sanchez *et al.*, (2006) indicate that the sCB WIN55212-2 concentration- and time-dependently induces apoptosis in encephalitogenic T cells during EAE, which may contribute to the anti-inflammatory propensity of WIN55212-2 in EAE models [321]. It is clear that cannabinoids have diverse effects on cellular viability which is dependent on the cell type examined (immune versus neural cells), the concentration and time of cannabinoid treatment, and the type of cannabinoid being tested (phyto- versus endo- versus sCB).

1.20 Cannabis based medicines

The development of cannabis-based therapeutics has advanced in recent decades due to increased evidence linking the cannabinoid system with disease pathogenesis. With relation to MS, cannabinoids have shown much therapeutic promise and have been shown to alleviate disease symptoms, particularly muscle spasticity, spasms, bladder dysfunction and pain in pwMS [322]. In addition, it is

widely accepted that some pwMS self-medicate with cannabis, and clinical trial evidence indicates that cannabis extracts benefit the patient in terms of controlling/alleviating the symptoms of MS [323].

Currently several cannabinoid-based therapies are in the clinic. Dronabinol and Nabilone are two approved cannabis-based medicines which were developed in the 1980's/1990's for the treatment of nausea in patients receiving chemotherapy [324], and as an appetite stimulant for patients with AIDs [325]. Dronabinol and Nabilone contain a synthetic form of THC [326], and some evidence, albeit limited, indicates that Dronabinol has efficacy as an analgesic in MS [327]. Epidiolex is a plant-derived highly purified CBD oral solution developed by GW Pharmaceuticals. Epidiolex is approved for the treatment of seizures associated with Dravet syndrome (DS), Lennox-Gastaut syndrome (LGS) and tuberous sclerosis complex (TSC) in the United States, and as an adjunctive treatment to clobazam for DS and LGS in Europe [328]. Sativex is another important cannabinoid-based medication containing CBD and THC as its most abundant phytocannabinoid components, in addition to other phytocannabinoids and non-phytocannabinoid components, and is administered as an oromucosal spray. Sativex is prescribed for pwMS with moderate to severe spasticity [329]. There is much clinical evidence of the efficacy and safety of the oral cannabinoid-based spray in terms of reducing spasticity, spasm frequency and pain [330]. In terms of potential pyschoactivity, Sativex is generally well tolerated, although dizziness, dry mouth and somnolence have been reported in a small percentage of users [331]. The oral administration of Sativex has the advantage of fast onset of action and high bioavailability [332]. Patients are given the freedom to self-titrate the dosage according to their need and tolerance of the drug. Sativex was first prescribed in Canada in 2005 and in 2010, Sativex gained regulatory approval in the UK and is now approved in over 20 European countries. In December 2016, the minister for health in Ireland commissioned a report from the Health Products Regulatory Authority (HPRA) to assess cannabis for medical use. The HPRA report concluded that cannabis-based medicines should be used in 'compassionate cases' when other medications are ineffective. Despite the development of cannabis-based therapeutics, much research is required to elucidate the cellular mechanisms of action of cannabinoids, including THC and CBD present in Sativex.

1.2 Hypothesis and aims

Hypothesis

The study hypothesis is that the botanically-derived phytocannabinoids, THC and CBD, are novel regulators of both viral (TLR3-mediated) and bacterial (TLR4-mediated) signalling pathways in monocyte and macrophage cell lines, and in PBMCs isolated from healthy donors and pwMS.

Overall aims

The specific aims of this study are:

1. To characterise TLR3 and TLR4 signalling in THP-1 monocyte and macrophage cell lines, in addition to primary PBMCs isolated from whole blood.
2. To investigate the proclivity of THC and CBD, when administered alone and in combination (1:1), to regulate TLR3 and TLR4 signalling mechanisms in monocytes, macrophages and primary human PBMCs from HC subjects and pwMS.
3. To determine if the impact of THC and CBD on TLR3 and TLR4 signalling events are mediated by $CB_{1/2}$ receptors.
4. To determine if differences exist in PBMCs isolated from HC volunteers and pwMS in terms of cellular responses to treatment with TLR3/4 ligands and the phytocannabinoids THC and CBD.
5. To determine the effect of a panel of botanically-derived cannabinoids (THC, CBD, CBDV, CBDA, THCV, THCA, CBG, CBC) on immune cell toxicity/viability.

Chapter 2: Materials and methods

2.1 Culture of cell lines

2.1.1 Culture of the THP-1 monocyte cell line

The human monocytic cell line (THP-1) were kind gifts from Prof. Marina Lynch and Prof. Andrew Bowie, Trinity College Dublin. These cells were originally derived from the peripheral blood of a 1-year-old male with acute monocytic leukaemia. THP-1 monocytes were maintained in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% (v/v) FBS (Sigma-Aldrich, Dorset, UK) and penicillin streptomycin (100 μg/ml) solution (Gibco) in a humidified environment at 37°C with 5% CO_2. Cells were passaged using dissociation every 2-3 days.

2.1.2 Phorbol myristate acetate (PMA)-induced THP-1 monocyte differentiation

PMA (Sigma-Aldrich) was dissolved in sterile ethanol (Sigma-Aldrich) and vortexed, giving a final concentration of 0.2 mg/ml. PMA was aliquoted and stored in the dark at -20ºC. Repeated freeze-thaw cycles were avoided. To induce THP-1 monocyte differentiation, THP-1 cells were resuspended and seeded in RPMI 1640 medium containing 10 ng/ml PMA. Cells were maintained in 5% CO_2 at 37°C for 48 h to allow for differentiation which is consistent with publications elsewhere [333].

2.2 Isolation of PBMCs

Written informed consent was obtained from each donor and the study received ethical approval from the School of Medicine Research Ethics Committee, TCD and Beaumont Hospital, Dublin (Appendix 2). Human PBMCs were collected from venous blood of HC donors (max 50 ml per donor) using a density gradient through lymphoprep (Axis-Shield, Oslo, Norway). Blood samples were initially diluted 1:1 with sterile PBS and gently overlaid onto lymphoprep. Each sample was subjected to centrifugation (800 g, 20 min, brake off) while in contact with the lymphoprep. Plasma was aliquoted and stored at -80°C until analysis and the PBMC layer was separated, diluted with PBS and centrifuged at 400 g for 10 min. The supernatant was discarded, and the pellet resuspended in PBS, prior to further centrifugation (400 g, 10 min). The pellet was resuspended in 1 ml of RPMI (10% FBS, 100 μg/ml of penicillin, and 100 μg/ml of streptomycin) and a cell count was performed.

PBMCs were plated at 1 x 10^6 cells/ml in 6- or 24-well plates for assessment of poly(I:C), LPS, and phytocannabinoid-induced signalling events. The remaining PBMCs (where applicable) were then cryopreserved (at 5-10 x 10^6 cells/ml in foetal bovine serum (FBS) containing 20% DMSO) in liquid nitrogen.

2.3 Drug treatments

2.3.1 LPS stimulation

THP-1 monocytes, THP-1-derived macrophages and human PBMCs were cultured at cell densities ranging from 0.2-1 x 10^6 cells/ml in 6-, 24-, and 96-well sterile cell culture plates. Stock LPS (10 µg/ml) (ALEXIS Biochemicals, USA) was diluted separately in RPMI media to the desired working concentrations (1-1000 ng/ml). Cells were stimulated for time points ranging from 10 min - 48 h. Control wells were incubated with RPMI media, using the same volume added to LPS-treated wells. At time zero, supernatants, cells and coverslips (if required) were harvested for ELISA, RT-qPCR and immunocytochemical analysis, respectively.

2.3.2 Poly(I:C) stimulation

THP-1 monocytes, THP-1-derived macrophages and human PBMCs were cultured at cell densities ranging from 0.2-1 x 10^6 cells/ml in 6-, 24-, and 96-well sterile cell culture plates. Stock poly(I:C) (1 mg/ml) (Invitrogen, France) was diluted separately in RPMI media to the desired working concentrations (0.5-50 µg/ml). Cells were stimulated for time points ranging from 10 min - 48 h. Control wells were incubated with RPMI media, using the same volume added to poly(I:C)-treated wells. At time zero, supernatants, cells and coverslips (if required) were harvested for ELISA, RT-qPCR and immunocytochemical analysis, respectively. Additionally, three different sources of poly(I:C) were used in some experiments (Fig. 3.1, 3.2, 3.3, 3.4, 3.7). Two sources were kind gifts from Prof. Aisling Dunne and Prof. Ursula Fearon, and the third source was the stock poly(I:C) mentioned previously. All sources of poly(I:C) were high molecular weight and from Invitrogen, France. Poly(I:C) 1 signified the drug compound from the laboratory, poly(I:C) 2 signified drug from Prof. Dunne, and poly(I:C) 3 signified drug from Prof. Fearon.

2.3.3 Cannabinoid treatment

THP-1 monocytes, THP-1-derived macrophages and human PBMCs were cultured at cell densities ranging from 0.2-1 x 10^6 cells/ml in 6-, 24-, and 96-well sterile cell culture plates. Eight phytocannabinoids, THC (batch no: THC/CG/1601), CBD (batch no: 6046727), CBDV (batch no: CBDV220914), CBDA (batch no: CBDA040912), THCV (batch no: THCV/CG/1005), THCA (THCA-CB-1001, CBG (batch no: CBG-CG-1501), and CBC (batch no: CBC/CG/0910) (supplied by GW Research Ltd, Cambridge, UK) were dissolved in sterile ethanol and stored protected from light at 4°C at a stock solution of 10 mM. The cannabinoids were diluted separately in RPMI media to the desired working concentrations (0.1-10 μM). For ELISA, RT-qPCR, and immunocytochemistry analysis, cells were pre-treated with either THC, CBD or a 1:1 combination of THC:CBD for 30-45 min prior to stimulation with either poly(I:C) or LPS for time points ranging from 30 min to 24 h. Control wells were incubated with RPMI media or RPMI media containing ethanol (0.1%), giving an overall same volume as the stimulated treatment wells. At time zero, supernatants, cells and coverslips (if required) were harvested for ELISA, RT-qPCR and immunocytochemical analysis, respectively. 5). In some experiments, cells were pre-treated with the CB_1 receptor antagonist SR141716 (N-[piperidin-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1-H-pyrazole-3-carboxamide], NIMH Chemical Synthesis Programme Batch 12,446–49-1; 1 μM for 1 h), the CB_2 receptor antagonist SR144528 ([N-[(1 s)-endo-1,3,3-timethylbicyclo[2.2.1]heptan-2-yl]5-(4-choro-3-methylpanyl)-1-(4-methlbenzyl)pyrazole-3-carboxamide] Chemical Synthesis Programme: Batch No. 11183–173-2; 1 μM for 1 h), or the PPARγ receptor antagonist T0070907 (Tocris Bioscience; 1 μM; 1 h) prior to treatment with the phytocannabinoids and LPS or poly(I:C).

2.4 Thiazoyl blue (MTT) cytotoxity assay

THP-1 monocytes (4 x 10^4 per well) or primary PBMCs (0.1 x 10^6 per well) were cultured in a 96-well plate in RPMI 1640 phenol red free media (Gibco, Life Technologies). For THP-1-derived macrophages, monocytes (4 x 10^4 per well) were allowed to differentiate to macrophages prior to analysis (see section 2.1.2) and cultured in RPMI 1640 media. Cells were maintained in culture at 37°C for

approximately 1 h before treatment with DMSO (0.1-2%), ethanol (0.1-1%), LPS (100 ng/ml), poly(I:C) (10-25 μg/ml), THC (0.1-10 μM), CBD (0.1-10 μM), THC:CBD (0.1-10 μM; equal concentration for both phytocannabinoids), CBDV (0.1-10 μM), CBDA (0.1-10 μM), THCV (0.1-10 μM), THCA (0.1-10 μM), CBG (0.1-10 μM), or CBC (0.1-10 μM) for 4 or 24 h. Triplicate wells were used for each treatment. Triton x100 (0.2%) (Sigma) was used as a positive control and was added 10 min prior to the addition of MTT to permeabilise the membrane. MTT (20 μl, 5 mg/ml dissolved in PBS, filter sterilised) was added to each well and incubated for 3.5 h. Finally, isopropanol (100 μl) (Hazardous material facility, TCD) was added to each well (for monocytes and PBMCs) to dissolve the purple formazan product. For macrophages, at time zero the RPMI media was aspirated and isopropanol was added to each well. The absorbance of each well was measured at 540-560 nm in a multiwell plate reader. Data was normalised to the control wells.

2.5 Immunocytochemistry

2.5.1 Preparation of sterile coverslips

To ensure sterility, 13mm diameter glass coverslips (VWR International, USA) were prepared. Coverslips were soaked in 70% ethanol and separated individually on sterile tissue in a laminar flow hood. Sterile coverslips were then placed into a petri dish (Sarstedt, Germany) containing filter sterilised poly-L-lysine (Sigma-Aldrich, Dorset, UK) solution (40 μg/ml in sterile H_2O), pushed individually to the base of the dish, and incubated for 3-4 h at 37°C to provide a suitable surface to which cells could adhere. Post 3-4 h incubation, coated coverslips were separated individually on sterile tissue in the laminar flow workstation and coverslips stored at 4°C in a sterile 50 ml falcon tube until required for use.

2.5.2 IRF-3/NF-κB immunocytochemistry

THP-1 cells were differentiated by resuspending in 1 ml of RPMI 1640 medium containing 10 ng/ml PMA following centrifugation. Cells were counted and cell suspension was made up to a final concentration of 1 x 10^6 cells/ml using PMA-treated media (10 ng/ml). Coated coverslips were positioned in a 24-well plate, the cell-suspension was added to each well (0.5 x 10^6 cells/well). Cells were maintained in 5% CO_2 at 37°C for 48 h. After 48 h, PMA-treated media was removed and

replaced with fresh RPMI media (250 µl/well). Stock LPS (10 µg/ml) or poly(I:C) (1 mg/ml) was diluted in RPMI media to a concentration of 200 ng/ml or 20 µg/ml, respectively. Cells were treated with 250 µl of LPS-, or poly(I:C)-containing media, giving a final LPS or poly(I:C) concentration in each well of 100 ng/ml or 10 µg/ml, respectively. Cells were stimulated with LPS or poly(I:C) for time points ranging from 10 min – 4 h. Control wells were treated with 250 µl/well RPMI media. Additionally, when cannabinoids treatments were added, THC (10 µM) and CBD (10 µM) were added as pre-treatments for 30 min prior to LPS (30 min) or poly(I:C) (60 min) treatment.

Following treatment, supernatants were removed from wells and stored at -20ºC for analysis by ELISA. Wells were carefully washed with ice-cold PBS and fixed in ice-cold 100% methanol (300 µl; Hazardous materials facility, TCD) at -20ºC for 10 min. Methanol was then removed and wells were washed three times with ice-cold PBS. Wells were flooded with PBS and coverslips were stored at 4ºC until analysis by fluorescence immunocytochemistry. Fixed cells were washed twice with PBS. Cells were permeabilised by adding 0.2% Triton X-100 in PBS (300 µl/well) for 10 min at room temperature (RT). Wells were then washed three times with PBS (5 min per wash). Non-specific staining was prevented by blocking cells with 250 µl normal goat serum (NGS) (10% v/v) (Sigma) in PBS for 2 h at RT. Following blocking, NGS was removed and primary antibody was added directly to fixed cells. Cells were incubated with rabbit polyclonal NF-κB p65 antibody or IRF3 (1:200 in 5% v/v NGS, Santa Cruz Biotechnology) (250 µl/well) overnight at 4°C. Negative controls were incubated with 5% v/v NGS in PBS in the absence of primary antibody. Following overnight incubation, primary antibody was removed by washing three times with PBS, 5 min per wash. Cells were then treated with 250 µl of secondary antibody solution containing goat anti-rabbit ALEXA 488 (1:1000, Life Technologies, USA) and DAPI (1:1000, Sigma) in 5% v/v NGS. Fixed cells were incubated in the dark for 1 h at RT. Coverslips were then washed 12 times with PBS, 10 min per wash in the dark. Coverslips were mounted onto 1.0 – 1.2 mm glass slides using mounting medium (Sigma-Aldrich, USA), sealed using nail varnish, and stored at 4ºC in the dark. Samples were viewed using an Olympus BX51P fluorescent microscope equipped with the appropriate filter sets. 4-5 fields of view were acquired for each treatment/coverslip and 40-60 cells analysed per

treatment. Images were analysed using ImageJ software with the CellMagicWand plug in and corrected total cell fluorescence (CTCF) was calculated for each cell.

2.6 Enzyme-linked Immunosorbent Assay (ELISA)

The expression of RANTES, TNFα, IL-6, CXCL8, IFN-β and CXCL10 protein were assessed by ELISA in all cell types including human THP-1 monocytes, THP-1-derived macrophages, and primary human PBMCs. Human RANTES, TNFα, IL-6, IL-8 (CXCL8), IFN-β and CXCL10 antibody was supplied from R&D systems®, Minneapolis, USA. Standard concentrations of the target were made from the stock standard (100 ng/ml) to range from 4000-7.8 pg/ml, depending on the cell line and target protein. RPMI was used to dilute each standard. The supernatants of LPS-, poly(I:C)- and phytocannabinoid-treated cells were used for analysis of cytokine, chemokine and IFN expression.

Day One

Human capture antibody was diluted to the working concentration (4 µg/ml) in PBS and 50 µl added to each well of the 96-well plate. Wells were coated overnight at RT.

Day Two

The 96-well plate was washed (x3) with 200 µl of the wash buffer (0.05% Tween in PBS) per well. Wells were blocked in blocking buffer (1% BSA in PBS) for at least 1 h at RT. The plate was washed (x3) and 50 µl of the standards and samples were added in duplicate to the plate and incubated at RT for 2 h. The plate was washed (x3) and 50 µl of the detection antibody (20 ng/ml in reagent diluent) added to each well for 2 h at RT. The plate was washed (x3), 50 µl of the Streptavidin-HRP solution (1:40 dilution) was added to each well and covered from light for 20 min. After washing (x3), 50 µl of Tetramethylbenzidine substrate solution was added to each well for a maximum of 30 min. The stop the reaction 25 µl of stop solution (0.18M H_2SO_4) was added to each well and the optical density of each well was read at 450 nm using a spectrophotometer (MULTISKAN FC, Thermo-Scientific) to give absorbance values.

2.7 Quantitative real-time polymerase chain reaction

THP-1 monocytes, THP-1 derived macrophages and human PBMCs were plated at a density of 0.5 x 10^6 cells per well in 24-well plates, or at a density of 2 x 10^6 cells in 6-well plates. Cells were treated with either poly(I:C) (10-25 μg/ml), LPS (100 ng/ml), THC (10 μM), or CBD (10 μM) for 2 - 6 h. RPMI media, or media containing ethanol (0.1%), was added to control wells. At time zero, the wells were triturated and the contents (supernatants and cells) removed using sterile filter tips and placed into RNAse free eppendorfs (Macherey-Nagel Inc., Geschaftsfuhrer, Germany). Eppendorfs were spun at 400 g for 3 min, and the supernatant carefully removed and stored at -20ºC. Each pellet was resuspended in 100 μl RA1 lysis buffer containing mercaptoethanol (1:100 dilution) and stored at -20°C. RNA was extracted from the three cell types using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Geschaftsfuhrer, Germany). The concentration of RNA was determined by placing 1 μl of the resulting solution onto a UV-vis spectrophotometer. Complementary DNA (cDNA) synthesis was performed on 0.1-1 μg of RNA using a High Capacity cDNA RT kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Equal amounts of cDNA were used for RT-qPCR amplification. Real-time PCR primers were delivered by Taqman® Gene Expression Assays containing forward and reverse primers, and a FAM-labelled MGB Taqman probe for each gene (Applied Biosystems). The primers used were as follows: IFN-β: Hs01077958_s1, TNFα: Hs01113624_g1, CB_1: Hs00275634_m1, CB_2: Hs00361490_m1, RANTES (CCL5): Hs00982282_m1, TLR3: Hs00152933_m1, TLR4: Hs00152939, and PPARγ: Hs00152933_m1 gene expression assays. Real-time PCR was performed on cDNA using Applied Biosystems 7300 Real-time PCR System. cDNA was mixed with qPCR™ Mastermix Plus (Applied Biosystems) and the respective gene assay in a 25 μl volume which contained 10 μl of cDNA, 12.5μl Taqman® Universal PCR Mastermix, 1.25μl target primer and 1.25μl 18S rRNA. Eukaryotic 18S rRNA (cat no: 4319413E) and the target primer of interest was used as non-template controls containing no cDNA. The samples were run in duplicate for a total of 40 cycles. Each cycle was as follows: 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, 1 min at 60°C. Gene expression was calculated to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. For cell line data, all control/untreated cells were grouped together to obtain an average ΔCt value. This average ΔCt value from

all groups was then subtracted from each control group individually to give a ΔΔCt value for each control, therefore the results of this analysis technique do not always result in a value of 1. For primary PBMCs, a ΔCt value was obtained specifically for each donor and each donors ΔCt was subtracted again from itself leading to a control group value of 1.

2.8 Western immunoblotting

THP-1 cells (0.5 x 10^6 cells/ml) were seeded in 6-well plates and differentiated for 48 h using PMA (10 ng/ml). Macrophages were incubated with LPS (100 ng/ml) or poly(I:C) (10 µg/ml) for timepoints ranging from 15 – 60 min, or 15 – 180 min, respectively. Cells were also pre-exposed (45 min) to THC (10 µM), CBD (10 µM) or a combination of both (1:1 ratio; each cannabinoid at 10 µM) (GW Research Ltd., Cambridge, UK) prior to LPS (100 ng/ml; 30 min) or poly(I:C) (10 µg/ml; 1 h) exposure. Following treatment, cells were washed (x3) in ice-cold PBS before being lysed on ice for 5 min in 70 µl of cytoplasmic lysis buffer (10 mM Tris-HCL, pH 7.5, containing 3 mM $MgCl_2$, 10mM NaCl, 0.5% Igepal, phosphatase inhibitor cocktail 2 and 3 (Sigma), protease inhibitor cocktail (Sigma)). Cells were scraped in cytoplasmic lysis buffer and maintained on ice for 5 min. Cell lysates were centrifuged (2000 g for 5 min at 4°C). The supernatants were carefully removed and stored at -80°C for future analysis. The remaining pellets were resuspended in nuclear extraction buffer (10 µl) (20 mM HEPES, pH 7.5, containing 5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 1mM DTT, glycerol (20%), phosphatase inhibitor cocktail 2 and 3 (Sigma), protease inhibitor cocktail (Sigma)), and subjected to liquid nitrogen – warm water, freeze-thaw to aid in nuclear membrane lysis. The resuspended pellet was centrifuged (16000 g for 20 min at 4°C). The supernatants (nuclear fractions) were stored at -80°C for future analysis. Stored lysate protein concentration was determined using the BCA method, with unknown protein concentrations interpolated from a bovine serum albumin (BSA) standard curve. Lysate protein concentration was equalized and mixed with denaturing buffer (0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 12.5% β-mercaptoethanol, and 0.0025% (w/v) bromophenol blue). Lysates were subjected to 95°C for 5 min to aid complete protein denaturing prior to electrophoresis. Lysates in denaturing buffer were loaded onto pre-casted gels (10% acrylamide), submerged in running buffer (25 mM Tris base, 190mM Glycine, 0.1% SDS, pH

8.3) and a current of 150V applied for approximately 90 min, or until the proteins had run off the end of the gel. Proteins were transferred to activated PVDF (Merch Millipore, Ireland) membranes (5s in methanol, 5 min in dH_2O, and 15 min in transfer buffer; 25 mM Tris-base, 190 mM glycine, 20% methanol, pH 8.3) after 2 h of 250 mA current passing through the membrane. PVDF was blocked for 2 h in 5% BSA in TBS-T. Membranes were incubated overnight at 4°C with rabbit monoclonal phospho-IRF3 antibody (1:2,000 in TBS-T, Cell Signalling Technology, USA), mouse monoclonal phospho-IκB-α antibody (1:1,000 in TBS-T; Cell Signalling Technology, USA), mouse monoclonal IκB-α (1:1000 in TBS-T, Cell Signalling Technology, USA)) or rabbit monoclonal phospho-NF-κB (1:500 in TBS-T, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with mouse monoclonal anti-β-actin antibody (1:20000; 1 h, Sigma, UK) as a loading control. Additionally, selected membranes were incubated with anti-histone H3 marker (1:5000; 1 h) on nuclear membranes (see Appendix 6). Membranes were washed and incubated with anti-mouse or anti-rabbit IRDye Infrared secondary antibody (1:10,000 in TBS-T; Li-Cor Biosciences) for 1 h in the dark at room temperature. The membranes were washed and immunoreactive bands were detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Molecular weight markers (Chameleon Duo pre-stained protein ladder, Li-Cor Biosciences) were used to calculate molecular weights of proteins represented by immunoreactive bands. Densitometry was performed using ImageStudioLite software, and values were normalized for protein loading relative to levels of β-actin.

2.9 Quick Inventory of Depressive Symptomatology (16-item) (self-report) ($QIDS-SR_{16}$) questionnaire, Quality of Life-54 (MSQOL-54) questionnaire and blood count profiling

Healthy volunteers with no history of autoimmune disease and RRMS patients attending the Neurology clinic at Beaumont Hospital, Dublin, were recruited to this study. At the time of blood donation, the volunteers completed the $QIDS-SR_{16}$ and MSQOL-54 questionnaires. The MSQOL is one of the most widely used MS-specific questionnaires and therefore was chosen for this study [334-336]. Additionally, the $QIDS-SR_{16}$ questionnaire is a self-report designed to provide an

indication of depressive symptom severity [337]. Collated data was scored using the relevant and accepted scoring system for each questionnaire. In addition, at the time of questionnaire completion, a blood sample (up to 50ml) of peripheral blood was collected by venepuncture. Blood samples were either stored at RT or on ice for up to 4 h. Whole blood composition was then assessed by applying approximately 25 µl of whole blood from each volunteer to the Sysmex Haematology Analyser at time points ranging from 0 – 4 h. The Sysmex Haematology analyser generates a readout of white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelets (PLTs), lymphocyte number, neutrophil number, and remaining immune cell number (mixed: MXD cells). HCT is a measure of the oxygen carrying capacity of blood and has effects on blood viscosity and flow resistance [338]. MCV is measure of the average size and volume of a RBC and can be useful in the classification of anaemia [339]. MCH and MCHC are a measure of the haemoglobin content of RBCs, specifically, MCH quantifies the amount of haemoglobin per RBC and MCHC quantifies the content of haemoglobin per unit volume [340].

2.10 Statistical Analysis

All data were analysed using GraphPad Prism 8 software. All data were tested for normality using the Shapiro-Wilk test. Data obtained from THP-1 monocytes and THP-1 derived macrophages (cell lines) was analysed using parametric testing. If cell line data contained two groups for analysis, then a student's t-test was used. If cell line data contained more than two groups, a one-way ANOVA followed by Dunnett's multiple comparisons test was performed. For analysis of data from primary human samples (HC donors and pwMS), data were also tested for normality using the Shapiro-Wilk normality test. If data was normally distributed, parametric testing was used via student's t-test (for two groups) or one-way ANOVA (for more than two groups) followed by Dunnett's multiple comparisons test. If data were not normally distributed in primary human samples non-parametric testing was employed using the Mann-Whitney test (for two groups) or the Krustal-Wallis test (for more than two groups) following by Dunn's multiple comparisons test. Two-way ANOVA followed by Bonferroni's post-hoc test was

performed when there was more than one variable to be analysed. Data are expressed as means ± standard errors of the mean (SEM) from at least three separate experiment cell passages, or from at least three HC or MS donors. Within each experiment, singlet/duplicate replicates were performed for each condition/drug treatment. Each experiment was repeated a minimum of three times. Significance was determined if p values were less than 0.05.

Chapter 3

Characterisation of TLR3 and TLR4 signalling events in THP-1 monocytes, THP-1-derived macrophages, and primary human peripheral blood mononuclear cells

3.1 Introduction

TLRs are PRRs that recognise specific conserved pathogen patterns, namely PAMPS and DAMPs. TLRs are expressed in immune cells and cells of the CNS [341, 342] and to date 10 functional TLRs have been discovered in humans and 12 in mice [5]. TLR3, 7, 8 and 9 are expressed on endosomal compartments while TLR1, 2, 4, 5, 6, and 10 are expressed on the cell membrane. TLR signalling mechanisms can be sub-categorised into 'shared' and 'specific' pathways. A shared signalling pathway is utilized by all TLRs, while specific pathways are only activated by certain TLRs [3]. Specifically, the shared signalling pathway (termed MyD88-dependent), used by all TLRs, has four essential components including the adaptor MyD88 [12], and in turn induces phosphorylation and the activation of NF-κB, JNK and p38 MAP kinase [15]. Conversely, TLR3 and TLR4 can signal via a MyD88-independent pathway, employing the use of TRIF instead of MyD88 to recruit adaptor proteins, which promotes the nuclear sequestration of IRFs and induction of inflammatory cytokines and type I IFNs [16]. TLR3 is expressed on populations of PBMCs [20] including monocytes [21] and DCs [343], and is a receptor for viral dsRNA [23]. The synthetic analogue of dsRNA, poly(I:C), is used for research purposes in the study of TLR3 signalling. dsRNA and poly(I:C) induce MyD88-independent antiviral immune responses through a signalling cascade which promotes IRF3 activation and translocation to the nucleus, with downstream production of both type I IFNs and inflammatory cytokines [24]. TLR4 is expressed on human immune cells including monocytes, macrophages, granulocytes and mature DCs [21]. TLR4 recognises bacterial infection and is activated by LPS to promote the induction of pro-inflammatory cytokines and chemokines, including TNFα and RANTES [39]. TLR4 signals via the MyD88-dependent pathway which regulates NF-κB activation, in addition to the MyD88-independent pathway (TRIF-TRAM), which activates IRF3 and type I IFNs [42].

TLR3 and TLR4 have been implicated in the pathogenesis of many diseases. For example, TLR3 has been shown to play a role in neurodegenerative disease, particularly MS. Indeed, poly(I:C) stimulation suppresses demyelination in EAE via induction of endogenous IFN-β [151]. TLR4 also plays a role in the pathogenesis of MS. Indeed, the Asp299Gly polymorphism on the TLR4 gene has been linked to the incidence of MS, with data indicating that PBMCs from pwMS

that are heterozygous for the Asp299Gly mutation showing reduced proliferative capacity, when compared to PBMCs from wild-type patients [157]. Furthermore, published data from our laboratory indicates that PBMCs from pwMS are hyper-sensitive to TLR4 stimulation with LPS, showing as increased production of TNFα and IL-8 [155]. Therefore, an improved understanding of TLR3 and TLR4 signalling mechanisms is key to elucidating their role in the development of immune/neurodegenerative diseases.

Aims

The specific aims of this chapter are as follows:

- To characterise key TLR3 signalling events in THP-1 monocytes, THP-1-derived macrophages, and primary human PBMCs.
- To characterise key TLR4 signalling events in THP-1 monocytes, THP-1-derived macrophages, and primary human PBMCs.
- To conduct time- and concentration-dependent analysis of the proclivity of poly(I:C) and LPS to induce TLR3 and TLR4 end-point readouts in THP-1 monocytes/macrophages and primary PBMCs, with focus on detection of TNFα production, RANTES production, IFN-β production, CXCL10 production, IRF3 nuclear sequestration, NF-κB nuclear sequestration and IκB-α degradation.
- To assess the potential cytotoxic effects of poly(I:C) and LPS in THP-1 monocytes, THP-1-derived macrophages and primary human PBMCs.

3.2 TLR3 is expressed in THP-1 monocytes and poly(I:C) does not regulate *TLR3* or *TLR4* mRNA expression

Poly(I:C) is a known activator of innate immune viral TLR3 signalling [23]. Initially, the expression profile of TLR3, and the effect of poly(I:C) on TLR3 signalling, was assessed using human THP-1 monocytes. THP-1 monocytes were cultured over three passages, RNA harvested, and RT-qPCR performed to determine relative *TLR3* mRNA expression. 18S ribosomal RNA (rRNA) was used as an endogenous control and Ct values determined. Data in Table 1 indicate that TLR3 is expressed, albeit at low levels, in this cell line, which is in agreement with the literature [60]. Following detection of *TLR3* mRNA in THP-1 monocytes, three different sources of the viral dsRNA mimetic poly(I:C) were used to determine if poly(I:C) treatment altered the expression of both *TLR3* mRNA and *TLR4* mRNA, as evidence indicates that activation of TLR3 using poly(I:C) can enhance *TLR3* mRNA [344]. THP-1 monocytes were cultured in the presence of three different batches of poly(I:C) (10 μg/ml) for 4 h. This timepoint was chosen as previous data from the laboratory indicates that poly(I:C) induces an IFN-β response in PBMCs after a minimum 3 h treatment [253]. However, data in Fig 3.1 suggest that treatment of monocytes with each source of poly(I:C) had no effect on relative *TLR3* (Fig. 3.1A) or *TLR4* (Fig. 3.1B) mRNA expression. TLR4 is not activated by poly(I:C) directly, and given that poly(I:C) did not modulate *TLR4* mRNA expression, this suggests that each source of poly(I:C) had no "off-target" transcriptional effects at this receptor.

Additionally, to determine whether activating TLR3 was cytotoxic to THP-1 monocytes, MTT cell viability assays were performed following treatment with the TLR3 agonist poly(I:C). Two concentrations of poly(I:C) were selected for testing (10 and 25 μg/ml), based on the manufacturers recommended concentration guidelines and on a large body of literature indicating the use of the TLR3 ligand at these concentrations *in vitro* [345, 346]. After 24 h incubation, poly(I:C) (10 μg/ml) had no effect on the viability in THP-1 monocytes (Fig. 3.1C). However, treatment with poly(I:C) (25 μg/ml) significantly reduced THP-1 cell viability when compared to control monocytes (Fig 3.1C). In all MTT assays, Triton x100 (0.2%) was added 10 min prior to the addition of the MTT assay to act as a positive control.

Table 1. TLR3 is expressed on THP-1 monocytes

Target	Average basal expression (Ct) (*n*=3)
TLR3	34.56 ± 0.39
18S rRNA	12.21 ± 0.34

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

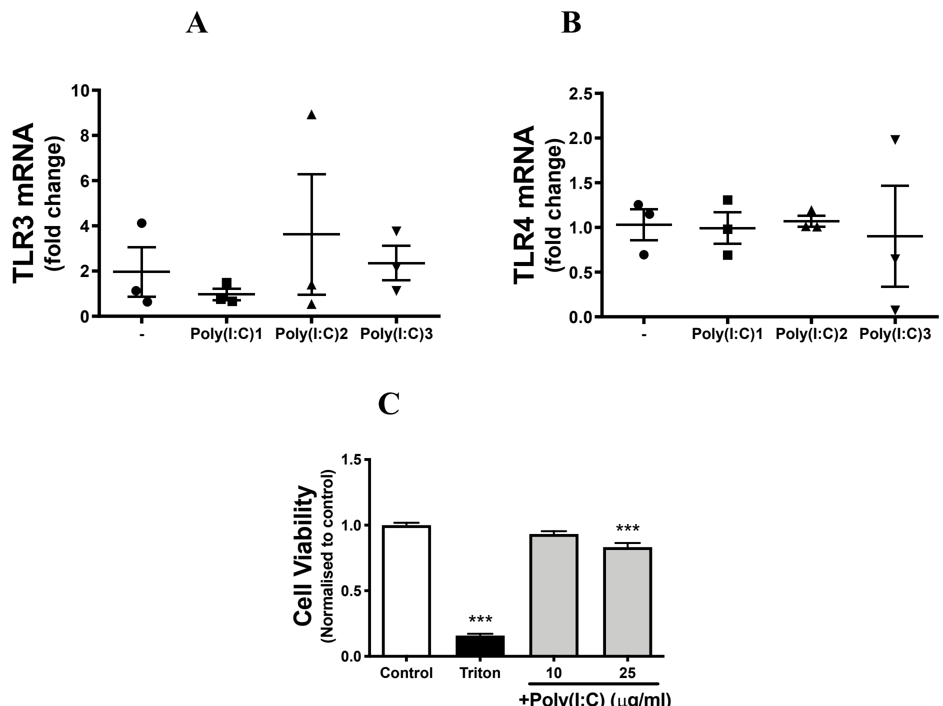


Figure 3.1. Poly(I:C) does not alter TLR3 or TLR4 mRNA expression in THP-1 monocytes. THP-1 monocytes were cultured in the presence of three different sources (1, 2, 3) of poly(I:C) (10 μg/ml; 4 h) and mRNA expression assessed using RT-qPCR. Poly(I:C) failed to impact **(A)** *TLR3* and **(B)** *TLR4* mRNA expression. (**C**) An MTT assay was used to determine the effect of poly(I:C) (at 10 and 25 μg/ml) on cell viability. Cells were treated 24 h prior to analysis. Triton x100 (0.2%) was added 10 min prior to addition of MTT to act as a positive control. Data were analysed using one-way ANOVA followed by Dunnett's post-hoc test. All data are presented as the mean ± S.E.M from 3 independent passages. ****$p<0.001$ versus* untreated control cells.

3.3 TLR3 activation does not modulate TNFα, RANTES or IFN-β expression in THP-1 monocytes

In order to characterise TLR3 signalling pathways in THP-1 monocytes, and to optimise the concentration and duration of treatment required to induce cytokine/chemokine protein and mRNA production using poly(I:C), THP-1 monocytes were cultured with or without poly(I:C) (0.5 – 50 μg/ml) for 8 h and 24 h for protein detection, and 2 - 48 h for mRNA detection. Following treatment, supernatants were collected and analysed for TNFα and RANTES protein expression by ELISA, and RNA harvested for analysis of *TNF*α*, RANTES* and *IFN-*β mRNA expression by RT-qPCR. Data demonstrate that poly(I:C) had no effect on TNFα (Fig. 3.2A, B, C) or RANTES (Fig. 3.2D, E, F) mRNA and protein expression in THP-1 monocytes cell cultures at each time point tested. Interestingly, a significant decrease in TNFα protein expression was determined following treatment with poly(I:C) at (10 μg/ml) for 24 h (Fig. 3.2C). Additionally, *IFN-*β mRNA levels were unchanged following poly(I:C) treatment at all timepoints tested (2 - 48 h) (Fig. 3.2G), and treatment with three different sources of poly(I:C) for 4 h had no effect on *IFN-*β mRNA expression (Fig. 3.2H). These data indicate that poly(I:C) does not induce TNFα, RANTES or IFN-β expression in THP-1 monocytes.

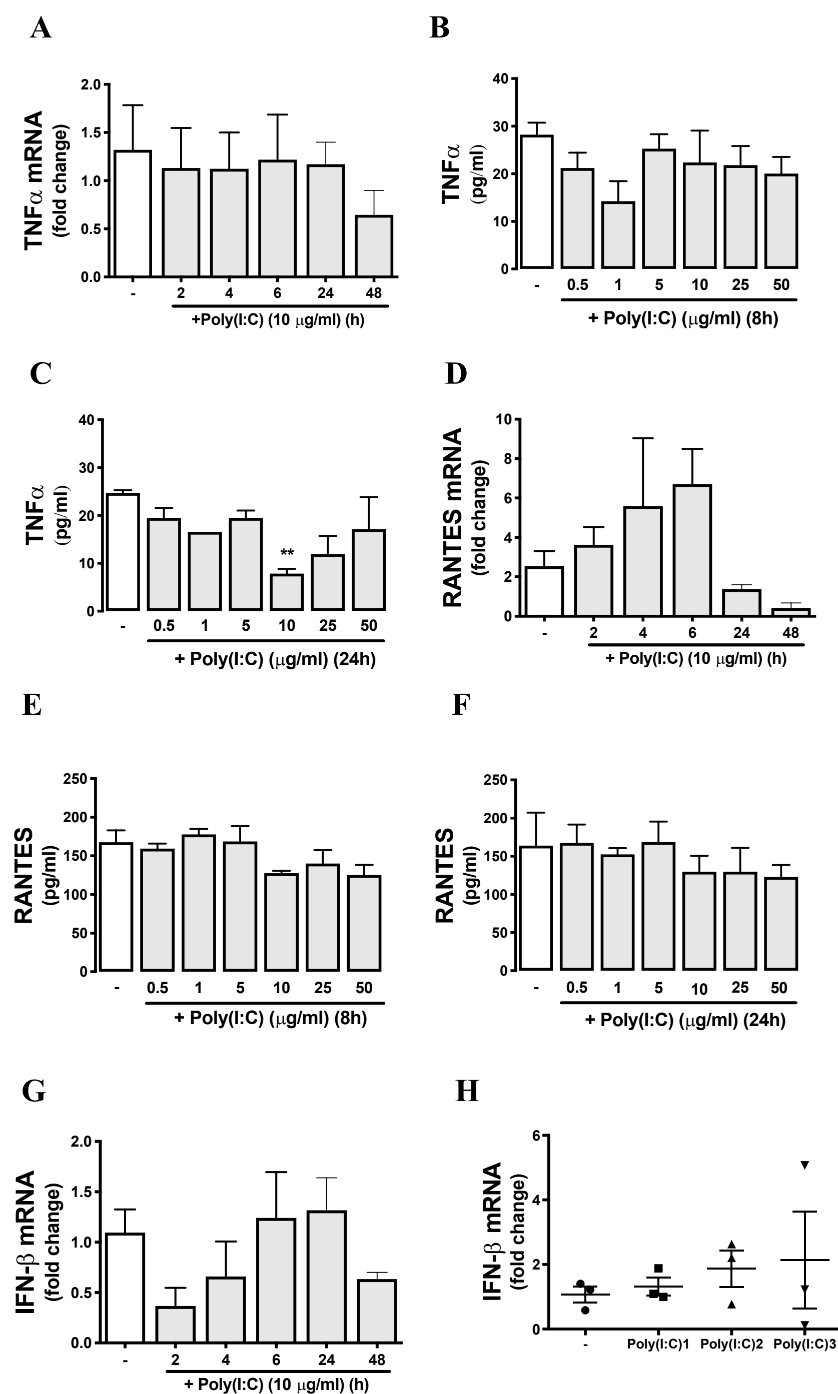


Figure 3.2. Poly(I:C) does not alter RANTES, TNFα and IFN-β expression in THP-1 monocytes. THP-1 monocytes were cultured with poly(I:C) over a range of concentrations (0.5 - 50 µg/ml) and timepoints (2 - 48 h). Supernatants were collected and analysed by ELISA, and RNA collected for analysis via RT-qPCR. Effect of poly(I:C) on **(A, B, C)** TNFα, **(D, E, F)** RANTES and **(G)** IFN-β expression at all timepoints tested up to 48 h. **(H)** Effect of treatment (for 4 h) with three different sources of poly(I:C) on *IFN-β* mRNA expression. **(C)** Poly(I:C) (10 µg/ml treatment at 24 h) significantly reduced TNFα protein expression. Data are presented as the mean ± S.E.M from 3 independent passages. Data were analysed using one-way ANOVA and Dunnett's post-hoc test. ***p*<0.01 *versus* untreated control cells.

3.4 TLR3 is expressed on THP-1-derived macrophages and treatment with poly(I:C) increases *TLR3*, but not *TLR4*, mRNA

The basal gene expression level of the poly(I:C) receptor, TLR3, was next determined in THP-1-derived macrophages. THP-1 monocytes were cultured in the presence of PMA (10 ng/ml; 4h h) to differentiate THP-1 monocytes to a macrophage-like phenotype. RNA was harvested and RT-qPCR performed to assess the expression of *TLR3* mRNA. 18S rRNA was determined as the endogenous control. Data presented in Table 2 indicates that *TLR3* mRNA is expressed in THP-1 macrophages.

Analysis of poly(I:C) treatment was conducted using three different sources of poly(I:C) to assess whether TLR3 signalling is functional in macrophages. PMA-treated THP-1 macrophages were treated separately with the three different sources (1, 2, 3) of poly(I:C) (outlined in methods) for 4 h. THP-1-derived macrophages were harvested in lysis buffer, RNA extracted and *TLR3* and *TLR4* mRNA expression analysed using RT-qPCR. Data presented in Fig 3.3 indicates that *TLR3* mRNA expression was increased, albeit insignificantly, following treatment with all three sources of poly(I:C) (Fig. 3.3A). In addition, poly(I:C) had no effect on *TLR4* mRNA expression levels (Fig. 3.3B), suggesting that each source of poly(I:C) tested in our experiments does not regulate TLR4 transcription in THP-1-derived macrophages.

Table 2: *TLR3* is expressed on THP-1-derived macrophages

Target	Average basal expression (Ct, *n*=3)
TLR3	33.22 ± 0.59
18S rRNA	13.43 ± 0.65

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

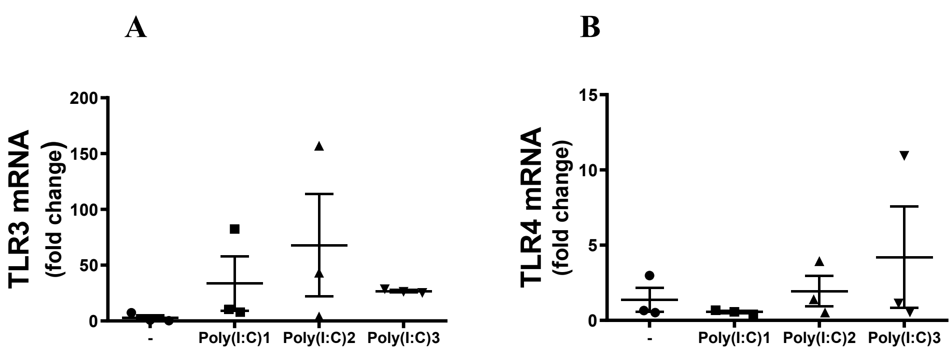


Figure 3.3. Effect of poly(I:C) on relative TLR3 and TLR4 mRNA expression in THP-1-derived macrophages. THP-1 monocytes were differentiated to a macrophage phenotype by treatment with PMA (10 ng/ml; 48 h). Macrophages were treated with three different sources (1, 2, 3) of poly(I:C) for 4 h and TLR3/4 expression assessed using RT-qPCR. Poly(I:C) promoted a trend towards increasing **(A)** *TLR3*, but not **(B)** *TLR4*, mRNA expression. Data were analysed using one-way ANOVA followed by Dunnett's post-hoc test. Data are presented as the mean ± S.E.M from 3 independent passages.

3.5 TLR3 activation enhances nuclear IRF3 expression and downstream induction of IFN-β and CXCL10 mRNA/protein in THP-1-derived macrophages

Due to the inability of poly(I:C) to activate TLR3 signalling in THP-1 monocytes (Fig. 3.2), we next determined if TLR3 signalling was operative in THP-1 monocytes differentiated to the macrophage-like phenotype by culturing cells in the presence of PMA (10 ng/ml) for 48 h. After 48 h, THP-1-derived macrophages were treated with poly(I:C) (10 μg/ml) for a range of time points (10 – 60 min) and the subcellular location of IRF3 analysed via immunocytochemistry. IRF3 expression was targeted as this transcription factor is known to promote the expression of type I IFNs [347]. Data presented in Fig. 3.4 indicate that poly(I:C) time-dependently increased IRF3 translocation to the nucleus, with a peak nuclear IRF3 immunofluorescence observed after 10 – 60 min (Fig. 3.4A, B). These data indicate that poly(I:C) triggers the sequestration of IRF3 in the nucleus and confirms that TLR3 signalling is functional in THP-1-derived macrophages.

Next, downstream targets of TLR3-IRF3 activation were assessed to confirm the activation of the viral innate immune pathway in THP-1 macrophages. The expression profile of the type I IFN, IFN-β, was determined at mRNA and protein levels, using RT-qPCR and ELISA, respectively, following treatment with three sources of poly(I:C) for 4 h. Data herein show that a significant induction of *IFN-β* mRNA was detected following treatment with poly(I:C) 2 (Fig. 3.4C), whereas poly(I:C) (all sources) significantly increased IFN-β protein expression (Fig. 3.4D). Additionally, the chemokine CXCL10 was chosen as a downstream target of TLR3-IRF3 signalling as this chemokine is a known responder to TLR3 activation [348]. Poly(I:C) treatment significantly increased CXCL10 mRNA and protein expression in THP-1-derived macrophages (Fig. 3.4E, F). These data suggest that THP-1 monocytes require differentiation to a macrophage phenotype to promote efficient TLR3 intracellular signalling events.

Finally, to assess the effect of TLR3 activation on the viability of macrophages, THP-1-derived macrophages were treated with poly(I:C) and cell viability was determined using MTT assays. Data in Fig. 3.4G indicates that poly(I:C) (4 h treatment) had no effect on the viability of THP-1-derived macrophages at both

concentrations tested (10 and 25 μg/ml) (Fig 3.4G). These data suggest that poly(I:C) is not toxic to THP-1-derived macrophages and that the effects of poly(I:C) on IRF3, IFN-β and CXCL10 expression are not associated with ligand cytotoxicity.

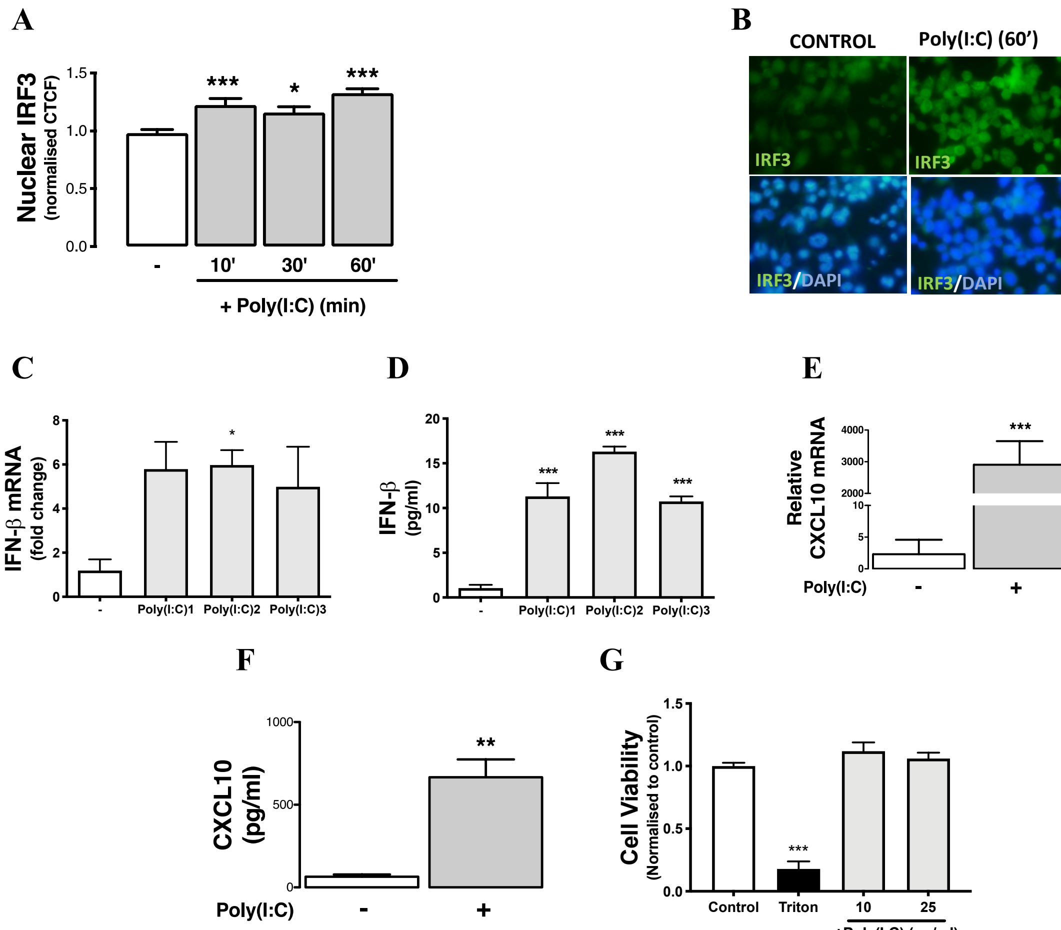


Figure 3.4. Poly(I:C) activates IRF3 and induces IFN-β/CXCL10 expression in THP-1-derived macrophages. (**A**) THP-1-derived macrophages were cultured on poly-L-lysine coated coverslips in the presence of poly(I:C) (10 μg/ml) for time points ranging from 10 - 60 min. Cells were stained with DAPI (bis-benzamide) and nuclear IRF-3 expression quantified using fluorescence immunocytochemistry. (**B**) Representative images of THP-1 macrophages showing IRF3 (green) alone and IRF3 merged with DAPI (blue). Immunofluorescence images taken at 60x magnification. The effect of three different sources (1, 2, 3) of poly(I:C) (10 μg/ml: 4 h) on the expression of IFN-β **(C)** mRNA and **(D)** protein. Poly(I:C) (10 μg/ml: 4 h) increased the expression of **(E)** *CXCL10* mRNA and **(F)** protein. **(G)** An MTT assay was used to determine the effect of poly(I:C) (10 and 25 μg/ml) on cell viability. Cells were treated 4 h prior to analysis. Triton x100 (0.2%) was used as a control. Data are presented as the mean ± S.E.M and are representative of 3-4 independent experiments. Data were analysed using Student's t-test or one-way ANOVA, followed by Dunnett's post-hoc test. *p<0.05, **p<0.01 and ***p<0.001 *versus* untreated cells.

3.6 TLR3 is expressed on primary human PBMCs, and treatment with poly(I:C) does not increase *TLR3* or *TLR4* mRNA in PBMCs

Given that TLR3 is expressed in both THP-1 monocytes (Table 1) and macrophages (Table 2), and differences in TLR3 signalling intermediates was determined between THP-1 monocytes and macrophages (Fig. 3.2 and 3.4) we next set out to characterise TLR3 signalling in primary immune cells. Given that TLR3 is expressed on T cells, B cells, monocytes, NK cells and DCs [21], we first determined the basal gene expression of TLR3 in primary PBMCs isolated from healthy volunteers. Three HC subjects donated whole blood via venepuncture and PBMCs were isolated using the filcoll-hypaque density gradient technique. Isolated PBMCs were cultured for 2 - 6 h, RNA harvested, and RT-qPCR performed to determine relative *TLR3* mRNA expression (Table 3). 18S rRNA was used as the endogenous control and Ct values are indicated. Data presented in Table 3 indicate that TLR3 is expressed on primary human PBMCs. In the next set of experiments, PBMCs were plated at a low density (0.5 x 10^6 cells/well), treated with a high concentration of poly(I:C) (25 μg/ml) for three timepoints (2, 4, and 6 h), and the expression profile of *TLR3* and *TLR4* mRNA assessed using RT-qPCR. Our findings indicate that poly(I:C) did not significantly impact *TLR3* (Fig. 3.5A) or *TLR4* (Fig 3.5B) mRNA in PBMCs from HC subjects.

Table 3. ***TLR3* is expressed in human PBMCs**

Target	Average basal expression (Ct, *n*=3)
TLR3	33.86 ± 0.59
18S rRNA	15.72 ± 0.33

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

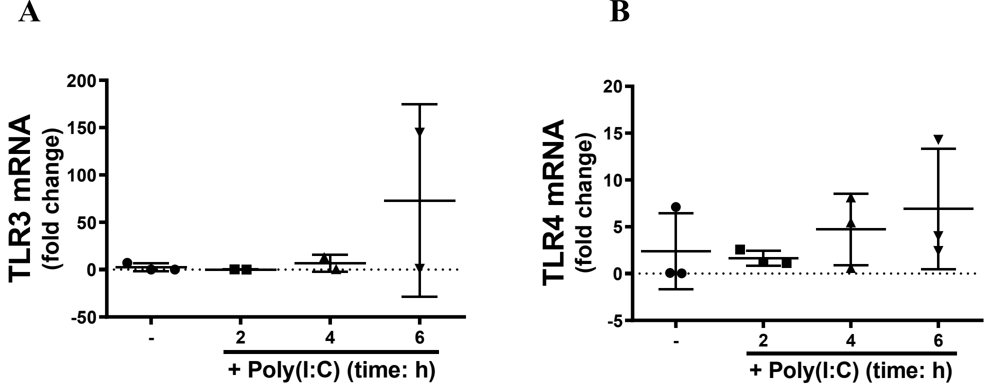


Figure 3.5. Effect of poly(I:C) on TLR3 and TLR4 mRNA expression in PBMCs plated at low cell densities. PBMCs isolated from healthy volunteers were cultured in the presence of poly(I:C) (25 µg/ml) for 2 - 6 h and the expression of (**A**) *TLR3* and (**B**) *TLR4* mRNA determined via RT-qPCR. Data are presented as the mean ± S.E.M from 2-3 HC subjects. Data were checked for normality using Shapiro-Wilk and analysed using one-way ANOVA, followed by Dunnett's post-hoc test.

3.7 TLR3 activation does not increase RANTES or TNFα protein, in addition to *IFN-β* mRNA, in PBMCs seeded at a low density

Given that poly(I:C) differentially modulates TLR3 signalling intermediates in THP-1 monocytes (Fig. 3.2) and THP-1-derived macrophages (Fig. 3.4), we next assessed the effect of TLR3 activation on key signalling read-outs in PBMCs from healthy volunteers. PBMCs from HC subjects were cultured with poly(I:C) to optimise the concentration/timepoints required to induce cytokine and chemokine production in this cell type. PBMCs were initially cultured at a cell density of 0.5 x 10^6 cells/well, with or without poly(I:C) (0.5 - 50 µg/ml), for 8 h and 24 h, and supernatants analysed for RANTES (Fig. 3.6A, B) and TNFα (Fig. 3.6C, D) protein expression via ELISA. Poly(I:C) did not promote RANTES expression in PBMCs, and significantly downregulated RANTES expression at a concentration of 5 µg/ml (8 h treatment) (Fig. 3.6A). Furthermore, poly(I:C) had no effect on the expression of the pro-inflammatory cytokine TNFα at both timepoints (8 and 24 h) assessed (Fig. 3.6C, D). Additionally, *IFN-β* mRNA expression levels were assessed after treatment with poly(I:C) (10 µg/ml) for a range of timepoints (2 - 6 h) by RT-qPCR. Data presented in Fig. 3.6E demonstrate that poly(I:C) did not significantly induce *IFN-β* mRNA expression in primary PBMCs at each timepoint tested.

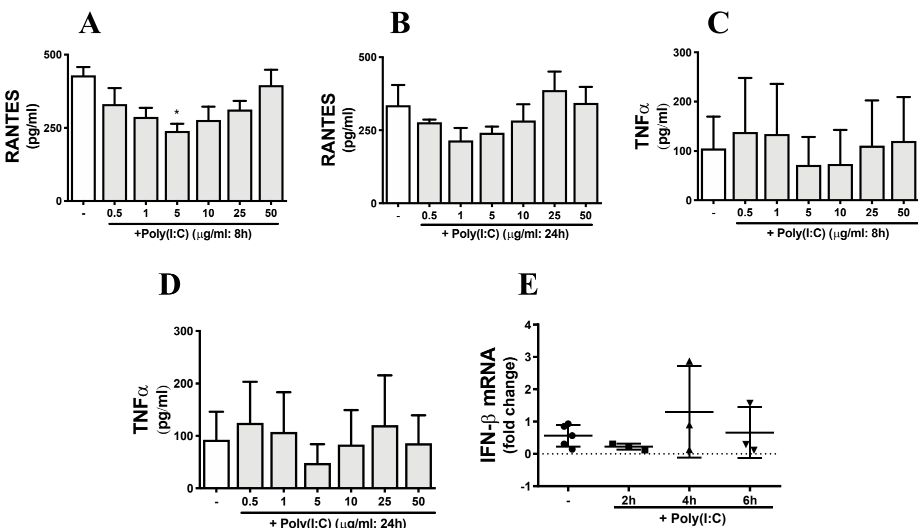


Figure 3.6. TLR3 activation does not promote TNFα, RANTES and IFN-β expression in PBMCs from healthy volunteers. The concentration of **(A, B)** RANTES and **(C, D)** TNFα protein was determined in primary human PBMCs cultured at a low cell density (0.5 x 10^6 PBMCs/well) following exposure to poly(I:C) (0.5 - 50 µg/ml) for either 8 h or 24 h. **(E)** Poly(I:C) had no effect on *IFN-β* mRNA (25 µg/ml: 2 - 6 h) expression. Data were analysed using one-way ANOVA followed by Dunnett's post-hoc test. Data are represented as the mean ± S.E.M from 2 - 4 HC donors. *p<0.05 *versus* untreated control cells.

3.8 Poly(I:C) treatment increases IFN-β and CXCL10 (mRNA and protein) expression, in addition to *TLR3* mRNA, in PBMCs seeded at a high density

Data presented in Fig. 3.6 indicates that when PBMCs are plated at a low density of 0.5 x 10^6 cells/well, and treated with a high concentration of poly(I:C) (25 μg/ml), no increase in RANTES, TNFα, or IFN-β was determined (Fig. 3.6). Furthermore, data previously presented indicates that poly(I:C) (4 h treatment at 10 μg/ml) promotes an induction of *TLR3* mRNA (Fig 3.3), IFN-β and CXCL10 (Fig 3.4) in THP-1-derived macrophages, when cells are seeded at a higher concentration of cells (1 x 10^6 cells/well). Therefore, in the next series of experiments primary human PBMCs were cultured at a high cell density (2 x 10^6 cells/well) and treated with three sources of poly(I:C) (poly(I:C) 1, 2, 3) (10 μg/ml) for 4 h. Supernatants were harvested for protein determination of cytokines and chemokines by ELISA, and RNA harvested, cDNA synthesized and gene expression of cytokines and chemokines determined by RT-qPCR. Using this approach (higher plating density), exposure of PBMCs to poly(I:C) promoted a trend towards increased *TLR3* mRNA expression (Fig. 3.7A), however one-way ANOVA analysis revealed there were no significant differences between group means ($p=0.1255$). Additionally, *IFN-β* mRNA showed a trend towards increased expression with all three sources of poly(I:C) tested (Fig 3.7B), however one-way ANOVA analysis revealed there were no significant differences between group means ($p=0.2856$). Poly(I:C) treatment (4 h) also promoted a trend towards induction of IFN-β protein expression in PBMCs from HC cases (Fig 3.7C). In our hands, this was the first data to suggest that TLR3 signalling can be activated by poly(I:C) in primary PBMCs. Therefore, a series of timecourse experiments were next conducted (0 - 24 h) to determine the optimal timepoint for induction of key signalling targets, namely IFN-β, CXCL10, and TNFα. Indeed, TLR3 activation promoted IFN-β (Fig 3.7D) and CXCL10 (Fig 3.7E) protein expression in PBMCs following treatment for 24 h. In contrast, TLR3 activation failed to promote TNFα protein expression in PBMCs at all timepoints assessed (Fig 3.7F). In addition, *CXCL10* and *TNFα* mRNA expression was assessed in PBMCs following poly(I:C) (10 μg/ml; 4 h) treatment. Data presented in Fig 3.7G, H indicate that *CXCL10* and *TNFα* mRNA expression was insignificantly increased by poly(I:C), respectively.

To determine whether the TLR3 agonist, poly(I:C), was cytotoxic in primary immune cells, MTT cell viability assays were performed in primary human PBMCs following treatment with poly(I:C). Isolated PBMCs were cultured with poly(I:C) (10 μg/ml) for 24 h. Data presented in Fig. 3.7I indicate that poly(I:C) had no effect on cell viability in PBMCs at the concentration tested in eight HC donors (Fig 3.7I). These data suggest that poly(I:C) does not affect the viability of primary human PBMCs isolated from HC volunteers. Overall, these data indicate that a high seeding density is required to determine clear cellular read-outs (IFN-β and CXCL10) for components of the TLR3 signalling pathway in response to poly(I:C) in primary PBMCs.

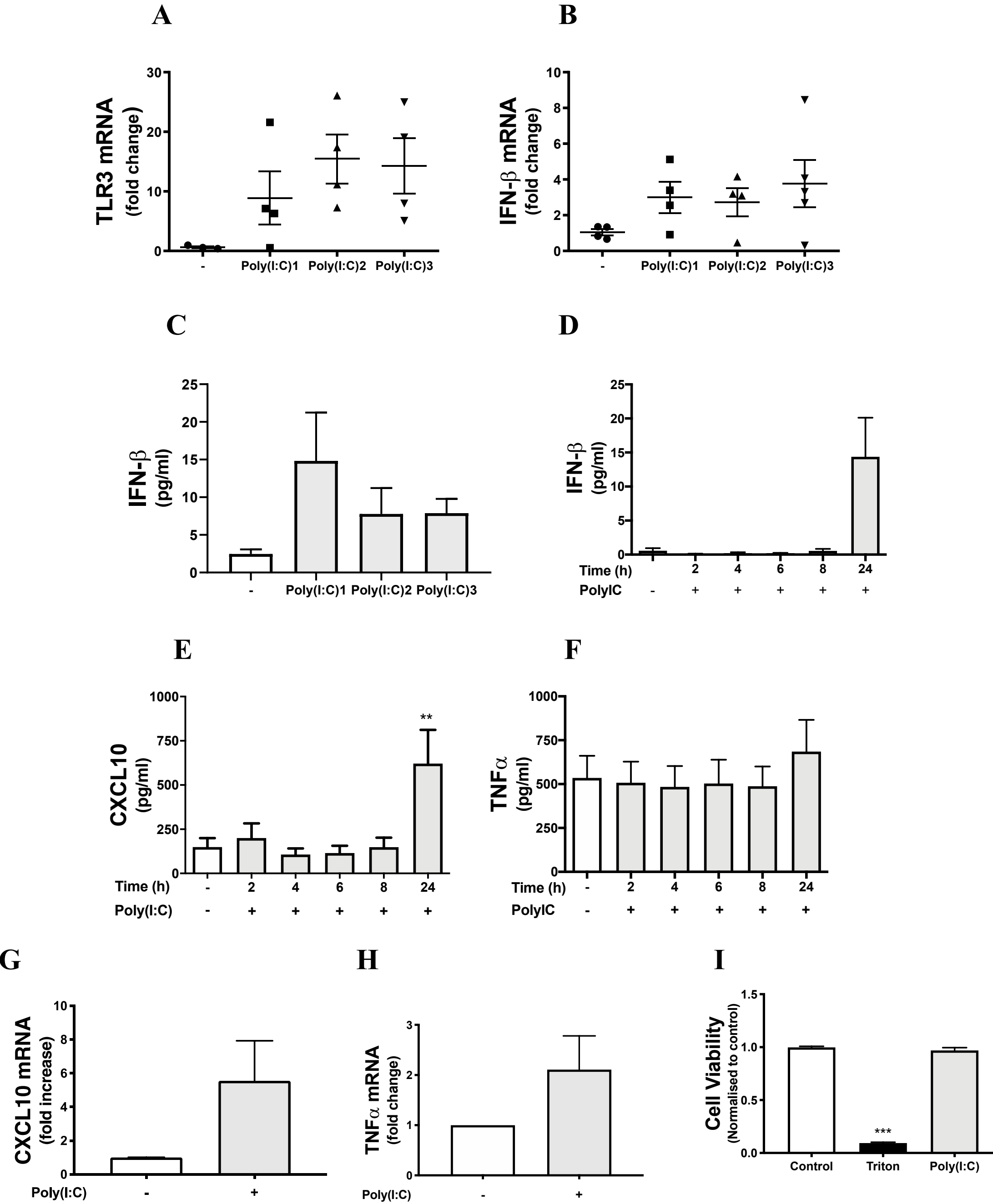


Figure 3.7. Poly(I:C) increases expression of CXCL10 in primary human PBMCs. Primary PBMCs isolated from HC subjects were cultured at a higher cell density (2 x 10^6 PBMCs/well) and treated with poly(I:C) (10 μg/ml) for 4 h (for mRNA) or 2 - 24 h (for protein expression). Effect of poly(I:C) on the expression of **(A)** *TLR3* and **(B)** *IFN-β* mRNA and **(C)** IFN-β protein expression using three different sources of poly(I:C). Poly(I:C) increased **(D)** IFN-β, **(E)** CXCL10, but not **(F)** TNFα, protein expression at 24 h. Effect of poly(I:C) on **(G)** *CXCL10* and **(H)** *TNFα* mRNA following poly(I:C) treatment (4 h). **(I)** An MTT assay was used to determine the effect of poly(I:C) (10 μg/ml) on PBMC viability. PBMCs were treated with poly(I:C) for 24 h and triton x100 (0.2%) was used as a positive control. Data are represented as the means ± S.E.M from 4 - 6 HC donors. Data were analysed using one-way ANOVA, followed by Dunnett's post-hoc test or using students t-test where appropriate. ***p*<0.01 and ****p*<0.001 *versus* untreated cells.

3.9 TLR4 is expressed on THP-1 monocytes

LPS is a potent activator of TLR4 signalling events [349]. The human monocytic cell line THP-1 was chosen as a model to characterise LPS stimulation prior to use of primary PBMCs given that LPS has been shown to promote COX-2 and NF-κB-related genes in THP-1 cells [350]. Initially, the basal gene expression profile of *TLR4* mRNA was determined in three passages of THP-1 monocytes (Table 4). 18S rRNA was used as an endogenous control and Ct values are shown. Data presented in Table 4 indicate that *TLR4* mRNA is abundantly expressed in THP-1 monocytes. To determine the effect of LPS on cell viability in THP-1 monocytes, MTT assays were performed. A concentration of 100 ng/ml of LPS was tested as a body of literature indicates that this concentration significantly promotes TLR4-induced inflammatory signalling events in monocytes [351]. Data presented in Fig 3.8 indicates that following a 24 h incubation, LPS had no effect on the viability of THP-1 monocytes (Fig. 3.8).

Table 4. *TLR4* expression in THP-1 monocytes.

Target	Average basal Ct expression (*n*=3)
TLR4	24.92 ± 0.18
18S rRNA	11.91 ± 0.34

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

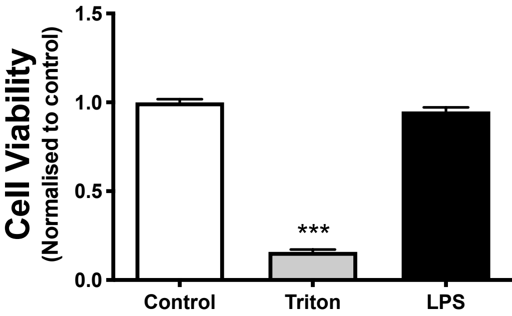


Figure 3.8. LPS does not affect THP-1 monocyte viability. MTT assays were used to determine the effect of LPS (100 ng/ml) on cell viability. THP-1 monocytes were treated with LPS for 24 h prior to analysis, and triton x100 (0.2%) was used as a positive control. Data are presented as the means ± S.E.M from three separate cell passages. Data were analysed using one-way ANOVA followed by Dunnett's post-hoc test. ***$p<0.001$ *versus* control cells.

3.10 TLR4 activation induces RANTES, TNFα and IFN-β expression in THP-1 monocytes

To characterise TLR4-induced inflammatory signalling in THP-1 monocytes, and to optimise the concentration and duration of treatment required to induce cytokine/chemokine expression in response to LPS, THP-1 monocytes were cultured with or without LPS (1 – 1000 ng/ml) for 8 h and 24 h, and supernatants analysed for TNFα and RANTES protein expression via ELISA. Additionally, the relative concentration of *TNFα*, *RANTES* and *IFN-β* mRNA in THP-1 monocytic cells was determined by RT-qPCR following exposure to LPS (100 ng/ml) for 2 - 6 h. Data presented indicates that LPS induced an increase in *TNFα* mRNA expression at 2, 4 and 6 h, with a significant increase in expression determined at 2 and 6 h post-treatment (Fig. 3.9A). There was a significant increase in TNFα protein expression at 8 h (Fig. 3.9B) for all concentrations tested, with the strongest effect observed with 50 ng/ml LPS treatment. Similarly, TNFα protein expression was significantly induced at all LPS concentrations tested, apart from the 1 ng/ml LPS concentration at 24 h (Fig. 3.9C), with the strongest induction achieved at a final concentration of 100 ng/ml. Furthermore, there was a significant increase in *RANTES* mRNA expression in THP-1 monocytes following LPS stimulation, with peak expression observed at 6 h post-LPS treatment (Fig. 3.9D). RANTES protein expression was significantly induced following treatment with LPS for 8 h (Fig. 3.9E), except at the 1000 ng/ml LPS concentration, with the strongest induction observed at 10 ng/ml. RANTES protein expression was also significantly induced at all concentrations of LPS at 24 h (Fig. 3.9F), with the strongest protein production determined at the 100 ng/ml LPS concentration. Interestingly, LPS-induced RANTES production was higher at the 24 h time point compared to the 8 h time point, while conversely, LPS-induced TNFα protein production was higher following 8 h incubation, compared to 24 h. *IFN-β* mRNA expression was also assessed given that LPS has been shown to induce IFN-β via IRF signalling in a macrophage cell line [352]. Data presented in Fig. 3.9G indicates that LPS induced *IFN-β* mRNA expression at 2 h and 6 h (albeit insignificant), with no induction determined at 4 h (Fig. 3.9G). These data suggest that THP-1 monocytes respond to TLR4 activation via LPS, which can initiate MyD88-dependent (TNFα) and MyD88-independent (RANTES and IFN-β) signalling

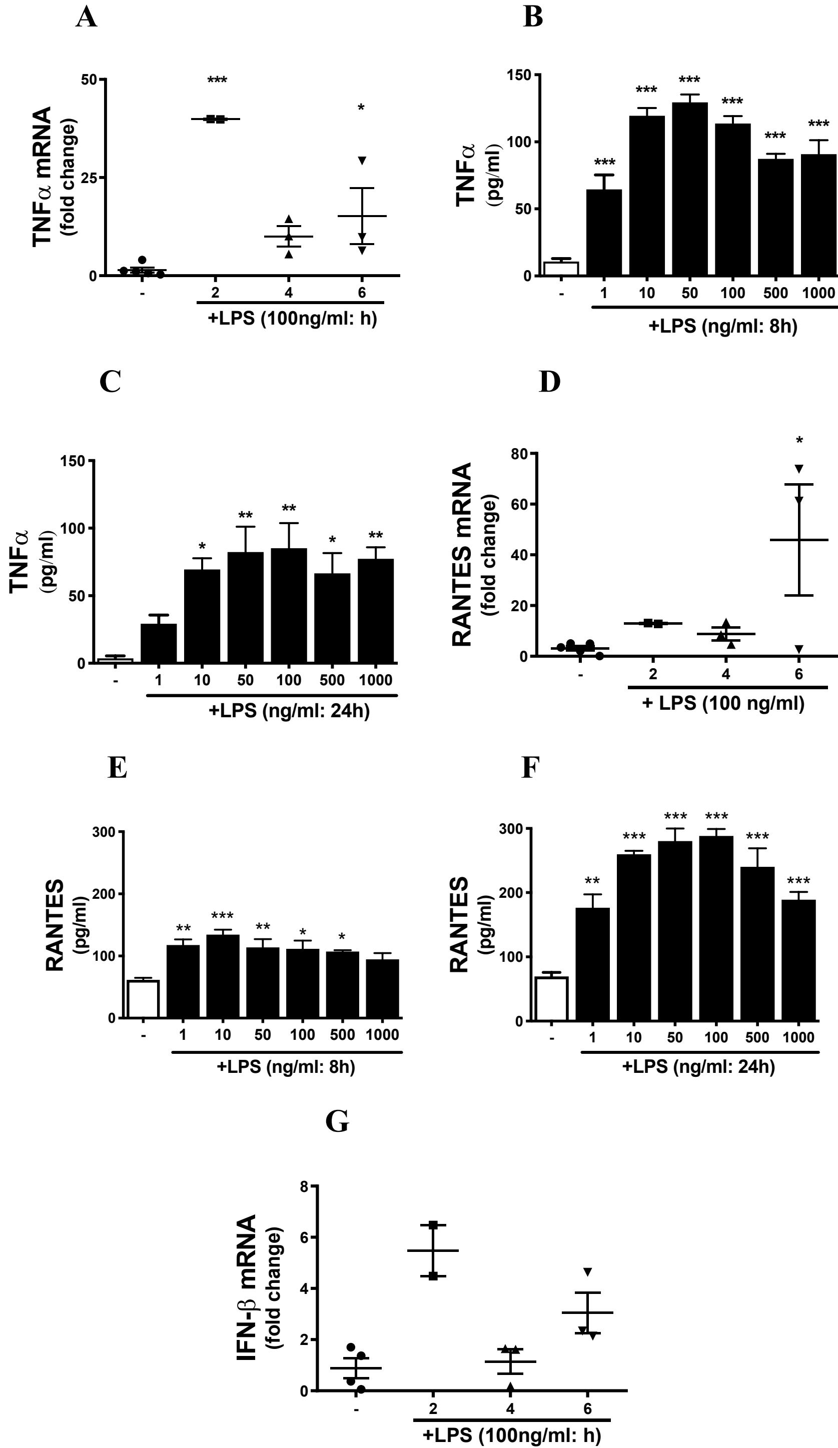


Figure 3.9. LPS promotes TNFα, RANTES and IFN-β expression in THP-1 monocytes. THP-1 monocytes were treated with a range of LPS concentrations (1-1000 ng/ml) for 8 or 24 h and supernatants analysed via ELISA. For mRNA detection, THP-1 monocytes were cultured with LPS (100 ng/ml) for 2, 4, or 6 h and gene expression determined via RT-qPCR. LPS promoted an increase in the expression of TNFα **(A)** mRNA and **(B, C)** protein, along with RANTES **(D)** mRNA and **(E, F)** protein, and **(G)** *IFN-β* mRNA. Data are presented as the mean ± S.E.M from 3 separate passages for protein detection, and 2-3 separate passages for mRNA detection. One-way ANOVA with Dunnets post-hoc test used. **p*<0.05, ***p*<0.01 and ****p*<0.001 *versus* untreated cells.

3.11 TLR4 is expressed in THP-1-derived macrophages

Macrophages are an important cell of the innate immune system, and play a key role in MS pathology by exerting both neuroprotective effects and contributing to tissue damage by production of inflammatory proteins [353]. As previously indicated, THP-1 monocytes can be differentiated to macrophage-like cells by treatment with a low concentration of PMA, and respond to LPS [56]. Indeed, previous data elucidated TLR3 signalling in monocytes (Fig. 3.2) and macrophages (Fig. 3.4) and showed that TLR3 signalling is operative in macrophages, but not monocytes. Therefore, a similar pattern of analysis was utilized to investigate TLR4 signalling. The basal gene expression level of the LPS receptor, TLR4, was determined in THP-1 macrophages. THP-1 monocytes were cultured in the presence of PMA (10 ng/ml) for 48 h, RNA was harvested, and RT-qPCR performed to assess *TLR4* mRNA expression in macrophages. 18S rRNA was determined as the endogenous control. Data presented in Table 5 indicates that *TLR4* mRNA was abundantly expressed in THP-1 macrophages. To determine the effect of LPS on cell viability in THP-1-derived macrophages, MTT assays were performed. A concentration of 100 ng/ml of LPS was tested as a body of literature indicates that this concentration significantly promotes inflammatory signalling events in monocytes [351], and previous data indicated herein employed the use of this concentration of LPS in THP-1 monocytes (Fig. 3.9). After 4 h incubation, LPS had no effect on cell viability in THP-1-derived macrophages (Fig. 3.10). This indicates that the effects of LPS on inflammatory read-outs are not associated with toxicity associated with LPS incubation.

Table 5. ***TLR4*** **expression in THP-1-derived macrophages.**

Target	Average basal Ct values (*n*=3)
TLR4	23.29 ± 0.33
18S rRNA	13.69 ± 0.67

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

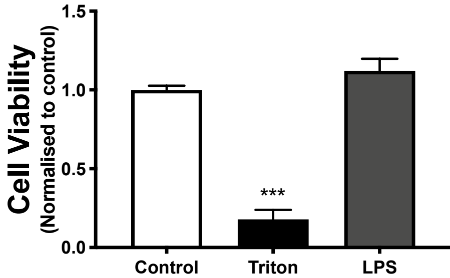


Figure 3.10. LPS does not alter THP-1-derived macrophage viability. An MTT assay was used to determine the effect of LPS (100 ng/ml; 24 h) on the viability of THP-1-dervied macrophages. Triton x100 (0.2%) was used as a positive control. Data are presented as mean ± S.E.M from three passages. Data were analysed using one-way ANOVA followed by Dunnett's post-hoc. ***p<0.001 *versus* control cells.

3.12 TLR4 activation induces NF-κB and IRF3 activation, IκB-α degradation, while promoting the downstream expression of TNFα, IFN-β and CXCL10 in THP-1-derived macrophages

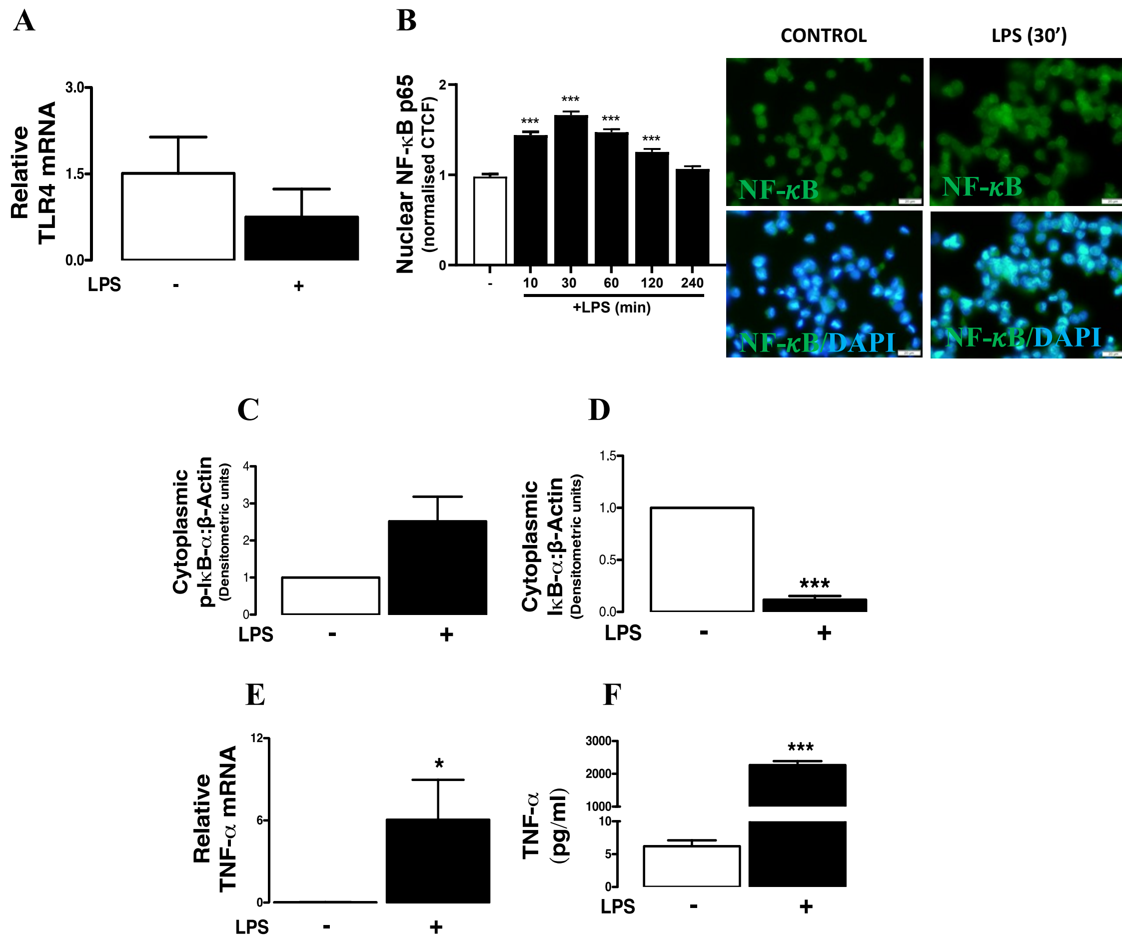
Following an investigation of LPS signalling in THP-1 monocytes (Fig. 3.9), we next set out to determine the kinetics of TLR4 signalling in macrophages using differentiated THP-1 cells. Both, TLR4-NF-κB and TLR4-IRF3 signalling were assessed. THP-1 monocytes were differentiated to a macrophage phenotype using PMA (10 ng/ml) for 48 h. The freshly differentiated THP-1-derived macrophages were then treated with LPS (100 ng/ml) for a range of timepoints (10 - 240 min) and analysed via immunocytochemistry (to assess nuclear NF-κB p65 and IRF3 expression). TNFα, CXCL10, and IFN-β protein and mRNA expression were determined via ELISA and RT-qPCR following LPS treatment (100 ng/ml; 4 h), respectively. Cytoplasmic expression of p-IκB-α and total IκB-α, in addition to cytoplasmic and nuclear expression of pIRF3, was determined by western immunoblot analysis.

In the first set of experiments, the effect of LPS on TLR4 expression was determined via RT-qPCR. Interestingly, LPS had no effect on *TLR4* mRNA at the timepoint assessed (4 h) (Fig. 3.11A). Data presented in Figure 3.11B demonstrates that LPS time-dependently induced NF-κB translocation to the nucleus, with a peak in nuclear expression observed at 30 min post-treatment. LPS treatment for 30 min promoted IκB-α phosphorylation (Fig. 3.11C), while significantly promoting IκB-α degradation (Fig. 3.11D). Data presented in Figure 3.11E, F demonstrates that LPS significantly induced TNFα mRNA and protein expression, indicating that THP-1-derived macrophages respond to LPS agonism and promote MyD88-dependent signalling events in this cell type.

Additionally, TLR4-induced MyD88-independent signalling was assessed in THP-1-derived macrophages in terms of IRF3 activation and CXCL10/IFN-β production. The effect of LPS (100 ng/ml), over a range of timepoints (0 - 60 min), on cytoplasmic and nuclear expression of pIRF3 was determined via western immunoblot analysis using β-actin as a housekeeping control. Peak expression of cytoplasmic (Fig. 3.11G) and nuclear (Fig. 3.11H) pIRF3 was determined at 60 min

following LPS treatment; therefore this timepoint was chosen for future LPS treatments to assess cannabinoid impact on TLR4-induced pIRF3. Next, the effect of LPS (100 ng/ml), over a range of timepoints (0 - 240 min), on total nuclear expression of IRF3 was determined via immunocytochemistry. LPS significantly induced peak nuclear expression of IRF3 at 30 min post-treatment (Fig. 3.11I); this timepoint was employed for future IRF3 immunocytochemistry analysis.

Finally, *IFN-β* and *CXCL10* mRNA and protein expression was determined in THP-1 macrophages, given that LPS has been shown to induce IFN-β via IRF proteins [352], and data also indicate that CXCL10 is activated via IRF3 transcription factor [354]. Indeed, our findings indicate that LPS significantly induced *IFN-β* mRNA and protein expression (Fig. 3.11J, K), and significantly increased CXCL10 mRNA and protein (Fig. 3.11L, M) expression in THP-1-derived macrophages. These findings indicate that THP-1-derived macrophages are a suitable *in vitro* model to assess TLR4 signalling mechanisms via MyD88-dependent and -independent mechanisms.



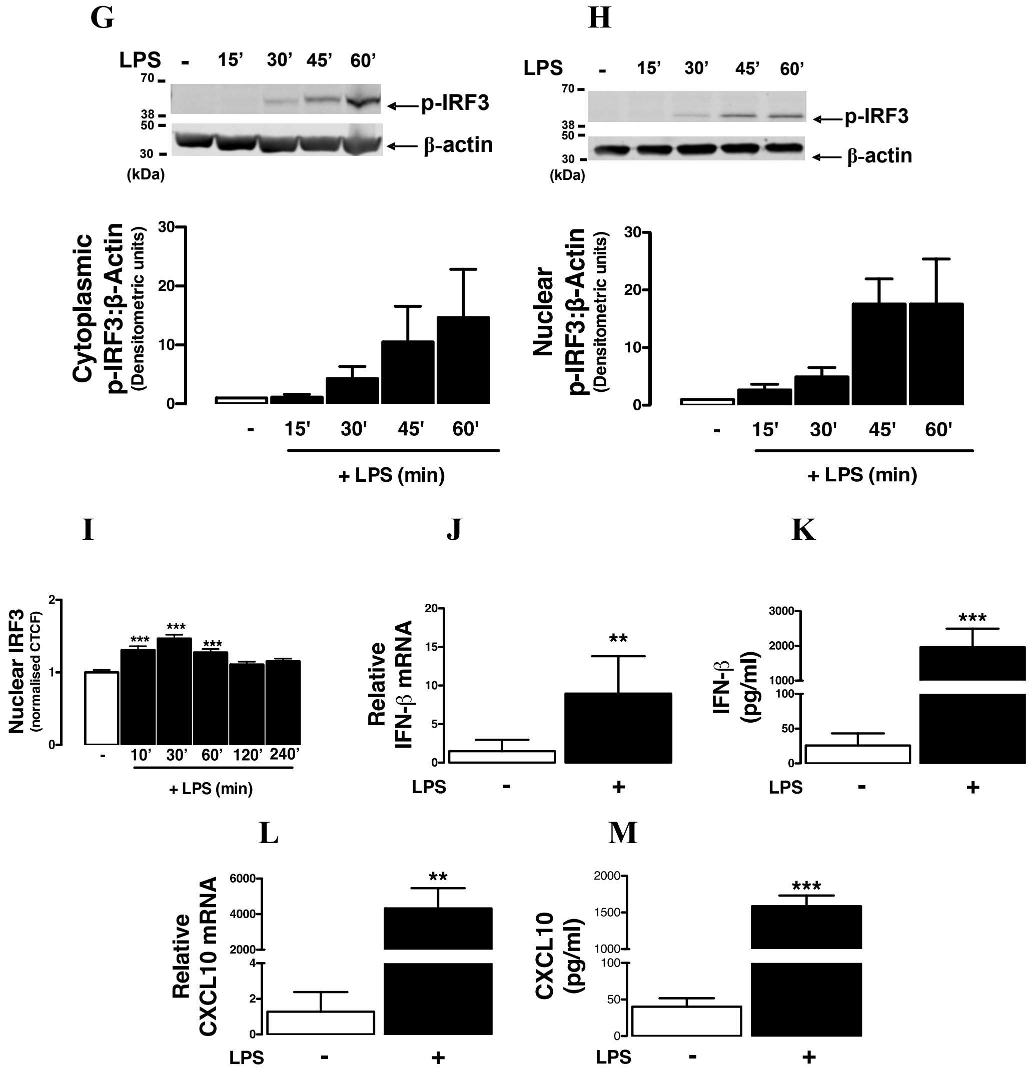


Figure 3.11. LPS promotes MyD88-dependent and independent signalling in THP-1-derived macrophages. Macrophages were treated with LPS (100 ng/ml) for 4 h (protein and mRNA detection), 0 - 240 min for immunocytochemical analysis or 0 - 60 min for western blot analysis. pIκB-α and IκB-α were detected after 30 min stimulations with LPS. **(A)** LPS did not alter *TLR4* mRNA. **(B)** LPS promoted NF-κB p65 nuclear sequestration, with representative images of cells showing DAPI (blue), NF-κB (green) and merge (blue and green) immunofluorescence images taken at 60x magnification. Scale bar = 20 μM. LPS promotes **(C)** IκB-α phosphorylation and **(D)** IκB-α degradation. LPS increased the expression of **(E)** *TNF*α mRNA and **(F)** TNFα protein. Time-dependent induction of **(G)** cytoplasmic and **(H)** nuclear expression of pIRF3 following LPS stimulation. LPS treatment promoted **(I)** IRF3 translocation to the nucleus. The expression of IFN-β and CXCL10 **(J, L)** mRNA and **(K, M)** protein was induced following LPS treatment. Data are expressed as mean ± S.E.M from 3-6 independent passages. Data were analysed using Student's t-test or one-way ANOVA, followed by Dunnett's post-hoc test as appropriate. *p<0.05, **p<0.01 and ***p<0.001 *versus* untreated cells.

3.13 TLR4 is expressed in primary human PBMCs and the effect of LPS treatment on TLR4 receptor expression in PBMCs

Considering that LPS signalling has been characterised in both THP-1 monocytes (Fig. 3.9) and THP-1-derived macrophages (Fig. 3.11), next TLR4 signalling was assessed in PBMCs isolated from healthy individuals. The PBMC population consists of several immune cell types including B cells (~15%), T cells (~70%) monocytes (~5%) and NK cells (~10%) [355], and produce cytokines/chemokines following LPS stimulation [356]. We first characterised the basal gene expression profile of the LPS receptor, TLR4, in primary human PBMCs isolated from HC subjects. Three HC subjects donated whole blood via venepuncture and PBMCs were isolated using the filcoll-hypaque technique. Isolated PBMCs were cultured for 24 h, RNA harvested, and RT-qPCR performed to determine relative *TLR4* mRNA expression (Table 6). 18S rRNA was used as the endogenous control and Ct values are also indicated. Data presented in Table 6 indicate that TLR4 is expressed in primary PBMCs.

In the next analysis, the relative concentration of *TLR3* and *TLR4* mRNA in healthy human PBMCs was determined via RT-qPCR following exposure to LPS (100 ng/ml) for 2 - 6 h. Data presented in Fig. 3.12A indicate that there was a trend towards increased *TLR4* mRNA expression at 2 h and 6 h post-LPS treatment (Fig. 3.12A). In addition, LPS had no significant effect on *TLR3* mRNA expression at the timepoints tested (2 - 6 h), however there was an increase in *TLR3* mRNA following LPS treatment (at 4 h) in one healthy donor (Fig. 3.12B). These data indicate that LPS has the proclivity to time-dependently regulate its own receptor mRNA expression in PBMCs isolated from HC volunteers.

To determine whether the TLR4 agonist, LPS, was cytotoxic to primary cells, an MTT cell viability assay was performed on primary PBMCs following treatment with LPS. A single concentration of LPS was tested (100 ng/ml). Human whole blood was obtained via venepuncture, PBMCs were isolated and cultured with LPS for 24 h. Data presented in Fig. 3.12C indicate that LPS had no effect on cell viability in PBMCs at the concentration tested in eight HC donors (Fig. 3.12C). These data suggest that LPS is not toxic to human primary PBMCs.

Table 6. TLR4 is expressed on healthy human PBMCs

Target	Average basal expression (Ct) (*n*=3)
TLR4	31.67 ± 0.20
18S rRNA	20.47 ± 0.82

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

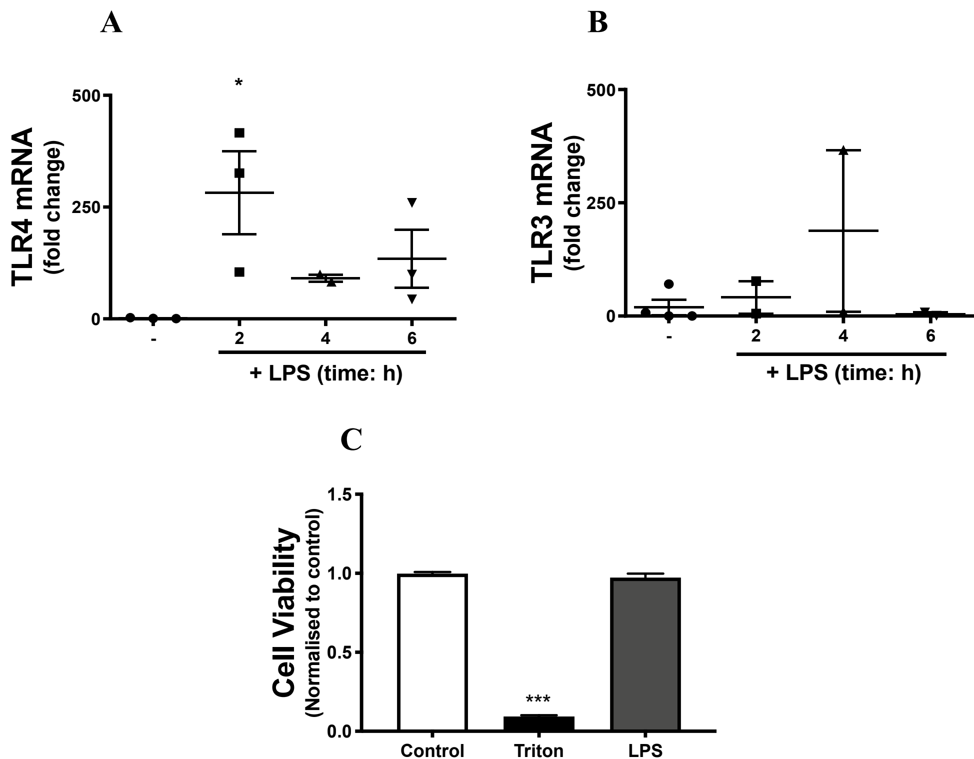


Figure 3.12. Effect of TLR4 activation on TLR3 and TLR4 mRNA expression in PBMCs from HC subjects. PBMCs were isolated from whole blood of healthy donors, plated at $1x10^6$ cells/ml and treated with LPS (100 ng/ml) for 2, 4, or 6 h. The expression of *TLR3* and *TLR4* mRNA was analysed by RT-qPCR. LPS had no significant effect on **(A)** *TLR4* and **(B)** *TLR3* mRNA in primary human PBMCs. **(C)** An MTT assay was employed to determine the effect of LPS (100 ng/ml) on cell viability. PBMCs were isolated from whole blood of HC donors and treated with LPS for 24 h. Triton x100 (0.2%) was used as a positive control. Data are presented as the mean ± S.E.M from **(A, B)** 2-3 or **(C)** 8 HC donors. Data were analysed using one-way ANOVA followed by Dunnett's post-hoc test. ***p<0.001 *versus* control cells.

3.14 Effect of LPS on TNFα, IFN-β and CXCL10 expression in primary PBMCs

To further characterise TLR4-induced signalling events, and to optimise the concentration/timepoints of LPS required to induce cytokine/chemokine production in primary human immune cells, primary PBMCs were cultured with or without LPS (1 – 1000 ng/ml) for 8 h and 24 h, and supernatants analysed for TNFα and RANTES protein expression by ELISA. LPS had no effect on RANTES protein production at both timepoints assessed (Fig. 3.13A, B). *TNFα* mRNA expression was significantly increased following LPS treatment (4 h: 100 ng/ml) in primary PBMCs (Fig. 3.13C). Furthermore LPS significantly increased the expression of TNFα protein at both timepoints tested (8 and 24 h), with a significant increase observed at 1, 10, 100 and 1000 ng/ml concentrations at 8 h (Fig. 3.13D), and for all concentrations of LPS tested at 24 h (Fig. 3.13E). A stronger signal for TNFα, reflected in a higher concentration of protein, was exhibited following LPS treatment for 24 h.

To further determine the optimal timepoint to treat PBMCs with LPS to promote inflammatory signalling, PBMCs from HC subjects were cultured with LPS (100 ng/ml) for a range of timepoints including 2, 4, 6, 8 and 24 h, and assessed for TNFα, IFN-β and CXCL8 protein expression. The expression of *IFN-β* and *CXCL10* mRNA was also assessed following LPS treatment by RT-qPCR. Data show the LPS significantly induced TNFα protein expression (at 24 h) (Fig. 3.13F), but not IFN-β (Fig. 3.13H), CXCL10 (Fig. 3.13J) or CXCL8 (Fig. 3.13K) protein expression. However, an insignificant increase in *IFN-β* mRNA was detected after 2 h LPS treatment (Fig. 3.13G), and a significant increase in *CXCL10* mRNA was found after 4 h LPS treatment (Fig. 3.13I). These data suggest that LPS preferentially activates MyD88-dependent targets (TNFα), but not MyD88-independent (CXCL10 and IFN-β), in primary human PBMCs.

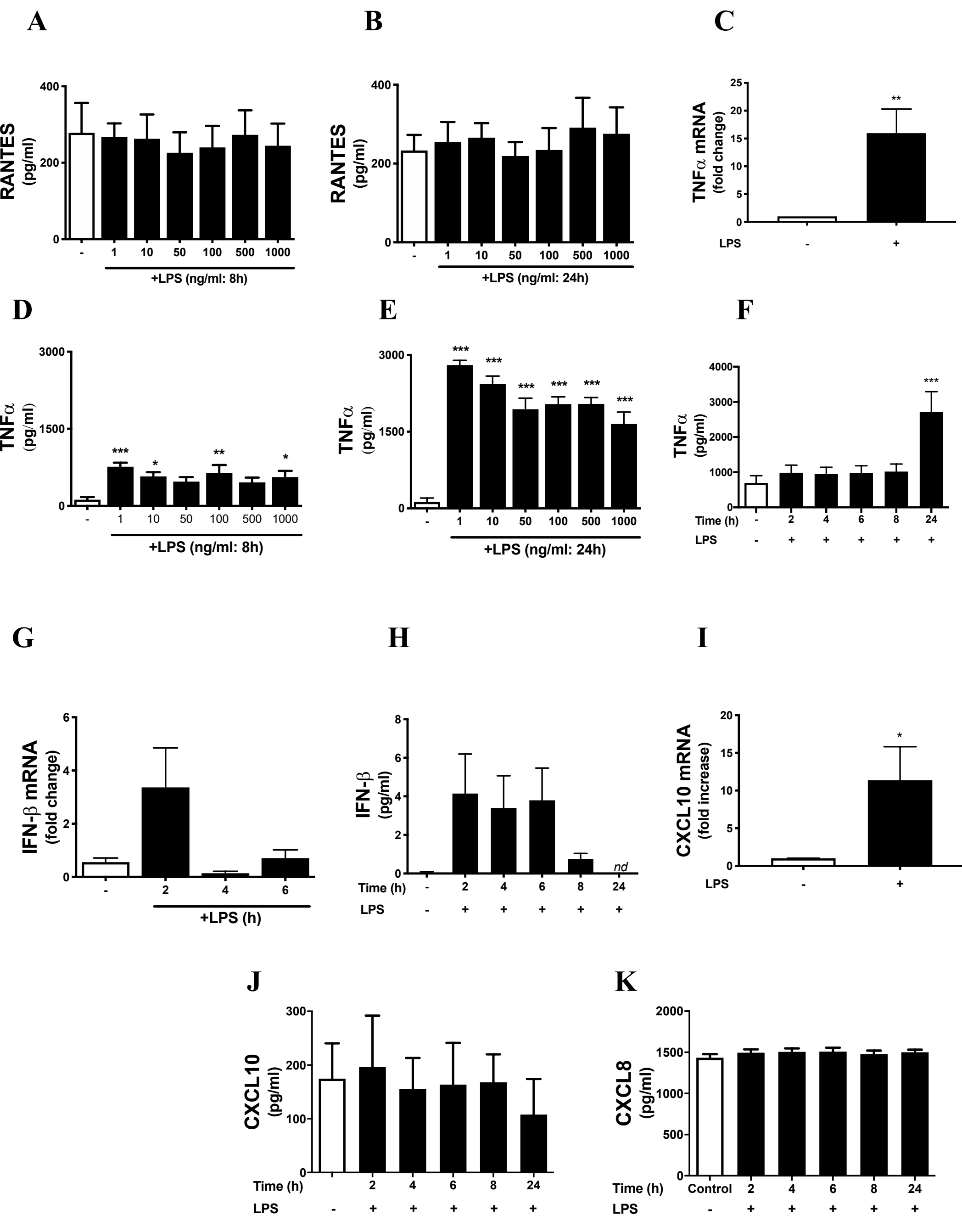


Figure 3.13. Effect of LPS on TNFα, IFN-β and CXCL10 expression in primary human PBMCs. Primary PBMCs were isolated from whole blood, plated at 1x10^{6} cells/ml and treated with LPS at a range of concentrations (1 - 1000 ng/ml) at the 2, 4, or 6 h timepoints (for mRNA detection), or up to 24 h (LPS concentration at 100 ng/ml) for protein determination. LPS did not induce **(A, B)** RANTES protein expression at 8 or 24 h. LPS promoted *TNFα* **(C)** mRNA and **(D, E, F)** protein expression. LPS increased **(G)** *IFN-β* and **(I)** *CXCL10* mRNA, but not **(H)** IFN-β, **(J)** CXCL10, or **(K)** CXCL8, protein expression. Data are represented as the mean ± S.E.M in PBMCs from 5 HC donors. Data were analysed using Student's t-test or one-way ANOVA, followed by Dunnett's post-hoc test as appropriate. *p<0.05, **p<0.01 and ***p<0.001 *versus* untreated cells.

3.15 Discussion

TLRs are PRRs that recognise PAMPs from microorganisms and DAMPs from damaged tissue. TLRs have been detected on cells of the immune system [357] and CNS [358], and play an important role in immune cell activation and downstream inflammatory responses, and are therefore implicated in many diseases including MS [359]. Initially, data herein aimed to characterise TLR3 and TLR4 signalling in THP-1 monocytes, THP-1-derived macrophages and human PBMCs in terms of TNFα, RANTES, CXCL8, CXCL10 and IFN-β expression. In addition, characterisation of MyD88-dependent signalling was determined by examination of p-IκB-α, IκB-α degradation, and nuclear expression of NF-κB p65. MyD88-independent signalling was characterised by detecting cytoplasmic and nuclear expression of pIRF3, in addition to nuclear expression of total IRF3. We found that THP-1 monocytes are a poor model for assessing TLR3 signalling, however differentiation of THP-1 monocytes to macrophages was sufficient to activate the TLR3-IRF-3-IFN-β signalling axis in this cell type. Data presented in this chapter also indicate that both THP-1 monocytes and macrophages are a suitable cell model for the study of LPS-induced TLR4 signalling. Finally, in terms of the analysis of TLR3/TLR4 signalling in primary immune cells, it was determined that appropriate cell plating densities are essential for the assessment of poly(I:C)-TLR3 and LPS-TLR4 signalling axes in primary human PBMCs *in vitro*.

Kinetics of response to TLR3 activation in human immune cells

TLR3 is expressed at low levels in cells of the immune system. Indeed, low levels of TLR3 have been detected on NK cells, T cells, monocytes and B cells [21]; however, a body of evidence also suggests that TLR3 expression is restricted to the DC population A growing body of literature suggests that TLR3 plays a crucial role in many disease types, including neurodegenerative diseases, particularly MS. TLR3 activation via the viral mimetic poly(I:C) can inhibit demyelination in an EAE model by inducing IFN-β [151]. Additionally, using IRF3 [152] and TRIF [172] deficiency studies, it is clear that IRF3 and adaptor molecules in the TLR3 signalling cascade play a role in EAE progression. Previous data from our laboratory indicates that PBMCs isolated from pwMS show decreased sensitivity to poly(I:C) stimulation [155], suggesting that this pathway is dysregulated in

pwMS. It is clear that TLR3 signalling is closely associated with the pathogenesis of disease, particularly MS.

TLR3 can signal in a MyD88-independent manner via recruitment of TRIF (which can also lead to NF-κB activation) and IRF3 activation, leading to expression of type I IFNs [33]. TLR3 expression on THP-1 monocytes is debated in the literature, with some studies indicating *TLR3* mRNA in THP-1 cells [360], and others failing to determine TLR3 expression in this cell type [59]. Our data (using RT-qPCR) indicate that TLR3 is expressed in THP-1 monocytes, THP-1-derived macrophages and human PBMCs, albeit at low levels in each cell type. However, our initial assessment of the proclivity of poly(I:C) to induce TLR3 signalling in THP-1 monocytes failed to indicate that poly(I:C) promotes TLR3 signalling, in terms of TNFα, RANTES and IFN-β expression. We hypothesize that failure of THP-1 cells to respond to TLR3 activation was due to the inability of poly(I:C) to gain sufficient access to the intracellular endosomal compartments of monocytes. This hypothesis is supported by the literature showing that THP-1 cells respond to poly(I:C) stimulation, but only following poly(I:C) transfection [62]. A body of literature also indicates that THP-1 cells efficiently respond to TLR3 activation following PMA differentiation to a macrophage-like phenotype [56]. Indeed, upon PMA-induced differentiation of THP-1 monocytes to macrophage-like cells, poly(I:C) promoted IRF3 translocation to the nucleus. The effect of poly(I:C) in THP-1-derived macrophages may be due to the phagocytic nature of this cell type [361], facilitating poly(I:C) binding to TLR3 on endosomal compartments. Additionally, high molecular weight poly(I:C) has been shown to be more efficient at inducing TLR3 signalling, when compared to low molecular weight poly(I:C) [362]. In this study we treated THP-1-derived macrophages with three different high molecular weight sources of poly(I:C) to ensure that a response, or lack thereof, was not batch specific. Our data shows that each source of poly(I:C) used in the present study induced the expression of *IFN-β* and *TLR3* mRNA, alongside IFN-β protein expression.

TLR4 mRNA was not altered following poly(I:C) treatment, indicating that poly(I:C) does not modulate TLR4 transcription. However, *TLR3* mRNA expression was increased following poly(I:C) treatment, and this is in accordance with data published elsewhere [363]. Additionally, poly(I:C) activates TLR3 which

can promote CXCL10 production [364], and TLR3-induced CXCL10 expression has been shown previously in THP-1-derived macrophages [62]. Therefore, the chemokine CXCL10 was targeted as an endpoint read-out of TLR3 activation across all cell types. Indeed, data reported herein indicate that poly(I:C) promoted the expression of *CXCL10* mRNA and protein in THP-1-derived macrophages. As a whole, the findings indicate that THP-1 monocytes require differentiation to a macrophage-like phenotype to facilitate cellular responses to poly(I:C), with IFN-β and CXCL10 mRNA/protein expression, in addition to IRF3 activation (nuclear sequestration), employed as indicators that TLR3 signalling is operative at a cellular level.

Following the characterisation of poly(I:C)-induced signalling in THP-1 monocytes and THP-1-derived macrophages, we next studied TLR3 signalling in primary human PBMCs. Previous reports have shown that $\gamma\delta$ T cells are indirectly activated by type I IFNs released by poly(I:C) activated DCs [345]. IFN responses have been detected in PBMCs [365] and in human macrophages [366] treated with poly(I:C). Previous findings from our laboratory indicate that in PBMCs from healthy donors, poly(I:C) promotes a significant induction of IFN-β, TNFα, and IL-8, however poly(I:C) treated PBMCs from pwMS are refractory, in terms of IFN-β induction [184]. Indeed, a diminished IFN-β production has been reported in immune cells from pwMS [367]. This evidence from the literature highlights TLR3 signalling as a target for investigation in MS. Our findings indicate that treatment of PBMCs from healthy volunteers with poly(I:C) gave no response in terms of TNFα and RANTES protein production. In addition, poly(I:C) failed to promote IL-6 and CXCL8 protein expression in PBMCs from healthy volunteers (Appendix 5). At the gene level, a minimal increase (albeit insignificant) in *IFN-β* and *TLR3* mRNA expression was determined in PBMCs following poly(I:C) treatment. We hypothesised that this was due to low seeding density of PBMCs (0.5 x 10^6 cells/well) and/or due to the source of poly(I:C) being ineffective. Additionally, cell viability assays in THP-1 monocytes showed that a high concentration of poly(I:C) (25 μg/ml) could significantly reduce cell viability. Therefore, in subsequent experiments, PBMCs were plated at a higher density (2 x 10^6 cells/well) and treated with three different sources of poly(I:C) at a lower concentration (i.e. 10 μg/ml). Under these new experimental conditions, the findings indicate that each source of

poly(I:C) increased the expression of *IFN-β* and *TLR3* mRNA. Similarly, poly(I:C) activation promoted an increase in the expression of *CXCL10* mRNA/protein in human primary PBMCs but had no effect on TNFα protein expression. Poly(I:C) induction of CXCL10 in human PBMCs has been reported elsewhere [368]. In conclusion, a specific cell plating density for PBMCs is required to elicit poly(I:C)-induced downstream signalling in terms of *IFN-β, CXCL10* and *TLR3* mRNA, in addition to IFN-β and CXCL10 protein expression, in primary human PBMCs.

Kinetics of response in human immune cells to TLR4 activation

Human immune cells including monocytes, macrophages, granulocytes and mature DCs express the bacterial detecting receptor TLR4 [21], and much evidence suggests that TLR4 may be a critical player in many diseases and neurodegenerative disorders, including MS [156]. Indeed, TLR4 knockout mice have diminished disease symptoms in EAE [158], and PBMCs from pwMS are highly sensitive to LPS stimulation [155], highlighting a crucial role for TLR4 in MS pathogenesis. TLR4 recognises LPS, a major component of the outer membrane of gram-negative bacteria, and can initiate two separate signalling cascades: recruitment of MyD88-dependent signalling via NF-κB, and MyD88-independent signalling through recruitment of TRIF, which promotes IRF3 activation and sequestration to the nucleus [42]. Activation of the TLR4 receptor promotes the induction of a range of pro-inflammatory cytokines and chemokines, including but not limited to, TNFα [369], RANTES [370], IL-6 and IL-8 [371], CXCL10 [372], in addition to the anti-inflammatory type I IFN, IFN-β [373].

TLR4 is expressed on THP-1 monocytes, differentiated THP-1 cells [57] and human PBMCs [20]. Our data is consistent with this literature indicating the detection of *TLR4* mRNA in THP-1 monocytes, THP-1-derived macrophages and on PBMCs from HC volunteers. In addition, LPS is known to increase the expression of TNFα protein and mRNA in THP-1 monocytes, THP-1-derived macrophages and PBMCs [351, 374], and these findings are consistent with data presented in this Chapter.

The pro-inflammatory chemokine RANTES is intricately regulated by two transcription factors, NF-κB [375] and IRF3 [376]. We observed a significant

increase in RANTES expression following LPS stimulation in THP-1 monocytes, however this result could not be replicated in PBMCs. This is in contrast to the literature reporting a positive induction of RANTES following LPS treatment in THP-1 monocytes and PBMCs [377, 378]. Data reported herein indicates that LPS failed to induce RANTES protein expression in primary PBMCs. These data may reflect the timepoints chosen to assess the effect of LPS on RANTES expression in the current study.

The activation of intracellular signalling proteins following TLR4 stimulation were assessed in THP-1-derived macrophages. LPS activation of TLR4 is known to phosphorylate IκB-α, leading to IκB-α degradation and activation of NF-κB [379]. Once NF-κB is released by IκB-α, NF-κB translocates to the nucleus and binds to the promoter of genes controlling the expression of a suite of cytokines/chemokines, including TNFα. In support of this, data shown here indicate that LPS promoted the phosphorylation and degradation of IκB-α in macrophages, and nuclear sequestration of NF-κB. These data suggest that THP-1-derived macrophages are a suitable model to assess LPS-induced TLR4 signalling mechanisms.

As discussed previously, LPS is a well characterised inducer of IFN-β via recruitment of the TRIF adaptor and activation of IRF3 [373]. We found that LPS induced *IFN-β* mRNA expression in THP-1 cells and primary PBMCs. Additionally, IFN-β protein levels were increased following treatment with LPS in THP-1-derived macrophages and PBMCs. These effects have also been documented in the literature in PBMCs and THP-1 cells [366, 380]. CXCL10 was targeted as another potential read out for LPS-TRIF-induced signalling [381]. Data presented in this Chapter indicate that LPS promoted a significant induction of *CXCL10* mRNA and protein expression in THP-1-derived macrophages, and a significant increase in *CXCL10* mRNA, but not protein, in primary PBMCs. LPS induction of CXCL10 has been reported in THP-1 cells [382] and primary neutrophils when treated in combination with IFN-γ [372]. Taken together, our data indicates the time and concentration-dependent induction of TNFα, RANTES, CXCL10 and IFN-β following exposure to LPS in three cell types, THP-1 monocytes, THP-1-derived macrophages and primary human PBMCs.

In conclusion, data presented herein indicate that both the TLR3-IRF3-IFN-β/CXCL10 and TLR4-NF-κB-TNFα signalling axes are operative in THP-1-derived macrophages and primary PBMCs. In addition, data presented herein indicate that THP-1 monocytes are not a suitable cell model for *in vitro* assessment of the TLR3-IRF3-IFN-β signalling axis. Based in these findings and conclusions, all further assessment of TLR3 signalling events was performed in THP-1-derived macrophages and primary human PBMCs, whereas TLR4 signalling events were examined in THP-1 monocytes, THP-1-derived macrophages and primary human PBMCs.

Results Chapter 4

THC and CBD differentially target TLR3 and TLR4 signalling events in THP-1 monocytes and THP-1-derived macrophages

4.1 Introduction

THC (the euphoric component of *C. sativa*) and CBD (a non-euphoric cannabinoid) are the most abundant phytocannabinoids derived from *C. sativa* extracts, and preclinical research has focused on THC and CBD to demonstrate their anti-inflammatory [194, 383], antioxidant [195, 384] and anti-excitotoxic efficacy [196, 385]. Some phytocannabinoids can act via G protein–coupled cannabinoid receptors CB_1 and CB_2 [386]. Indeed, THC is a CB_1 and CB_2 receptor partial agonist, with *in vitro* evidence indicating that THC binds to CB_1 and CB_2 with K_i values in the low nanomolar range [387, 388]. Unlike THC, CBD demonstrates minimal agonist activity (and very low affinity) for both CB_1 and CB_2 [220, 389]. $CB_{1/2}$-independent mechanisms of action for CBD have also been extensively studied and have identified several receptor targets for this cannabinoid, including PPARγ. Cannabinoids modulate multiple intracellular signal transduction pathways involving adenylyl cyclase, MAP kinases, phosphoinositide 3-kinase/protein kinase B, mTOR, caspases, NF-κB, JAK/STAT and voltage-dependent ion channels (K^+, Ca^{2+}, Na^+) [283, 317, 390, 391], acting via cannabinoid receptor-dependent and -independent mechanisms.

Various studies have demonstrated that cannabinoids, including phytocannabinoids, the endocannabinoids and sCB compounds, modify innate immune responses via TLR-mediated signalling in various cell and tissue types. Indeed, TLR2, TLR3, TLR4, TLR7 and TLR8 signalling is sensitive to cannabinoid ligands and endocannabinoid signalling [274], identifying TLRs as a cannabinoid target. Specifically in terms of TLR4, the phytocannabinoids (THC, CBD), sCBs (*R*(+)WIN55212, HU-210, CP55,940), and endocannabinoids (AEA, 2-AG, NADA) impact TLR4-induced signalling in various cell types including endothelia, astrocytes and microglia [283, 286, 299, 392-395]. In terms of TLR3, the sCB *R*(+)WIN55212 has been shown to regulate TLR3-induced signalling in immune cells and astrocytes [184], while a range of phytocannabinoids, including CBD, CBG, CBC, THCV, and cannabigevarin (CBGV) inhibit TLR3 signalling in keratinocytes [194]. Furthermore, systemic and central administration of a FAAH inhibitor has been shown to regulate TLR3 signalling in hippocampal tissue [295]. Therefore, a full investigation of the effects of THC and CBD on TLR3 and TLR4

signalling in human immune cells was undertaken to further elucidate the anti-inflammatory and therapeutic potential of the phytocannabinoids THC and CBD.

Aims

The specific aims of this chapter are as follows:

- To determine if the phytocannabinoids, THC and CBD, when delivered alone or in a 1:1 combination, regulate key TLR3 and TLR4 signalling events (identified in Chapter 3) in human THP-1 monocytes and THP-1-derived macrophages.
- To determine if the effects of THC and CBD on TLR3/4 signalling are mediated by the classic CB_1 and CB_2 cannabinoid receptors, in addition to the putative PPARγ receptor, in THP-1 immune cells.

4.2 CB_1 and CB_2 cannabinoid receptors are expressed in THP-1 monocytes and THP-1-derived macrophages

Given that the key aim of this study was to determine the impact of phytocannabinoids on the TLR3/4 signalling axis in immune cell lines, and that cannabinoids classically signal via CB_1 and CB_2 receptors [396], the basal expression profile of CB_1 and CB_2 mRNA expression was first determined in THP-1 monocytes and THP-1-derived macrophages. RT-qPCR was conducted in THP-1 monocytes and THP-1-derived macrophages to determine relative CB_1 and CB_2 mRNA in both cell types. Table 7 below demonstrates the Ct values for each target (CB_1 or CB_2) alongside their corresponding endogenous control (18S ribosomal RNA). CB_1 and CB_2 were both detected in THP-1 monocytes and THP-1-derived macrophages, however CB_1 expression levels are higher (i.e. lower Ct values) than CB_2 in both cell types. This result is interesting considering that the CB_1 receptor is predominately expressed in the CNS, while CB_2 receptor is abundantly expressed on immune cells [397].

Table 7. CB_1 and CB_2 receptor expression in THP-1 monocytes and THP-1-derived macrophages.

Target gene	THP-1 monocytes (*n*=3)	THP-1-derived macrophages (*n*=3)
CB_1 (Ct)	26.56 ± 0.18	27.71 ± 0.26
18S rRNA (Ct)	9.94 ± 0.32	10.02 ± 0.17
CB_2 (Ct)	37.61 ± 0.28	37.97 ± 0.35
18S rRNA (Ct)	10.08 ± 0.24	9.56 ± 0.47

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

4.3 THC and CBD do not attenuate TLR4-induced TNFα or RANTES expression in THP-1 monocytes

There is some evidence, albeit limited, that cannabinoids modulate TLR signalling events in immune cells [274]. Initially, the effect of THC and CBD on basal TNFα and RANTES protein expression in the absence of LPS was determined. THP-1 monocytes were cultured with THC or CBD at a range of concentrations (0.001 - 10 μM) for 8 h and supernatants harvested for protein detection via ELISA. THC treatment alone had no effect on TNFα (Fig. 4.1A) or RANTES (Fig. 4.1G) protein expression at all concentrations tested. Similarly, CBD did not alter TNFα (Fig. 4.1B) or (Fig. 4.1H) RANTES protein expression at the range of concentrations used.

Considering previous data indicating that LPS (1 and 100 ng/ml) treatment (8 h) promotes a significant induction of the pro-inflammatory cytokine TNFα and chemokine RANTES in THP-1 monocytes (Fig. 3.9), we next examined the effect of THC and CBD on LPS-TLR4-TNFα/RANTES signalling. The ability of THC or CBD to regulate LPS-induced TNFα (LPS at 1 or 100 ng/ml) and RANTES (LPS at 1 ng/ml) protein expression was determined. THP-1 monocytes were pre-treated (45 min) with either THC or CBD (0.001 - 10 μM) prior to stimulation with LPS (1 or 100 ng/ml; 8 h), and TNFα or RANTES ELISAs were performed on harvested supernatants. Data indicate that THC (Fig. 4.1C, D) and CBD (Fig. 4.1E, F) failed to impact LPS-induced TNFα expression at each concentration tested. However, it is important to note that the lower concentration of LPS (1 ng/ml) did not significantly induce TNFα protein expression, as was determined previously following LPS treatment for 24 h (Fig. 3.9C), but is in contrast to data seen previously after LPS stimulation for 8 h (Fig. 3.9B). Furthermore, it was previously reported that LPS at (1 ng/ml) was sufficient to significantly induce RANTES protein expression in THP-1 monocytes (Fig. 3.9E, F); however, this finding could not be replicated in Fig. 4.1I, J. In addition, both THC (Fig. 4.1I) and CBD (Fig. 4.1J) failed to significantly impact LPS-induced RANTES expression at all concentrations tested, confirming that THC and CBD do not target TLR4-induced signalling events in THP-1 monocytes.

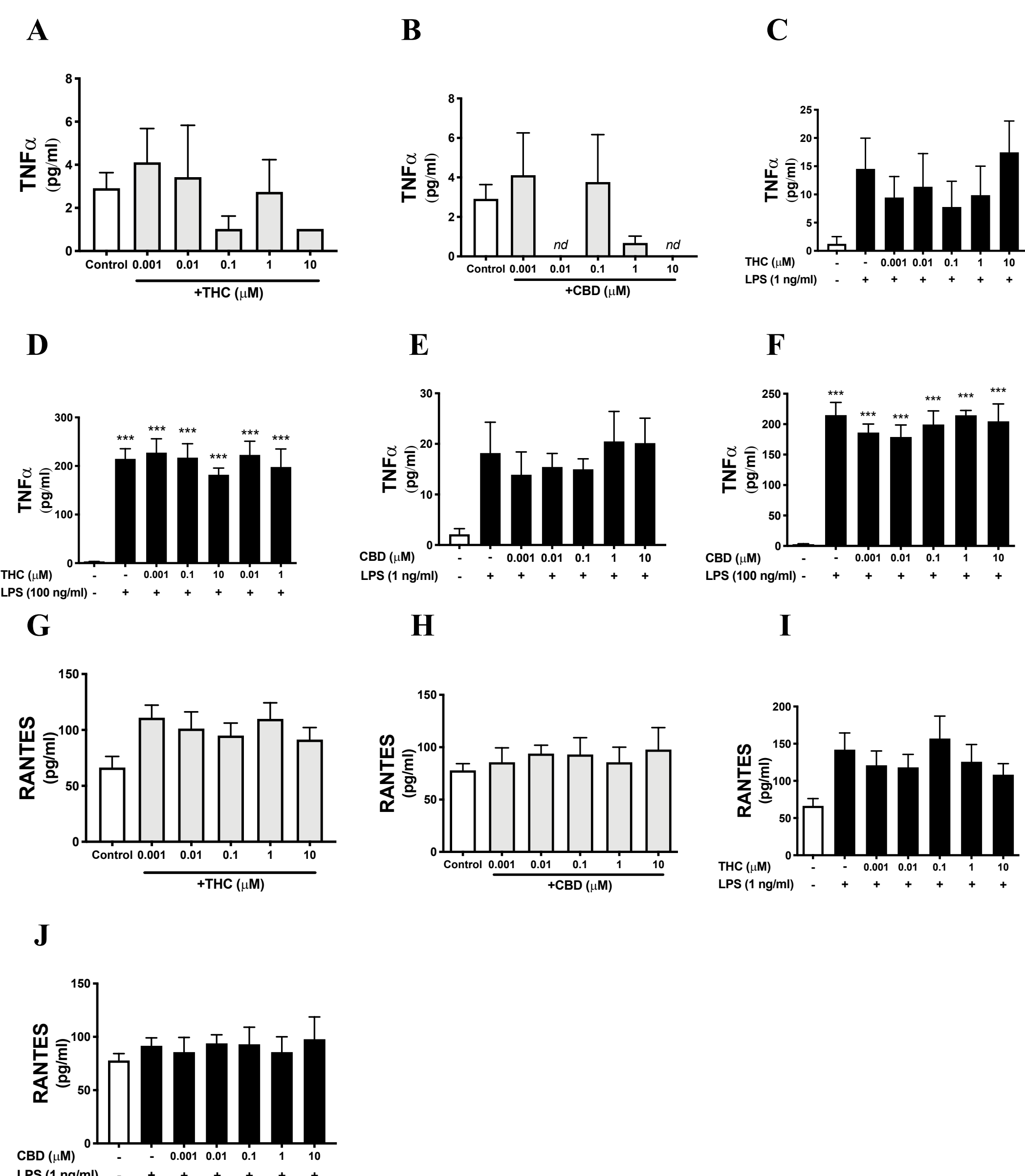


Figure 4.1. The effect of THC and CBD on LPS-induced TNFα and RANTES protein expression in THP-1 monocytes. THP-1 monocytes were cultured with THC or CBD (0.001 - 10 μM) alone or in combination with LPS (1 or 100 ng/ml; 8 h), supernatants harvested and analysed for RANTES and TNFα protein expression via ELISA. THC and CBD had no effect on basal **(A, B)** TNFα and **(G, H)** RANTES protein expression when administered alone. LPS-induced TNFα expression was not modulated by **(C, D)** THC or **(E, F)** CBD at all concentrations of cannabinoid and LPS tested. The effect of LPS on RANTES expression was not modulated by **(I)** THC and **(J)** CBD at all concentrations assessed. Data are presented as the mean ± S.E.M and are representative of 3 independent experiments.

Data were analysed using one-way ANOVA, followed by Dunnett's post-hoc test. ***$p<0.001$ *versus* untreated cells.

4.4 The impact of THC, CBD, and a THC:CBD combination on THP-1 monocyte viability

To determine the effect of the phytocannabinoids THC and CBD, when delivered alone and in a 1:1 combination (THC:CBD), on the viability of monocytes, MTT cell viability assays were performed in THP-1 monocytes treated with the phytocannabinoids at a high concentration of 10 μM. After 24 h incubation, THC (10 μM), CBD (10 μM), and a THC:CBD combination (both phytocannabinoids at a final concentration of 10 μM), had no significant effect on cell viability in THP-1 monocytes (Fig. 4.2). Triton x100 (0.2%) was added 10 min prior to the addition of MTT to act as a positive control. This finding indicates that THC and CBD, when delivered alone and in a 1:1 combination (THC:CBD) at 10 μM, do not negatively regulate THP-1 monocyte cell viability.

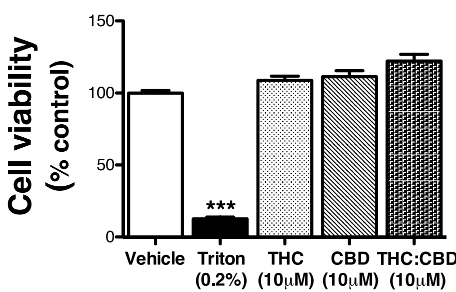
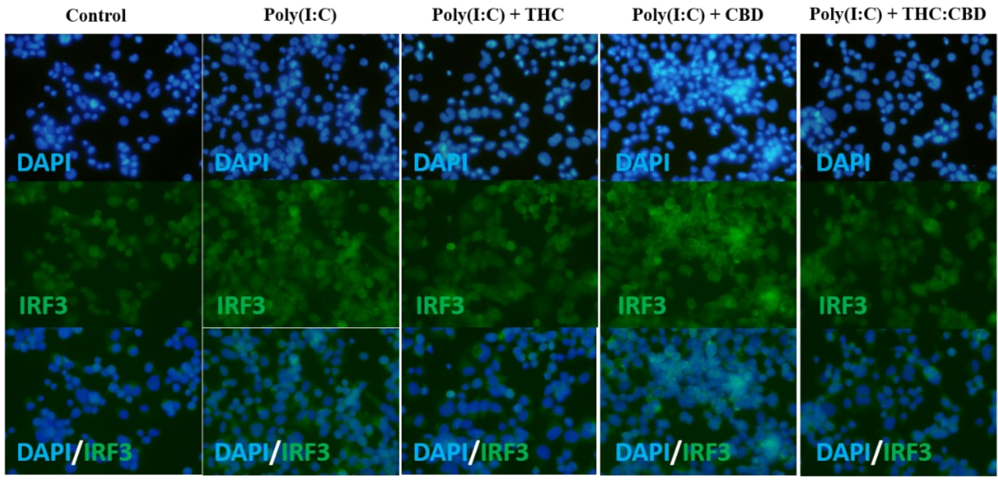


Figure 4.2. THC, CBD and a THC:CBD combination are not toxic to THP-1 monocytes.
An MTT assay was employed to determine the effect of THC, CBD, and a THC:CBD combination (all at 10 μM) on monocyte viability. Cells were treated with cannabinoids for 24 h prior to analysis. Triton x100 (0.2%) was used as a positive control. Data are presented as the mean ± S.E.M for 3 independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used for analysis. ***$p<0.001$ *versus* control cells.

4.5 CBD, THC and THC:CBD (1:1) inhibit TLR3-induced IRF3 activation and induction of CXCL10/IFN-β in THP-1 macrophages

Given that TLR3 signalling independent of MyD88 is operative in THP-1 macrophages (Chapter 3), we next examined the impact of the phytocannabinoids, THC and CBD, alone and in a 1:1 combination, on key TLR3-induced signalling intermediates in THP-1 macrophages. Firstly, macrophages were pre-treated with THC (10 μM), CBD (10 μM) and THC:CBD (1:1 both phytocannabinoids at a final concentration of 10 μM) for 45 min prior to poly(I:C) (10 μg/ml) exposure for 60 min (timepoint based in data in Fig. 3.4A), and nuclear expression of IRF3 measured by fluorescence microscopy. Data presented in Fig. 4.3A indicates that poly(I:C) significantly promoted the accumulation of IRF3 in the nucleus. Pre-exposure to THC, CBD and THC:CBD (each cannabinoid at a final concentration of 10 μM) attenuated TLR3-induced IRF3 activation, returning nuclear IRF3 expression to basal levels (Fig. 4.3A).

As the IRF3 transcription factor can induce the downstream expression of type I IFNs [347] and CXCL10 [35], the sensitivity of CXCL10/IFN-β to THC and CBD in response to poly(I:C) was next evaluated. Pre-exposure to THC, CBD and THC:CBD (all at final concentrations of 10 μM) significantly attenuated TLR3-induced *CXCL10* mRNA (Fig. 4.3B) and protein (Fig. 4.3C) expression. Furthermore, Fig. 4.3D demonstrates that THC, CBD and THC:CBD (all at 10 μM) attenuated poly(I:C)-induced *IFN-β* mRNA, but not IFN-β protein, expression (Fig. 4.3E), in macrophages. These findings indicate that both THC and CBD can negatively regulate TLR3 signalling to IRF3, CXCL10 and IFN-β in macrophages.



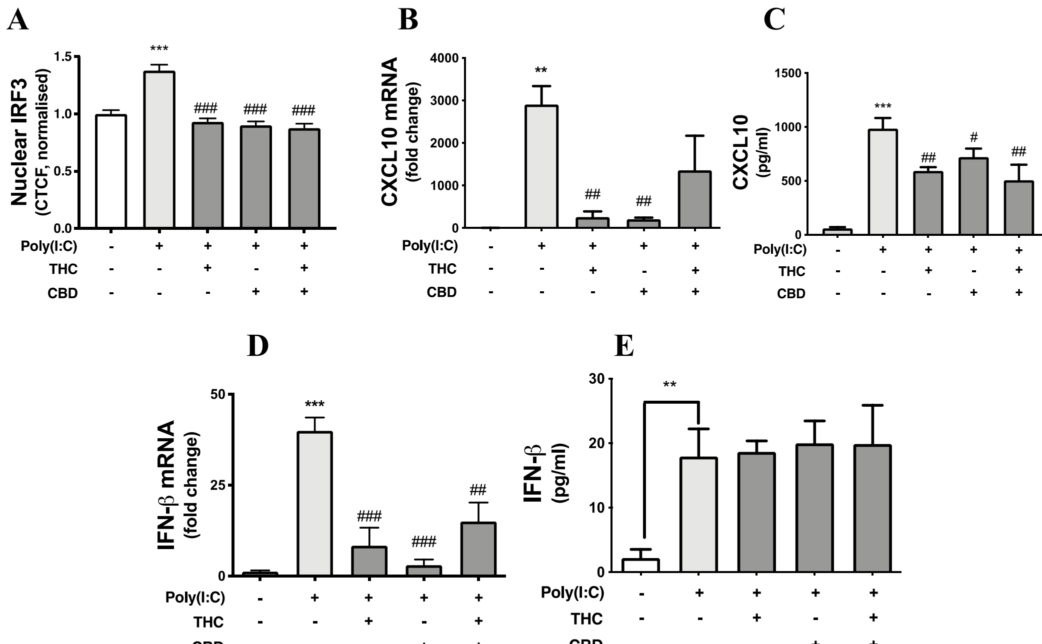


Figure 4.3. THC, CBD, and THC:CBD (1:1 combination) inhibit MyD88-independent signalling via TLR3 in THP-1-derived macrophages. (A) THC, CBD and the combination (1:1) of phytocannabinoids (final concentration of 10 µM for each cannabinoid; 45 min pre-treatment) inhibited poly(I:C)-induced (10 µg/ml; 60 min) IRF3 translocation to the nucleus. Cells were stained with DAPI (bis-benzamide) and nuclear IRF3 expression quantified using fluorescence immunocytochemistry and CTCF calculated. Representative images of cells showing DAPI (blue), IRF3 (green) and both channels merged (blue and green). Immunofluorescence images taken at 40x magnification. THC, CBD and the combination (all at 10 µM; 45 min pre-treatment) attenuated poly(I:C)-induced (10 µg/ml; 4 h) **(B)** *CXCL10* mRNA and **(C)** CXCL10 protein expression in addition to **(D)** *IFN-β* mRNA expression. **(E)** THC and CBD did not impact TLR3-induced IFN-β protein expression. Data are expressed as means ± S.E.M from 3-8 independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used for analysis. **$p<0.01$, ***$p<0.001$ *versus* control groups and #$p<0.05$, ##$p<0.01$ and ###$p<0.001$ *versus* poly(I:C)-treated groups.

4.6 Effect of CBD and THC on TLR4-induced IκB-α degradation, NF-κB nuclear sequestration and TNFα/CXCL8 protein production in THP-1-derived macrophages

Given that both THC and CBD can negatively regulate TLR3 signalling to IRF3, CXCL10 and IFN-β (Fig. 4.3), we next examined the proclivity of phytocannabinoids to impact TLR4-induced signalling mediated by the MyD88 adaptor. Firstly, macrophages were pre-treated with THC (10 μM), CBD (10 μM) and THC:CBD (both at a final concentration of 10 μM) for 45 min prior to LPS treatment (100 ng/ml: 30 min), and cytoplasmic fractions assessed for IκB-α phosphorylation (Fig. 4.4A, B), IκB-α degradation (Fig. 4.4A, C) and nuclear NF-κB p-p65 expression (Fig. 4.4D, E) via immunoblotting. Interestingly, THC (10 μM), CBD (10 μM) and THC:CBD (both at a final concentration of 10 μM) failed to inhibit LPS-induced IκB-α phosphorylation (Fig. 4.4B) and degradation (Fig. 4.4C) in cytoplasmic fractions. However, THC, CBD, and the combination treatment insignificantly attenuated LPS-induced NF-κB-p-p65 (Fig. 4.4D, E). Furthermore, immunocytochemical analysis indicated that LPS-induced nuclear expression of the NF-κB p65 subunit was significantly attenuated by THC, CBD and THC:CBD (Fig. 4.4F). In addition, pre-exposure to THC, CBD, and THC:CBD (at 10 μM) failed to impact TLR4-induced TNFα (Fig. 4.4G) and CXCL8 (Fig. 4.4H) protein expression. These findings suggest that both THC and CBD do not regulate TLR4-induced pro-inflammatory proteins (TNFα and CXCL8) but may alter NF-κB p-p65 and NF-κB p65 nuclear expression in THP-1 macrophages. Therefore, the ability of THC, CBD, and THC:CBD to attenuate LPS-induced NF-κB, but not downstream inflammatory protein production, may be due to the effect of phytocannabinoids on other LPS-activated target(s) (i.e. MAPK). These findings highlight the complex interaction of phytocannabinoids with signalling intermediates in the TLR4 pathway.

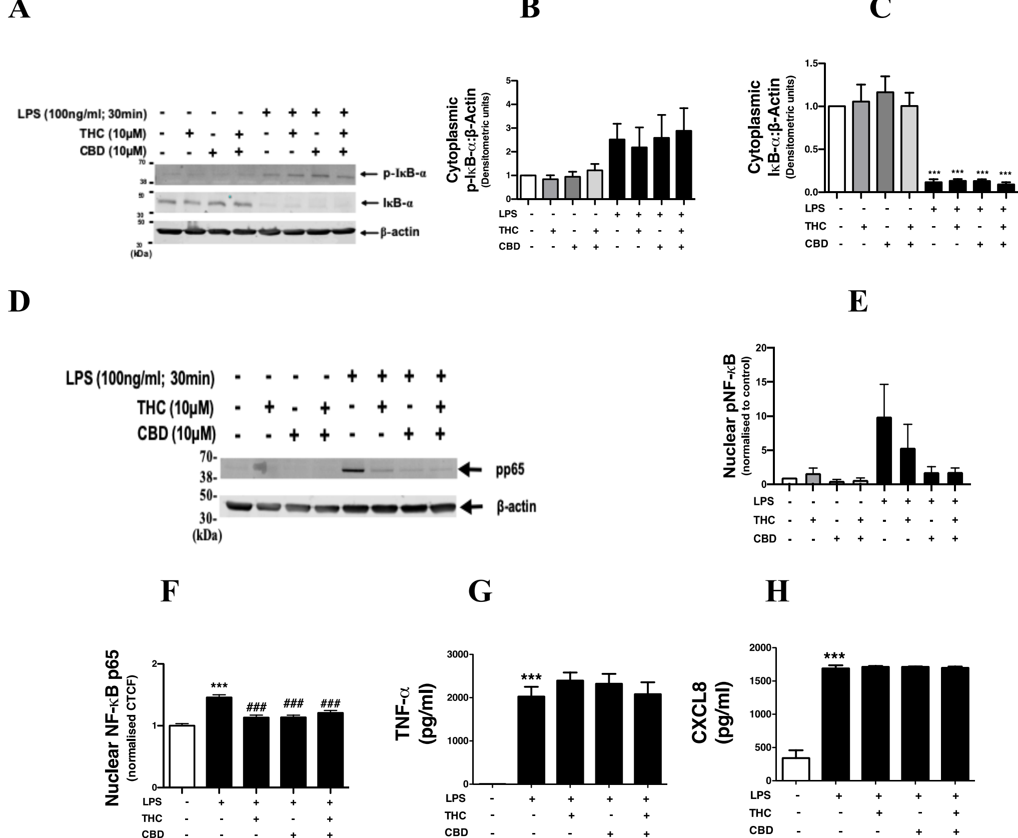


Figure 4.4. The effect of THC, CBD and THC:CBD on TLR4 signalling in THP-1-derived macrophages. (A) Representative immunoblot of I*κ*B-α, pI*κ*B-α and the endogenous control β-actin after pre-treatment with THC, CBD, or THC:CBD and stimulation with LPS. Treatment with THC and CBD, alone and in a 1:1 combination (all at a final concentration of 10 µM; 45 min pre-treatment) had no impact on LPS (100 ng/ml; 30 min)-induced I*κ*B-α **(B)** phosphorylation and **(C)** degradation in cytoplasmic fractions, but reduced the expression of **(E)** pNF-κB in nuclear fractions. **(D)** Representative immunoblot of NF-κB p-p65 expression and β-actin after pre-treatment with THC, CBD or THC:CBD and stimulation with LPS. **(F)** THC, CBD, and THC:CBD significantly attenuated LPS-induced nuclear NF-κB expression. THC and CBD (10 µM; 45 min pre-treatment) did not impact LPS-induced (100ng/ml; 4 h) **(G)** TNF*α* or **(H)** CXCL8 expression. Data are expressed as mean ± S.E.M from 3-4 independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used to determine statistical differences. ****p*<0.001 *versus* control groups and ###*p*<0.001 *versus* LPS-treated groups.

4.7 CBD and THC regulate TLR4-induced IRF3, CXCL10 and IFN-β expression in THP-1-derived macrophages

Next, the ability of THC/CBD to regulate TLR4 signalling independent of the MyD88 adaptor was assessed. Macrophages were pre-treated with THC (10 μM), CBD (10 μM) and THC:CBD (both at 10 μM: 45 min) prior to LPS treatment (100 ng/ml: 60 min), and the phosphorylation of IRF3 determined in cytoplasmic (Fig. 4.5A, B) and nuclear (Fig. 4.5C, D) fractions via immunoblotting. LPS promoted the phosphorylation of IRF3 in cytoplasmic (Fig. 4.5B) and nuclear (Fig. 4.5D) fractions, and pre-treatment with THC:CBD in combination partially reversed the LPS effect, insignificantly reducing IRF3 phosphorylation in both the cytoplasm and nucleus. To investigate this finding further, nuclear expression of endogenous IRF3 was measured by fluorescence microscopy (Fig. 4.5E, F). LPS promoted the accumulation of IRF3 in the nucleus and pre-treatment to THC, CBD and THC:CBD (at 10 μM) attenuated TLR4-induced nuclear sequestration of IRF3 (Fig. 4.5F). Furthermore, THC, CBD and THC:CBD (all at 10 μM) attenuated TLR4-induced CXCL10 (Fig. 4.5H) and IFN-β (Fig. 4.5J) protein expression. However, THC, CBD and THC:CBD did not significantly attenuate *CXCL10* (Fig. 4.5G) or *IFN-β* (Fig. 4.5I) mRNA expression, although a minor decrease in mRNA levels was detected. These findings indicate that THC and CBD can negatively regulate the MyD88-independent pathways induced by TLR4 to control the production of CXCL10 and IFN-β in THP-1 macrophages.

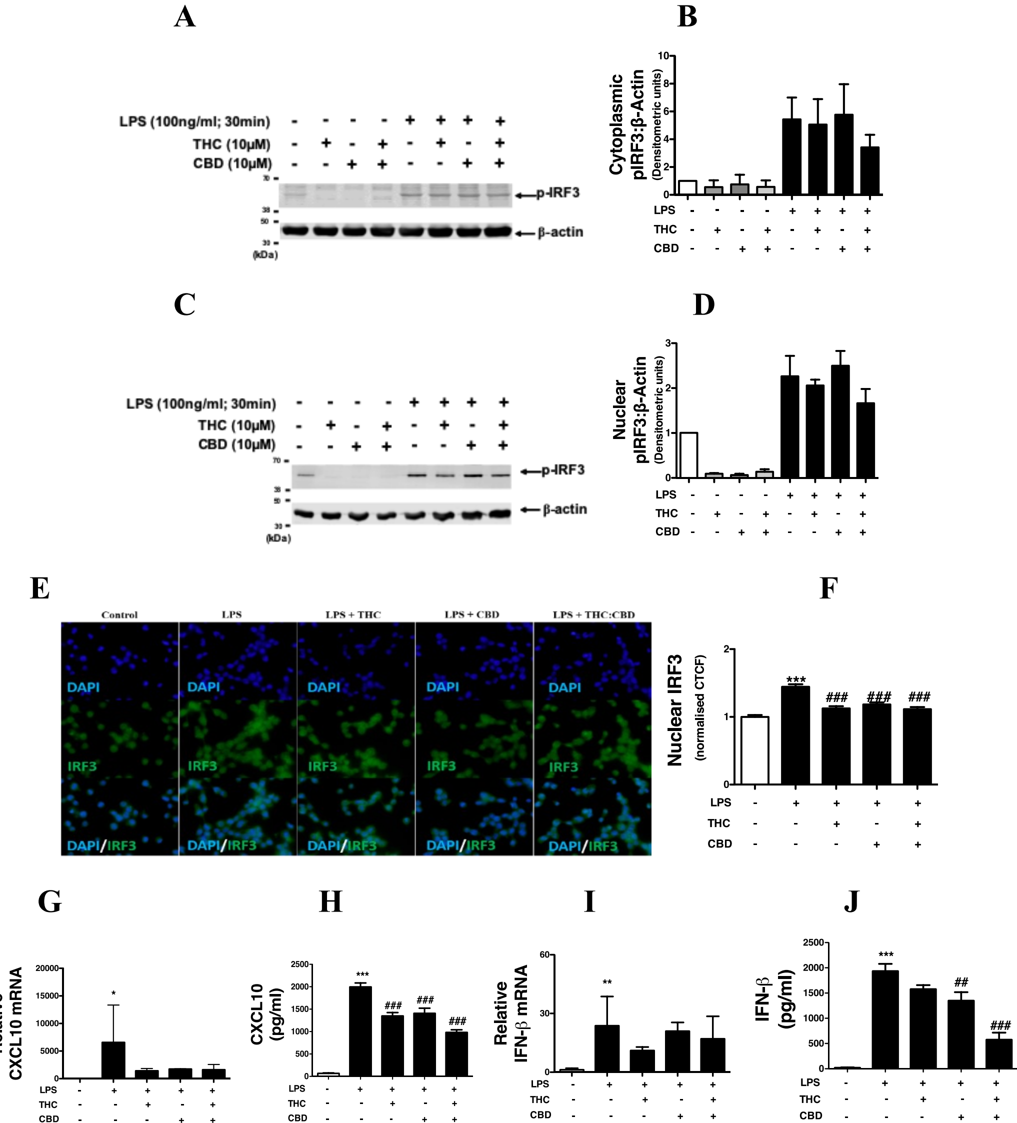


Figure 4.5. CBD, THC and THC:CBD inhibit TLR4-induced IRF3, CXCL10 and IFN-β expression in THP-1-derived macrophages. (A, C) Representative immunoblots of pIRF3 and β-actin after pre-treatment with THC, CBD or THC:CBD (all cannabinoid treatments for figure at a final concentration of 10 µM; 45 min pre-treatment) and stimulation with LPS in **(A)** cytoplasmic and **(C)** nuclear fractions. LPS (100 ng/ml; 30 min) treatment promoted the phosphorylation of IRF3 in **(B)** cytoplasmic and **(D)** nuclear fractions and treatment with THC and CBD in a 1:1 combination partially reversed the LPS effect. **(E)** Cells were stained with DAPI (bis-benzamide) and nuclear IRF3 expression quantified using fluorescence ICC. Representative images of cells showing DAPI (blue), IRF3 (green) and both channels merged (blue and green). Immunofluorescence images taken at 40x magnification. **(F)** Treatment with THC and CBD, alone and in a 1:1 combination, inhibited LPS-induced (100 ng/ml; 30 min) IRF3 translocation to the nucleus. Effect of THC, CBD and the THC:CBD combination on LPS-induced (100 ng/ml; 4 h) *CXCL10* **(G)** mRNA and CXCL10 **(H)** protein expression, in addition to *IFN-β* **(I)** mRNA and IFN-β **(J)** protein expression. Data are expressed as mean ± S.E.M from 3-8 independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used for analysis. *p<0.05, **p<0.01, ***p<0.001 *versus* control groups and $^{##}p$<0.01 and $^{###}p$<0.001 *versus* LPS-treated cells.

4.8 The role of CB_1 and CB_2 cannabinoid receptors, and the nuclear PPAR*γ* receptor, in mediating the effects of CBD and THC on TLR4-induced CXCL10 and IFN-β, and TLR3-induced CXCL10 expression

The cannabinoid pharmacology underlying the above effects was next assessed. CB_1 and CB_2 cannabinoid receptor expression was confirmed on THP-1 macrophages by PCR (Table. 7), and receptor involvement was addressed by employing the use of the CB_1 and CB_2 antagonists, SR141716 and SR144528, respectively. Pre-exposure to SR141716 or SR144528 (both at 1 μM for 1 h), failed to impact the proclivity of THC (10 μM), CBD (10 μM) and THC:CBD (both at 10 μM) to regulate LPS-induced IFN-β (Fig. 4.6A) and CXCL10 (Fig. 4.6B) protein expression. This indicates that both THC and CBD, when delivered alone and in combination (1:1), impacts the signalling pathways leading from TLR4 to IFN-β and CXCL10 independently of CB_1/CB_2 receptors. Both CB_1 and CB_2 antagonists had no effect of TLR3 signalling when delivered independently (Fig. 4.6C). The effect of pre-exposure to SR141716 or SR144528 and THC and CBD on LPS-, and poly(I:C)-, induced *IFN-β* and *CXCL10* mRNA (Appendix 4) was also determined.

Both CB_1- and CB_2-independent effects of THC [283, 398] and CBD [283, 399] have also been demonstrated, with evidence that phytocannabinoids can act via PPARs. PPARγ was next assessed as a potential phytocannabinoid target in our culture system given that previous studies highlighted PPARγ as a cannabinoid target [400, 401]. Firstly, PPARγ was detected on THP-1 macrophages (Table. 8). We then employed the use of the PPARγ antagonist T0070907 to determine if this receptor mediates the impact of THC/CBD on TLR signalling. Pre-exposure to the PPARγ antagonist T0070907 (at 1 μM) failed to reverse the inhibitory effect of THC (10 μM), CBD (10 μM) and THC:CBD (both at 10 μM) on TLR3-induced CXCL10 expression (Fig. 4.6D), indicating that THC and CBD impacts the signalling pathways leading from TLR3 to CXCL10 independently of PPARγ.

Table 8. Constitutive expression of PPARγ in THP-1-derived macrophages

Target gene	THP-1-derived macrophages (*n*=3)
PPARγ (Ct)	22.52 ± 0.21
18S rRNA (Ct)	9.86 ± 0.83

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

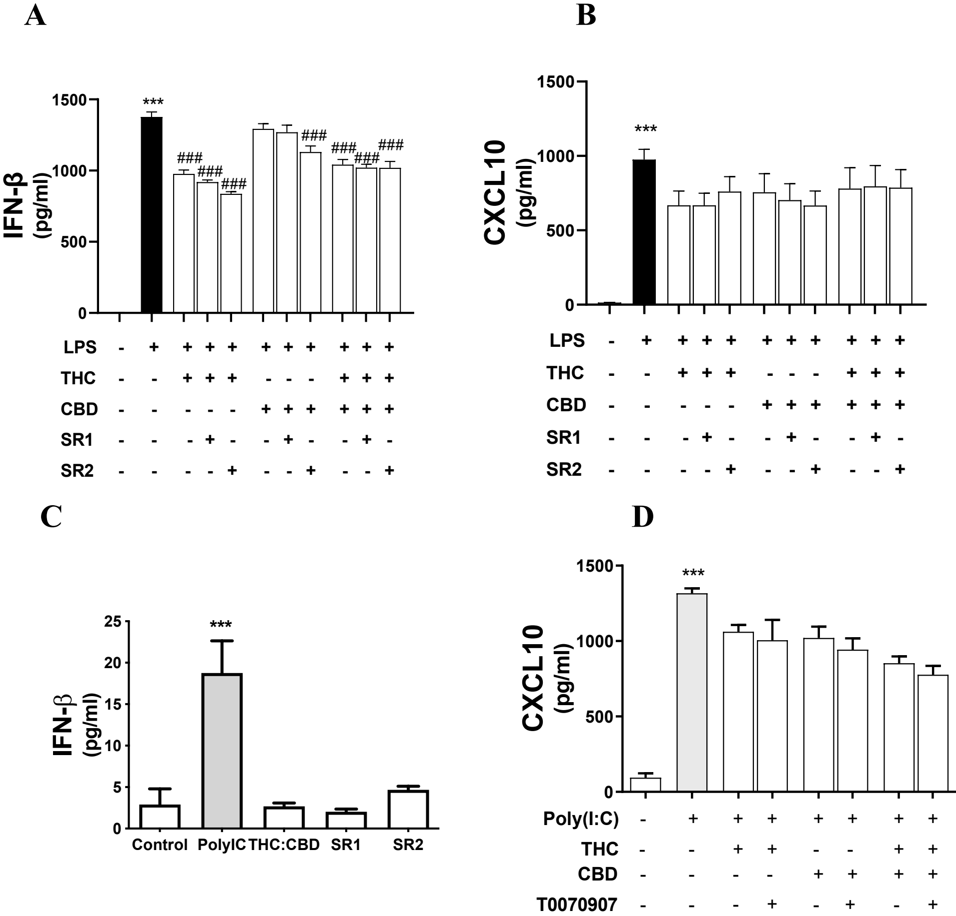


Figure 4.6. THC and CBD do not act via CB1, CB2 or the PPARγ receptor to modulate TLR signalling. THP-1 macrophages were pre-treated with SR141716 (SR1), SR144528 (SR2) or T0070907 (all 1 μM; 1 h), followed by treatment with phytocannabinoids (all at 10 μM for 45 min) and stimulation with LPS (100 ng/ml) or poly(I:C) (10 μg/ml) for 4 h. Pre-exposure to SR141716 and SR144528 failed to impact the proclivity of THC, CBD and THC:CBD to inhibit LPS-induced **(A)** IFN-β and **(B)** CXCL10 protein expression. **(C)** SR1, SR2 and THC:CBD, when delivered alone, did not alter IFN-β protein levels. **(D)** Pre-treatment with T0070907 failed to impact the proclivity of THC, CBD and THC:CBD to inhibit poly(I:C)-induced CXCL10. Data are expressed as mean ± S.E.M from 3-4 independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used for statistical assessment. ***p<0.001 *versus* control groups. $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 *versus* LPS- or poly(I:C)-treated groups.

4.9 Discussion

This study set out to determine if THC and CBD could target TLR3 and TLR4 signalling in a human monocytic cell line and a monocyte-derived macrophage cell line. We identified that THC and CBD do not target TLR4 signalling in terms of RANTES and TNFα protein production in monocytes. However, it was identified that THC and CBD, when delivered alone and in a 1:1 combination, have the proclivity to differentially target TLR3 and TLR4 inflammatory events in THP-1 macrophages. The significant finding is that both phytocannabinoids preferentially targeted MyD88-independent signalling via TLR3 and TLR4 to inhibit poly(I:C)- and LPS-induced IRF3 activation and the expression of CXCL10 and IFN-β. Interestingly, both phytocannabinoids failed to impact MyD88-dependent signalling via TLR4 signalling pathways controlling phosphorylation/degradation of IκB-α, and the downstream production of TNFα and CXCL8. However, CBD and THC, alone and in a 1:1 combination, inhibited NF-κB translocation to the nucleus and nuclear expression of pNF-κB. $CB_{1/2}$ and PPARγ receptor dependent effects of CBD and THC on TLR-induced CXCL10 and IFN-β expression were determined, and these data suggested a potential cannabinoid and PPARγ receptor-independent effect. However, these data were incomplete and variable and have not fully excluded these receptors as targets of cannabinoids during TLR modulation .

Given that LPS activates TLR4 signalling in THP-1 monocytes (Chapter 3), the effect of THC and CBD on TLR4 signalling in monocytes was initially examined. Firstly, the expression of CB_1 and CB_2 was detected on THP-1 monocytes, which is in line with data elsewhere [227]. Our findings indicate that THC and CBD, when administered alone, do not alter RANTES and TNFα protein expression in monocytes over a range of concentrations tested (0.001 - 10 μM). Our data are consistent with findings from elsewhere which have shown that THC does not alter LPS-induced IκB-α or NF-κB p65 expression, in addition to TNFα or CXCL8 mRNA and protein levels, in primary monocytes [402]. Furthermore, the absence of effect of both phytocannabinoids in our study cannot be explained by the potential toxic characteristics of THC [403] or CBD [404], as both THC and CBD, when delivered alone and in a 1:1 combination at a concentration of 10 μM, did not impact THP-1 monocyte viability.

Data presented previously in Chapter 3 determined that both the TLR3-IRF3-IFN-β/CXCL10, TLR4-NF-κB-TNFα and TLR4-IRF3-IFN-β/CXCL10 signalling axes are operative in THP-1-derived macrophages. Therefore, THC and CBD were assessed for their ability to regulate such signalling pathways in THP-1 macrophages. A key objective of this study was to determine if the cellular actions of THC and CBD on TLR signalling differed when cannabinoids were delivered independently or in combination. Overall, data herein indicate that the same degree of anti-inflammatory efficacy was seen following treatment with THC and CBD alone, compared to in combination, with the exception of TLR4-induced CXCL10/IFN-β and IRF3 phosphorylation. Indeed, both THC and CBD inhibited LPS-induced CXCL10 and IFN-β protein expression, although an exaggerated inhibition was determined when THC and CBD were delivered in a 1:1 combination. Similarly, THC and CBD exerted an inhibitory effect on TLR4-induced pIRF3, only when delivered in a 1:1 combination. The mechanistic basis of this remains to be elucidated. However, in support of this, it was recently shown that a 1:1 combination of THC:CBD was more effective in the restoration of motor function in the Theiler's murine encephalomyelitis virus (TMEV)-induced demyelination model, when compared to the administration of botanical extracts of CBD, and in particular THC, alone [405]. Indeed, a 1:1 combination of CBD botanical extract (containing 64.8% CBD, 2.3% THC, 1.1% CBG, 3.0% CBC, 1.5% other phytocannabinoids) and THC botanical extract (containing 67.1% THC, 0.3% CBD, 0.9% CBG, 0.9% CBC, 1.9% other phytocannabinoids) was more effective at improving motor deficits in the chronic phase of TMEV infection than administration of CBD or THC botanical extract alone.

We report that THC, CBD and a 1:1 combination of both cannabinoids attenuated MyD88-independent signalling via both TLR3 and TLR4, in terms of IRF3 activation and production of CXCL10/IFN-β in macrophages. *In vitro* data elsewhere has elucidated a role for sCB in regulating poly(I:C)-induced IRF3 activation and induction of IFN-β [184], and an array of phytocannabinoids (including CBD, CBC, THCV, CBG) have been shown to inhibit poly(I:C)-induced MCP-2 production in human keratinocytes [194]. THC has also been shown to inhibit TLR7 signalling to TBK1 and IRF7 in primary human plasmacytoid DCs [289]. Furthermore, *in vivo* administration of CBD (10 mg/kg) attenuates cognitive

and social interaction deficits induced by pre-natal poly(I:C) exposure in rats [406], while Peres and colleagues (2016) have shown that peripubertal treatment with CBD (1 mg/kg) attenuates hyperlocomotion induced by prenatal exposure to poly(I:C) [407]. However, to our knowledge, our findings represent the first evidence to indicate that the phytocannabinoids, THC and CBD, can specifically target viral signalling induced by activation of TLR3 in macrophages, and identifies potential new molecular targets for both THC and CBD.

TLR4 can initiate MyD88-independent signalling via TRIF by employing the bridging adaptor TRAM [408]. In terms of TLR4-induced MyD88-independent signalling, data herein also indicate that both THC and CBD have the proclivity to inhibit TLR4 signalling to IRF3, IFN-β and CXCL10. This is supported by *in vitro* evidence in the BV-2 microglial cell line indicating that both THC and CBD inhibit LPS-induced IFN-β expression [283], which further suggests that phytocannabinoids preferentially target TRIF-dependent signalling independently of MyD88.

Cannabinoids have well-characterized anti-inflammatory propensity by targeting TLR4 signalling, and it has been reported that the endogenous cannabinoid anandamide (2.5 μM) can attenuate TLR4-induced pro-inflammatory signalling *in vitro* in monocytes isolated from healthy subjects [163], while THC and CBD inhibit TLR4-induced IL-1β production in the BV-2 microglial cell line [283]. Surprisingly, our findings indicate that TLR4-induced signalling via MyD88 to the phosphorylation and degradation of IκB-α, and production of TNFα and CXCL8, was refractory to phytocannabinoids. This is in contrast with evidence indicating that CBD inhibits TLR4-induced NF-κB activation in BV2 microglia [282, 283] and that THC can inhibit NF-κB activation in human T cells [409]. However, we found that THC, CBD and the THC:CBD combination were sufficient to attenuate LPS-induced NF-κB and pNF-κB expression in the nucleus. These data are intriguing and requires further investigation to be fully understood. Our findings indicating that THC/CBD failed to inhibit LPS-induced phosphorylation and degradation of IκB-α, and LPS-induced TNFα and CXCL8 expression, but inhibited NF-κB, may reflect differential roles of cannabinoids, or indeed MyD88

and TRIF adaptor molecules, in various cell types (T cells versus microglia versus macrophages). Furthermore, LPS can activate MAPK family members in THP-1 macrophages [410], therefore the inhibition of NF-κB translocation to nucleus by THC and CBD without subsequent inhibition of TNFα and CXCL8 protein expression, may be due to LPS-induction of pro-inflammatory proteins via MAPKs. Given that MAPK signalling cascades play key roles in the inflammatory responses in macrophages [411], further experiments are required to determine the role of MAPKs in determining the effect of both THC and CBD on inflammatory cytokine expression in THP-1 cells.

Cannabinoids exert their cellular effects via an array of molecular targets including ion channels (including K^+, Ca^{2+}, Na^+ and transient receptor potential channels) [211, 389, 390], transporters (including neurotransmitter transporters, anandamide membrane transporters) [412, 413] and intracellular signal transduction pathways (including MAP kinases, JAK/STATs) [283, 390], and can act via cannabinoid receptor-dependent- and -independent mechanisms involving PPARγ, GPR55 and 5-HT receptors [220, 235, 387-389, 414-416]. CBD and THC differ in their pharmacology at the classic cannabinoid receptors, CB_1 and CB_2 [220, 387-389]. To determine the pharmacological targets for THC/CBD in mediating their effects on TLR signalling, we confirmed the expression of CB_1/CB_2 on THP-1 macrophages, and then employed the use of selective CB_1 and CB_2 receptor antagonists. Our findings potentially indicate that neither antagonist reversed the inhibitory effect of THC and CBD on TLR4-induced CXCL10 and IFN-β expression, suggesting that CB_1 and CB_2 receptors do not mediate the anti-inflammatory propensity of phytocannabinoids in this cellular model of macrophage inflammation. However, data highlighting the cannabinoid receptor independent effects were variable and incomplete and do not conclusively determine cannabinoid receptor independent effects. It is important to note that both CB_1- and CB_2-independent effects of THC [283, 398] and CBD [283, 399] have been demonstrated, with evidence that phytocannabinoids can act via PPARs [417]. In this study PPARγ was detected on THP-1 macrophages, and furthermore the PPARγ antagonist T0070907 failed to reverse the inhibitory effect of THC and CBD on TLR3-induced signalling events. This potentially indicates that CB_1, CB_2

and PPARγ do not mediate the effects of THC and CBD on TLR signalling in this macrophage cell line. Both THC and CBD are lipid soluble phytocannabinoids [418], and hence their cellular effect on TLR signalling in macrophages may be attributed to their lipophilicity due to their direct partitioning into cellular membranes. Further research is required to pinpoint the pharmacological target(s) of THC and CBD in modulating signalling induced by TLR3 and TLR4 activation.

Chapter 5

Examining the proclivity of THC and CBD to modulate TLR3/4 signalling in primary human PBMCs from healthy control subjects and pwMS

5.1 Introduction

MS is a chronic progressive inflammatory autoimmune disease of the CNS associated with the activation of peripheral immune cells (T cells, B cells, monocytes) which, upon activation, infiltrate the CNS and promote neuroinflammation, demyelination and axonal damage [78, 87]. There are several treatment options available to patients that show reasonable and varying efficacy that have been approved by the FDA and the EMA, but no cure for the disorder currently exists. Approved medications include Beta-interferon (IFN-β; Plegridy, Avonex, Betaseron), Glatiramer acetate (Copaxone), Natalizumab (Tysabri), Fingolimod (Gilenya), Rituximab (Rituxan), Mitoxantrone (Novatrone), and Sativex (Nabiximols). Of particular relevance to this study is Sativex. Sativex is an oromucosal spray containing CBD and THC as its most abundant phytocannabinoid components, in addition to other phytocannabinoids and non-phytocannabinoid components, that has shown efficacy for pwMS with moderate to severe spasticity [329]. There is much clinical evidence of the efficacy and safety of this oral cannabinoid-based spray in terms of reducing spasticity, spasm frequency and pain [330]. However, much further research is required to elucidate the mechanisms of action of THC and CBD, as little is known about the precise cellular signalling mechanisms targeted by Sativex in pwMS.

As discussed previously, TLRs are key components of the innate immune system and are linked to neuroinflammation [419] and the pathogenesis of MS [420]. There is growing evidence that cannabinoids may alter TLR signalling and downstream inflammatory cytokine/chemokine production. For example, using the sCB *R*(+)WIN55,212-2, Downer and colleagues (2011) showed evidence that *R*(+)WIN55,212-2 is a novel regulator of TLR3 and TLR4 signalling. Specifically, *R*(+)WIN55,212-2 inhibited the pro-inflammatory signalling axis activated by TLR3 and TLR4, while amplifying the activation of the IRF3 protein and consequently, IFN-β production, with resulting anti-inflammatory effects in EAE [184]. pwMS demonstrate reduced expression of IFN-stimulated genes which suggests an underlying defect in type I IFN signalling in MS [177]. IFN-β therapy is proposed to have anti-inflammatory properties, in addition to positive effects on BBB permeability [121]. Studies such as these demonstrate the therapeutic potential of cannabinoids in EAE progression and, more importantly, MS, by modulating

signalling pathways associated with the production of IFN-β. Studies elsewhere have shown that THC and CBD can inhibit the development of EAE and ameliorate clinical signs of EAE [421, 422]. These effects were correlated with significantly less inflammation in the spinal cord, diminished axonal damage, in addition to a decline in microglial activation and T-cell recruitment [262, 263]. Therefore, it is of great interest to understand the mechanisms by which the phytocannabinoids THC and CBD exert their anti-inflammatory and immunosuppressive effect(s), and to determine if these effects are mediated by targeting TLR signalling events in peripheral immune cells isolated from HC subjects and pwMS.

Aims

The specific aims of this chapter are as follows:

- To profile whole blood cellular composition and physical/mental health parameters in HC subjects and pwMS.
- To determine if THC and CBD, when administered alone or in combination, can regulate TLR3 and TLR4 signalling events in PBMCs from HC volunteers and pwMS.
- To assess the effects of cannabinoid treatment, in the absence of TLR activation, on basal cytokine and chemokine production in PBMCs isolated from both study cohorts.
- To profile TLR3/4 and $CB_{1/2}$ receptor expression in PBMCs from HC subjects and pwMS.
- To assess the impact of DMTs on primary immune cell responses to TLR3/4 activation and THC/CBD treatment.

5.2 Whole blood cell composition profiles of HC subjects

Whole blood collected from healthy volunteers was analysed using a Sysmex Haematology analyser to provide a complete blood profile immediately post-venepuncture, and to track cellular changes over time (0 – 4 h) to account for delays in sample processing. Initially the impact of temperature (RT or ice) on whole blood cellular profiles was assessed to determine the optimal method of transport of whole blood from the Neurology clinic at Beaumont Hospital Dublin to the laboratory, without compromising the cellular composition of the sample. Immediately post-venepuncture, whole blood samples were maintained on ice or at RT and analysed at 0, 2, and 4 h post-venepuncture. Data presented in Fig. 5.1 indicate that no significant change in WBC number (Fig. 5.1A, D), RBC number (Fig. 5.1B, E) or percentage lymphocytes (Fig. 5.1C, F) were observed in each sample at 2 h- and 4 h-post blood draw when blood samples were maintained on ice (Fig. 5.1A, B, C) and at RT (Fig. 5.1D, E, F). Full blood cell composition profiles including WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, and the number of lymphocytes and neutrophils obtained from the samples processed at RT and on ice, at 0, 2 and 4 h post-venepuncture, are presented in Table 9 and Table 10.

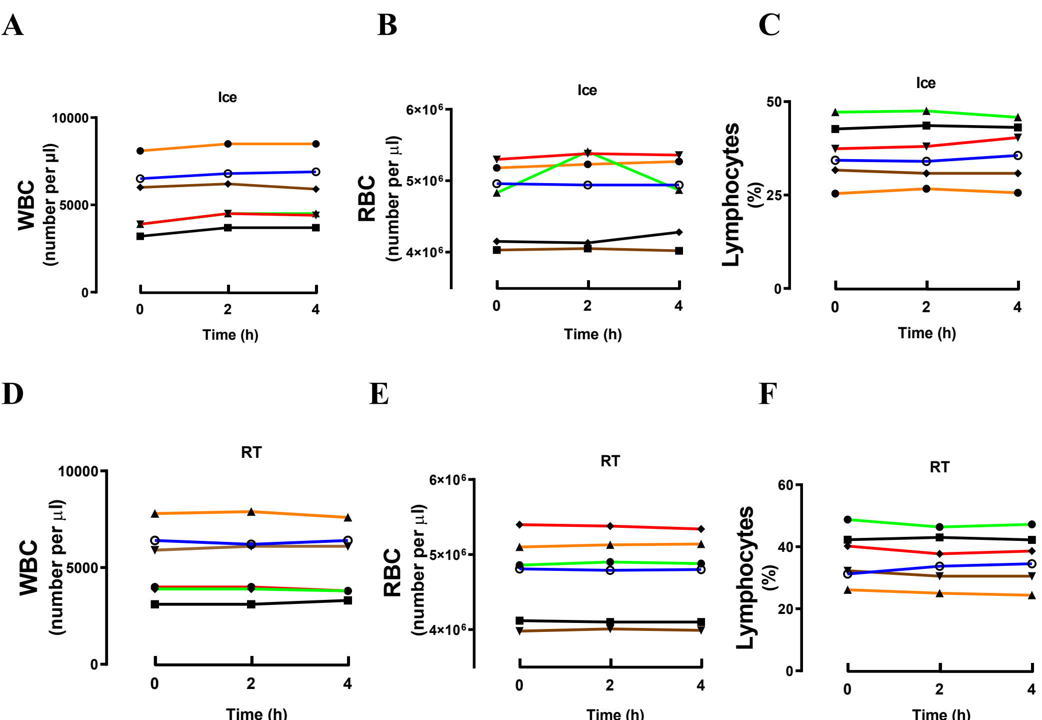


Figure 5.1. Blood cell profiles in samples collected from healthy volunteer's post-venepuncture. The impact of storing fresh blood samples on (**A, B, C**) ice or at (**D, E, F**) RT was determined by assessing cell profiles using the Sysmex Haematology analyser. The number of (**A, D**) WBCs, (**B, E**) RBCs and (**C, F**)

percentage of lymphocytes in each sample was determined. Graphs are representative of data from 6 HC subjects, with each coloured line representative of one HC donor.

Table 9. Blood profiles in samples from healthy subjects maintained at RT post-venepuncture.

Maintained at RT	0 h	2 h	4 h
n	6	6	6
WBC (per µL)	$5.02 \times 10^3 \pm 0.90 \times 10^3$	$5.48 \times 10^3 \pm 0.86 \times 10^3$	$5.4 \times 10^3 \pm 0.85 \times 10^3$
RBC (per µL)	$4.69 \times 10^6 \pm 0.26 \times 10^6$	$4.84 \times 10^6 \pm 0.31 \times 10^6$	$4.76 \times 10^6 \pm 0.27 \times 10^6$
HGB (g/dL)	14.56 ± 0.62	15.08 ± 0.88	14.92 ± 0.59
HCT (%)	42.12 ± 1.73	43.4 ± 2.33	42.66 ± 1.79
MCV (fL)	89.96 ± 1.5	89.96 ± 1.54	89.94 ± 1.48
MCH (pg)	31.08 ± 0.5	31.22 ± 0.72	31.52 ± 1.70
MCHC (g/dL)	34.56 ± 0.32	34.74 ± 0.51	35.06 ± 1.04
PLT (per µL)	$2.44 \times 10^5 \pm 0.16 \times 10^5$	$2.22 \times 10^5 \pm 0.2 \times 10^5$	$2.27 \times 10^5 \pm 0.18 \times 10^5$
Lymphocyte (%)	36.88 ± 3.87	37.32 ± 3.87	37.14 ± 3.84
Neutrophil (%)	54.78 ± 4.96	54.24 ± 4.29	55.48 ± 4.82
Lymphocyte (per µL)	$1.74 \times 10^3 \pm 0.13 \times 10^3$	$1.92 \times 10^3 \pm 0.13 \times 10^3$	$1.9 \times 10^3 \pm 0.11 \times 10^3$
Neutrophil (per µL)	$2.90 \times 10^3 \pm 0.76 \times 10^3$	$3.14 \times 10^3 \pm 0.73 \times 10^3$	$3.14 \times 10^3 \pm 0.79 \times 10^3$

Table 10. Blood profiles in samples from control subjects maintained on ice post-venepuncture.

Maintained on ice	0 h	2 h	4 h
n	6	6	6
WBC (per µL)	$4.94 \times 10^3 \pm 0.85 \times 10^3$	$5.0 \times 10^3 \pm 0.88 \times 10^3$	$4.88 \times 10^3 \pm 0.85 \times 10^3$
RBC (per µL)	$4.69 \times 10^6 \pm 0.28 \times 10^6$	$3.98 \times 10^6 \pm 0.92 \times 10^6$	$4.69 \times 10^6 \pm 0.27 \times 10^6$
HGB (g/dL)	14.6 ± 0.62	15.14 ± 0.7	14.54 ± 0.62
HCT (%)	42.02 ± 1.93	42.3 ± 1.9	42.28 ± 1.93
MCV (fL)	89.88 ± 1.51	90.24 ± 1.59	90.42 ± 1.36
MCH (pg)	31.26 ± 0.69	31.02 ± 0.61	31.12 ± 0.62
MCHC (g/dL)	34.8 ± 0.48	34.4 ± 0.49	34.42 ± 0.43
PLT (per µL)	$2.4 \times 10^5 \pm 0.17 \times 10^5$	$2.39 \times 10^5 \pm 0.17 \times 10^5$	$2.44 \times 10^5 \pm 0.18 \times 10^5$
Lymphocyte (%)	37.94 ± 3.96	36.52 ± 3.94	36.74 ± 4.14
Neutrophil (%)	54.72 ± 4.52	53.86 ± 5.03	54.58 ± 4.74
Lymphocyte (per µL)	$1.76 \times 10^3 \pm 0.14 \times 10^3$	$1.72 \times 10^3 \pm 0.14 \times 10^3$	$1.68 \times 10^3 \pm 0.12 \times 10^3$
Neutrophil (per µL)	$1.76 \times 10^3 \pm 0.72 \times 10^3$	$2.84 \times 10^3 \pm 0.75 \times 10^3$	$2.8 \times 10^3 \pm 0.78 \times 10^3$

WBC, white blood cell; RBC, red blood cell; HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; PLT, platelet. Data presented as the mean ± S.E.M.

5.3 Demographics of healthy donors and pwMS

Healthy volunteers and pwMS attending the Neurology clinic at Beaumont Hospital, Dublin, Ireland, were recruited to this study. Written informed consent was obtained from each participant and the study received ethical approval from the Beaumont Hospital Ethics (Medical Research) and the Faculty of Health Sciences Research Ethics Committee, Trinity College Dublin, Ireland (PIL and consent form indicated in Appendix 3). All pwMS had RRMS and were currently taking immunomodulatory treatments including Plegridy, Gilenya, Dimethyl fumarate, Avonex, Capaxone, Tysabri and Rituximab. HC donors had no history of autoimmune, cardiovascular, respiratory, or degenerative diseases. HC participants were matched on the basis of age and gender where possible. Additionally, HC volunteers and pwMS completed the MS-QOL-54 and QIDS-SR_{16} questionnaires (Appendix 3) at the time of blood draw. The MSQOL-54 questionnaire is a MS-specific instrument that consists of 54 questions that measure the subject's own perception based on two categories: physical health and mental health composite scores. Physical health is assessed under the following sub-categories: physical function, health perceptions, energy/fatigue, physical role limitations, pain, sexual function, social function and health distress. The mental health composite score is assessed under the following sub-categories: health distress, overall QOL, emotional well-being, emotional role limitations and cognitive function. The MSQOL is one of the most widely used MS-specific questionnaires and therefore was chosen for this study [334-336]. Additionally, the QIDS-SR_{16} questionnaire is a self-report designed to provide an indication of depressive symptom severity [337]. The QIDS-SR_{16} assesses depressive symptomatology in subjects across nine domains: sad mood, concentration, self-criticism, suicidal ideation, interest, energy/fatigue, sleep disturbance, decrease/increase in appetite/weight and psychomotor agitation/retardation. This test is regarded as an accurate screening measure for major depressive disorder (MMD) [423], and previously, the laboratory has published MSQOL-54 and QIDS-SR_{16} questionnaire data in HC and MS study cohorts [424]. Data herein demonstrate that pwMS reported significantly reduced physical health (Fig. 5.2A), mental health (Fig. 5.2B) and energy levels (Fig. 5.2C), when compared to HC subjects. Additionally, pwMS reported increased pain scores (Fig. 5.2D) and depressive symptoms (Fig. 5.2E), when compared to HC participants.

Table 11. Demographic data from HC subjects and pwMS included in the study.

Baseline demographics	HC	MS	*P value*
n	32	32	-
Sex (F/M)	20/10 (2 not reported)	28/4	-
Age (Year ± SEM)	33.90 ± 2.41	38.34 ± 1.70	-
MSQoL-54: -Physical Health (%)	93.49 ± 0.82	61.19 ± 3.64***	*$p<0.001$*
-Mental Health (%)	90.39 ± 1.47	65.48 ± 3.90***	*$p<0.001$*
QIDS-SR_{16}	2.19 ± 0.40	6.92 ± 0.82***	*$p<0.001$*
EDSS	N/A	2.15 ± 0.39	-
Disease Duration (months)	N/A	62.88 ± 9.30	-
Other Medical conditions • Blood disorder, *n* (%) • Thyroid disease, *n* (%) • Non-MS autoimmune disease, *n* (%) • Allergies, n (%)	20 HC donors completed 0 (0%) 2 (10%) 2 (10%) 7 (35%)	23 pwMS completed section 1 (4.35%) 1 (4.35%) 2 (8.70%) 7 (30.43%)	-
Infection, *n* (%)	0 (0%)	3 (13.04%)	-
Smoker, *n* (%)	0 (0%)	9 (39.13%)	-
Cannabis user, *n* (%)	0 (0%)	2 (8.70%)	-
MS DMT at time of blood draw	N/A	Tysabri, Gilenya, Rituximab, Capaxone Plegridy, Tecfidera, Avonex	-

N/A = Not applicable

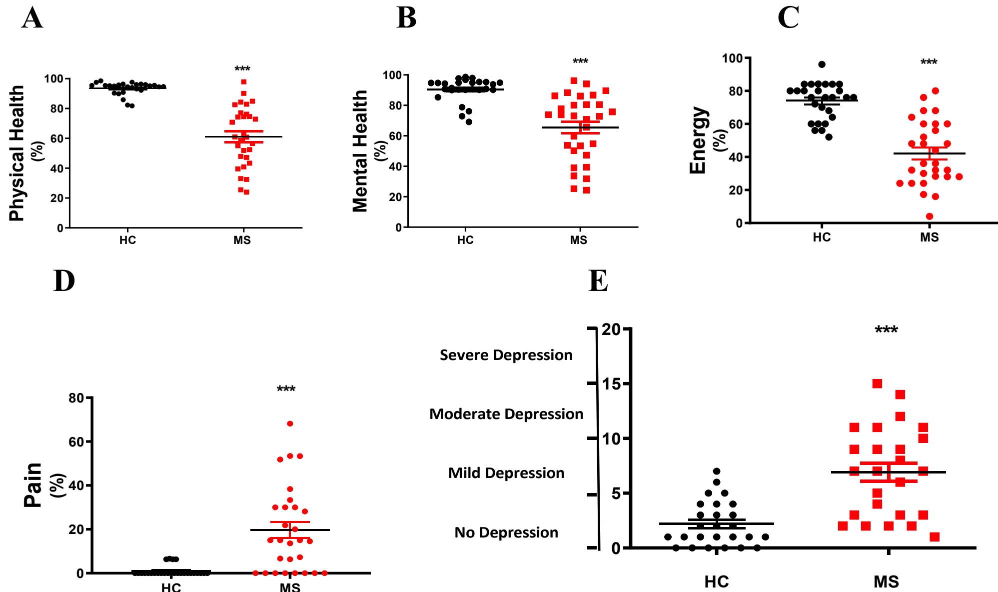


Figure 5.2. MSQOL-54 and QIDS-SR16 data in HC and MS cohorts. HC subjects and pwMS completed the MSQOL-54 and QIDS-SR_{16} questionnaires within one day following blood donation. Questionnaire data indicate that pwMS reported reduced **(A)** physical health, **(B)** mental health and **(C)** energy scores, compared to HC subjects. pwMS reported increased **(D)** pain scores and **(E)** depressive symptomatology when compared to HC subjects. Data are presented as individual dot-plots and the mean ± S.E.M. Data were checked for normality using Shapiro-Wilk. Data was analysed using Mann-Whitney test. ****p*<0.001 versus HC group.

5.4 Comparison of the cellular composition of whole blood from HC and pwMS participants

Much data has reported alterations in whole blood cellular composition in samples isolated from pwMS when compared to healthy volunteers. Indeed, reports indicate that the red blood cell distribution width (RDW), which is related to MCH, MCV and MCHC, is higher in pwMS when compared to HC subjects [425]. In addition, mild thrombocytopenia has been reported in pwMS [426]. However, to our knowledge, there is no published evidence comparing baseline cellular composition values in pwMS compared to HC cases. Therefore, to determine the cellular composition of whole blood in HC cases and pwMS in our study cohorts, whole blood from each subject was analysed using a Sysmex Haematology analyser to collate data regarding WBC, RBC, HGB, HCT, MCV, MCH, MCHC and PLT. Data presented in Fig. 5.3 indicate that there was no significant difference in WBC and RBC number (Fig. 5.3A, B), HGB and HCT levels (Fig. 5.3C, D), MCHC levels (Fig. 5.3G), PLT number (Fig. 5.3H), in addition to the number and percentage of lymphocytes/neutrophils (Fig. 5.3I, J, K, L), between HC and MS subjects. Interestingly, a significant increase in MCV (Fig. 5.3E) and MCH (Fig. 5.3F) was determined in pwMS, compared to the control group. In addition, significant differences were found in the MXD (mixed WBCs without lymphocytes and neutrophils) population between the two study cohorts (Fig. 5.3M, N). Importantly, the Sysmex Haematology analyser did not identify alterations in the exact WBC population (i.e. T cells, B cells, monocytes, eosinophils, basophils) between the groups, and hence caution must be applied when interpreting these data. Future work will pinpoint if alterations in populations of specific cell subsets occur in pwMS.

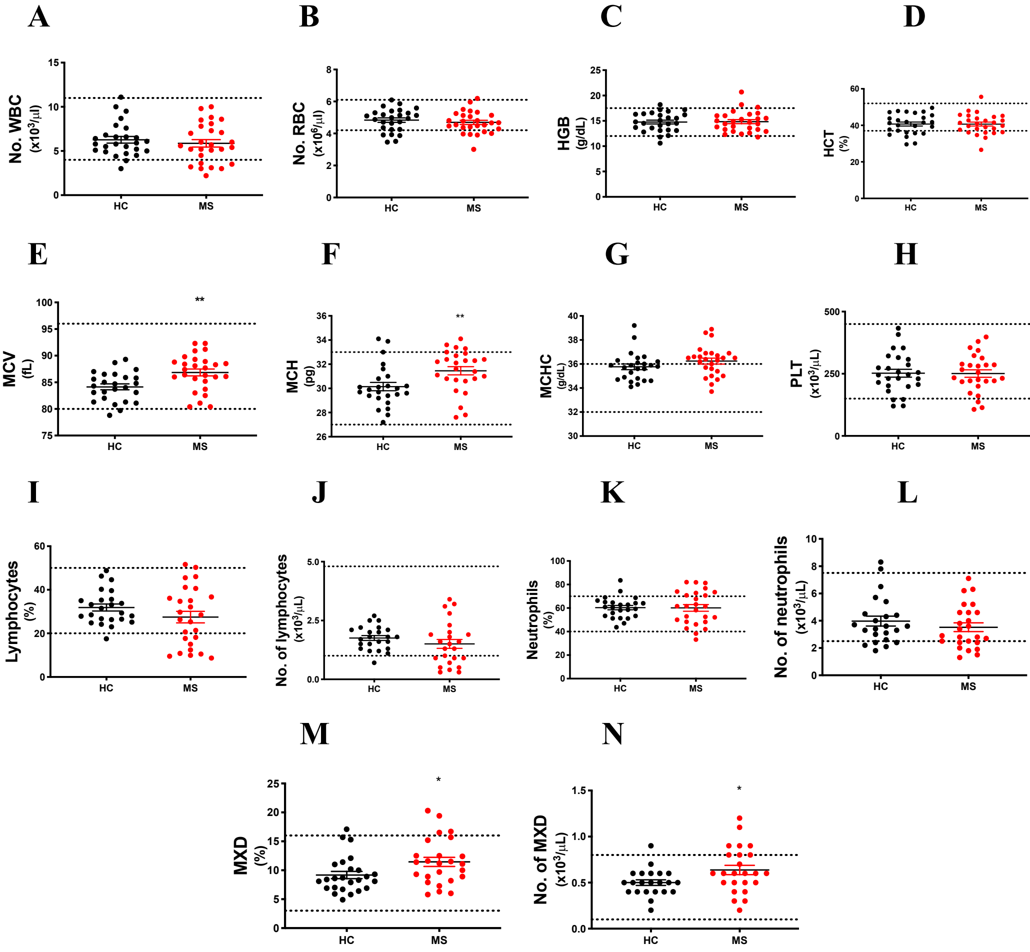


Figure 5.3. Whole blood cellular profiles in samples from HC cases and pwMS. Whole blood was isolated via venepuncture and analysed to assess cellular composition using the Sysmex Haematology analyser. The number of **(A)** WBC and **(B)** RBC was unchanged between HC subjects and pwMS. **(C)** HGB, **(D)** HCT, **(G)** MCHC and **(H)** PLT levels were similar between HC cases and pwMS. Blood samples from pwMS demonstrated increased levels of **(E)** MCV and **(F)** MCH. There was no difference between both cohorts in terms of the percentage and number of **(I, J)** lymphocytes and **(K, L)** neutrophils. Blood samples from pwMS reported an increased percentage and number of **(M, N)** MXD cells in whole blood, when compared to samples from HC volunteers. Data are shown as dot-plots for each donor and as the mean ± S.E.M. Dashed lines represent accepted 'normal ranges' for each measurement. Data were assessed for normality using Shapiro-Wilk and analysed using students t-tests. *p<0.05 and **p<0.01 versus HC group.

5.5 PBMCs from pwMS are desensitised in terms of TLR3-induced CXCL10 expression, when compared to cells from healthy subjects

Data shown previously (Chapter 3: Fig. 3.7) provided evidence that primary immune cells respond to TLR3 stimulation in terms of producing CXCL10 and IFN-β. Therefore, we set out to determine if PBMCs isolated from HC subjects and pwMS demonstrate differential responses to TLR3 agonism. Peripheral whole blood was collected via venepuncture, and PBMCs were isolated using lymphoprep over density gradient. PBMCs were cultured with poly(I:C) (10 μg/ml) for 4 (mRNA) or 24 h (protein), RNA harvested for RT-qPCR analysis, and supernatants collected for protein analysis via ELISA. Initially, the expression profile of the TLR3 receptor was determined in untreated PBMCs from HC volunteers and pwMS. Data in Fig. 5.4 indicate that there was no significant difference in basal *TLR3* mRNA expression in PBMCs from HC and MS individuals (Fig. 5.4A). Next, PBMCs from HC cases and pwMS were treated with poly(I:C) and the expression of CXCL10 determined. Data in Fig. 5.4B indicate that poly(I:C) treatment insignificantly increased *CXCL10* mRNA expression 5.5-fold in PBMCs from HC subjects; conversely, poly(I:C) treatment had no effect on *CXCL10* mRNA expression in PBMCs from pwMS (Fig. 5.4B). In support of CXCL10 mRNA data from HC and MS cohorts, Fig 5.4C shows that MS was not associated with a difference in basal expression of CXCL10 protein in PBMCs. Indeed, post hoc analysis revealed that PBMCs from pwMS were associated with a significant reduction in poly(I:C)-induced CXCL10 protein expression, when compared to poly(I:C)-induced CXCL10 production in PBMCs from HC volunteers. Importantly, poly(I:C) promoted a significant induction of CXCL10 protein in the HC cohort. However, only a minor insignificant increase of CXCL10 protein expression was observed in the PBMCs from MS cohort following poly(I:C) treatment. Two-way ANOVA analysis revealed a significant influence of poly(I:C) treatment ($p < 0.001$) and disease status ($p < 0.01$), in addition to a significant interaction of these factors ($p < 0.01$) (Fig. 5.4C). This data suggests that PBMCs from pwMS are desensitised to TLR3 agonism. However, no significant difference was determined between HC and MS poly(I:C) stimulated PBMCs in terms of *IFN-β* mRNA (Fig. 5.4D) and IFN-β protein expression (Fig. 5.4E). However, PBMCs from pwMS significantly increased IFN-β protein after poly(I:C) treatment, an effect not observed in HC PBMCs (Fig. 5.4E).

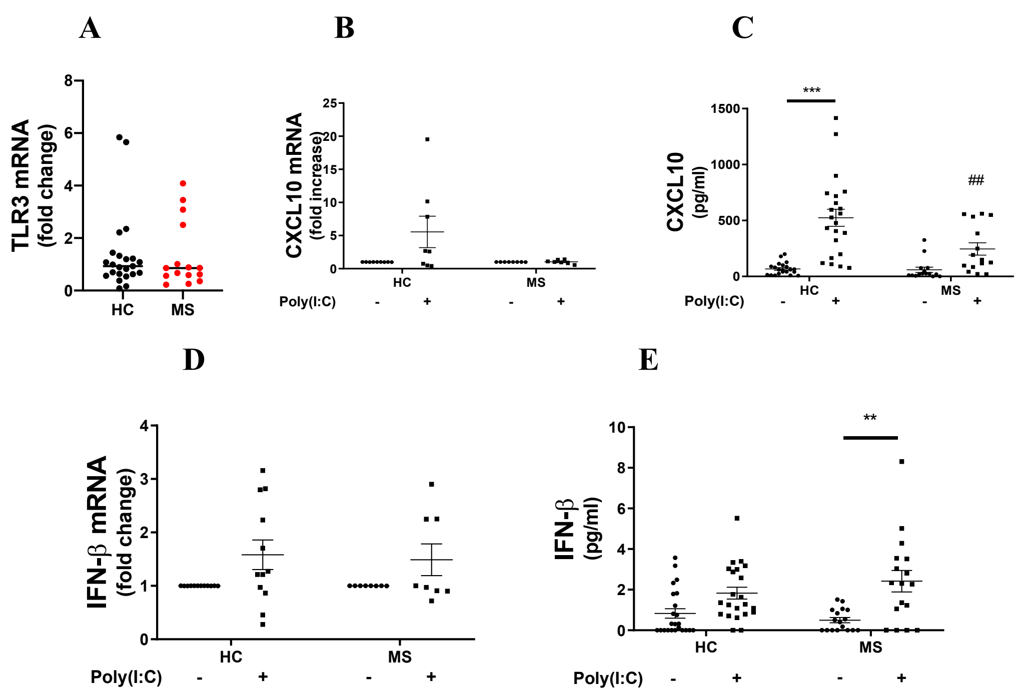


Figure 5.4. Effect of TLR3 activation on CXCL10 and IFN-β expression in PBMCs from HC and MS subjects. PBMCs from HC volunteers and pwMS were isolated and cultured with poly(I:C) (10 µg/ml) for 4 (mRNA) or 24 h (protein) and analysed from CXCL10 and IFN-β expression. **(A)** No significant difference was determined in *TLR3* mRNA expression in PBMCs from HC subjects and pwMS. PBMCs from pwMS are desensitised to poly(I:C) in terms of CXCL10 **(B)** mRNA and **(C)** protein expression. PBMCs from HC cases and pwMS respond to poly(I:C)-induced **(D)** *IFN-β* mRNA and **(E)** IFN-β protein expression equivalently. Data are represented as the mean ± S.E.M from 9-24 HC subjects and 8-18 MS donors. Students t-test was used when comparing 2 groups. Two-way ANOVA followed by Bonferroni's post-hoc test was performed when there was more one variable. $^{**}p<0.01$ and $^{***}p<0.001$ *versus* indicated groups and $^{\#\#}p<0.01$ *versus* poly(I:C)-treated PBMCs from HC subjects.

5.6 THC and CBD target TLR3-induced CXCL10 and IFN-β expression in primary PBMCs from HC subjects and pwMS

Given that THC and CBD, when administered alone and in combination, can target TLR3-induced CXCL10 and IFN-β in THP-1 macrophages (Chapter 4: Fig. 4.2), we next set out to determine the effect of both phytocannabinoids on TLR3 signalling in primary immune cells isolated from HC subjects and pwMS. Peripheral whole blood was isolated via venepuncture, and PBMCs were extracted using lymphoprep over density gradient. PBMCs were pre-treated with THC, CBD or a 1:1 combination (all at 10 μM) for 45 min prior to stimulation with poly(I:C) (10 μg/ml) for 4 (for mRNA analysis) or 24 h (for protein analysis). THC, CBD and a 1:1 combination promoted a trend towards reducing poly(I:C)-induced *CXCL10* mRNA expression in PBMCs from HC subjects (Fig. 5.5A). Indeed, this result translated to CXCL10 protein expression, where both cannabinoids, delivered in combination, significantly attenuated poly(I:C)-induced CXCL10 protein expression (Fig. 5.5B). Interestingly, the combination treatment was significantly more effective at inhibiting poly(I:C)-induced CXCL10 than the cannabinoid alone treatments (Fig. 5.5B). PBMCs isolated from pwMS did not produce *CXCL10* mRNA after poly(I:C) stimulation (which is in line with data in Fig 5.4C); however, THC:CBD treatment, in the presence of poly(I:C), significantly reduced basal *CXCL10* mRNA expression in PBMCs from pwMS (Fig. 5.5C). Interestingly, THC:CBD combination treatment significantly attenuated poly(I:C)-induced CXCL10 protein expression in PBMCs from pwMS (Fig. 5.5D).

In terms of IFN-β expression, poly(I:C) enhanced *IFN-β* mRNA expression, albeit insignificantly, in PBMCs from HC subjects, and THC and CBD failed to significantly modulate poly(I:C)-induced *IFN-β* mRNA expression (Fig. 5.5E). In contrast, treatment of cells with the THC:CBD combination, but not the cannabinoids alone, significantly reduced poly(I:C)-induced IFN-β protein expression in PBMCs from HC subjects (Fig. 5.5F). Finally, PBMCs from pwMS did not respond to poly(I:C) in terms of *IFN-β* mRNA expression (Fig. 5.5G) and the THC:CBD combination significantly inhibited poly(I:C)-induced IFN-β protein expression in PBMCs from pwMS (Fig. 5.5H). These data suggest that phytocannabinoids can inhibit TLR3 signalling in primary PBMCs, with the

THC:CBD combination treatment being most effective in PBMCs from HC cases and pwMS.

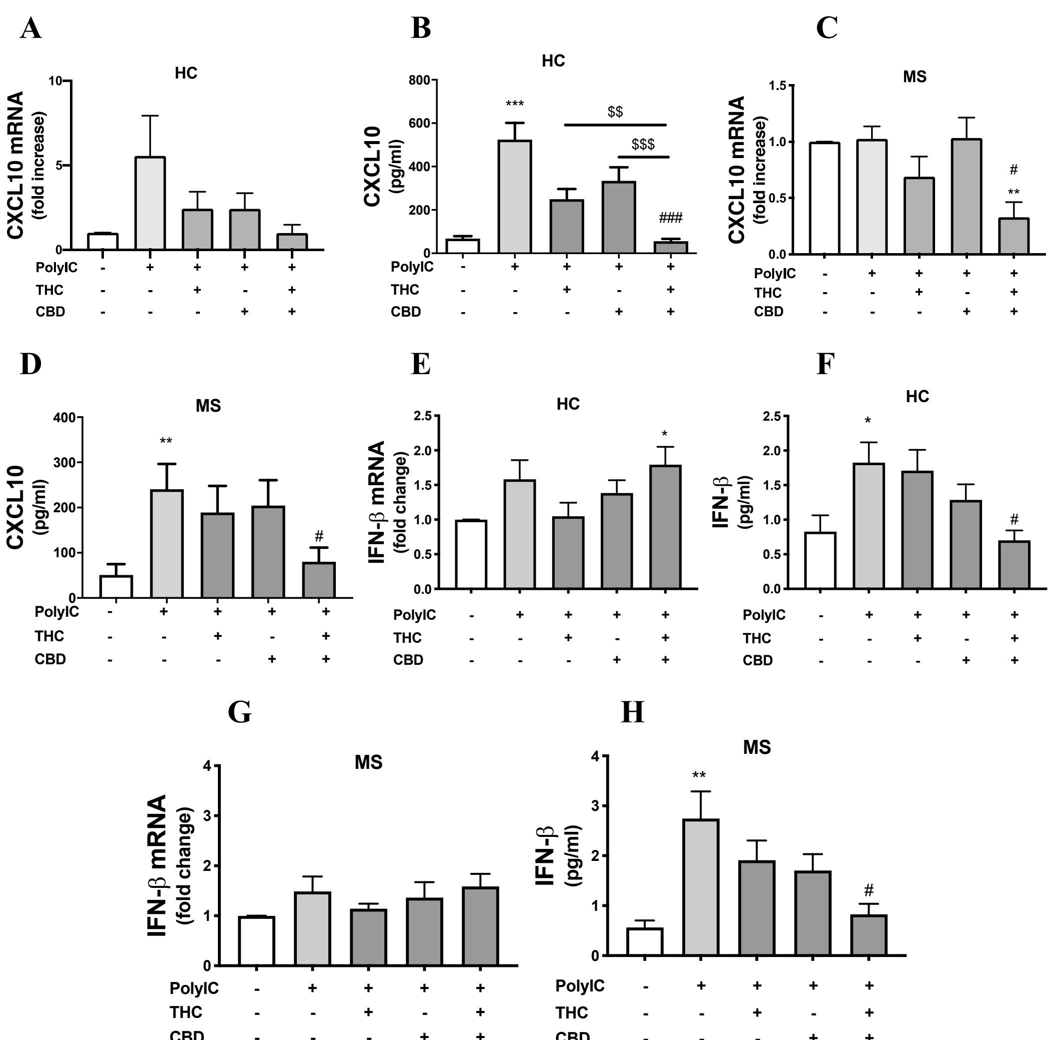


Figure 5.5. Effect of THC, CBD, and a 1:1 combination on TLR3-induced CXCL10 and IFN-β expression in PBMCs from HC cases and pwMS. Effect of THC, CBD and the combination (1:1) of phytocannabinoids on poly(I:C)-induced **(A)** *CXCL10* mRNA (n=9), in addition to **(B)** CXCL10 protein expression, in PBMCs from HC subjects (n=23). THC:CBD combination inhibited **(C)** basal *CXCL10* mRNA expression (n=8) and **(D)** TLR3-induced CXCL10 protein expression (n=15) in PBMCs from pwMS. Effect of THC and CBD on **(E)** *IFN-β* mRNA expression in PBMCs from HC subjects (n=12). **(F)** The combination of THC and CBD reduced TLR3-induced IFN-β protein expression in PBMCs from HC subjects (n=14). **(G)** Poly(I:C), THC and CBD did not alter *IFN-β* mRNA expression in PBMCs from pwMS (n=8). **(H)** THC:CBD attenuated poly(I:C)-induced IFN-β protein expression in PBMCs from pwMS (n=13). Data are expressed as means ± S.E.M. Data that passed the Shapiro-Wilk normality test were analysed by one-way ANOVA followed by Dunnett's multiple comparison test. For non-parametric data, Kruskal-Wallis followed by Dunn's multiple comparison test was performed. *p<0.05, **p<0.01, ***p<0.001 *versus* untreated groups. $^{\#}p$<0.05 and $^{\#\#\#}p$<0.001 *versus* poly(I:C)-treated groups. $^{\$\$}p$<0.01 and $^{\$\$\$}p$<0.001 *versus* THC:CBD combination treated groups.

5.7 A 1:1 combination of THC:CBD inhibits basal CXCL10 expression in PBMCs

Given the effects of THC/CBD on TLR3-induced signalling in PBMCs (Fig. 5.5), we next set out to determine the effects of THC, CBD and THC:CBD on basal CXCL10 and IFN-β expression in PBMCs isolated from HC cases and pwMS. Peripheral whole blood was isolated, PBMCs were separated over a density gradient and were treated with THC, CBD or a 1:1 combination (all at 10 μM) for 4 (mRNA) or 24 h (protein). RNA was harvested for analysis via RT-qPCR, and supernatants collected for protein analysis via ELISA. Initially, cannabinoid receptor expression was determined in PBMCs isolated from HC subjects and pwMS. Data in Fig. 5.6A, B demonstrate that there was no significant difference in CB_1 and CB_2 mRNA expression between the groups, with a trend towards increased CB_1 (Fig. 5.6A) and CB_2 (Fig. 5.6B) mRNA expression determined in PBMCs from the MS group. Importantly, the THC:CBD combination, but not THC and CBD alone, significantly inhibited *CXCL10* mRNA (Fig. 5.6C) and protein (Fig. 5.6D) expression, when compared to vehicle-treated cells, in PBMCs from HC cases. In PBMCs from pwMS the phytocannabinoids had no significant effect on *CXCL10* mRNA (Fig. 5.6E) expression; however, THC:CBD reduced CXCL10 protein (Fig. 5.6F) expression, albeit insignificantly, in PBMCs from pwMS. Furthermore, there was no significant effect of the phytocannabinoids on *IFN-β* mRNA (Fig. 5.6G, I) and IFN-β protein (Fig. 5.6H, J) expression in PBMCs from HC volunteers and pwMS, however there was a minor insignificant increase in IFN-β protein after phytocannabinoid treatment in PBMCs from pwMS. This suggests that the 1:1 combination of THC and CBD, but not the phytocannabinoids alone, inhibit the basal expression of the pro-inflammatory chemokine CXCL10 in primary PBMCs.

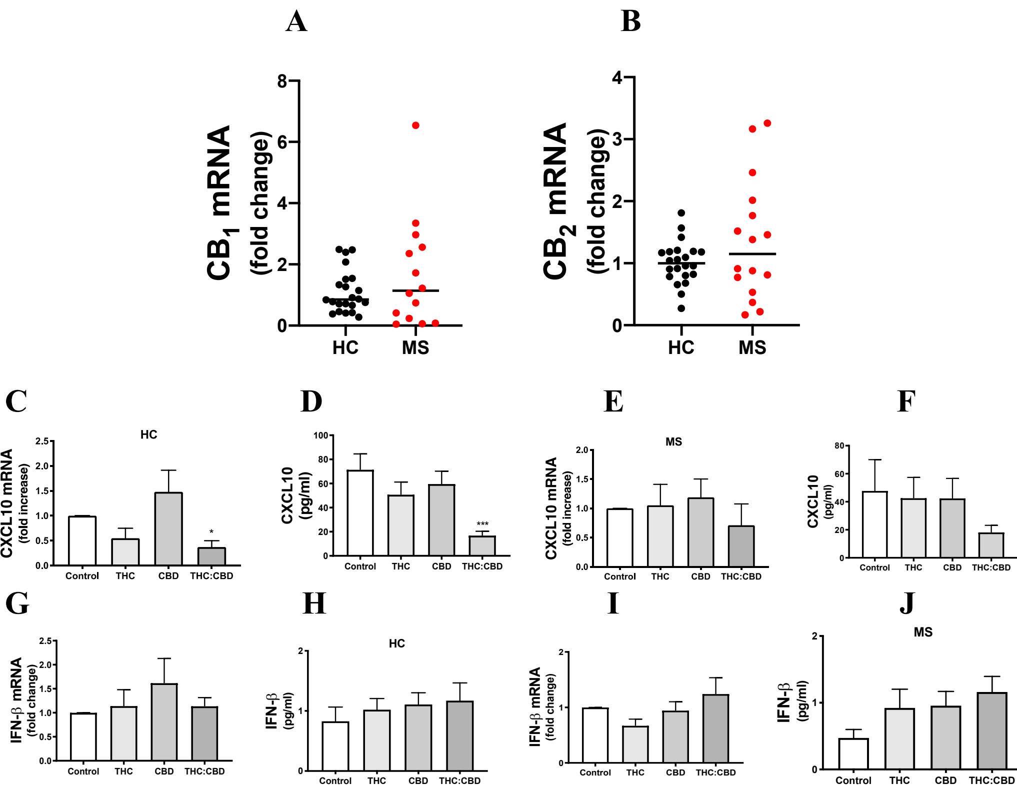


Figure 5.6. A THC:CBD (1:1) combination of phytocannabinoids inhibit CXCL10 expression in primary PBMCs. Primary PBMCs from HC cases and pwMS were isolated from whole blood and treated with THC (10 μM), CBD (10 μM) or a 1:1 combination (10 μM of each) for 4 (RNA) or 24 h (protein). No difference was detected in **(A)** *CB_1*, or **(B)** *CB_2* mRNA expression levels between HC and MS groups. THC:CBD significantly inhibited **(C)** *CXCL10* mRNA and **(D)** CXCL10 protein expression in PBMCs from HC cases. **(E)** THC and CBD had no effect on *CXCL10* mRNA expression in PBMCs from pwMS. **(F)** THC:CBD reduced (albeit insignificantly) CXCL10 protein levels in PBMCs from pwMS. THC, CBD and THC:CBD had no effect on **(G, I)** *IFN-β* mRNA and **(H, J)** IFN-β protein expression in PBMCs isolated from HC subjects and pwMS. Data are means ± S.E.M from 8-24 HC and 4-18 pwMS. Students t-test was used when comparing 2 groups that passed the Shapiro-Wilk test, if 2 groups did not pass Mann-Whitney test was used. All data with more than 2 groups passed the Shapiro-Wilk normality test and were analysed by one-way ANOVA followed by Dunnett's multiple comparison test. *p<0.05, and ***p<0.001 *versus* untreated cells.

5.8 Stratifying the responses of PBMCs from pwMS to poly(I:C), THC and CBD treatment in terms of DMT use at the time of analysis

As indicated previously in Table 11, recruited pwMS to this study were currently prescribed a variety of immunomodulatory DMTs, including Interferon-β 1a, Dimethyl fumarate, Fingolimod, Rituximab, and Natalizumab (DMTs reviewed in introduction section 1.10). Therefore, it was of interest to determine the effect of THC and CBD on TLR3 signalling with respect to various DMTs. Whole blood was taken from pwMS via venepuncture and PBMCs isolated over density gradient. PBMCs were then placed in culture and pre-treated with THC, CBD or a 1:1 combination (all at 10 μM: 45 min) prior to poly(I:C) (10 μg/ml: 24 h) treatment. Supernatants were then collected and assessed for IFN-β and CXCL10 protein analysis via ELISA. In the clinical cohort, a total of six pwMS with a RR phenotype reported the use of IFN-β 1a therapy at the time of blood draw, two pwMS were taking Natalizumab at the time of blood draw, one individual took Rituximab, one individual was prescribed Fingolimod and one individual was prescribed Dimethyl fumarate at the time of blood donation.

In terms of IFN-β 1a therapy, the data indicate that PBMCs from each patient responded to poly(I:C) (to varying degrees) by increasing IFN-β and CXCL10 expression (Fig. 5.7A, B). Furthermore, in this MS cohort, THC, CBD and the 1:1 combination promoted a trend towards attenuating poly(I:C)-induced IFN-β and CXCL10 expression in PBMCs, with the combination treatment being most effective (Fig. 5.7A, B). Furthermore, in the study cohort one or two pwMS were prescribed either Natalizumab, Rituximab, Fingolimod, and Dimethyl fumarate therapies at the time of blood draw. Therefore, the data presented below for each DMT are indicative of potential responses. Two pwMS were prescribed Natalizumab therapy at the time of recruitment to this study, and our findings indicate that PBMCs from one MS case responded strongly, while PBMCs from a further MS case responded weakly, to TLR3 activation (Fig 5.7C, D). THC, CBD, and THC:CBD promoted some degree of inhibition in terms of poly(I:C)-induced IFN-β and CXCL10 in PBMCs from both cases (Fig. 5.7C, D). Poly(I:C)-induced IFN-β and CXCL10 was reduced by THC/CBD, with the combination treatment being most effective, in PBMCs from an MS case prescribed Rituximab (Fig. 5.7E, F). In terms of Fingolimod therapy, PBMCs from one MS case responded weakly

to TLR3 treatment; however, THC, CBD, and THC:CBD again promoted a trend towards attenuating IFN-β and CXCL10 expression in PBMCs (Fig. 5.7G, H). Finally, in terms of Dimethyl Fumarate therapy, PBMCs isolated from this MS case responded to TLR3 activation in terms of IFN-β and CXCL10 expression, while THC, CBD, and THC:CBD attenuated poly(I:C)-induced IFN-β and CXCL10 expression, with the combination treatment being most effective, in this case (Fig. 5.7I, J).

These data are of particular interest as the cannabinoid-based therapy Sativex, an oromucosal spray containing CBD and THC as its most abundant phytocannabinoid components, in addition to other phytocannabinoids and non-phytocannabinoid components, is currently prescribed to pwMS in certain countries as an add-on adjunctive therapy to known DMTs in pwMS [330, 427]. These data highlight the potential effects of DMTs on cellular responses to TLR3 agonism, in addition to the effects of DMTs on primary immune cellular responses to phytocannabinoids.

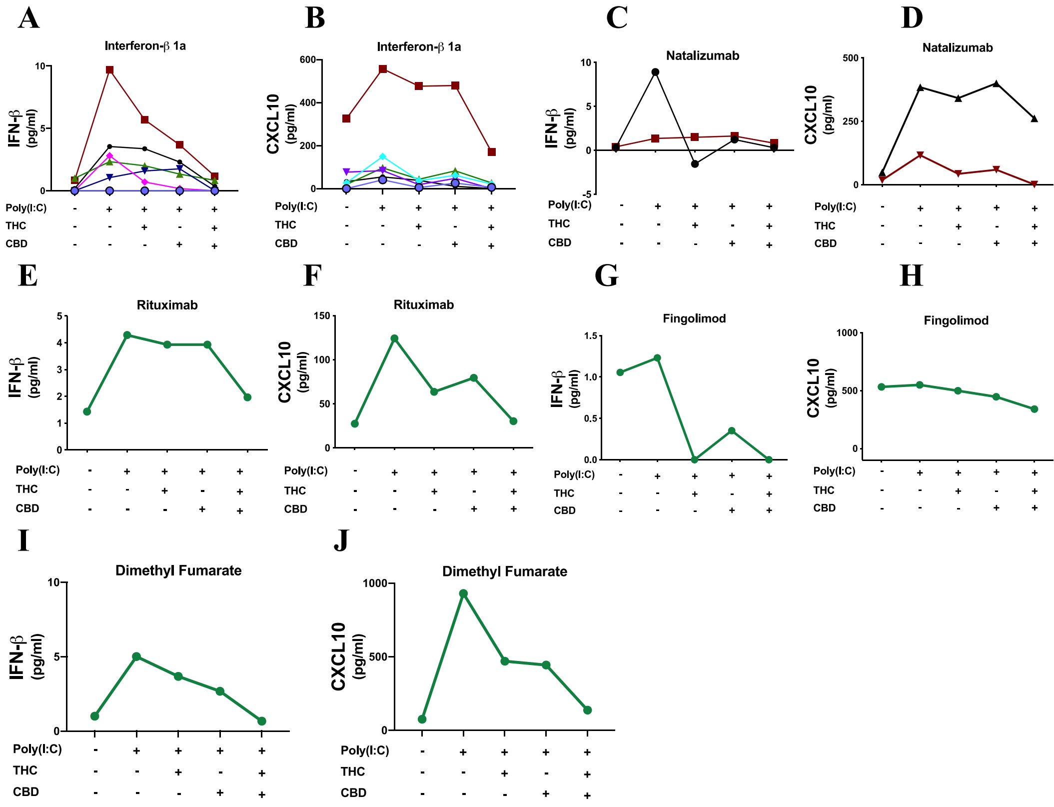


Figure 5.7. Analysis of the effect of DMTs on the cellular responses of PBMCs from pwMS to poly(I:C), THC and CBD treatment. PBMCs from pwMS were isolated from whole blood and pre-treated with THC (10 µM), CBD (10 µM) or a 1:1 combination (10 µM of each) for 45 min prior to stimulation with poly(I:C) (10 µg/ml) for 24 h. IFN-β and CXCL10 protein levels were determined via ELISA. The effect of THC, CBD, and THC:CBD on poly(I:C)-induced **(A)** IFN-β and **(B)** CXCL10 expression in PBMCs isolated from pwMS receiving Interferon-β 1a therapy (*n*=6). The effect of the cannabinoids on poly(I:C)-induced **(C)** IFN-β and **(D)** CXCL10 expression in PBMCs isolated from pwMS receiving Natalizumab therapy (*n*=2). The effect of THC and CBD on poly(I:C)-induced **(E, G, I)** IFN-β and **(F, H, J)** CXCL10 expression in PBMCs isolated from pwMS receiving **(E, F)** Rituximab, **(G, H)** Fingolimod and **(I, J)** Dimethyl Fumarate therapies (*n*=1). Data are expressed as line graphs with each line representing one MS case.

5.9 THC and CBD potentiate LPS-induced TNFα expression in PBMCs

Given that THC and CBD, when administered alone and in combination, do not target TLR4-induced TNFα expression in THP-1 monocytes (Fig. 4.2) and macrophages (Fig. 4.5), we next set out to determine if the lack of effect of cannabinoids on TNFα expression translates to primary immune cells. Peripheral whole blood was collected via venepuncture and PBMCs were isolated over a density gradient. PBMCs were pre-treated with THC, CBD or a 1:1 combination (all at 10 μM) for 45 min prior to stimulation with LPS (100 ng/ml) for 4 (mRNA) or 24 h (protein). RNA (TNFα/TLR4) and protein (TNFα) expression were determined by RT-qPCR and ELISA, respectively. Initially, the basal expression level of *TLR4* mRNA was assessed in PBMCs from HC cases and pwMS. Data in Fig. 5.8A indicate that TLR4 is expressed in PBMCs, and disease did not significantly alter relative TLR4 expression. Next, data in Fig. 5.8B indicates that LPS significantly increased TNFα protein expression to comparable levels in both HC and MS groups (Fig. 5.8B). Indeed, Two-way ANOVA analysis revealed that there was a significant influence of drug (LPS) treatment ($p<0.001$), but no influence of disease status ($p=0.48$), in addition to no significant interaction of these factors ($p=0.07$). Data in Fig. 5.8A, B suggest that TLR4 expression and signalling was comparable in PBMCs from HC cases and pwMS. Next, the effect of cannabinoid administration alone, and in conjunction with LPS, was assessed in PBMCs from the HC group in terms of *TNFα* mRNA and TNFα protein expression. Firstly, THC, CBD, and a THC:CBD combination treatment did not alter basal expression of *TNFα* mRNA (Fig. 5.8C) and protein (Fig. 5.8D) expression in PBMCs from the HC group. However, when the phytocannabinoids were administered with LPS, THC:CBD treatment promoted a trend towards potentiating LPS-induced *TNFα* mRNA expression in PBMCs from the HC group (Fig. 5.8E) with one-way ANOVA analysis revealing a significant difference between group means ($p=0.001$), and this translated to a statistically significant potentiation of LPS-induced TNFα protein expression in PBMCs from HC volunteers (Fig. 5.8F).

Following characterisation of the effect of the cannabinoids in PBMCs from HC subjects, next, the effect of THC, CBD, and THC:CBD, alone and in combination with LPS, was assessed in PBMCs from pwMS. Data indicate that THC, CBD, and THC:CBD promoted a trend towards reducing basal *TNFα* mRNA expression in

PBMCs from pwMS (Fig. 5.8G). Importantly, THC, CBD, and THC:CBD significantly inhibited TNFα protein expression in PBMCs from pwMS (Fig. 5.8H). Interestingly, this inhibitory effect of cannabinoids on basal TNFα expression was not detected in PBMCs from HC cases, suggesting that pwMS are more sensitive to the effects of the phytocannabinoids in terms of targeting the cellular mechanisms controlling TNFα expression. Furthermore, CBD, but not THC and THC:CBD, promoted a trend towards increasing LPS-induced *TNFα* mRNA expression in PBMCs from pwMS (Fig. 5.8I) and one-way ANOVA analysis revealed a significant difference between group means ($p=0.03$). However, THC, CBD, and THC:CBD, significantly potentiated LPS-induced TNFα protein expression in PBMCs from pwMS, with the combination treatment being most effective (Fig. 5.8J). These data highlight the heightened sensitivity of PBMCs from pwMS, compared to PBMCs from HC donors, to the cellular effects of cannabinoids in terms of targeting mechanisms controlling TNFα expression .

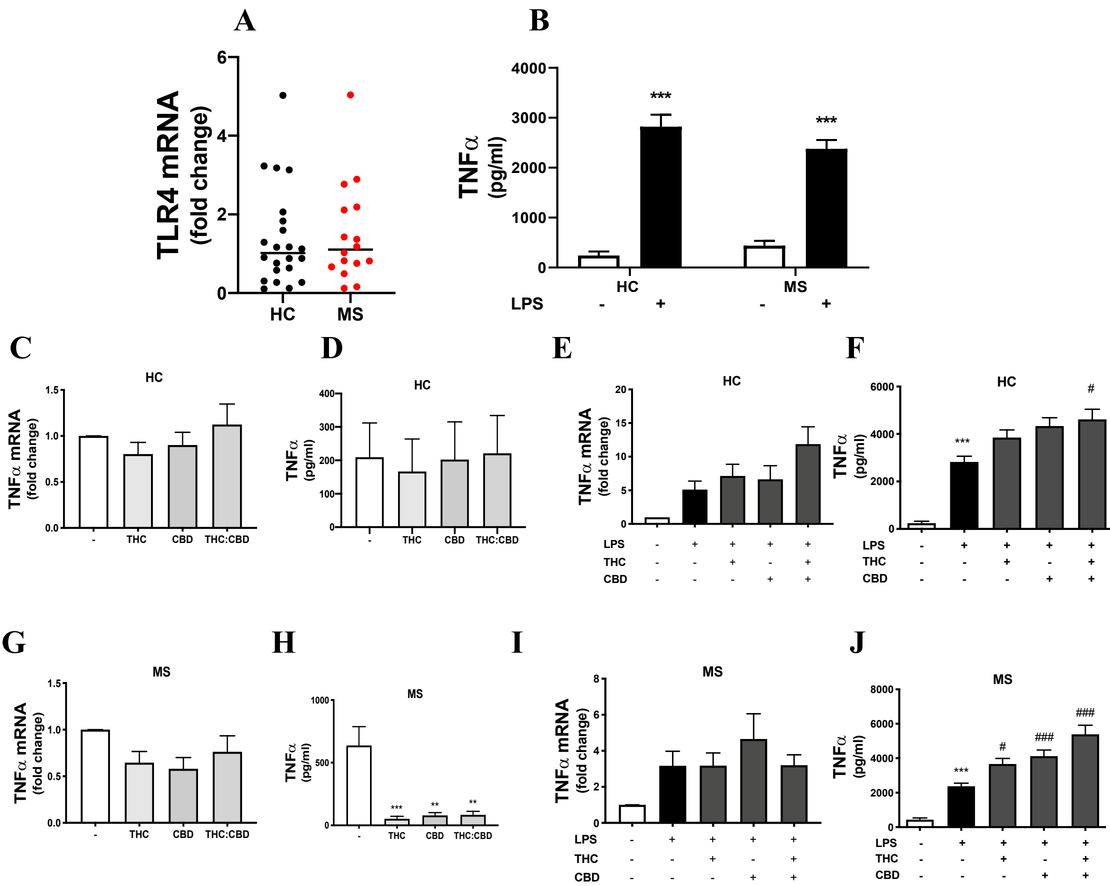


Figure 5.8. THC, CBD, and THC:CBD differentially target TNFα production in PBMCs from HC and MS cohorts. Human primary PBMCs from HC subjects and pwMS were isolated from whole blood and pre-treated with THC (10 μM), CBD (10 μM) or a 1:1 combination (10 μM of each) prior to stimulation with LPS (100 ng/ml) for 4 h (RNA) or 24 h (protein). **(A)** *TLR4* mRNA expression in PBMCs from HC and MS cases. **(B)** PBMCs from HC and MS subjects respond in a similar manner in terms of LPS-induced TNFα protein expression. THC, CBD, and THC:CBD do not alter **(C)** *TNFα* mRNA and **(D)** TNFα protein expression in PBMCs from HC subjects. Effect of THC and CBD on LPS-induced **(E)** *TNFα* mRNA and **(F)** TNFα protein expression in PBMCs from HC subjects. THC, CBD, and THC:CBD inhibit **(G)** *TNFα* mRNA and **(H)** TNFα protein expression in PBMCs from pwMS. Effect of THC and CBD on LPS-induced **(I)** *TNFα* mRNA and **(J)** TNFα protein expression in PBMCs from pwMS. Data are represented as the means ± S.E.M from 19-27 HC and 10-19 MS donors. Students t-test was used when comparing 2 groups. Data that passed the Shapiro-Wilk normality test was analysed by one-way ANOVA followed by Dunnett's multiple comparison test. For non-parametric data, the Kruskal-Wallis followed by Dunn's multiple comparison test was performed. Two-way ANOVA followed by Bonferroni's post-hoc test was performed when there was more than one variable. $^{**}p<0.01$ and $^{***}p<0.001$ *versus* untreated groups and $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ *versus* LPS-treated cells.

5.10 THC and CBD differentially target MyD88-independent signalling events regulated by TLR4 in PBMCs from HC and MS subjects

Previously we have shown that THC and CBD target the LPS-TRIF-IFN-β/CXCL10 signalling axis in THP-1 macrophages (Fig. 4.6). Therefore, the effect of THC, CBD, and THC:CBD, on TLR4 signalling, independent of MyD88, was determined in PBMCs from HC cases and pwMS. PBMCs were isolated from whole blood and were pre-treated with THC, CBD or a 1:1 combination (all at 10 μM) for 45 min prior to stimulation with LPS (100 ng/ml) for 4 h (mRNA) or 24 h (protein). Gene and protein expression of IFN-β and CXCL10 were assessed by RT-qPCR and ELISA, respectively. Firstly, in PBMCs from HC subjects LPS promoted a trend towards increasing *IFN*-β mRNA expression, THC, CBD, and THC:CBD had no effect on LPS-induced *IFN-β* mRNA expression (Fig. 5.9A), and one-way ANOVA analysis revealed no significant difference between group means ($p=0.17$). LPS treatment in the absence and presence of cannabinoids, had no effect on IFN-β protein levels in PBMCs from HC subjects (Fig. 5.9B). Next, the effect of the cannabinoids on LPS-induced CXCL10 expression was assessed. Data in Fig. 5.9C demonstrate that LPS insignificantly increased *CXCL10* mRNA expression in PBMCs from HC subjects, and THC, CBD, and THC:CBD, all promoted a trend towards attenuating LPS-induced CXCL10 expression (Fig. 5.9C), one-way ANOVA analysis of this data found no significant difference between group means ($p=0.24$). However, the proclivity of LPS to induce *CXCL10* mRNA did not translate to protein expression in PBMCs from HC subjects, and THC, CBD, and THC:CBD attenuated basal CXCL10 protein expression in PBMCs from HC subjects (Fig. 5.9D).

Next, the proclivity of THC and CBD to alter LPS-IFN-β/CXCL10 expression in PBMCs from pwMS was assessed. Data show that LPS increased (albeit insignificantly) *IFN-β* mRNA expression, with THC, CBD, and THC:CBD having no significant effect on this signalling axis, in PBMCs from pwMS (Fig. 5.9E). However, it is worth noting that the phytocannabinoid combination treatment insignificantly potentiated LPS-induced *IFN-β* mRNA expression in PBMCs from pwMS (Fig. 5.9E), however one-way ANOVA analysis did not reveal any significant differences between group means ($p=0.21$). Again, this did not translate to IFN-β protein expression, where LPS did not increase IFN-β protein, and THC,

CBD, and THC:CBD had no effect on IFN-β expression, in PBMCs from pwMS (Fig. 5.9F).

Finally, the effect of the cannabinoids on LPS-induced CXCL10 expression was determined in PBMCs from pwMS. LPS insignificantly increased *CXCL10* mRNA in PBMCs from pwMS, and THC and THC:CBD, attenuated this effect (Fig. 5.9G). Interestingly, CBD had the opposite effect, and potentiated LPS-induced *CXCL10* mRNA expression in PBMCs from pwMS. The proclivity of CBD to potentiate of LPS-induced *CXCL10* mRNA expression was significantly different when compared to PBMCs treated with the combination of THC:CBD, or PBMCs treated with THC in the presence of LPS (Fig. 5.9G). These data suggest that THC, CBD, and THC:CBD may differentially target TLR4-induced MyD88-independent signalling events in PBMCs from HC volunteers and pwMS.

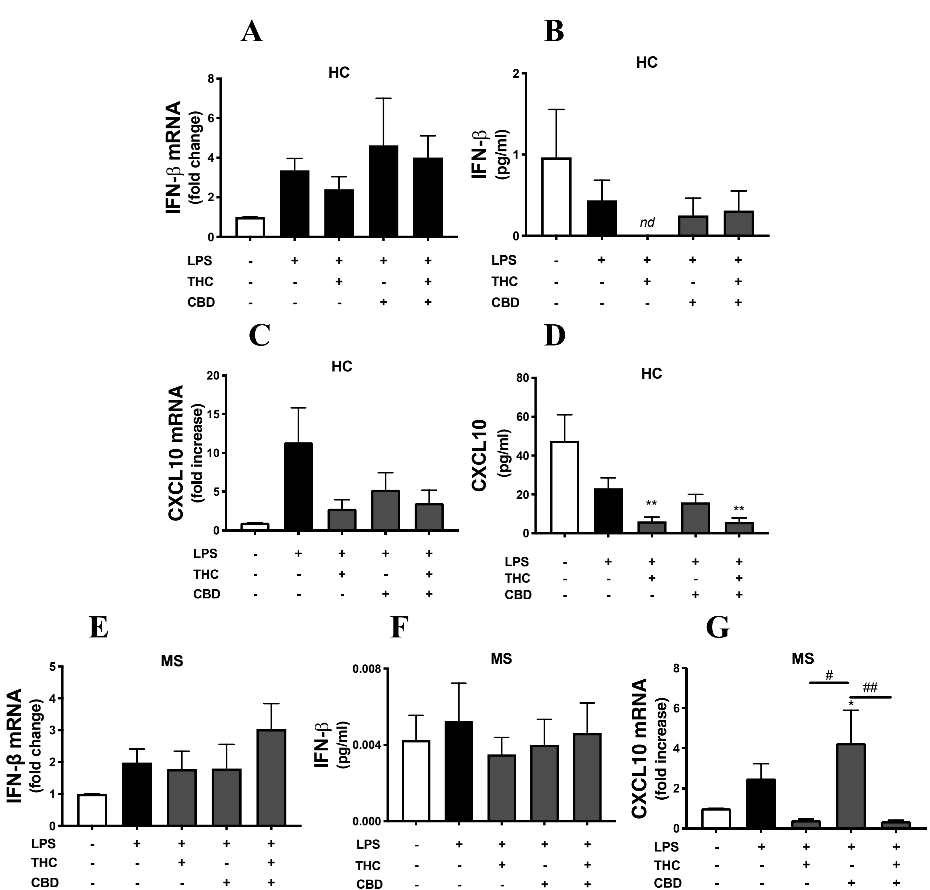


Figure 5.9. The effect of THC and CBD on TLR4 signalling independent of MyD88 in PBMCs from HC cases and pwMS. Primary PBMCs from HC subjects and pwMS were pre-treated with THC (10 μM), CBD (10 μM) or a 1:1 combination (10 μM of each) and stimulated with LPS (100 ng/ml) for 4 h (RNA) or 24 h (protein). THC, CBD and the THC:CBD combination did not alter **(A)** *IFN-β* mRNA and **(B)** IFN-β protein expression in PBMCs from HC cases. THC, CBD and THC:CBD attenuated **(C)** *CXCL10* mRNA (albeit insignificantly) and **(D)** CXCL10 protein expression in PBMCs from HC cases. **(E)** THC:CBD, but not THC or CBD, promoted a trend towards potentiating LPS-induced *IFN-β* mRNA expression. **(F)** LPS, THC and CBD had no effect of IFN-β protein expression in PBMCs from pwMS. **(G)** THC and THC:CBD attenuated, while CBD potentiated, LPS-induced *CXCL10* mRNA expression in PBMCs from pwMS. Data are represented as the means ± S.E.M from 5-12 HC and 4-5 MS donors. Data that passed the Shapiro-Wilk normality test was analysed by one-way ANOVA followed by Dunnett's multiple comparison test. For non-parametric data, the Kruskal-Wallis followed by Dunn's multiple comparison test was performed. *$p<0.05$ and **$p<0.01$ *versus* LPS-treated groups and $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *versus* LPS co-treated with THC or THC:CBD.

5.11 Stratifying the response of PBMCs to LPS, THC and CBD treatment in cells from pwMS in terms of current DMT use

As shown previously in Table 11, and in parallel to data in section 5.8, pwMS recruited to this study were diagnosed with RRMS and were prescribed a variety of immunomodulatory DMTs at the time of enrolment to the study. Therefore, it was of interest to determine the effect of THC and CBD on TLR4-induced TNFα protein expression with respect to various therapies, including Interferon-β 1a, Dimethyl fumarate, Fingolimod, Rituximab, and Natalizumab.

PBMCs from pwMS were isolated using lymphoprep over a density gradient, pre-treated with THC, CBD or a 1:1 combination (all at 10 μM: 45 min) and then stimulated with LPS (100 ng/ml: 24 h). Supernatants were collected and frozen for TNFα protein analysis via ELISA. There was a total of five pwMS prescribed IFN-β 1a therapy, two taking Natalizumab, one individual was prescribed Rituximab, one individual taking Fingolimod and a further individual prescribed Dimethyl fumarate at the time of blood donation. The data for IFN-β 1a therapy (Fig. 5.10A) demonstrate that PBMCs from each patient responded to LPS (to varying degrees) by increasing TNFα protein expression. Furthermore, THC, CBD and the 1:1 combination of phytocannabinoid promoted a trend towards potentiating LPS-induced TNFα protein expression in PBMCs, with the combination treatment being most effective overall in terms of potentiation (Fig. 5.10A).

Furthermore, one or two individuals were prescribed Natalizumab, Rituximab, Fingolimod, and Dimethyl fumarate therapies at the time of immune cell analysis. Therefore, data herein are indicative of potential responses, and further patient recruitment will aim to increase patient numbers to increase the power for full analysis of the effect of DMTs on cell responses to LPS/phytocannabinoids. At the time of analysis two pwMS were prescribed Natalizumab therapy and PBMCs from both individuals responded to LPS-TLR4 activation, in terms of TNFα expression (Fig 5.10B). In addition, PBMCs from both individuals were sensitive to THC, CBD, and THC:CBD, potentiation of TLR4-induced TNFα protein expression (Fig. 5.10B). PBMCs from pwMS prescribed Rituximab (Fig. 5.10C), Fingolimod (Fig. 5.10D) or Dimethyl Fumarate (Fig. 5.10E) therapy responded to TLR4 activation in terms of TNFα expression, and again THC, CBD, and THC:CBD potentiated

LPS-induced TNFα expression, with the combination of cannabinoid treatment being most effective. This is of particular interest in terms of the cellular effects of Sativex, an oromucosal spray containing CBD and THC as its most abundant phytocannabinoid components, in addition to other phytocannabinoids and non-phytocannabinoid components, as an add-on adjunctive therapy to known DMTs in pwMS [330, 427]. These data highlight the effect of DMTs on cellular responses to LPS and phytocannabinoids in PBMCs from pwMS.

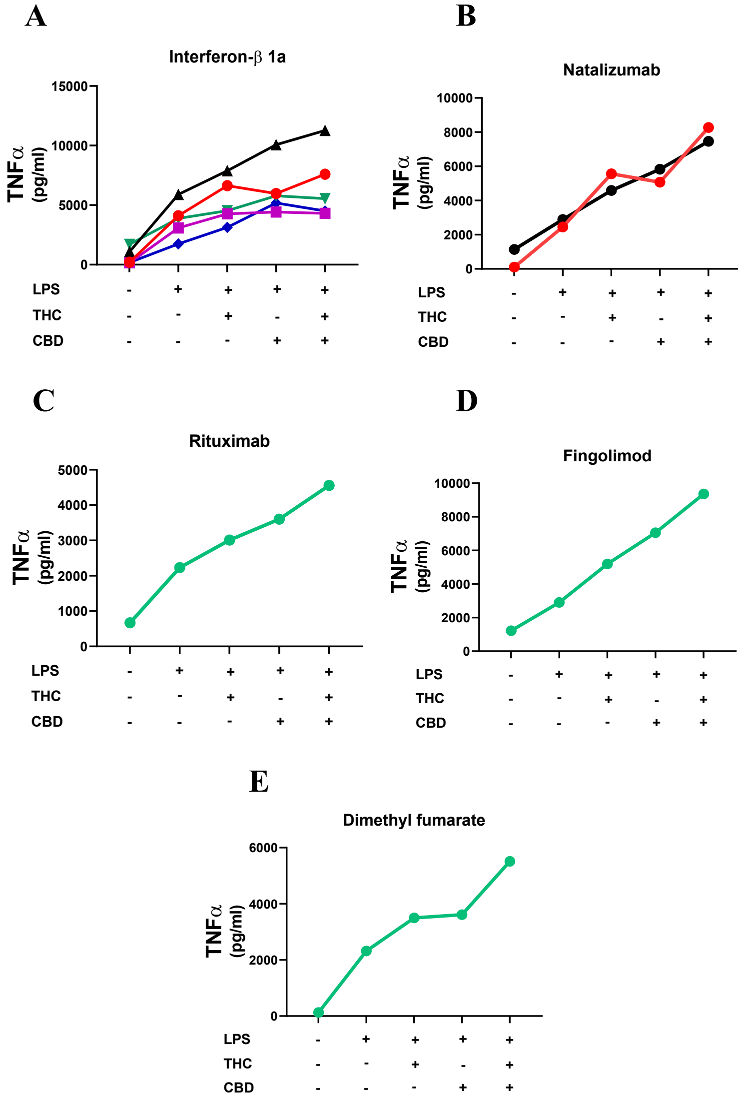


Figure 5.10. Effect of DMTs on cellular responses to LPS, THC and CBD. Human primary PBMCs from pwMS were isolated from whole blood and pre-treated with THC (10 µM), CBD (10 µM) or a 1:1 combination (10 µM of each) prior to treatment with LPS (100 ng/ml) for 24 h. TNFα protein expression was determined by ELISA. The effect of THC, CBD, and THC:CBD on LPS-induced TNFα expression in PBMCs from pwMS prescribed **(A)** Interferon-β 1a (*n*=6), **(B)** Natalizumab (*n*=2), **(C)** Rituximab (*n*=1), **(D)** Fingolimod (*n*=1), and **(E)** Dimethyl Fumarate (*n*=1) therapy. Data are expressed as line graphs with each line representing one patient.

5.12 Discussion

This study set out to assess TLR3/4 signalling in immune cells from HC subjects and pwMS, and to determine the effect of the phytocannabinoids THC and CBD on TLR3/4 signalling in PBMCs from HC cases and pwMS. Data herein indicate that PBMCs from pwMS demonstrate a desensitised response to TLR3-induced CXCL10 expression, but not IFN-β, when compared to PBMCs from healthy volunteers. However, there was no difference in TLR4-induced TNFα expression in PBMCs isolated from both study groups. Data herein indicate that THC, CBD, and the THC:CBD combination was sufficient to attenuate TLR3-induced CXCL10 and IFN-β expression in PBMCs from HC cases and pwMS. Interestingly, THC, CBD, and the THC:CBD combination, potentiated LPS-induced TNFα expression in PBMCs isolated from both study groups. The 1:1 combination of THC:CBD was most effective at inhibiting TLR3 responses and potentiating TLR4 responses in the immune cell populations from both HC and MS cohorts. Cannabinoid treatments differentially modulated TLR4 signalling in PBMCs independent of the adaptor MyD88, as assessed through TLR4-induced CXCL10 and IFN-β. Specifically, the cannabinoids had no significant effect on LPS-induced IFN-β expression in PBMCs from both groups; however THC, CBD and THC:CBD inhibited LPS-induced CXCL10 production in PBMCs from healthy subjects. In addition, THC, and the combination treatment (THC:CBD) inhibited LPS-induced CXCL10 expression in PBMCs from pwMS, while CBD potentiated CXCL10 expression in this PBMC population. Furthermore, MSQOL-54 and QIDS-SR_{16} questionnaire data indicate that pwMS have reduced physical and mental health, and increased depressive symptoms, when compared to the HC study cohort. Finally, whole blood levels of mixed immune cells, MCV and MCH was higher in pwMS when compared to the HC subjects assessed in this project.

Initially, the effect of storing whole blood samples immediately post-venepuncture at RT, or on ice for up to 4 h, on cellular composition was determined. This initial screen on storage conditions for blood sampling was conducted given that pwMS were recruited from Beaumont hospital, Dublin, resulting in up to a 4 h delay in processing each patient blood sample in the laboratory on the University campus. We found no time-dependent change in the cellular composition of whole blood samples, in terms of WBC, RBC and lymphocyte number, following storage of

whole blood at RT or on ice. Additionally, no time- or temperature-dependent differences were found in HGB, HCT, MCV, MCH, MCHC and PLT levels. Based on these findings, all samples from pwMS collected at Beaumont hospital Dublin were stored at RT post-venepuncture until further processing of the PBMCs in the laboratory on campus.

An important aspect of the study was to determine if differences exist in the cellular composition of whole blood isolated from HC cases and pwMS. Indeed, no differences were found in WBC, RBC, PLT and neutrophil numbers between both cohorts. However, a decrease, albeit insignificant, was found in lymphocyte number, and percentage of immune cells, in the MS cohort, when compared to the control subjects. As mentioned previously, at the time of blood draw, each RRMS patient in the study was prescribed an immunomodulatory DMT which targeted peripheral immune cells, therefore a decrease in lymphocyte number was expected in the MS group [428]. MCV determines the average size and volume of RBCs, whereas MCH is the average mass of haemoglobin in each RBC. Interestingly, blood cells from pwMS demonstrated an increase in the levels of MCV and MCH, when compared to blood cells from the HC group. Data from elsewhere has found no difference in MCV, but a decrease in MCH levels, between MS and HC groups [425]. pwMS enrolled in our study were prescribed a variety of DMTs which may account for the differences observed. Additionally, PBMCs from pwMS demonstrated significantly higher levels of MXD immune cells, when compared to PBMCs from HC subjects. The MXD population consists of monocytes, eosinophils and basophils [429]. Unfortunately, the sysmex haematology analyser used in the present study did not differentiate between monocytes, eosinophils, and basophils, therefore, we could not identify the exact population(s) impacted by disease in our analysis. Future work will aim to identify the precise cellular alterations in immune cell profiles in MS.

At the time of recruitment each participant completed the MSQOL-54 and QUIDS-SR_{16} questionnaires to gain insight regarding mental/physical health and depressive symptoms in each study participant at the time of blood draw. Indeed, an assessment of QOL measurements is vital as such measurements can aid in evaluating disease progression, treatment and management strategies in pwMS [430, 431]. MSQOL-

54 and QUIDS-SR_{16} measurements are widely accepted questionnaires that provide important information regarding the wellbeing of patient cohorts [336]. Indeed, published data from the laboratory employing the MSQOL-54 and QUIDS-SR_{16} measurements has shown that pwMS report reduced physical and mental health scores, and increased depressive symptoms, when compared to HC subjects, however 8 weeks of moderate exercise was sufficient to improve these endpoints [424]. Data from our study supports this, where a reduction in physical and mental health, and an increase in depressive symptomatology, was reported in pwMS. Future work will aim to correlate MSQOL-54 and QUIDS-SR_{16} scores with plasma protein, immune cell function, and cellular composition.

Dysregulation of TLR signalling in EAE models of MS have been reported, and a growing body of literature suggests that TLR signalling is a key player in MS pathogenesis [359] which will be discussed below. Indeed, TLR/adaptor knockout studies in EAE have highlighted the complex nature of TLR signalling in neuroinflammation associated with MS. Reports indicate that TLR2 [432], TLR9 [159], MyD88 [433], and IRF3 [152] deficiency is protective in murine models of neuroinflammation. Conversely, TLR4 [161], TLR2 [434] and TRIF [435] deficiency can exacerbate EAE, highlighting the potential for TLR-based therapies in MS. Additionally, several human studies demonstrate abnormal TLR signalling events in cells from pwMS. Indeed, PBMCs from pwMS have an enhanced responsiveness to TLR2 stimulation in terms of TNFα expression, when compared to non-MS controls [436]. Data presented herein indicate that PBMCs from pwMS were less sensitive to TLR3 activation with poly(I:C) in terms of CXCL10 production, but not IFN-β expression. This desensitisation was not due to reduced expression of the TLR3 receptor in PBMCs from pwMS. In addition, data presented also indicate that there was no difference in relative TLR4 expression and signalling, in PBMCs from the HC and MS cohorts. This is in contrast to published data from our laboratory that showed that PBMCs from treatment-naïve RRMS patients are hypersensitive to TLR4 activation, in terms of TNFα production [155]. The difference observed here may be due to the effect of DMTs on cellular responses to LPS in the MS subjects assessed in the current study.

Cannabinoids have potential efficacy in managing and treating the symptoms of MS. This is based on a large number of pre-clinical studies using the murine model of the disease, EAE, in addition to clinical trial data. Indeed, THC, *R*(+)WIN55,212, JWH-133 and methanandamide can ameliorate EAE [262, 437]. Elsewhere, CBD was shown to reduce the severity of EAE by decreasing axonal damage, microglial activation and T-cell recruitment [263]. As discussed, Sativex is an oromucosal spray containing CBD and THC as its most abundant phytocannabinoid components, in addition to other phytocannabinoids and non-phytocannabinoid components, and is prescribed for pain and spasticity related to MS [438]. The THC:CBD ratio treatment used in this study aimed to mimic a 1:1 combination of THC:CBD in an *in vitro* model. Data from our study found that PBMCs treated with the combination of THC and CBD was sufficient to inhibit the pro-inflammatory chemokine CXCL10 expression in PBMCs from HC subjects. This inhibition was not replicated when THC and CBD were administered alone. Interestingly, PBMCs from pwMS also demonstrated a decrease in CXCL10 protein expression following treatment with the THC:CBD (1:1) cannabinoid combination, however this was not significant. Importantly, the 1:1 combination of THC:CBD treatment was more effective at inhibiting TLR3-, and TLR4-, induced CXCL10 expression in PBMCs, when compared to THC or CBD treatment alone. Therefore, this 1:1 combination of THC:CBD may give insight into the cellular mechanism of action of Sativex in immune cells from pwMS. Finally, THC, CBD, and the combination treatment, did not alter IFN-β expression in PBMCs from healthy volunteers; however, an insignificant increase in IFN-β protein expression was detected in PBMCs from pwMS following treatment with phytocannabinoids. This is of particular interest as one of the first line prescribed treatments for RRMS is IFN-β [167]. Therefore, elucidating the mechanisms by which cannabinoid treatment may increase endogenous IFN-β tone in pwMS may be of therapeutic value.

There is increasing evidence that cannabinoids may alter TLR signalling events (for review see Fitzpatrick and Downer., 2017 [274]). In brief, the sCB *R*(+)WIN55,212-2, and the endocannabinoid NADA, can attenuate TLR2- and TLR4-induced pro-inflammatory cytokines in endothelia [286], and data elsewhere indicate that CB_1 antagonists can blunt TLR4-induced pro-inflammatory cytokine

expression in adipocytes [287]. Indeed, *R*(+)WIN55,212-2 can prevent TLR3-induced inflammatory cytokine production in PBMCs from HC subjects and pwMS, and can increase IFN-β expression in PBMCs from pwMS, but not in PBMCs from HC cases [184]. Data elsewhere indicate that THC and CBD differentially inhibit TLR4 activated NF-κB and IFN-β/STAT pro-inflammatory pathways in BV-2 microglial cells, and that this effect was independent of the cannabinoid receptors [283].

Data from our study suggest that THC, CBD, and the combination treatment, can attenuate TLR3-induced CXCL10 expression in PBMCs from healthy subjects. This inhibition was only reported following THC:CBD treatment in PBMCs isolated from pwMS. The combination treatment was also effective at inhibiting TLR3-induced IFN-β expression in PBMCs from HC cases and from pwMS. Interestingly, THC, CBD, and THC:CBD had the opposing effect on TLR4-induced TNFα expression. Surprisingly, the cannabinoids, when administered alone and in combination, potentiated TLR4-induced TNFα protein expression, with the combination treatment increasing TNFα expression to the greatest extent. This effect was observed in PBMCs from HC subjects and pwMS, however, PBMCs from pwMS demonstrated an increased sensitivity to all cannabinoid treatments (alone and in combination), significantly enhancing TLR4-induced TNFα protein expression. The complex nature of these data is highlighted by the effect of the cannabinoids on TNFα production when administered without TLR4 agonism. Indeed, treatment with THC, CBD, and THC:CBD, in the absence of LPS, did not alter TNFα expression in PBMCs from HC volunteers, however, a significant inhibition of TNFα protein was detected in PBMCs from pwMS. These data were unexpected and, to the best of our knowledge, represents a novel finding which has not been reported elsewhere. Previous data from chapter 4 indicated that THC and CBD inhibited LPS-induced NF-κB translocation to the nucleus, with no effect on downstream pro-inflammatory proteins in macrophages. This suggests that the inhibition of NF-κB is compensated for by other TLR4 targets, resulting in the expression of the pro-inflammatory proteins. This could help explain the effect observed by cannabinoids on TLR4-induced TNFα expression in PBMCs. It is known that TLR4 activation promotes NF-κB and MAPK activation [379], therefore it is reasonable to suggest that the cannabinoids tested in this study may

target MAPK signalling to modulate TNFα production. Future work will look to mechanistically delineate these results further, focusing on the effect of THC and CBD, alone and in combination, on MAPK and NF-κB signalling mechanisms in primary human PBMCs.

pwMS recruited to the study were prescribed a variety of DMTs. It was of interest to determine whether patients on various DMTs would have a different TLR3/4 signature, and if these DMTs effected how PBMCs from pwMS would respond to cannabinoid exposure. At the time of recruitment to the student, pwMS reported the use of a variety of DMTs, including IFN-β 1a, Natalizumab, Rituximab, Fingolimod, Glatiramer acetate, and Dimethyl fumarate. In total, six pwMS recruited were prescribed IFN-β 1a therapy. Data reported herein indicate that each patient responded to TLR3 and TLR4 activation in terms of CXCL10, IFN-β and TNFα expression, respectively. Indeed, each donor demonstrated a similar pattern of response to cannabinoid interference in these pathways, with THC, CBD and the combination treatment all promoting a trend towards attenuating poly(I:C)-induced CXCL10 and IFN-β expression, and potentiating LPS-induced TNFα expression. This analysis was limited by participant number, as one or two of patients reported the use of Natalizumab, Rituximab, Fingolimod, Glatiramer acetate and Dimethyl fumarate, therefore future patient recruitment will aim to target pwMS receiving these therapies to aid our understanding of how different DMTs may interfere with TLR and cannabinoid signalling events.

Chapter 6

Examining the effect of highly purified botanically-derived phytocannabinoids on immune cell viability

6.1 Introduction

The *C. sativa* plant contains a group of over 100 classified phytocannabinoids [439], including THC, CBD, CBDA, CBDV, THCA, THCV, CBG, and CBC, all of which were assessed for the effects on cellular viability in this Chapter. In addition, the effect of the TLR3 and TLR4 agonists, poly(I:C) and LPS respectively, on immune cell viability were assessed in primary human PBMCs. It is well established that cannabinoids can modulate immune responses through studies in disease models such as MS, diabetes and RA, among others [440, 441]. Cannabinoids are thought to exert their immunomodulatory effects through four main mechanisms: (a) regulating apoptosis, (b) inhibiting proliferation, (c) inhibiting cytokine and chemokine production, and (d) modulating regulatory T cells (Tregs) [442]. THC has been shown to trigger apoptosis in murine macrophages and T cells by targeting Bcl-2 and caspase activity [276]. Furthermore, CBD can trigger apoptosis in murine $CD4^+$ and $CD8^+$ T cells in a time-and concentration-dependent manner [443]. Studies elsewhere have also shown that cannabinoids can alter the viability of DCs and monocytes [279, 444], and cannabinoids can increase proliferation of B cells [445]. Overall, understanding how cannabinoids affect immune cell viability is critical in understanding the mechanism(s) by which cannabinoids exert their immunomodulatory effect(s).

There is also increasing evidence that cannabinoids can alter cellular metabolism, which is an important consideration in terms of the clinical development of cannabinoid-based medicines. For instance, cannabinoids have been demonstrated to activate AMPK, which plays a key role in cellular energy homeostasis [446]. Specifically, THC and JWH-015 activate AMPK and downstream induction of autophagy through CB_2 in hepatocellular carcinoma [312]. Furthermore, data elsewhere indicates that LPS-induced NLRP3 inflammasome activation in macrophages is attenuated by the CB_2 agonist HU308, and this is through the AMPK-mTOR-P70S6 K signalling pathway [316]. Therefore, in immune cells, there is a potential for cannabinoids to alter metabolism by activating AMPK, which inhibits mTOR [447] and stimulates OXPHOS [303].

In this Chapter, the MTT viability assay was utilised to study the effects of cannabinoids on immune cell viability. This assay measures cell viability in terms

of reductive activity by assessing the cell capacity to convert a tetrazolium compound to a formazan product using dehydrogenases found in the mitochondria, although lysosomal/endosomal compartments have also been implicated [448]. Therefore, studying the effect of cannabinoids on immune cell viability using the MTT assay may give insights on the potential effects of cannabinoids on immune cell metabolism.

Aims

The specific aims of the following Chapter are as follows:

- To determine the effect of purified botanically-derived phytocannabinoids THC, CBD, THC:CBD, CBDA, CBDV, THCA, THCV, CBG, and CBC on the viability of THP-1 monocytes, THP-1-derived macrophages, and PBMCs from HC subjects and pwMS.
- To determine the effect of the TLR3 and TLR4 agonists, poly(I:C) and LPS, on the viability of PBMCs isolated from HC volunteers and pwMS.

6.2 The effect of DMSO, ethanol and a panel of eight highly purified phytocannabinoid extracts on the viability of THP-1 monocytes

Cannabinoids are highly lipophilic molecules and therefore require reconstitution in organic solvents such as DMSO or ethanol prior to use in experimental conditions [449]. DMSO is a dipolar aprotic solvent that can dissolve many otherwise insoluble polar and nonpolar molecules and is frequently used in research studies [450]. Therefore, examining the effect of DMSO and ethanol on THP-1 viability was essential to determine the effect of solvent on cell viability in our studies. THP-1 monocytes were cultured in the presence of DMSO (0.1-2%) and ethanol (0.1-1%) for 24 h and immune cell viability determined using MTT assays. Data demonstrate that DMSO concentration-dependently reduced THP-1 monocyte viability, while conversely, ethanol had no effect on THP-1 monocyte viability at all concentrations examined (Fig. 6.1A). Therefore, in all subsequent experiments, all phytocannabinoid extracts were reconstituted in ethanol as data suggest that ethanol does not alter immune cell viability in our culture system.

Next the effect of THC, CBD, a combination of THC and CBD, CBDV, CBDA, THCV, THCA, CBG, and CBC on THP-1 monocyte viability was determined. THP-1 monocytes were cultured in the presence of phytocannabinoid extracts over a range of concentrations (0.1-10 µM) for 24 h and following treatment MTT assays performed. Triton x-100 (0.2%) was used a positive control to induce cell death. THC (Fig. 6.1B) and CBD (Fig. 6.1C) had no effect on cellular viability for all concentrations tested. Interestingly, THC:CBD had no effect on THP-1 monocyte viability at higher concentrations (1 and 10 µM), however, a significant reduction in viability was detected after treatment with THC:CBD at [0.1 µM:0.1 µM] (Fig. 6.1D). Interestingly, CBDV, but not CBDA (Fig. 6.1F), concentration-dependently increased THP-1 monocyte cell viability (Fig. 6.1E), and similarly THCA and THCV concentration-dependently increased THP-1 monocyte viability, with a significant increase in viability detected following treatment at the higher 10 µM concentration of both THCA and THCV, when compared to cells treated with vehicle alone (Fig. 6.1G, H). Furthermore, data presented herein suggest that CBG and CBC both increased monocyte viability. Indeed, CBG significantly increased THP-1 monocyte viability at concentrations of 1 µM and 10 µM (Fig. 6.1I), while the highest concentration of CBC (10 µM) significantly increased monocyte

viability (Fig. 6.1J), with lower concentrations having no effect. Overall, all eight highly purified botanical phytocannabinoids, tested over a range of concentrations, were not cytotoxic to the monocyte cell line, with the exception of CBDA and THC:CBD at final concentrations of [1 μM] and [0.1 μM], respectively.

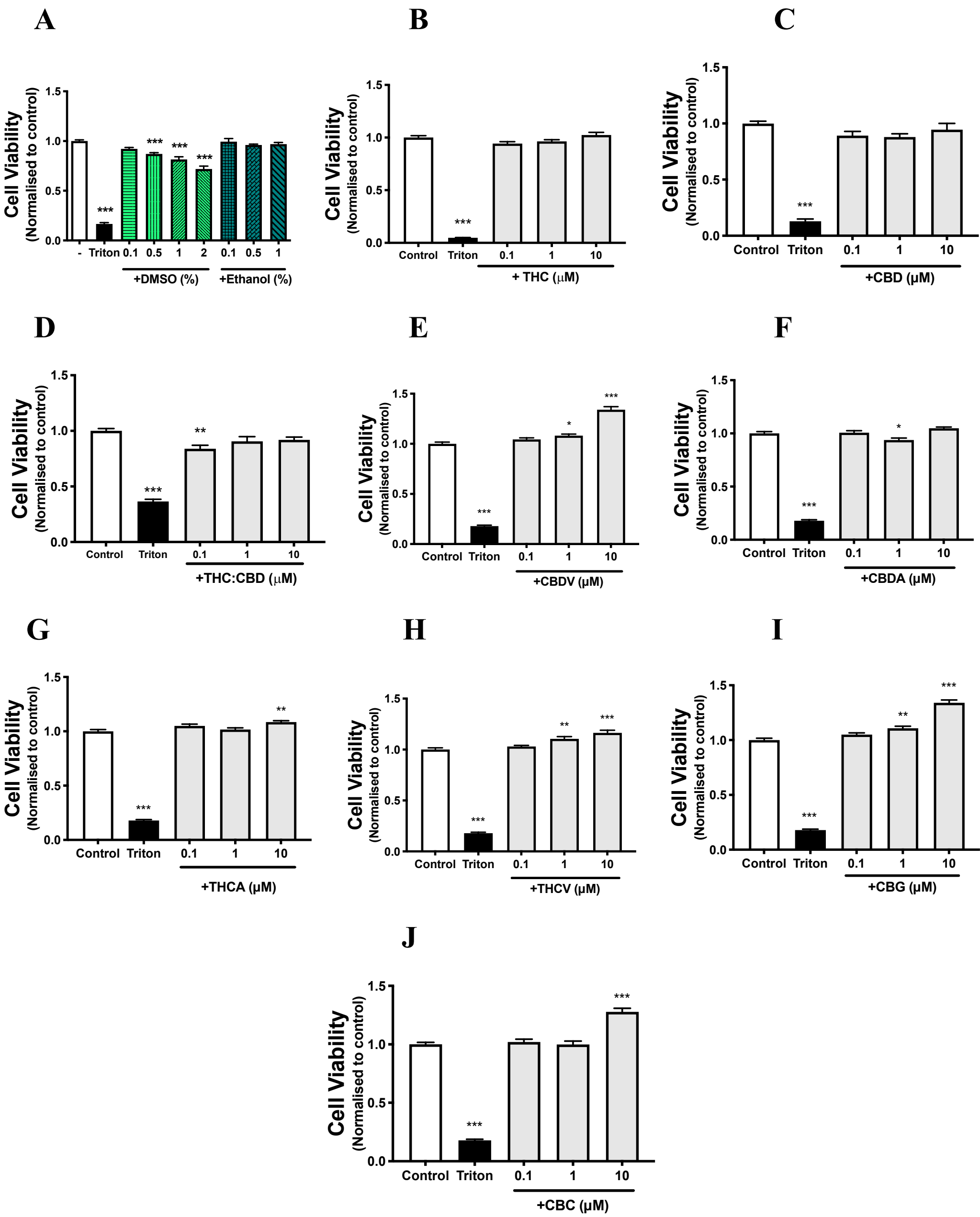


Figure 6.1. The effect of DMSO, ethanol, THC, CBD, CBDA, CBDV, THCA, THCV, CBG and CBC on THP-1 monocyte cell viability. THP-1 monocytes were cultured with DMSO, ethanol or eight phytocannabinoids for 24 h and MTT assays performed. Triton x-100 (0.2%) was added to cells (10 min prior to addition of MTT) as a positive control in all MTT assays. **(A)** DMSO, but not ethanol, dose-dependently reduced cell viability. **(B)** THC and **(C)** CBD did not alter cell viability. **(D)** A low concentration of THC:CBD (0.1 μM) decreased cell viability. **(E)** CBDV concentration-dependently increased cell viability. **(F)** CBDA (1 μM) decreased cell viability. **(G)** THCA and **(H)** THCV concentration-dependently increased cell viability. **(I)** CBG and **(J)** CBC concentration-dependently increased THP-1 monocyte viability. Data are presented as the mean ± S.E.M for three independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used for analysis. **p*<0.05, ***p*<0.01 and ****p*<0.001 *versus* control cells.

6.3 The effect of a panel of eight highly purified phytocannabinoid extracts on the viability of THP-1-derived macrophages

Given that the effect of eight purified phytocannabinoids on THP-1 monocyte viability was initially determined (Fig. 6.1), in the next series of experiments we set out to determine the toxicity profile of the phytocannabinoids in THP-1-derived macrophages. Initially, THP-1 monocytes were cultured with PMA (10 ng/ml) for 48 h to allow differentiation to a macrophage phenotype. After differentiation, THP-1-derived macrophages were cultured with THC, CBD, THC:CBD, CBDV, CBDA, THCV, THCA, CBG and CBC (all cannabinoids at 0.1-10 μM) for 24 h, and MTT cell viability assays were performed. Data indicate that THC (Fig. 6.2A), CBD (Fig. 6.2B) and the THC:CBD (Fig. 6.2C) all significantly increased macrophage viability at [10 μM], with lower concentrations having no effect on viability (apart from THC:CBD at 0.1 μM). In addition, CBDV increased macrophage cell viability at [10 μM] but had no effect at lower concentrations (Fig. 6.2D), whereas CBDA did not alter macrophage viability at any concentration tested (Fig. 6.2E). THCV concentration-dependently increased THP-1 macrophage viability, with concentrations of 1 and 10 μM significantly increasing viability, when compared to control cells (Fig. 6.2F). Interestingly, the acid variant of THC, THCA, had no effect on macrophage viability at all concentrations tested (Fig. 6.2G). Finally, CBG (Fig. 6.2H) and CBC (Fig. 6.2I) concentration-dependently increased macrophage viability, with the highest concentration of each phytocannabinoid tested (i.e. 10 μM) being most effective. Data here highlight that THP-1-derived macrophages are sensitive to phytocannabinoid treatment, with certain cannabinoids, particularly at high concentrations, significantly increasing macrophage viability using MTT assays as a read-out.

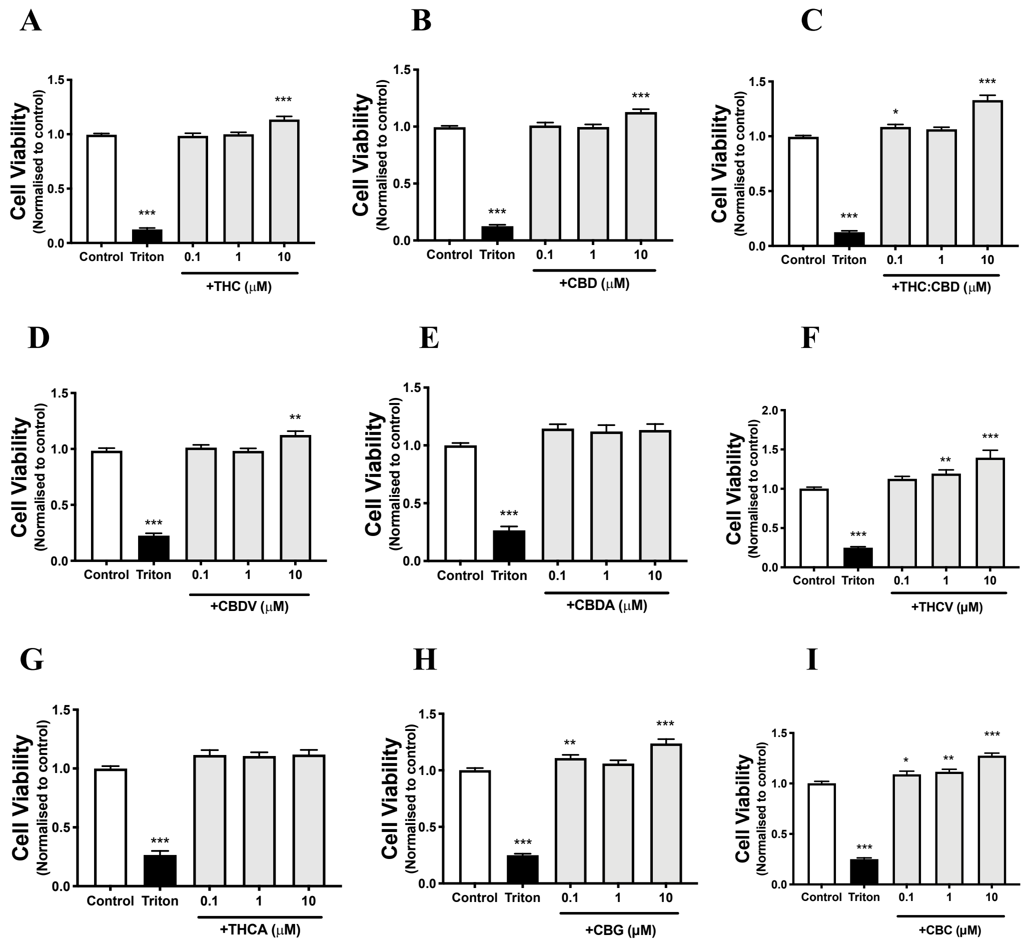


Figure 6.2. The effect of THC, CBD, CBDA, CBDV, THCA, THCV, CBG and CBC on THP-1-derived macrophage cell viability. THP-1 monocytes were differentiated to macrophages using PMA (10 ng/ml) for 48 h. THP-1-derived macrophages were cultured with eight purified phytocannabinoids for 24 h and MTT assays performed. Triton x-100 (0.2%) was added to cells (10 min prior to addition of MTT) as a positive control in all MTT assays. **(A)** THC, **(B)** CBD **(C)** THC:CBD and **(D)** CBDV all increased cell viability at [10 μM]. **(E)** CBDA had no effect on cell viability at the concentrations tested. **(F)** THCV concentration-dependently increased cell viability. **(G)** THCA did not alter cell viability at all concentrations. **(H)** CBG and **(I)** CBC concentration-dependently increased cell viability. Data are presented as the mean ± S.E.M for 9-14 independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used for analysis. **p*<0.05, ***p*<0.01 and ****p*<0.001 *versus* control cells.

6.4 Examining the proclivity of TLR3/4 agonists, and eight purified phytocannabinoids, to alter the viability of primary human PBMCs isolated from healthy volunteers

Following an analysis of the effect of a panel of phytocannabinoids on the viability in THP-1 monocytes (Fig. 6.1) and macrophages (Fig. 6.2), next, the toxicity profile of all phytocannabinoids was screened in primary human PBMCs. Whole blood from HC subjects was isolated via venepuncture and PBMCs isolated using a density gradient. Isolated PBMCs were cultured with poly(I:C) (10 µg/ml), LPS (100 ng/ml), THC, CBD, THC:CBD, CBDV, CBDA, THCV, THCA, CBG and CBC (all cannabinoids at 0.1-10 µM) for 24 h, and cell viability determined by MTT assay. Given that the TLR3 and TLR4 agonists, poly(I:C) and LPS respectively, were employed to stimulate TLR signalling in PBMCs (Chapter 5), we firstly assessed the effect of these ligands on the viability of primary PBMCs. Data in Fig. 6.3A indicate that poly(I:C) and LPS (at recommended concentrations employed in Chapter 5) did not alter the viability of PBMCs isolated from HC subjects (Fig. 6.3A).

In the next series of experiments, all eight targeted phytocannabinoids were again assessed. It is noteworthy that, in contrast to THP-1 monocytes and macrophages, THC (Fig. 6.3B) and CBD (Fig. 6.3C) promoted a significant decrease in the viability of PBMCs at final concentrations of [0.1 µM] and [1 µM]; however, treatment with a high concentration of THC and CBD (10 µM) did not affect PBMC viability. Interestingly, when THC and CBD were administered in combination (THC:CBD), there was a significant increase in PBMC viability at [10 µM], when compared to control cells, with no effect determined at the lower concentrations (Fig. 6.3D). This highlights the concentration-specific nature of the effects of THC and CBD, and that delivery of THC and CBD, alone and in combination, may differentially impact immune cell function.

Furthermore, CBDV (Fig. 6.3E), CBDA (Fig. 6.3F) and THCV (Fig. 6.3H) all significantly decreased PBMC viability at [0.1 µM] and [1 µM], when compared to control cells. In contrast, the higher concentration (i.e. 10 µM) of CBDV (Fig. 6.3E) and CBDA (Fig. 6.3F) did not affect PBMC viability, when compared to control cells. Furthermore, THCA increased PBMC viability at the highest concentration

tested (10 μM) but had no effect at lower concentrations (Fig. 6.3G). In addition, CBG treatment significantly increased PBMC viability, again at the highest concentration tested (10 μM), and the lower concentrations of CBG (0.1 μM and 1 μM) had no effect on PBMC viability. Finally, data presented in Fig 6.3J indicates that all three concentrations of CBC tested significantly reduced the viability of PBMCs. These data highlight the differential cannabinoid-specific effects in PBMCs, with certain cannabinoids increasing viability (THC:CBD, THCA and CBG) and certain cannabinoids decreasing viability (THC, CBD, CBDV, CBDA, THCV and CBC) at specific concentrations tested.

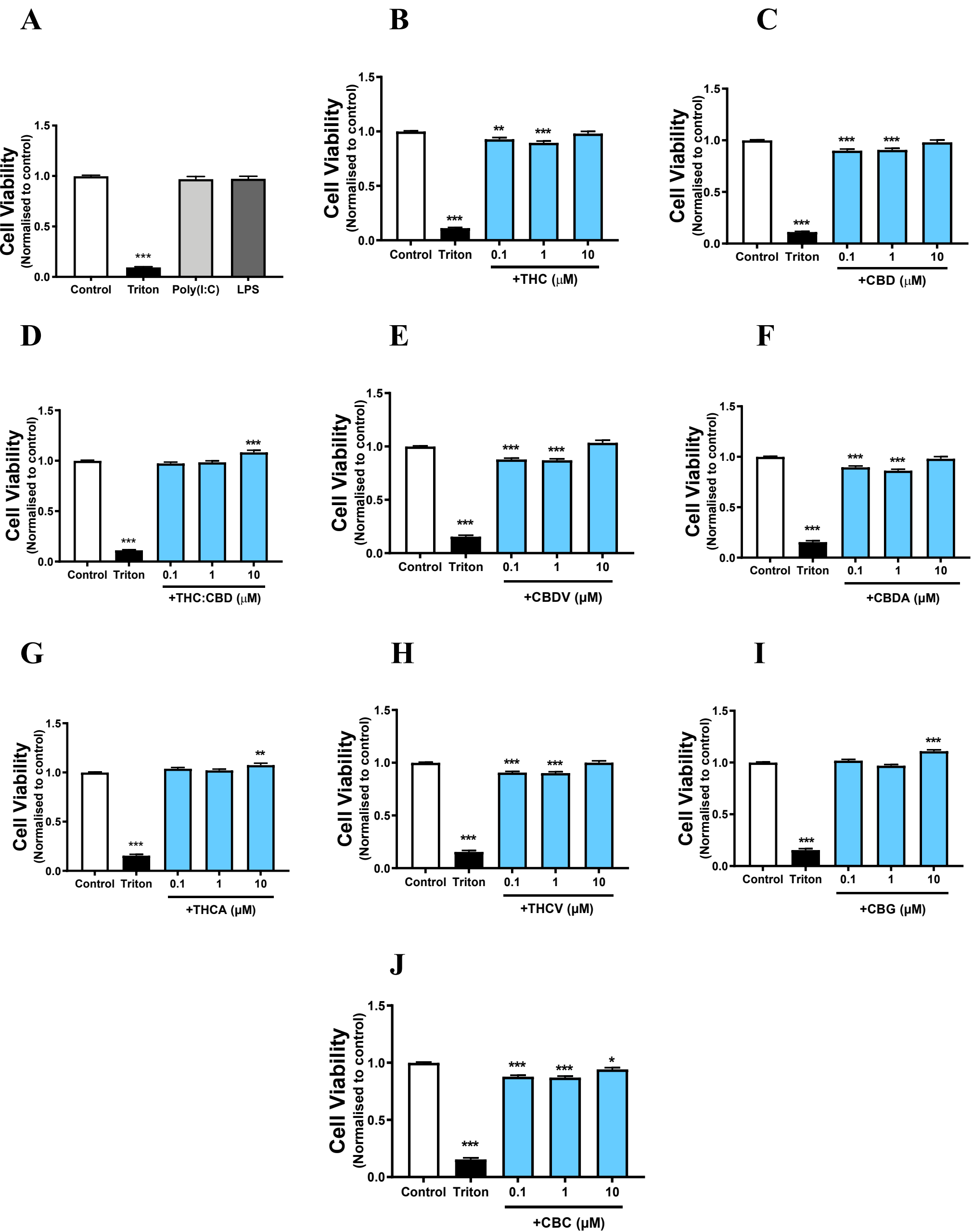


Figure 6.3. The effect of poly(I:C), LPS, THC, CBD, CBDA, CBDV, THCA, THCV, CBG and CBC on the viability of primary human PBMCs isolated from healthy volunteers. Primary PBMCs were cultured with poly(I:C), LPS or eight purified phytocannabinoids for 24 h and MTT assays performed. Triton x-100 (0.2%) was used as a positive control. **(A)** Poly(I:C) and LPS were not cytotoxic. **(B)** THC and **(C)** CBD reduced PBMC viability in the low concentration range. **(D)** THC:CBD increased cell viability at [10 μM]. **(E)** CBDV and **(F)** CBDA reduced cell viability at lower, but not the highest, concentrations tested. **(G)** THCA increased cell viability at [10 μM]. **(H)** THCV reduced PBMC viability at low, but not high, concentrations of cannabinoid. **(I)** CBG increased PBMC viability at [10 μM]. **(J)** CBC concentration-dependently decreased cell viability. Data are presented as the mean ± S.E.M from 15-20 HC subjects. One-way ANOVA followed by Dunnett's post-hoc test was used for analysis. **p*<0.05, ***p*<0.01 and ****p*<0.001 *versus* control cells.

6.5 Examining the proclivity of TLR3/4 agonists and eight botanically-derived cannabinoid extracts to alter the viability of primary PBMCs from pwMS

Previously, the TLR3/4 agonists and a range of eight phytocannabinoids were assessed for their impact on the viability of PBMCs isolated from HC subjects (Fig. 6.3). Therefore, the next set of experiments assessed the proclivity of phytocannabinoids to regulate the viability of PBMCs from pwMS, with a view to determining whether MS affects the cellular responses to cannabinoid exposure in terms of viability. Patient recruitment was from the Neurology clinic at Beaumont Hospital Dublin, and full participant demographics are indicated in Chapter 5, Table 11. Whole blood from individuals with RRMS was isolated via venepuncture and PBMCs isolated using a density gradient. PBMCs were cultured with poly(I:C) (10 μg/ml), LPS (100 ng/ml), THC, CBD, THC:CBD, CBDV, CBDA, THCV, THCA, CBG and CBC (all cannabinoids at 0.1-10 μM) for 24 h, and cell viability determined via MTT assay. The TLR3 and TLR4 agonists, poly(I:C) and LPS respectively, were first assessed for their effects on the viability of PBMCs from pwMS, given that they were previously employed to activate TLR3/4 signalling in PBMCs (Chapter 5). MTT assay data in Fig. 6.4A indicate that treatment with poly(I:C) and LPS did not alter the viability of PBMCs from pwMS. Furthermore, the phytocannabinoid THC significantly reduced the viability of PBMCs at the concentration of 1 μM but had no effect at the other concentrations tested (i.e. 0.1 μM and 10 μM) (Fig. 6.4B). Interestingly, CBD reduced the viability of PBMCs from pwMS at low concentrations (i.e. 0.1 μM), when compared to control cells. However treatment with CBD at a concentration of 1 μM had no effect on cell viability, while treatment with CBD at a final concentration of [10 μM] significantly increased the viability of PBMCs from pwMS (Fig. 6.4C), highlighting the concentration-specific nature of the effect of CBD on primary immune cell viability. When THC and CBD were delivered in a 1:1 combination at [10 μM] the phytocannabinoids significantly increased the viability of PBMCs from pwMS, when compared to control cells (Fig. 6.4D). Lower concentrations of THC:CBD (i.e. at 0.1 μM and 10 μM) had no effect on viability (Fig. 6.4D).

The effects of CBDV were concentration-dependent, demonstrating a significant decrease in viability at [0.1 μM] when compared to control cells, but a significant increase in PBMC viability at a final concentration of 10 μM (Fig. 6.4E). In terms

of CBDA, this phytocannabinoid was cytotoxic to PBMCs from pwMS at [0.1 μM] and [1 μM], when compared to control (Fig. 6.4F). However, treatment of PBMCs from pwMS with CBDA at [10 μM] did not alter cell viability, which was recovered when compared to the lower concentrations of CBDA (Fig. 6.4F). THCV (Fig. 6.4G) and THCA (Fig. 6.4H) both significantly increased the viability of PBMCs from pwMS at [10 μM], compared to control cells. However, data indicate that THCV significantly reduced the viability of PBMCs from pwMS at lower concentrations of [0.1 μM], while THCA increased PBMC viability at a concentration of [0.1 μM] (Fig. 6.4G, H). CBG (Fig. 6.4I), but not CBC (Fig. 6.4J), significantly increased the viability of PBMCs from pwMS at [10 μM] and had no effect at lower concentrations. However, CBC significantly reduced the viability of PBMCs at [0.1 μM], compared to control (Fig. 6.4J).

Overall, PBMCs from pwMS, in addition to PBMCs from HC volunteers (Fig. 6.3), demonstrate differential responses to cannabinoids in terms of viability. This highlights the importance of selecting the appropriate cannabinoid concentrations for *in vitro* studies, in addition to the potential of cannabinoids to concentration-dependently regulate metabolic processes in immune cells.

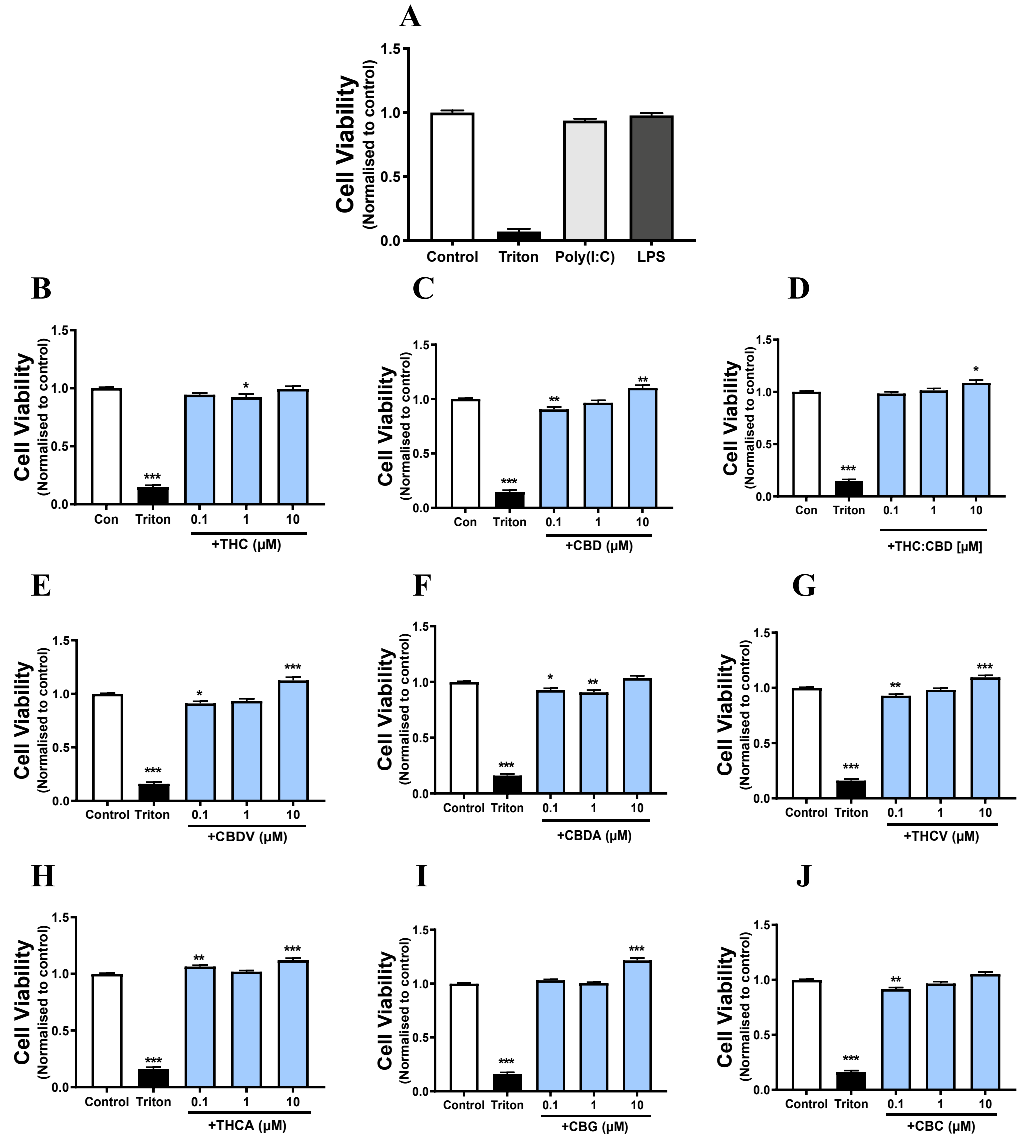


Figure 6.4. The effect of poly(I:C), LPS, THC, CBD, CBDA, CBDV, THCA, THCV, CBG and CBC on the viability of primary PBMCs from pwMS. Primary PBMCs from pwMS were cultured with poly(I:C), LPS or eight pure phytocannabinoids for 24 h. Following treatments, MTT assays were performed to assess cell viability, and Triton x-100 (0.2%) was used as a positive control. **(A)** Poly(I:C) and LPS were not cytotoxic to PBMCs. **(B)** THC reduced viability at [1 μM]. **(C)** CBD reduced PBMC viability at [0.1 μM], but increased PBMC viability at [10 μM]. **(D)** THC:CBD increased cell viability at [10 μM]. **(E)** CBDV reduced viability at [0.1 μM], but increased cell viability at [10 μM]. **(F)** CBDA reduced cell viability at [0.1 μM] and [1 μM]. **(G)** THCV reduced cell viability at [0.1 μM] and increased cell viability at [10 μM]. **(H)** THCA increased the viability of PBMCs from pwMS at [0.1 μM] and [10 μM] concentrations. **(I)** CBG increased PBMC viability at [10 μM]. **(J)** CBC decreased cell viability at [0.1 μM]. Data are presented as the mean ± S.E.M from 2 (Fig. 6.4A) and 14 pwMS (Fig. 6.4B-J). One-way ANOVA followed by Dunnett's post-hoc test was used for analysis. *p<0.05, **p<0.01 and ***p<0.001 *versus* control cells.

6.6 Discussion

This Chapter set out primarily to determine the effect of a range of phytocannabinoids on the viability of immune cells, with focus on THP-1 monocytes/macrophages and primary PBMCs. Additionally, the toxicity profile of the TLR3 and TLR4 agonists, poly(I:C) and LPS, were determined in primary immune cells. There were two distinct research aims in this study: (1) to determine the effect of a panel of highly purified phytocannabinoids on immune viability, and (2) to determine if the effect of phytocannabinoids on viability differ between THP-1 monocytes, THP-1 macrophages and primary PBMCs. To adequately assess these aims, four different populations of immune cells were employed: THP-1 monocytes, THP-1-derived macrophages, PBMCs from HC volunteers and PBMCs from pwMS. Initially, all phytocannabinoids were screened in the THP-1 monocyte and macrophage cell line, followed by testing in primary PBMCs from HC subjects and pwMS. Data show that each cannabinoid behaves uniquely from a pharmacological viewpoint, in terms of impacting viability, which is dependent upon the cell type and the concentration of cannabinoid administered.

Data herein indicates that the effect of THC on immune cell viability was cell type specific. Indeed, in monocytes, THC had no effect on cell viability, whereas treatment of THP-1 macrophages with THC at [10 μM] significantly increased macrophage viability. This suggests THP-1 cell differentiation alters cellular sensitivity to THC administration, in terms of viability. In addition, primary PBMCs from HC subjects and pwMS responded in a similar manner in terms of the impact of THC treatment on cell viability. PBMCs isolated from both groups demonstrated a significant decrease in viability following treatment with THC at [1 μM] treatment, with no effect observed at [10 μM]. In addition, a significant decrease in the viability of PBMCs from HC volunteers was also determined following treatment with THC at [0.1 μM]. Previous studies have shown that THC can induce apoptosis in murine macrophages and T cells [276], and in cultured cortical neurons [317], however, it is not known by what mechanism THC decreases viability in primary PBMCs used in our study. Data from our study also highlights that PBMCs from HC subjects and pwMS respond to THC treatment in a similar manner, whereby lower concentrations of THC may be toxic to immune cells from both cohorts, while higher concentrations of phytocannabinoid (i.e. 10

μM) do not alter viability. This is surprising given that high concentrations of THC have been shown to reduce cell viability [451]. However, previous studies have also shown the biphasic nature of cannabinoids, in terms of concentration-dependent effects on neurotransmission. For example, the CB_1 agonist *R*(+)WIN55,212-2 regulates hippocampal cholinergic neurotransmission in a concentration-dependent biphasic pattern, with a low concentration of this sCB stimulating transmission and a high concentration inhibiting transmission [452]. However, to the best of our knowledge, there is no report of biphasic effects of cannabinoids on immune cell viability in the literature.

CBD is one of the most commonly studied non-euphoric cannabinoids and is in clinical development as a therapeutic. Data presented herein indicate that CBD had varying effects on immune cell viability which were dependent upon CBD concentration and the cell type under examination. Indeed, our findings indicate that CBD had no effect on the viability of THP-1 monocytes at all concentrations tested. In contrast, in THP-1 macrophages, a significant increase in macrophage viability was determined following treatment with CBD at [10 μM]. Again, this highlights the difference in monocytes versus macrophages, in terms of the proclivity of cannabinoids to alter immune cell viability. Both PBMCs from HC subjects and pwMS showed a significant decrease in viability following treatment with CBD at [0.1 μM]. However, treatment of PBMCs with CBD at higher concentrations (i.e. 10 μM) was sufficient to significantly increase the viability of PBMCs from pwMS, but not HC volunteers, where no effect on viability was detected with CBD. These data are important as it indicates a potential protective effect of CBD at higher concentrations, that is restricted to PBMCs from pwMS. Previous studies have shown that CBD can induce apoptosis in murine $CD4^+$ and $CD8^+$ T cells in a concentration- and time-dependent manner [443]. The decrease in viability detected in primary PBMCs at lower concentrations of CBD could be due to activation of classic apoptotic pathways. Future studies will assess the mechanisms by which CBD affects cell viability, and potentially metabolic activity, in our cell culture systems.

Sativex contains CBD and THC as its most abundant phytocannabinoid components, in addition to other phytocannabinoids and non-phytocannabinoid

components, and is approved for managing spasticity related to MS [453]. Therefore, in our *in vitro* model, a combination of THC:CBD (1:1) was employed to study effects of phytocannabinoids on immune cell viability at a cellular level. Data herein indicate that the effects of THC:CBD treatments differed in monocytes and macrophages, in terms of MTT assay read-outs. Indeed, in monocytes, delivery of THC:CBD at [0.1 μM] significantly decreased cell viability; however higher concentrations of THC:CBD had no effect on monocyte viability. Conversely, in macrophages, treatment with THC:CBD at [0.1 μM and 10 μM] significantly increased immune cell viability, with the concentration of 10 μM being most efficacious. In primary PBMCs, the results were identical between both cohorts of participants investigated. Indeed, treatment of PBMCs from HC subjects and pwMS with THC:CBD at [10 μM] significantly increased PBMC viability, and the lower concentrations of phytocannabinoid had no significant effect on viability. This suggests that the combination of THC:CBD (1:1) may enhance cell viability (at certain concentrations) in PBMCs from both groups. Few viability studies have been conducted employing the use of the THC:CBD combination, and such studies have been restricted to cancer cells. Indeed, the combination of THC:CBD has been shown to decrease the viability in a multiple myeloma cell line by inducing autophagic-dependent necrosis [454]. This is in contrast to the data reported in our study using PBMCs, where an increase in viability was observed by an unknown mechanism.

CBDV is a non-euphoric cannabinoid that has potential as an anti-inflammatory [455] and anti-convulsant [456]. Data show that, in both monocytes and macrophages, CBDV (10 μM) treatment was sufficient to significantly increase cell viability, highlighting the potential protective effect of CBDV in THP-1 cell lines. However, in primary PBMCs, treatment of PBMCs from HC subjects and pwMS with CBDV at [0.1 μM] significantly decreased viability. In contrast, a significant decrease in cell viability in PBMCs from HC subjects, but not pwMS, was determined following treatment with CBDV at [1 μM]. The decrease in viability observed in PBMCs from HC subjects was fully recovered following CBDV administration at [10 μM], and conversely, in PBMCs from pwMS, CBDV treatment at [10 μM] significantly increased the viability of PBMCs. These data highlight the concentration-specific nature of the effects of cannabinoids and

suggest that PBMCs from pwMS are more sensitive to the effects of CBDV in terms of viability. To our knowledge CBDV viability studies in immune cells are limited. However, Russo *et al.,* (2019) recently examined the effect of CBDV on a human liver cell line and in buccal-derived cells and found that CBDV treatment promoted DNA damage in these cell types [457]. Future work will assess the mechanisms by which CBDV alters immune cell viability by investigating death pathways and the effect(s) of phytocannabinoids on mitochondrial function.

CBDA is the acidic precursor to CBD and has potential as an anti-inflammatory and anti-hyperalgesia compound [202]. Therefore, it is of interest to understand how CBDA interacts with immune cells, particularly in terms of viability. Data presented in this study indicates that CBDA had no effect on macrophage viability at all concentrations tested, however, there was a significant decrease in the viability of monocytes following CBDA treatment at [1 μM]. Furthermore, PBMCs from HC subjects and pwMS responded to CBDA treatment in a similar manner in terms of viability. Indeed, treatment of PBMCs from HC subjects and pwMS with CBDA at [0.1 μM and 1 μM] significantly reduced PBMC viability in both groups, however, CBDA (10 μM) did not alter PBMC viability. This indicates that the effects of CBDA on PBMC function *in vitro* are reliant on the concentration of this cannabinoid. Again, CBDA viability studies are limited, however, CBDA has been shown to increase the number of viable bone marrow-derived mesenchymal stem cells [458]. This is in contrast to results found here and may reflect differential cell responses to CBDA.

THCA is the acidic precursor to THC and produces THC following decarboxylation [459]. Interestingly, THCA is not psychotropic, unlike its decarboxylated form, and has potential as a neuroprotective agent through involvement of the PPARγ receptor [197]. Data herein indicate that THCA was well tolerated by all immune cell types studied. Specifically, treatment of monocytes and PBMCs from HC subjects and pwMS with THCA at [10 μM] significantly increased cell viability, however, this increase was not detected in macrophages. All other concentrations (0.1 μM and 1 μM) of THCA assessed did not alter the viability of each immune cell type tested, which highlights the concentration-specific nature of the effects of THCA. Previous studies have shown that THCA does not affect the viability of

primary dopaminergic neurons, and furthermore THCA (10 μM) can increase the viability of neuroblastoma cells [207]. This study correlates with data presented here. Again, further research is required to elucidate the mechanism by which THCA increases immune cell viability.

Data presented in this results Chapter indicate that monocytes and macrophages both responded to THCV in a similar manner, in terms of the impact of this phytocannabinoid on cell viability. Indeed, treatment of monocytes/macrophages with THCV at [1 and 10 μM] significantly increased viability in both cell types. Furthermore, treatment of PBMCs from HC subjects and pwMS with THCV at [0.1 μM] significantly decreased cell viability in both study cohorts. Conversely, treatment of PBMCs from pwMS, but not HC subjects, with THCV at [10 μM] increased PBMC viability. This is of particular interest as it indicates the capacity of THCV to protect immune cells from pwMS, and hence this phytocannabinoid may be worthy of further investigation as a potential therapeutic agent in the disorder. In support of this, data elsewhere indicates that the number of viable bone marrow-derived mesenchymal stem cells are increased following THCV treatment [458]. Data from our study agrees with this, however, more experimentation is required to elucidate the mechanism(s) employed by THCV to increase/decrease immune cell viability.

Given that the non-euphoric cannabinoid, CBG, has potential as an antioxidant [460] and anti-inflammatory agent [461], our study also investigated the effects of CBG on immune cell viability. CBG treatment at the highest concentration of [10 μM] was sufficient to significantly increase the viability of monocytes, macrophages, and PBMCs from HC volunteers and pwMS. Lower concentrations of CBG had no effect on the viability of PBMCs from both study cohorts, however, treatment of macrophages and monocytes with CBG at [0.1] and [1 μM] increased cellular viability. To date, to our knowledge, there are no studies examining the direct *in vitro* effects of CBG on immune cells. However, data elsewhere indicates that CBG is protective in the mouse model of multiple sclerosis, EAE [462], and additionally, using MTT assays, Gugliandolo and colleagues (2018) found that CBG was protective to NSC-34 motor neurons against the toxicity induced by the

medium of LPS-treated macrophages [209]. These data are in line with results found in our study.

Clear differences in response to CBC treatment between primary and non-primary (cell lines) immune cells were determined. Indeed, all three concentrations of CBC tested (0.1 μM, 1 μM and 10 μM) significantly increased the viability of macrophages, while the proclivity of CBC to increase the viability in monocytes was restricted to CBC at [10 μM], with the lower concentration having no effect on viability. Conversely, all concentrations of CBC tested significantly reduced the viability of PBMCs from HC subjects, but only the lowest concentration of CBC tested (i.e. 0.1 μM) reduced the viability of PBMCs from pwMS. This highlights potential cell-specific effects of CBC, and also suggests that research findings in cell line studies commonly do not translate to primary cells. It is worth noting here that the PBMC population contains predominantly T (approx. 70%) and B (approx. 15%) cells, with approximately 5% of the population consisting of monocytes [355], therefore differences between the groups would be expected. A decrease in the viability of PBMCs from HC cases and pwMS was determined following CBC treatment, suggesting that CBC could be an immunotoxic compound. Data elsewhere indicate that CBC treatment increased the viability of neural stem progenitor cells (NSPCs) via stimulation of ERK1/2 and an up-regulation of ATP synthesis [463]. These data are in line with data found in our study, where an increase in the viability of monocytes and macrophages was determined following incubation with CBC.

The technique used to detect changes in viability, the MTT assay, is an important consideration that must be made for this Chapter. This assay relies upon an enzymatic conversion utilising mitochondrial enzymes. Specifically, MTT is reduced to MTT-formazan and this reduction is catalysed by mitochondrial succinate dehydrogenase. Hence, the assay involves mitochondrial respiration [464], and therefore, an increase or decrease in viability may reflect the direct effect of cannabinoids on mitochondrial function. Furthermore, understanding how the cannabinoids may interact with immune cell metabolism is critical for clarifying the results in this Chapter. Indeed, data elsewhere suggests that cannabinoids activate AMPK. Cellular energy levels are regulated by AMPK, which can promote

energy production by increasing mitochondrial biogenesis, as well as inducing catabolic processes and downregulating anabolic processes, such as inhibiting mTOR [465]. For example, THC and the sCB JWH-015, have been shown to activate AMPK in hepatocellular carcinoma cell lines, with downstream induction of autophagy [312]. Additionally, findings elsewhere have shown that cannabinoids can activate AMPK in pancreatic cancer cells, which was dependent on a ROS-induced increase in the AMP/ATP ratio; this effect lead to a decrease in glycolysis [313]. Furthermore, there is increasing evidence that cannabinoids can regulate immune cell metabolism as a mechanism to suppress inflammation. Indeed, Chan and colleagues (2017) reported that knockout of the CB_2 receptor in B cells resulted in an increase in glucose uptake and ATP production [315]. Indeed, cannabinoids can attenuate the NLRP3 inflammasome in macrophages [316], activate AMPK (which suppresses mTOR) and stimulate oxidative phosphorylation [303]. Given much evidence linking cannabinoids to cell metabolism, future studies will investigate the increase/decrease in MTT absorbance values detected in this study. Apoptotic and proliferation markers, in addition to live/dead staining of the immune cells, analysed via flow cytometry, will aid in elucidating the effects cannabinoids indicated herein. Indeed, elucidating the effect of phytocannabinoids on glycolysis and oxidative phosphorylation, would further increase our understanding of how cannabinoids may regulate immunosuppression in the cell types investigated in our studies.

Chapter 7: Discussion

7.1 General Discussion

TLRs are highly conserved PRRs found primarily on immune cells, and upon activation in response to infection, promote the activation of a cascade of signalling events that control the production of pro- and anti-inflammatory cytokines and chemokines to combat the infectious insult. There is growing evidence that TLRs play pivotal roles in many diseases, and aberrant activation of TLRs has been implicated in exacerbating many disease states, including MS [466]. Therefore, understanding the mechanisms which control TLR expression and function is of vital importance in treating and managing diseases associated with inflammation. Indeed, extracts of the *C. sativa* plant, the phytocannabinoids, are emerging as potential regulators of TLR signalling events in many immune cell types, and therefore offer a novel therapeutic approach to control dysregulated inflammatory signalling pathways in many disease states (for review see Fitzpatrick and Downer, 2017 [274]). Furthermore, there is some evidence, albeit limited, for cannabinoid regulation of viral TLR3 signalling and bacterial TLR4 signalling in macrophages [467], PBMCs [184], microglia [283], pDCs [291] and monocytes [163]. Accordingly, the primary objective of this thesis was to determine if the euphoric phytocannabinoid, THC, the non-euphoric phytocannabinoid CBD, and a combination of THC and CBD, have the proclivity to alter TLR3 and TLR4 signalling when administered alone and in combination, in a monocyte and macrophage cell line, in addition to primary human PBMCs extracted from HC donors and pwMS. This study also aimed to screen the effect of a panel of highly purified botanically-derived phytocannabinoids on the viability of immune cells.

The data presented in this thesis indicates that (a) THP-1-derived macrophages are a suitable model for studying TLR3 and TLR4 signalling events, (b) primary human PBMCs respond to TLR3 and TLR4 stimulation via poly(I:C) and LPS, respectively, in terms of IFN-β, CXCL10, and TNFα production, (c) THC, CBD, and a 1:1 combination of THC and CBD, target MyD88-independent signalling via TLR3 and 4 via inhibition of IRF3, IFN-β, and CXCL10 expression in THP-1-derived macrophages, (d) THC and CBD, when administered alone and in combination, attenuate TLR3-induced CXCL10 and IFN-β expression and potentiate TLR4-induced TNFα expression, in primary human PBMCs from HC donors and pwMS, and (e) a range of highly purified botanically-derived

phytocannabinoids (THC, CBD, THC:CBD, CBDA, CBDV, THCA, THCV, CBC and CBG) differentially alter the viability of THP-1 monocytes, THP-1-derived macrophages, and primary human PBMCs from HC subjects and pwMS, which is dependent upon the cell type examined, the cannabinoid being tested, and the concentration utilised. Taken together, these findings demonstrate that phytocannabinoids have the proclivity to modulate TLR3/4 signalling in immune cells and suggest that phytocannabinoids may have broad therapeutic potential in inflammatory disorders where TLR signalling is dysregulated. This discussion will address the primary findings of this thesis and discuss how the data presented herein add to the body of knowledge regarding cannabinoid regulation of TLR signalling mechanisms, with particular relevance to MS.

THC and CBD target MyD88-independent signalling via TLR3 and TLR4 in THP-1-derived macrophages

The viral mimetic poly(I:C), and the gram-negative endotoxin LPS, activate TLR3 and TLR4, respectively, and upon activation promote the production of a range of cytokines and chemokines, including IFN-β, CXCL10 and TNFα. Indeed, data presented in Chapter 3 characterised TLR3-induced signalling via the MyD88-independent pathway, and TLR4 signalling via MyD88-dependent and -independent mechanisms, in THP-1 monocytes, macrophages and primary human PBMCs. Overall, data presented herein indicate that THP-1 monocytes did not respond to TLR3 agonism in terms of IFN-β, CXCL10 and TNFα expression, however, poly(I:C) increased expression of IFN-β and CXCL10, but not TNFα, in macrophages and primary PBMCs. Furthermore, all three cell types employed in this research study responded to TLR4 activation using LPS, based on the increased expression of TNFα (in monocytes, macrophages and PBMCs) and IFN-β (in macrophages only), further highlighting the proclivity of TLR4 to signal via MyD88-dependent and independent pathways. Considering that THP-1 monocytes did not respond to poly(I:C) treatment, while THP-1-derived macrophages responded to both poly(I:C) and LPS stimulation, THP-1-derived macrophages were chosen as a suitable *in vitro* cell line model to study the effects of the phytocannabinoids THC and CBD on TLR3 and TLR4 signalling mechanisms.

Data presented in this thesis demonstrate that THC and CBD do not target TLR4-induced TNFα or RANTES expression in THP-1 monocytes, and furthermore do not target TLR4-induced TNFα or CXCL8 in THP-1-derived macrophages. Indeed, TLR4-induced IκB-α degradation was unaltered by THC and CBD treatment, however, NF-κB translocation to the nucleus, in addition to phosphorylation of the p65-NF-κB subunit, was blocked by addition of THC and/or CBD. This suggests that THC and CBD, both alone and in combination, have the proclivity to inhibit NF-κB activation, while not impacting downstream production of pro-inflammatory cytokines/chemokines. It is reasonable to suggest that TLR4-induced activation of the AP-1 transcription factor, which can regulate pro-inflammatory signalling [11], may be compensating for the inactivation of NF-κB in our cell culture model.

TLR4-induced MyD88-independent signalling was inhibited by THC, CBD, and a 1:1 combination of both phytocannabinoids. Indeed, THC and CBD inhibited TLR3 and TLR4-induced IRF3, IFN-β and CXCL10 expression, highlighting that THC and CBD preferentially target the MyD88-independent pathway induced by LPS and poly(I:C). Figure 7.1 outlines the proposed mechanism of action of THC and CBD on TLR3/4 signalling in THP-1-derived macrophages. Data presented herein also suggests that pharmacological inhibition of the cannabinoid receptors, in addition to blocking the nuclear receptor PPARγ, do not ameliorate the inhibitory effects of THC and CBD on TLR3- and TLR4-induced MyD88-independent signalling in THP-1-derived macrophages. This is in accordance with data presented elsewhere where CB_1- and CB_2-independent effects of THC [398] and CBD [399] have been demonstrated. Indeed, many other pharmacological targets of cannabinoids have been suggested, including 5-HT [468], TRPVs [469], PPARs [253], in addition to modulating many cellular macromolecules such as ion channels, transporters, and enzymes [470, 471]. Both THC and CBD are lipid soluble phytocannabinoids [418], and thus their cellular effect on TLR signalling may be attributed to their lipophilicity due to their direct partitioning into cellular membranes. Further research is necessary to elucidate the pharmacological target(s) of THC and CBD in modulating TLR3 and TLR4 signalling.

One of the primary goals of this project was to determine whether the proclivity of THC and CBD to impact TLR3 and TLR4 signalling was influenced by the

administration of each cannabinoid alone or in a 1:1 combination. Interestingly, some data presented in this thesis suggest that the combination of THC and CBD was more efficacious than treatments alone at inhibiting TLR3/4 signalling mechanisms. This is of interest given that the cannabinoid-based therapeutic Sativex, which contains CBD and THC as its most abundant phytocannabinoid components, in addition to other phytocannabinoids and non-phytocannabinoid components, is prescribed for spasticity related to MS [438]. The precise cellular mechanisms of action of Sativex are unknown, and this thesis offers insights into potential mechanisms of actions of Sativex through analysis of the effect of a 1:1 ratio of THC:CBD on the modulation of TLR3 and TLR4 signalling events in macrophages.

Macrophages play a key role in the pathogenesis of MS [472] and EAE [473], which together with microglia can contribute to key neuroinflammatory events associated with MS. Indeed, during the acute phase of RRMS, evidence indicates that macrophages switch to an M1 pro-inflammatory state and release pro-inflammatory cytokines/chemokines to promote tissue damage, demyelination and neuronal death [474]. Macrophages can also broadly present as anti-inflammatory M2 macrophages, which are characterised by the release of anti-inflammatory proteins including IL-4, IL-10, IL-13, IL-33 and transforming growth factor (TGF)-β, which are linked to the suppression of EAE [475]. Unfortunately, the polarisation of macrophages following TLR3/4 activation and phytocannabinoid exposure was beyond the scope of this study. However, our data suggest that the activation of macrophages following treatment with poly(I:C) or LPS, was suppressed by both THC and CBD. Future research will address the proclivity of phytocannabinoids to polarise macrophages towards an M2 anti-inflammatory phenotype, as this may highlight mechanisms to suppress peripheral macrophage activation that may be beneficial in treating MS.

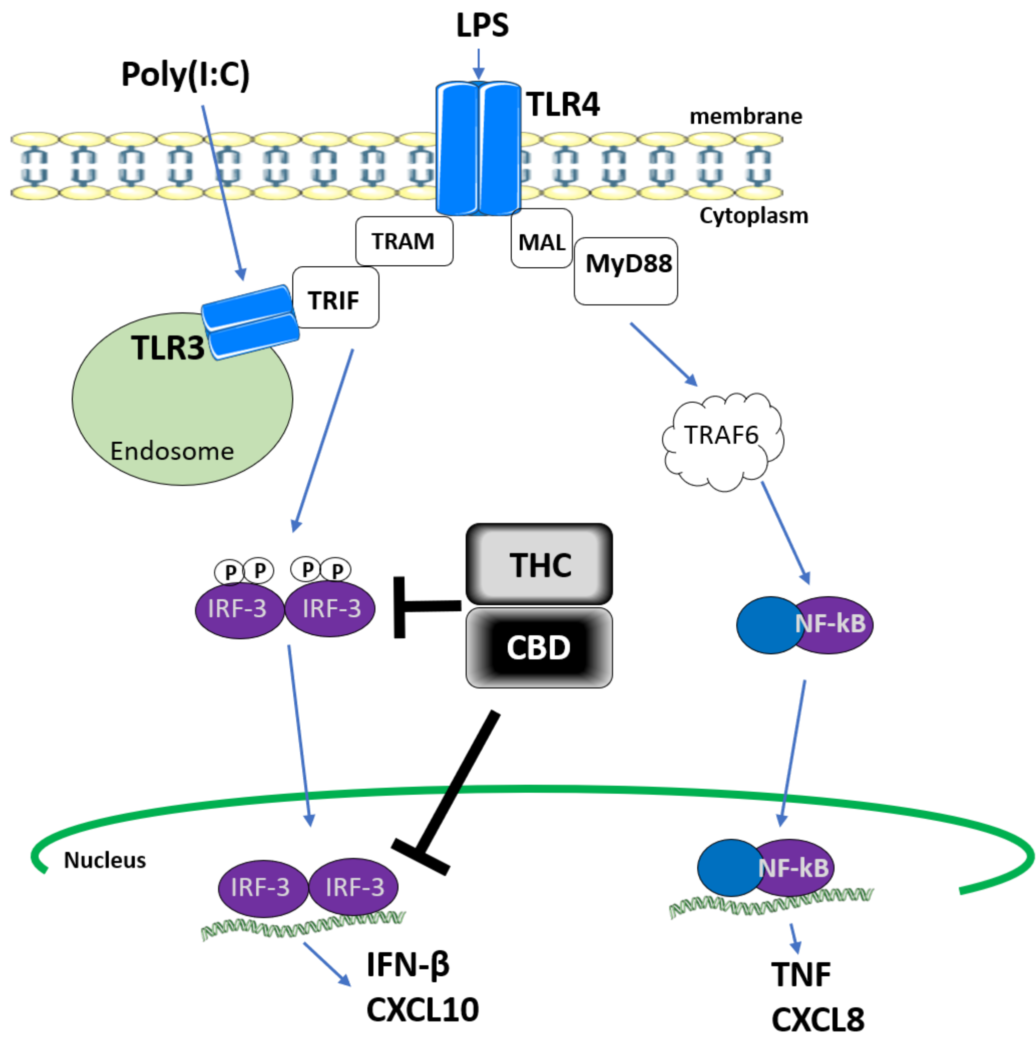


Figure 7.1. Proposed mechanism by which THC and CBD target TLR3/4 signalling in THP-1 macrophages. LPS-induced TLR4 activation induces the expression of MyD88-dependent and MyD88-independent cytokines and chemokines in THP-1-derived macrophages. Administration of poly(I:C) activates the MyD88-independent signalling pathway only, which promotes IRF3 activation and translocation to the nucleus, and the expression of the type I IFN-β and CXCL10 chemokine. Treatment with THC and CBD, alone and in combination, attenuated TLR3- and TLR4-induced IRF3 activation and expression of IFN-β and CXCL10. Conversely, THC and CBD had no clear effect of TLR4-induced MyD88-dependent signalling in THP-1-derived macrophages.

Inhibits = ⊣

THC and CBD attenuate TLR-induced MyD88-independent signalling, but potentiate TLR4-induced MyD88-dependent TNFα expression, in primary PBMCs from HC donors and pwMS

MS is a chronic inflammatory autoimmune disease associated with CNS infiltration by peripheral immune cells and subsequent neuroinflammation, demyelination and degradation of axons. Therefore, it is of critical importance to understand the mechanisms leading to peripheral immune cell activation and to develop therapeutics targeting the peripheral immune response. Indeed, many DMTs for MS including Fingolimod, Natalizumab and IFN-β target peripheral immune cell trafficking across the BBB [476] (see introduction for full review). Approved therapies for MS have partial efficacy, and not all pwMS respond to approved DMTs. Therefore, there it is a requirement to develop novel therapeutics for treating MS. As previously discussed, cannabinoids offer potential as a novel therapeutic for MS. There is increasing evidence that cannabinoids can alter peripheral immune cell activation and function. Data outlined in this thesis present evidence for cannabinoid modulation of TLR3 and TLR4 signalling mechanisms in PBMCs from HC donors and pwMS. PBMCs from pwMS were desensitized in terms of cellular responses to TLR3 activation (for CXCL10 production), when compared to PBMCs from HC cases. Both groups (HC and pwMS) responded similarly to TLR4 agonism in terms of TNFα production. These data are contrary to previous data from our laboratory indicating that PBMCs from pwMS are hypersensitive to LPS treatment in terms of TNFα expression [155]. Such differences may be due to the fact that pwMS recruited to the present study reported the use of a variety of DMTs, while previous assessments were conducted in newly diagnosed, treatment naïve pwMS.

Overall, data presented herein in Chapter 5 demonstrate a role for THC, CBD and a 1:1 combination, in modulating TLR3-induced CXCL10 and IFN-β expression, in addition to TLR4-induced TNFα expression, in PBMCs from HC donors and pwMS. Unexpected findings in this Chapter suggest that phytocannabinoids, when administered alone and in combination, exacerbate TLR4-induced TNFα expression. In addition, administration of the phytocannabinoids alone in the absence of LPS, significantly decreased basal TNFα expression, a finding unique to PBMCs from pwMS. Studies elsewhere have shown that THC can inhibit TNFα

production in a macrophage cell line [477] and can decrease the constitutive production of IL-8, MIP-1α, MIP-1β, RANTES and TNFα in a NK cell line [478]. Additionally, *R*(+)WIN55,212-2 and THC have been shown to decrease LPS-induced TNFα expression in the bronchoalveolar lavage fluid of mice [479]. Data presented in this thesis support these studies given that an inhibition of TNFα expression was detected in unstimulated PBMCs from pwMS. However, data presented herein indicating that THC and/or CBD potentiate LPS-induced TNFα expression is in contrast to the published literature. This is, to the best of our knowledge, a novel finding and highlights that PBMCs from pwMS may have increased sensitivity to cannabinoid modulation of TLR4 signalling. Indeed, although immune cell activation states were not assessed in this study, PBMCs from pwMS would be expected to have an activated phenotype, given that peripheral immune activation is crucial in MS pathogenesis, while PBMCs from HC donors would not share this characteristic. This may aid in understanding the increased sensitivity of PBMCs from pwMS to cannabinoids. Figure 7.2 outlines potential targets of THC and CBD in TLR3/4 signalling cascades in primary human PBMCs.

IFN-β therapy is considered a first-line DMT for RRMS. The exact mechanism of action of IFN-β therapy is unknown, however, it has been shown to reduce relapse rate in pwMS, in addition to having anti-viral and anti-inflammatory properties, including BBB modulation [120]. An interesting finding from this thesis was that administration of THC, CBD, and the combination of THC:CBD (1:1) alone (without TLR agonism) was sufficient to increase basal IFN-β tone in PBMCs from pwMS, albeit insignificantly. The phytocannabinoids tested did not alter IFN-β expression in PBMCs from HC donors. This finding suggests that phytocannabinoids have the proclivity to activate pathways leading to increased IFN-β expression, such as engaging IFNARs, or activating/modulating TLR pathways leading to IFN-β production in pwMS. Previous data has established a role for the sCB *R*(+)WIN55,212-2 in regulating IFN-β expression following TLR activation in PBMCs from pwMS [184]. This finding was not replicated in this study using phytocannabinoids, but further investigation of the effect of phytocannabinoids on endogenous IFN-β tone warrants full investigation. These data, when considered alongside data suggesting that phytocannabinoids significantly inhibit basal TNFα expression in PBMCs from pwMS, suggest that

PBMCs from pwMS are uniquely sensitive to the effects of phytocannabinoids on intracellular inflammatory signalling mechanisms. Thus, data in this thesis suggest that phytocannabinoids can increase IFN-β while simultaneously decreasing TNFα expression in unstimulated PBMCs from pwMS.

Following TLR3 activation with poly(I:C), and co-treatment with THC, CBD, and THC:CBD combination, our data indicate that the phytocannabinoids attenuated TLR3-induced IFN-β protein expression in PBMCs from pwMS, with the combination of THC:CBD (1:1) significantly attenuating IFN-β production. These data suggest that phytocannabinoid therapy in pwMS may blunt the viral response by inhibiting TLR3/viral signalling, thereby decreasing efficient IFN-β expression. Similarly, this is true for bacterial infections, mimicked through activation of TLR4 using LPS. In the absence of LPS, THC and CBD inhibited TNFα expression in PBMCs, while in the presence of LPS the phytocannabinoids potentiated LPS-induced TNFα expression. This may suggest that administering phytocannabinoids to a patient who currently harbours a bacterial infection may exacerbate the immune response to the infection by producing excessive TNFα and thereby further increasing inflammation.

Throughout this project we have focused on the effects of THC and CBD when administered alone, in addition to combining THC and CBD at a 1:1 ratio. Some data presented in Chapter 5 indicate that combining THC and CBD at a 1:1 ratio was most effective at attenuating cellular responses to poly(I:C) or potentiating cellular responses to LPS. Indeed, the combination of THC:CBD (1:1) was most effective at inhibiting CXCL10 protein expression, in addition to increasing IFN-β protein expression, in PBMCs from HC donors and pwMS. A key objective of this study was to determine if the cellular actions of THC and CBD on TLR signalling differed when cannabinoids were delivered independently or in combination. Studies elsewhere have highlighted that a combination of THC:CBD (1:1) was more effective therapeutically, when compared to the administration of CBD, and THC, alone. For example, in an acute model of colitis in rats, THC and CBD in combination was more effective than treatments with cannabinoids in isolation, at reducing inflammation [480]. Elsewhere, a combination therapy of THC and CBD, but not when given alone, attenuated EAE by reducing neuroinflammation and suppression of Th17 and Th1 cells [422].

Finally, data presented herein (in Chapter 5) also characterised the effectiveness of phytocannabinoid regulation of TLR3/4 responses in PBMCs isolated from pwMS with respect to the use of DMTs. pwMS recruited to this study indicated that they were prescribed a variety of DMTs at time of blood-draw, therefore it was of interest to determine if a specific DMT altered the cellular response of PBMCs to both TLR3/4 activation and phytocannabinoid treatment. Data presented in this thesis suggest that DMTs did not alter the responsiveness of PBMCs to TLR3/4/phytocannabinoid treatment, although a full interpretation of these data is limited given the low *n* number for each DMT assessed (one or two pwMS recruited for each specific DMT apart from IFN-β which included six pwMS in our analysis). Future studies will aim to recruit more pwMS reporting the use of a range of DMTs to clarify these results.

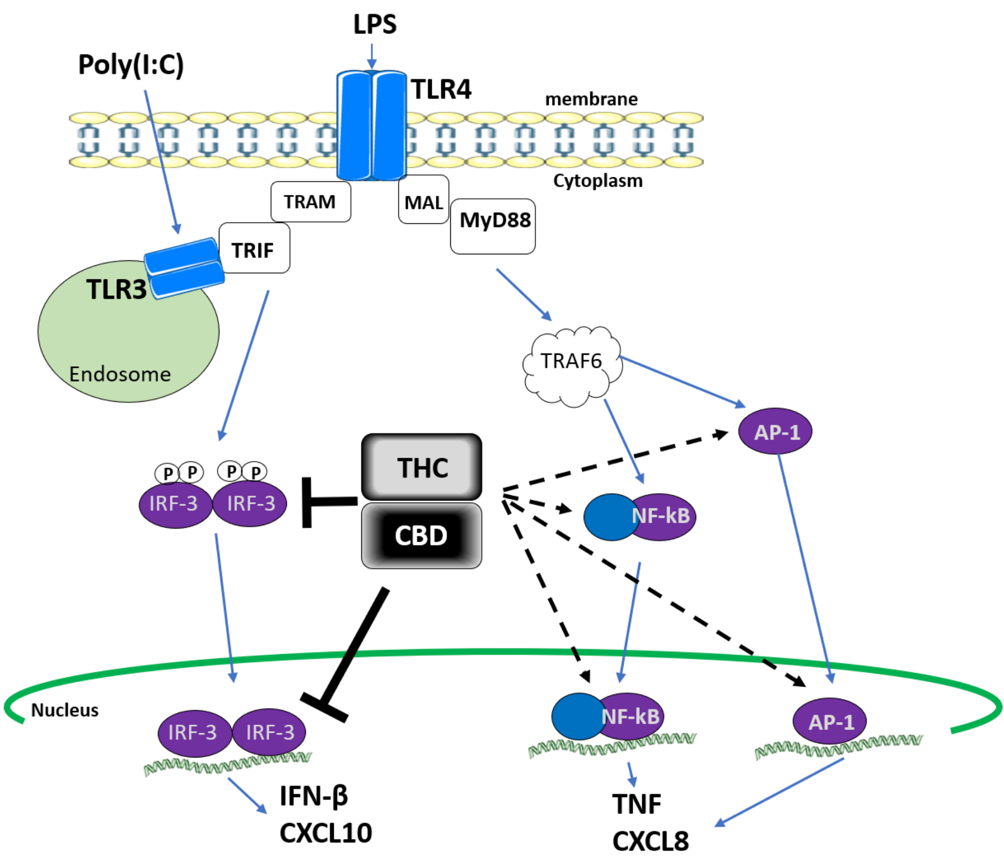


Figure 7.2. Schematic outlining the primary findings in Chapter 5. LPS can activate MyD88-dependent and -independent signalling pathways. Poly(I:C) activates MyD88-independent signalling via recruitment of TRIF. Administration

of THC and CBD, alone and in combination, attenuated TLR3 and TLR4 TRIF-dependent signalling however, the phytocannabinoids potentiated TLR4-induced TNFα expression in primary PBMCs isolated for HC donors and pwMS.

Inhibits = ━━┫ Potential induction by THC and CBD = ▬ ▬ ▬ ▬▶

Phytocannabinoids can alter immune cell viability, which is dependent upon the cannabinoid tested, the concentration *of cannabinoid, and type of immune cell examined*

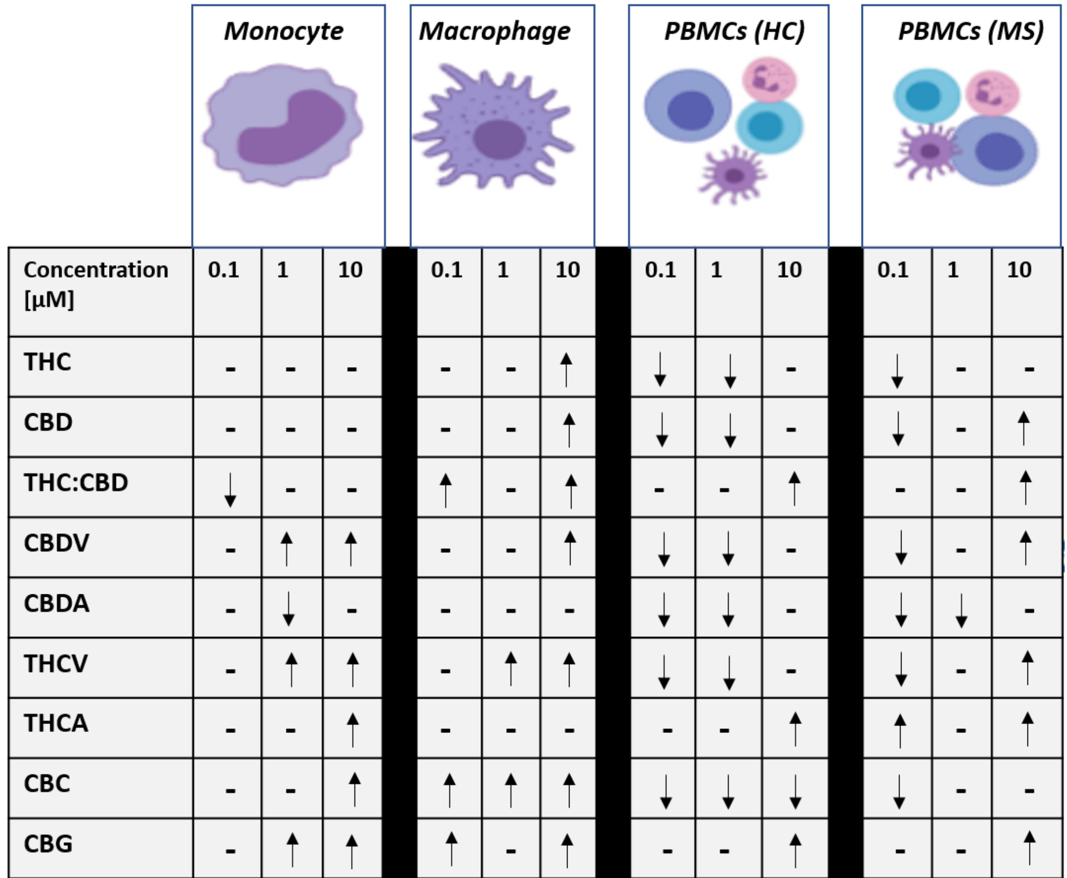
Results presented in Chapters 4 and 5 demonstrated that the phytocannabinoids THC and CBD can modulate TLR3 and TLR4 signalling mechanisms in THP-1-derived macrophages and primary PBMCs from HC donors and pwMS. Given the proclivity of the phytocannabinoids to alter TLR3 and TLR4 signalling events, experiments outlined in Chapter 6 investigated the impact of THC and/or CBD on immune cell viability to determine if the effects of both phytocannabinoids in our study could be explained by the potential toxic characteristics of THC and CBD. Data presented herein indicate that at a final concentration of [10 μM], THC and/or CBD increased THP-1-derived macrophage viability. In PBMCs from HC cases, THC and CBD, when administered alone at [10 μM], did not alter the viability of PBMCs, while the combination of THC:CBD (1:1) at [10 μM] increased the viability of PBMCs from HC donors. In PBMCs from pwMS, THC at [1 and 10 μM] did not affect viability, whereas CBD at [10 μM] and the combination of THC:CBD (1:1) at [10 μM] combination increased viability. Once again, the combination of THC and CBD was more effective at increasing the viability of immune cells, when compared to treating cells with THC or CBD alone.

A range of novel botanically-derived pure cannabinoids (THCA, THCV, CBDA, CBDV, CBC, and CBG) were also assessed for their effect on the viability of THP-1 monocytes, THP-1 macrophages, and PBMCs from HC donors and pwMS. Overall, all six phytocannabinoids tested were well tolerated by all immune cells examined. However, the effect of phytocannabinoids on viability was dependent upon the concentration of phytocannabinoid tested. For example, in primary PBMCs from HC volunteers and pwMS there was a significant decrease in PBMC viability after treatment with THC, CBD, CBDA, CBDV, THCV and CBC at [0.1 μM]. Interestingly, when THC, CBD, CBDA, CBDV, THCV and CBC were each

administered to PBMCs isolated from both HC cases and pwMS at a concentration of 10 μM, this effect was lost. Specifically, in PBMCs from pwMS all phytocannabinoids at [10 μM] (apart from THC, CBDA, and CBC) increased the viability of the cells, when compared to vehicle-treated cells, and therefore appeared to promote cell survival. In comparison, only THC:CBD, THCA and CBG increased the viability of PBMCs from HC cases at [10 μM]. These data highlight the increased efficacy that phytocannabinoids may impart on phenotypically activated immune cells.

Much research is required to fully understand the effects of the phytocannabinoids tested in our studies on the function of immune cells from HC donors and pwMS. Given that the PBMC population consists of B cells, T cells, NK cells, monocytes and DCs [355], the cell type(s) targeted by cannabinoids in our primary PBMC studies is unclear. Data elsewhere indicate that THC can induce apoptosis in murine macrophages and T cells [276], and that CBD can induce apoptosis in murine T cells [443]; however there are limited studies assessing the effects of the range of phytocannabinoids listed here on the viability of primary PBMCs (or cell subsets), and to our knowledge there is no published literature on the effect of phytocannabinoids on the viability of PBMCs from individuals with RRMS. Figure 7.3 summarises the concentration-dependent effect of the phytocannabinoids tested in this study on the viability of THP-1 monocytes, THP-1-derived macrophages, and primary PBMCs from HC donors and pwMS.

Overall, these data demonstrate that phytocannabinoids can alter immune cell viability, and potentially their function, which is highly dependent on the phytocannabinoid under investigation, the concentration administered, and the immune cell under investigation. Understanding how phytocannabinoids modulate or alter primary human immune cell viability is of critical importance in developing novel cannabis-based therapeutics, and these data may aid in that development.



Concentration [µM]	Monocyte			Macrophage			PBMCs (HC)			PBMCs (MS)		
	0.1	1	10	0.1	1	10	0.1	1	10	0.1	1	10
THC	-	-	-	-	-	↑	↓	↓	-	↓	-	-
CBD	-	-	-	-	-	↑	↓	↓	-	↓	-	↑
THC:CBD	↓	-	-	↑	-	↑	-	-	↑	-	-	↑
CBDV	-	↑	↑	-	-	↑	↓	↓	-	↓	-	↑
CBDA	-	↓	-	-	-	-	↓	↓	-	↓	↓	-
THCV	-	↑	↑	-	↑	↑	↓	↓	-	↓	-	↑
THCA	-	-	↑	-	-	-	-	-	↑	↑	-	↑
CBC	-	-	↑	↑	↑	↑	↓	↓	↓	↓	-	-
CBG	-	↑	↑	↑	-	↑	-	-	↑	-	-	↑

Figure 7.3. Schematic outlining the effect of THC, CBD, CBDV, CBDA. THCV, THCA, CBC and CBG (concentration range 0.1, 1, 10 µM) on the viability of immune cells using MTT assays. Four subsets of immune cells were examined: THP-1 monocytes, THP-1-derived macrophages, PBMCs from HC cases and PBMCs from pwMS. Cells were treated with nine phytocannabinoid formulations (listed on the left of table) over three concentrations (0.1 – 10 µM). No effect = — ; Decrease in viability = ↓ ; Increase in viability = ↑

7.2 Limitations of the studies

The work presented in this thesis has added a significant contribution to the scientific knowledge of phytocannabinoid regulation of TLR signalling events, however a number of limitations of the study exist and should be addressed in future studies. These include:

- Results presented in Chapters 3 and 4 indicate data from THP-1 monocytes and THP-1-derived macrophage cell lines. It is important to note that the use of cell lines does not always adequately model the function and cell responses of primary immune cells. However, data presented in this thesis indicate that primary PBMCs responded to TLR3/4 activation in a similar manner in THP-1 cell lines, and furthermore phytocannabinoid modulation of these pathways (particularly TLR3) was comparable in both cell lines and primary PBMCs.
- Data presented in this thesis demonstrate the proclivity of phytocannabinoids to modulate TLR3/4 signalling events in immune cells at specific time points. Therefore, an analysis of further time points would provide a clear profile on the mechanism of action of phytocannabinoids in our cell culture system. Data presented throughout this project targeted the impact of phytocannabinoids on TLR signalling events within well characterised temporal profiles, (i.e. nuclear sequestration studies at 30-60 min; mRNA analysis at 4 h; protein expression assessment at 24 h).
- Throughout the thesis, alterations in mRNA expression of a targeted marker did not always correspond with an alteration in protein expression of the corresponding marker. Indeed, the expression profiles for mRNA and protein did not stringently reflect one another. However, much data indicate that mRNA expression does not correlate with protein expression through various mechanisms including post-translational modifications. This may be the case observed in this thesis. Future work will investigate such discrepancies in more detail.
- Data presented in Chapter 5 of this thesis outlined the effect of phytocannabinoids on primary PBMCs isolated from HC donors and pwMS. The clinical aspect of this project set out to recruit and assess PBMCs isolated from treatment-naïve newly diagnosed individuals with

RRMS. However, recruitment of such cases via the Neurology Clinic at Beaumont Hospital was difficult. Therefore, pwMS recruited to this study reported the use of a variety of DMTs, all of which may alter immune cell function. To monitor to effect of DMT on PBMC read-outs, we clearly presented the effect of each DMT on cellular responses to LPS/poly(I:C) and phytocannabinoid treatment.

- Data presented in Chapter 6 demonstrate the effects of phytocannabinoids on immune cell viability using MTT assays. A cautionary note should be applied when consider this as "viability" data, as MTT assays may reflect cell proliferation, metabolic changes in cell function and(or) alterations in mitochondrial function. In the current study we employed the use of MTT assays as a screening tool to provide insight on the potential effects of phytocannabinoids on immune cell viability, and these data highlight phytocannabinoids that warrant further investigation in terms of their impact on immunometabolism.

7.3 Future studies

Data presented in this thesis has added to the body of literature examining the effects of phytocannabinoids on immune cell function. Based on these findings, several new avenues of research warrant further investigation. These include:

- A more detailed examination of the impact of phytocannabinoids on MyD88-dependent signalling. Data presented in this thesis have shown the complex nature of phytocannabinoid regulation of TLR4-induced NF-κB expression. Therefore, further studies should aim to assess the impact of phytocannabinoids on LPS-induced signalling to Raf-MEK1/2-ERK1/2, MEK4-JNK, MEK3/6-p38, and the regulation of AP-1 transcription factor. Such analysis would improve our understanding of the mechanisms by which phytocannabinoids regulate cytokine production in response to LPS.
- An investigation of the role of other non-classical cannabinoid receptor targets in mediating the effects of THC and/or CBD on TLR3- and TLR4-induced inflammatory events. Targets for consideration include PPARβ/δ, TRPV1 and GPR55.

- A targeted investigation of the effect of phytocannabinoids on the signalling proteins involved in TLR3/4 inflammatory cascades (IRF3, NF-κB and MAPKs) in primary PBMCs.
- Examine the proclivity of phytocannabinoids to alter TLR signalling in primary $CD14^{+}$ monocytes and primary monocyte-derived macrophages.
- Investigate the effects of THC, CBD, THC:CBD, THCA, THCV, CBDA, CBDV, CBC, and CBG on primary PBMC viability using a range of techniques including flow cytometry to stain for apoptotic, proliferative, and dead/live markers. Furthermore, an investigation of the effects of the listed phytocannabinoids on mitochondrial function such as altered AMPK, OXPHOS, ROS production and glycolysis, warrants further analysis.

References

1. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
2. O'Neill, L.A., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors - redefining innate immunity.* Nat Rev Immunol, 2013. **13**(6): p. 453-60.
3. Medzhitov, R., *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-45.
4. Zarember, K.A. and P.J. Godowski, *Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines.* J Immunol, 2002. **168**(2): p. 554-61.
5. Kawai, T. and S. Akira, *The roles of TLRs, RLRs and NLRs in pathogen recognition.* Int Immunol, 2009. **21**(4): p. 317-37.
6. Rock, F.L., et al., *A family of human receptors structurally related to Drosophila Toll.* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 588-93.
7. Chaturvedi, A. and S.K. Pierce, *How location governs toll-like receptor signaling.* Traffic, 2009. **10**(6): p. 621-8.
8. Ting, J.P., et al., *The NLR gene family: a standard nomenclature.* Immunity, 2008. **28**(3): p. 285-7.
9. Thoma-Uszynski, S., et al., *Induction of direct antimicrobial activity through mammalian toll-like receptors.* Science, 2001. **291**(5508): p. 1544-7.
10. Kawasaki, T. and T. Kawai, *Toll-like receptor signaling pathways.* Front Immunol, 2014. **5**: p. 461.
11. Troutman, T.D., J.F. Bazan, and C. Pasare, *Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K.* Cell Cycle, 2012. **11**(19): p. 3559-67.
12. Wesche, H., et al., *MyD88: an adapter that recruits IRAK to the IL-1 receptor complex.* Immunity, 1997. **7**(6): p. 837-47.
13. Burns, K., et al., *Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor.* Nat Cell Biol, 2000. **2**(6): p. 346-51.
14. Muzio, M., et al., *IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling.* Science, 1997. **278**(5343): p. 1612-5.
15. Wang, C., et al., *TAK1 is a ubiquitin-dependent kinase of MKK and IKK.* Nature, 2001. **412**(6844): p. 346-51.
16. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science, 2003. **301**(5633): p. 640-3.
17. Honda, K., et al., *IRF-7 is the master regulator of type-I interferon-dependent immune responses.* Nature, 2005. **434**(7034): p. 772-7.
18. Du B, J.Q., Cleveland J, Liu DR, Zhang D, *Targeting toll-like receptors against cancer.* Journal of Cancer Metastasis Treatments, 2016. **2**: p. 463-470.
19. Mikami, T., et al., *Molecular evolution of vertebrate Toll-like receptors: evolutionary rate difference between their leucine-rich repeats and their TIR domains.* Gene, 2012. **503**(2): p. 235-43.
20. Siednienko, J. and S.M. Miggin, *Expression analysis of the Toll-like receptors in human peripheral blood mononuclear cells.* Methods Mol Biol, 2009. **517**: p. 3-14.

21. Hornung, V., et al., *Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides.* J Immunol, 2002. **168**(9): p. 4531-7.
22. Muzio, M., et al., *Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells.* J Immunol, 2000. **164**(11): p. 5998-6004.
23. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-8.
24. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.
25. Ozinsky, A., et al., *The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13766-71.
26. Kawai, T. and S. Akira, *Toll-like receptor and RIG-I-like receptor signaling.* Ann N Y Acad Sci, 2008. **1143**: p. 1-20.
27. Jiang, Z., et al., *Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3533-8.
28. Meylan, E., et al., *RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation.* Nat Immunol, 2004. **5**(5): p. 503-7.
29. Razani, B., A.D. Reichardt, and G. Cheng, *Non-canonical NF-kappaB signaling activation and regulation: principles and perspectives.* Immunol Rev, 2011. **244**(1): p. 44-54.
30. Honda, K. and T. Taniguchi, *IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors.* Nat Rev Immunol, 2006. **6**(9): p. 644-58.
31. Hacker, H., et al., *Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6.* Nature, 2006. **439**(7073): p. 204-7.
32. Homma, T., et al., *Cooperative activation of CCL5 expression by TLR3 and tumor necrosis factor-alpha or interferon-gamma through nuclear factor-kappaB or STAT-1 in airway epithelial cells.* Int Arch Allergy Immunol, 2010. **152 Suppl 1**: p. 9-17.
33. Lundberg, A.M., et al., *Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems.* Blood, 2007. **110**(9): p. 3245-52.
34. Brownell, J., et al., *Independent, parallel pathways to CXCL10 induction in HCV-infected hepatocytes.* J Hepatol, 2013. **59**(4): p. 701-8.
35. Brownell, J., et al., *Direct, interferon-independent activation of the CXCL10 promoter by NF-kappaB and interferon regulatory factor 3 during hepatitis C virus infection.* J Virol, 2014. **88**(3): p. 1582-90.
36. Ivashkiv, L.B. and L.T. Donlin, *Regulation of type I interferon responses.* Nat Rev Immunol, 2014. **14**(1): p. 36-49.
37. Stark, G.R. and J.E. Darnell, Jr., *The JAK-STAT pathway at twenty.* Immunity, 2012. **36**(4): p. 503-14.
38. Qaisar, N., et al., *A Critical Role for the Type I Interferon Receptor in Virus-Induced Autoimmune Diabetes in Rats.* Diabetes, 2017. **66**(1): p. 145-157.

39. Li, W., et al., *Lipopolysaccharide-Induced Profiles of Cytokine, Chemokine, and Growth Factors Produced by Human Decidual Cells Are Altered by Lactobacillus rhamnosus GR-1 Supernatant.* Reprod Sci, 2014. **21**(7): p. 939-947.
40. Shimazu, R., et al., *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.* J Exp Med, 1999. **189**(11): p. 1777-82.
41. Gioannini, T.L., et al., *Monomeric endotoxin:protein complexes are essential for TLR4-dependent cell activation.* J Endotoxin Res, 2005. **11**(2): p. 117-23.
42. Vaure, C. and Y. Liu, *A comparative review of toll-like receptor 4 expression and functionality in different animal species.* Front Immunol, 2014. **5**: p. 316.
43. Guven-Maiorov, E., et al., *The Architecture of the TIR Domain Signalosome in the Toll-like Receptor-4 Signaling Pathway.* Sci Rep, 2015. **5**: p. 13128.
44. Zanoni, I., et al., *CD14 controls the LPS-induced endocytosis of Toll-like receptor 4.* Cell, 2011. **147**(4): p. 868-80.
45. Tsuchiya, S., et al., *Establishment and characterization of a human acute monocytic leukemia cell line (THP-1).* Int J Cancer, 1980. **26**(2): p. 171-6.
46. Bosshart, H. and M. Heinzelmann, *THP-1 cells as a model for human monocytes.* Ann Transl Med, 2016. **4**(21): p. 438.
47. Kim, J.I., et al., *Crystal structure of CD14 and its implications for lipopolysaccharide signaling.* J Biol Chem, 2005. **280**(12): p. 11347-51.
48. Park, B.S., et al., *The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex.* Nature, 2009. **458**(7242): p. 1191-5.
49. Bosshart, H. and M. Heinzelmann, *Lipopolysaccharide-mediated cell activation without rapid mobilization of cytosolic free calcium.* Mol Immunol, 2004. **41**(10): p. 1023-8.
50. Jiang, M.X., et al., *Expression profiling of TRIM protein family in THP1-derived macrophages following TLR stimulation.* Sci Rep, 2017. **7**: p. 42781.
51. Le, H.V. and J.Y. Kim, *Stable Toll-Like Receptor 10 Knockdown in THP-1 Cells Reduces TLR-Ligand-Induced Proinflammatory Cytokine Expression.* Int J Mol Sci, 2016. **17**(6).
52. Tsuchiya, S., et al., *Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester.* Cancer Res, 1982. **42**(4): p. 1530-6.
53. Schwende, H., et al., *Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3.* J Leukoc Biol, 1996. **59**(4): p. 555-61.
54. Chen, Q. and A.C. Ross, *Retinoic acid regulates cell cycle progression and cell differentiation in human monocytic THP-1 cells.* Exp Cell Res, 2004. **297**(1): p. 68-81.
55. Chen, Q., M.C. DeFrances, and R. Zarnegar, *Induction of met proto-oncogene (hepatocyte growth factor receptor) expression during human monocyte-macrophage differentiation.* Cell Growth Differ, 1996. **7**(6): p. 821-32.
56. Park, E.K., et al., *Optimized THP-1 differentiation is required for the detection of responses to weak stimuli.* Inflamm Res, 2007. **56**(1): p. 45-50.
57. Wan, J., et al., *NF-kappaB inhibition attenuates LPS-induced TLR4 activation in monocyte cells.* Mol Med Rep, 2016. **14**(5): p. 4505-4510.

58. Harrison, L.M., et al., *Chemokine expression in the monocytic cell line THP-1 in response to purified shiga toxin 1 and/or lipopolysaccharides.* Infect Immun, 2005. **73**(1): p. 403-12.
59. Carpenter, S., et al., *Toll-like receptor 3 (TLR3) signaling requires TLR4 Interactor with leucine-rich REPeats (TRIL).* J Biol Chem, 2011. **286**(44): p. 38795-804.
60. Pan, Z.K., et al., *Bacterial LPS up-regulated TLR3 expression is critical for antiviral response in human monocytes: evidence for negative regulation by CYLD.* Int Immunol, 2011. **23**(6): p. 357-64.
61. Rajan, J.V., et al., *The NLRP3 inflammasome detects encephalomyocarditis virus and vesicular stomatitis virus infection.* J Virol, 2011. **85**(9): p. 4167-72.
62. Lyons, C., et al., *Engagement of Fas on Macrophages Modulates Poly I:C induced cytokine production with specific enhancement of IP-10.* PLoS One, 2015. **10**(4): p. e0123635.
63. Takashiba, S., et al., *Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor kappaB.* Infect Immun, 1999. **67**(11): p. 5573-8.
64. Mirshafiey, A. and M. Kianiaslani, *Autoantigens and autoantibodies in multiple sclerosis.* Iran J Allergy Asthma Immunol, 2013. **12**(4): p. 292-303.
65. Greenfield, A.L. and S.L. Hauser, *B-cell Therapy for Multiple Sclerosis: Entering an era.* Ann Neurol, 2018. **83**(1): p. 13-26.
66. Bruck, W., *The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage.* J Neurol, 2005. **252 Suppl 5**: p. v3-9.
67. Vosoughi, R. and M.S. Freedman, *Therapy of MS.* Clin Neurol Neurosurg, 2010. **112**(5): p. 365-85.
68. Gaby, A., *Multiple sclerosis.* Glob Adv Health Med, 2013. **2**(1): p. 50-6.
69. Orton, S.M., et al., *Sex ratio of multiple sclerosis in Canada: a longitudinal study.* Lancet Neurol, 2006. **5**(11): p. 932-6.
70. Popescu, C.D., *Multiple sclerosis and pregnancy.* Rev Med Chir Soc Med Nat Iasi, 2014. **118**(1): p. 28-32.
71. Minagar, A., *Current and future therapies for multiple sclerosis.* Scientifica (Cairo), 2013. **2013**: p. 249101.
72. Wallin, M.T., et al., *Global, regional, and national burden of multiple sclerosis 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016.* The Lancet Neurology, 2019. **18**(3): p. 269-285.
73. Rosati, G., *The prevalence of multiple sclerosis in the world: an update.* Neurol Sci, 2001. **22**(2): p. 117-39.
74. O'Connell, K., et al., *Incidence of multiple sclerosis in the Republic of Ireland: A prospective population-based study.* Mult Scler Relat Disord, 2017. **13**: p. 75-80.
75. Mackenzie, I.S., et al., *Incidence and prevalence of multiple sclerosis in the UK 1990-2010: a descriptive study in the General Practice Research Database.* J Neurol Neurosurg Psychiatry, 2014. **85**(1): p. 76-84.
76. Miller, D.H., D.T. Chard, and O. Ciccarelli, *Clinically isolated syndromes.* Lancet Neurol, 2012. **11**(2): p. 157-69.
77. Mowry, E.M., *Natural history of multiple sclerosis: early prognostic factors.* Neurol Clin, 2011. **29**(2): p. 279-92.

78. Trapp, B.D. and K.A. Nave, *Multiple sclerosis: an immune or neurodegenerative disorder?* Annu Rev Neurosci, 2008. **31**: p. 247-69.
79. Ontaneda, D. and R.J. Fox, *Progressive multiple sclerosis.* Curr Opin Neurol, 2015. **28**(3): p. 237-43.
80. Thompson, A.J., et al., *Primary progressive multiple sclerosis.* Brain, 1997. **120 (Pt 6)**: p. 1085-96.
81. Dobson, R. and G. Giovannoni, *Multiple sclerosis - a review.* Eur J Neurol, 2019. **26**(1): p. 27-40.
82. Omerhoca, S., S.Y. Akkas, and N.K. Icen, *Multiple Sclerosis: Diagnosis and Differential Diagnosis.* Noro Psikiyatr Ars, 2018. **55**(Suppl 1): p. S1-S9.
83. McDonald, W.I., et al., *Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis.* Ann Neurol, 2001. **50**(1): p. 121-7.
84. Thompson, A.J., et al., *Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria.* Lancet Neurol, 2018. **17**(2): p. 162-173.
85. Noseworthy, J.H., et al., *Interrater variability with the Expanded Disability Status Scale (EDSS) and Functional Systems (FS) in a multiple sclerosis clinical trial. The Canadian Cooperation MS Study Group.* Neurology, 1990. **40**(6): p. 971-5.
86. Sen, S., *Neurostatus and EDSS Calculation with Cases.* Noro Psikiyatr Ars, 2018. **55**(Suppl 1): p. S80-S83.
87. Compston, A. and A. Coles, *Multiple sclerosis.* Lancet, 2008. **372**(9648): p. 1502-17.
88. Block, M.L. and J.S. Hong, *Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism.* Prog Neurobiol, 2005. **76**(2): p. 77-98.
89. Van Kaer, L., et al., *Innate, innate-like and adaptive lymphocytes in the pathogenesis of MS and EAE.* Cell Mol Immunol, 2019. **16**(6): p. 531-539.
90. Hauser, S.L., et al., *Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions.* Ann Neurol, 1986. **19**(6): p. 578-87.
91. Gilliet, M. and Y.J. Liu, *Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells.* J Exp Med, 2002. **195**(6): p. 695-704.
92. Kabelitz, D. and R. Medzhitov, *Innate immunity--cross-talk with adaptive immunity through pattern recognition receptors and cytokines.* Curr Opin Immunol, 2007. **19**(1): p. 1-3.
93. Tai, Y., et al., *Molecular Mechanisms of T Cells Activation by Dendritic Cells in Autoimmune Diseases.* Front Pharmacol, 2018. **9**: p. 642.
94. Ghoreschi, K., et al., *Fumarates improve psoriasis and multiple sclerosis by inducing type II dendritic cells.* J Exp Med, 2011. **208**(11): p. 2291-303.
95. Kurtzke, J.F., *A reassessment of the distribution of multiple sclerosis. Part one.* Acta Neurol Scand, 1975. **51**(2): p. 110-36.
96. Martyn, C.N., M. Cruddas, and D.A. Compston, *Symptomatic Epstein-Barr virus infection and multiple sclerosis.* J Neurol Neurosurg Psychiatry, 1993. **56**(2): p. 167-8.
97. Levin, L.I., et al., *Multiple sclerosis and Epstein-Barr virus.* JAMA, 2003. **289**(12): p. 1533-6.

98. Pakpoor, J., et al., *The risk of developing multiple sclerosis in individuals seronegative for Epstein-Barr virus: a meta-analysis.* Mult Scler, 2013. **19**(2): p. 162-6.
99. Handel, A.E., et al., *An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis.* PLoS One, 2010. **5**(9).
100. Marrie, R.A., *Environmental risk factors in multiple sclerosis aetiology.* Lancet Neurol, 2004. **3**(12): p. 709-18.
101. Holick, M.F., *Vitamin D: A millenium perspective.* J Cell Biochem, 2003. **88**(2): p. 296-307.
102. Sintzel, M.B., M. Rametta, and A.T. Reder, *Vitamin D and Multiple Sclerosis: A Comprehensive Review.* Neurol Ther, 2018. **7**(1): p. 59-85.
103. O'Connell, K., et al., *Effects of vitamin D3 in clinically isolated syndrome and healthy control participants: A double-blind randomised controlled trial.* Mult Scler J Exp Transl Clin, 2017. **3**(3): p. 2055217317727296.
104. Kurtzke, J.F., *Epidemiology in multiple sclerosis: a pilgrim's progress.* Brain, 2013. **136**(Pt 9): p. 2904-17.
105. Harirchian, M.H., et al., *Worldwide prevalence of familial multiple sclerosis: A systematic review and meta-analysis.* Mult Scler Relat Disord, 2018. **20**: p. 43-47.
106. Compston, D.A., J.R. Batchelor, and W.I. McDonald, *B-lymphocyte alloantigens associated with multiple sclerosis.* Lancet, 1976. **2**(7998): p. 1261-5.
107. Hollenbach, J.A. and J.R. Oksenberg, *The immunogenetics of multiple sclerosis: A comprehensive review.* J Autoimmun, 2015. **64**: p. 13-25.
108. Marrosu, M.G., et al., *HLA-DQB1 genotype in Sardinian multiple sclerosis: evidence for a key role of DQB1 *0201 and *0302 alleles.* Neurology, 1992. **42**(4): p. 883-6.
109. Lundmark, F., et al., *Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis.* Nat Genet, 2007. **39**(9): p. 1108-13.
110. International Multiple Sclerosis Genetics, C., et al., *Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis.* Nat Genet, 2013. **45**(11): p. 1353-60.
111. Gold, R., et al., *Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis.* N Engl J Med, 2012. **367**(12): p. 1098-107.
112. Montalban, X., et al., *Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis.* N Engl J Med, 2017. **376**(3): p. 209-220.
113. Amor, S., et al., *SARS-CoV-2 and Multiple Sclerosis: Not All Immune Depleting DMTs are Equal or Bad.* Ann Neurol, 2020. **87**(6): p. 794-797.
114. Foerch, C., et al., *Severe COVID-19 infection in a patient with multiple sclerosis treated with fingolimod.* Mult Scler Relat Disord, 2020. **42**: p. 102180.
115. Natarajan, V., et al., *Sphingosine-1-phosphate, FTY720, and sphingosine-1-phosphate receptors in the pathobiology of acute lung injury.* Am J Respir Cell Mol Biol, 2013. **49**(1): p. 6-17.
116. Huang, C., et al., *Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China.* Lancet, 2020. **395**(10223): p. 497-506.
117. Croze, E., et al., *Interferon-beta-1b-induced short- and long-term signatures of treatment activity in multiple sclerosis.* Pharmacogenomics J, 2013. **13**(5): p. 443-51.

118. Swiecki, M. and M. Colonna, *Type I interferons: diversity of sources, production pathways and effects on immune responses.* Curr Opin Virol, 2011. **1**(6): p. 463-75.
119. Harrison, D.M. and P.A. Calabresi, *Promising treatments of tomorrow for multiple sclerosis.* Ann Indian Acad Neurol, 2009. **12**(4): p. 283-90.
120. Shirani, A., et al., *Association between use of interferon beta and progression of disability in patients with relapsing-remitting multiple sclerosis.* JAMA, 2012. **308**(3): p. 247-56.
121. Minagar, A., et al., *Emerging roles of endothelial cells in multiple sclerosis pathophysiology and therapy.* Neurol Res, 2012. **34**(8): p. 738-45.
122. Durelli, L., et al., *T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta.* Ann Neurol, 2009. **65**(5): p. 499-509.
123. Mitsdoerffer, M. and V. Kuchroo, *New pieces in the puzzle: how does interferon-beta really work in multiple sclerosis?* Ann Neurol, 2009. **65**(5): p. 487-8.
124. Haas, J., et al., *Glatiramer acetate improves regulatory T-cell function by expansion of naive CD4(+)CD25(+)FOXP3(+)CD31(+) T-cells in patients with multiple sclerosis.* J Neuroimmunol, 2009. **216**(1-2): p. 113-7.
125. Johnson, K.P., et al., *Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group.* Neurology, 1995. **45**(7): p. 1268-76.
126. Moharregh-Khiabani, D., et al., *Fumaric Acid and its esters: an emerging treatment for multiple sclerosis.* Curr Neuropharmacol, 2009. **7**(1): p. 60-4.
127. Mandala, S., et al., *Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists.* Science, 2002. **296**(5566): p. 346-9.
128. Kappos, L., et al., *A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis.* N Engl J Med, 2010. **362**(5): p. 387-401.
129. Cohen, J.A., et al., *Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis.* N Engl J Med, 2010. **362**(5): p. 402-15.
130. Palmer, A.M., *Teriflunomide, an inhibitor of dihydroorotate dehydrogenase for the potential oral treatment of multiple sclerosis.* Curr Opin Investig Drugs, 2010. **11**(11): p. 1313-23.
131. Korn, T., et al., *Modulation of effector cell functions in experimental autoimmune encephalomyelitis by leflunomide--mechanisms independent of pyrimidine depletion.* J Leukoc Biol, 2004. **76**(5): p. 950-60.
132. Confavreux, C., et al., *Oral teriflunomide for patients with relapsing multiple sclerosis (TOWER): a randomised, double-blind, placebo-controlled, phase 3 trial.* Lancet Neurol, 2014. **13**(3): p. 247-56.
133. Ruck, T., et al., *Alemtuzumab in Multiple Sclerosis: Mechanism of Action and Beyond.* Int J Mol Sci, 2015. **16**(7): p. 16414-39.
134. Sheremata, W.A., et al., *The role of alpha-4 integrin in the aetiology of multiple sclerosis: current knowledge and therapeutic implications.* CNS Drugs, 2005. **19**(11): p. 909-22.
135. Miller, D.H., et al., *A controlled trial of natalizumab for relapsing multiple sclerosis.* N Engl J Med, 2003. **348**(1): p. 15-23.
136. McAllister, L.D., P.G. Beatty, and J. Rose, *Allogeneic bone marrow transplant for chronic myelogenous leukemia in a patient with multiple sclerosis.* Bone Marrow Transplant, 1997. **19**(4): p. 395-7.

137. Sormani, M.P., et al., *Autologous hematopoietic stem cell transplantation in multiple sclerosis: A meta-analysis.* Neurology, 2017. **88**(22): p. 2115-2122.
138. Correale, J. and M. Fiol, *BHT-3009, a myelin basic protein-encoding plasmid for the treatment of multiple sclerosis.* Curr Opin Mol Ther, 2009. **11**(4): p. 463-70.
139. Bar-Or, A., et al., *Induction of antigen-specific tolerance in multiple sclerosis after immunization with DNA encoding myelin basic protein in a randomized, placebo-controlled phase 1/2 trial.* Arch Neurol, 2007. **64**(10): p. 1407-15.
140. Cappellano, G., et al., *Subcutaneous inverse vaccination with PLGA particles loaded with a MOG peptide and IL-10 decreases the severity of experimental autoimmune encephalomyelitis.* Vaccine, 2014. **32**(43): p. 5681-9.
141. Park, Y., et al., *Association of the polymorphism for Toll-like receptor 2 with type 1 diabetes susceptibility.* Ann N Y Acad Sci, 2004. **1037**: p. 170-4.
142. Hong, J., et al., *TLR2, TLR4 and TLR9 polymorphisms and Crohn's disease in a New Zealand Caucasian cohort.* J Gastroenterol Hepatol, 2007. **22**(11): p. 1760-6.
143. Lafyatis, R. and M. York, *Innate immunity and inflammation in systemic sclerosis.* Curr Opin Rheumatol, 2009. **21**(6): p. 617-22.
144. Connolly, D.J. and L.A. O'Neill, *New developments in Toll-like receptor targeted therapeutics.* Curr Opin Pharmacol, 2012. **12**(4): p. 510-8.
145. Wittebole, X., D. Castanares-Zapatero, and P.F. Laterre, *Toll-like receptor 4 modulation as a strategy to treat sepsis.* Mediators Inflamm, 2010. **2010**: p. 568396.
146. Ospelt, C., et al., *Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis.* Arthritis Rheum, 2008. **58**(12): p. 3684-92.
147. Brentano, F., et al., *RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3.* Arthritis Rheum, 2005. **52**(9): p. 2656-65.
148. Roelofs, M.F., et al., *Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis.* J Immunol, 2006. **176**(11): p. 7021-7.
149. Hossain, M.J., et al., *The Soluble Form of Toll-Like Receptor 2 Is Elevated in Serum of Multiple Sclerosis Patients: A Novel Potential Disease Biomarker.* Front Immunol, 2018. **9**: p. 457.
150. Trudler, D., D. Farfara, and D. Frenkel, *Toll-like receptors expression and signaling in glia cells in neuro-amyloidogenic diseases: towards future therapeutic application.* Mediators Inflamm, 2010. **2010**.
151. Touil, T., et al., *Cutting Edge: TLR3 stimulation suppresses experimental autoimmune encephalomyelitis by inducing endogenous IFN-beta.* J Immunol, 2006. **177**(11): p. 7505-9.
152. Fitzgerald, D.C., et al., *Interferon regulatory factor (IRF) 3 is critical for the development of experimental autoimmune encephalomyelitis.* J Neuroinflammation, 2014. **11**: p. 130.

153. Wang, X., et al., *Role of TRIF Small Interference RNA (siRNA) in Chronic Experimental Allergic Encephalomyelitis (EAE).* Med Sci Monit, 2015. **21**: p. 2583-7.
154. Natarajan, C., S.Y. Yao, and S. Sriram, *TLR3 Agonist Poly-IC Induces IL-33 and Promotes Myelin Repair.* PLoS One, 2016. **11**(3): p. e0152163.
155. Crowley, T., et al., *Modulation of TLR3/TLR4 inflammatory signaling by the GABAB receptor agonist baclofen in glia and immune cells: relevance to therapeutic effects in multiple sclerosis.* Front Cell Neurosci, 2015. **9**: p. 284.
156. Liu, Y., et al., *TLR2 and TLR4 in autoimmune diseases: a comprehensive review.* Clin Rev Allergy Immunol, 2014. **47**(2): p. 136-47.
157. Kroner, A., et al., *Impact of the Asp299Gly polymorphism in the toll-like receptor 4 (tlr-4) gene on disease course of multiple sclerosis.* J Neuroimmunol, 2005. **165**(1-2): p. 161-5.
158. Reynolds, J.M., et al., *Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation.* Proc Natl Acad Sci U S A, 2012. **109**(32): p. 13064-9.
159. Prinz, M., et al., *Innate immunity mediated by TLR9 modulates pathogenicity in an animal model of multiple sclerosis.* J Clin Invest, 2006. **116**(2): p. 456-64.
160. Andersson, A., et al., *Pivotal advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis.* J Leukoc Biol, 2008. **84**(5): p. 1248-55.
161. Marta, M., et al., *Unexpected regulatory roles of TLR4 and TLR9 in experimental autoimmune encephalomyelitis.* Eur J Immunol, 2008. **38**(2): p. 565-75.
162. Miranda-Hernandez, S., et al., *Role for MyD88, TLR2 and TLR9 but not TLR1, TLR4 or TLR6 in experimental autoimmune encephalomyelitis.* J Immunol, 2011. **187**(2): p. 791-804.
163. Chiurchiu, V., et al., *Modulation of monocytes by bioactive lipid anandamide in multiple sclerosis involves distinct Toll-like receptors.* Pharmacol Res, 2016. **113**(Pt A): p. 313-319.
164. Cusson-Hermance, N., et al., *Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation.* J Biol Chem, 2005. **280**(44): p. 36560-6.
165. Thanos, D. and T. Maniatis, *Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome.* Cell, 1995. **83**(7): p. 1091-100.
166. Noppert, S.J., K.A. Fitzgerald, and P.J. Hertzog, *The role of type I interferons in TLR responses.* Immunol Cell Biol, 2007. **85**(6): p. 446-57.
167. Clerico, M., G. Contessa, and L. Durelli, *Interferon-beta1a for the treatment of multiple sclerosis.* Expert Opin Biol Ther, 2007. **7**(4): p. 535-42.
168. Constantinescu, C.S., et al., *Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS).* Br J Pharmacol, 2011. **164**(4): p. 1079-106.
169. Shinohara, M.L., et al., *Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin.* Immunity, 2008. **29**(1): p. 68-78.

170. Yong, V.W., et al., *Interferon beta in the treatment of multiple sclerosis: mechanisms of action.* Neurology, 1998. **51**(3): p. 682-9.
171. Arbour, N., et al., *Upregulation of TRAIL expression on human T lymphocytes by interferon beta and glatiramer acetate.* Mult Scler, 2005. **11**(6): p. 652-7.
172. Wang, Q. and Y. Mao-Draayer, *Interferon beta (IFN-beta) treatment exerts potential neuroprotective effects through neurotrophic factors and novel neurotensin/neurotensin high affinity receptor 1 pathway.* Neural Regen Res, 2015. **10**(12): p. 1932-3.
173. van Baarsen, L.G., et al., *A subtype of multiple sclerosis defined by an activated immune defense program.* Genes Immun, 2006. **7**(6): p. 522-31.
174. Comabella, M., et al., *A type I interferon signature in monocytes is associated with poor response to interferon-beta in multiple sclerosis.* Brain, 2009. **132**(Pt 12): p. 3353-65.
175. Bustamante, M.F., et al., *Implication of the Toll-like receptor 4 pathway in the response to interferon-beta in multiple sclerosis.* Ann Neurol, 2011. **70**(4): p. 634-45.
176. Axtell, R.C., et al., *T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis.* Nat Med, 2010. **16**(4): p. 406-12.
177. Feng, X., et al., *Low expression of interferon-stimulated genes in active multiple sclerosis is linked to subnormal phosphorylation of STAT1.* J Neuroimmunol, 2002. **129**(1-2): p. 205-15.
178. van der Voort, L.F., et al., *Spontaneous MxA mRNA level predicts relapses in patients with recently diagnosed MS.* Neurology, 2010. **75**(14): p. 1228-33.
179. Johnston LD, O.M.P., Bachman HG, *Monitoring the Future National Results on Adolescent Drug Use: Overview of Key Findings, 2011.* Ann Arbor, MI: Institute for Social Research, The University of Michagan, 2012.
180. Mailleux, P. and J.J. Vanderhaeghen, *Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and in situ hybridization histochemistry.* Neuroscience, 1992. **48**(3): p. 655-68.
181. O'Shaughnessy, W., *New remedy for tetanus and other convulsive disorders.* The Boston Medical and Surgical Journal, 1840. **23**: p. 153-155.
182. Ryz, N.R., D.J. Remillard, and E.B. Russo, *Cannabis Roots: A Traditional Therapy with Future Potential for Treating Inflammation and Pain.* Cannabis Cannabinoid Res, 2017. **2**(1): p. 210-216.
183. Killestein, J., B.M. Uitdehaag, and C.H. Polman, *Cannabinoids in multiple sclerosis: do they have a therapeutic role?* Drugs, 2004. **64**(1): p. 1-11.
184. Downer, E.J., et al., *Identification of the synthetic cannabinoid R(+)WIN55,212-2 as a novel regulator of IFN regulatory factor 3 activation and IFN-beta expression: relevance to therapeutic effects in models of multiple sclerosis.* J Biol Chem, 2011. **286**(12): p. 10316-28.
185. Flores-Sanchez, I.J. and R. Verpoorte, *Secondary metabolism in cannabis.* Phytochemistry Reviews, 2008. **7**(3): p. 615-639.
186. Sirikantaramas, S., et al., *Recent advances in Cannabis sativa research: biosynthetic studies and its potential in biotechnology.* Curr Pharm Biotechnol, 2007. **8**(4): p. 237-43.

187. Fellermeier, M. and M.H. Zenk, *Prenylation of olivetolate by a hemp transferase yields cannabigerolic acid, the precursor of tetrahydrocannabinol.* FEBS Lett, 1998. **427**(2): p. 283-5.
188. Sirikantaramas, S., et al., *The gene controlling marijuana psychoactivity: molecular cloning and heterologous expression of Delta1-tetrahydrocannabinolic acid synthase from Cannabis sativa L.* J Biol Chem, 2004. **279**(38): p. 39767-74.
189. Taura, F., et al., *Production of Delta(1)-tetrahydrocannabinolic acid by the biosynthetic enzyme secreted from transgenic Pichia pastoris.* Biochem Biophys Res Commun, 2007. **361**(3): p. 675-80.
190. Taura, F., et al., *Cannabidiolic-acid synthase, the chemotype-determining enzyme in the fiber-type Cannabis sativa.* FEBS Lett, 2007. **581**(16): p. 2929-34.
191. Hill, A.J., et al., *Phytocannabinoids as novel therapeutic agents in CNS disorders.* Pharmacol Ther, 2012. **133**(1): p. 79-97.
192. Devinsky, O., et al., *Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders.* Epilepsia, 2014. **55**(6): p. 791-802.
193. El-Remessy, A.B., et al., *Neuroprotective effect of (-)Delta9-tetrahydrocannabinol and cannabidiol in N-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite.* Am J Pathol, 2003. **163**(5): p. 1997-2008.
194. Petrosino, S., et al., *Anti-inflammatory Properties of Cannabidiol, a Nonpsychotropic Cannabinoid, in Experimental Allergic Contact Dermatitis.* J Pharmacol Exp Ther, 2018. **365**(3): p. 652-663.
195. Sun, S., et al., *Cannabidiol attenuates OGD/R-induced damage by enhancing mitochondrial bioenergetics and modulating glucose metabolism via pentose-phosphate pathway in hippocampal neurons.* Redox Biol, 2017. **11**: p. 577-585.
196. Khaksar, S. and M.R. Bigdeli, *Anti-excitotoxic effects of cannabidiol are partly mediated by enhancement of NCX2 and NCX3 expression in animal model of cerebral ischemia.* Eur J Pharmacol, 2017. **794**: p. 270-279.
197. Nadal, X., et al., *Tetrahydrocannabinolic acid is a potent PPARgamma agonist with neuroprotective activity.* Br J Pharmacol, 2017. **174**(23): p. 4263-4276.
198. Verhoeckx, K.C., et al., *Unheated Cannabis sativa extracts and its major compound THC-acid have potential immuno-modulating properties not mediated by CB1 and CB2 receptor coupled pathways.* Int Immunopharmacol, 2006. **6**(4): p. 656-65.
199. Rock, E.M., et al., *Tetrahydrocannabinolic acid reduces nausea-induced conditioned gaping in rats and vomiting in Suncus murinus.* Br J Pharmacol, 2013. **170**(3): p. 641-8.
200. Bolognini, D., et al., *The plant cannabinoid Delta9-tetrahydrocannabivarin can decrease signs of inflammation and inflammatory pain in mice.* Br J Pharmacol, 2010. **160**(3): p. 677-87.
201. Garcia, C., et al., *Symptom-relieving and neuroprotective effects of the phytocannabinoid Delta(9)-THCV in animal models of Parkinson's disease.* Br J Pharmacol, 2011. **163**(7): p. 1495-506.
202. Rock, E.M., C.L. Limebeer, and L.A. Parker, *Effect of cannabidiolic acid and (9)-tetrahydrocannabinol on carrageenan-induced hyperalgesia and*

edema in a rodent model of inflammatory pain. Psychopharmacology (Berl), 2018. **235**(11): p. 3259-3271.
203. Ligresti, A., et al., *Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma.* J Pharmacol Exp Ther, 2006. **318**(3): p. 1375-87.
204. Morales, P., D.P. Hurst, and P.H. Reggio, *Molecular Targets of the Phytocannabinoids: A Complex Picture.* Prog Chem Org Nat Prod, 2017. **103**: p. 103-131.
205. Huizenga, M.N., A. Sepulveda-Rodriguez, and P.A. Forcelli, *Preclinical safety and efficacy of cannabidivarin for early life seizures.* Neuropharmacology, 2019. **148**: p. 189-198.
206. Rock, E.M., et al., *Evaluation of the potential of the phytocannabinoids, cannabidivarin (CBDV) and Delta(9) -tetrahydrocannabivarin (THCV), to produce CB1 receptor inverse agonism symptoms of nausea in rats.* Br J Pharmacol, 2013. **170**(3): p. 671-8.
207. Rosenthaler, S., et al., *Differences in receptor binding affinity of several phytocannabinoids do not explain their effects on neural cell cultures.* Neurotoxicol Teratol, 2014. **46**: p. 49-56.
208. Brierley, D.I., et al., *Cannabigerol is a novel, well-tolerated appetite stimulant in pre-satiated rats.* Psychopharmacology (Berl), 2016. **233**(19-20): p. 3603-13.
209. Gugliandolo, A., et al., *In Vitro Model of Neuroinflammation: Efficacy of Cannabigerol, a Non-Psychoactive Cannabinoid.* Int J Mol Sci, 2018. **19**(7).
210. Udoh, M., et al., *Cannabichromene is a cannabinoid CB2 receptor agonist.* Br J Pharmacol, 2019. **176**(23): p. 4537-4547.
211. De Petrocellis, L., et al., *Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8.* J Pharmacol Exp Ther, 2008. **325**(3): p. 1007-15.
212. Romano, B., et al., *The cannabinoid TRPA1 agonist cannabichromene inhibits nitric oxide production in macrophages and ameliorates murine colitis.* Br J Pharmacol, 2013. **169**(1): p. 213-29.
213. Appendino, G., et al., *Antibacterial cannabinoids from Cannabis sativa: a structure-activity study.* J Nat Prod, 2008. **71**(8): p. 1427-30.
214. DeLong, G.T., et al., *Pharmacological evaluation of the natural constituent of Cannabis sativa, cannabichromene and its modulation by Delta(9)-tetrahydrocannabinol.* Drug Alcohol Depend, 2010. **112**(1-2): p. 126-33.
215. Maione, S., et al., *Non-psychoactive cannabinoids modulate the descending pathway of antinociception in anaesthetized rats through several mechanisms of action.* Br J Pharmacol, 2011. **162**(3): p. 584-96.
216. El-Alfy, A.T., et al., *Antidepressant-like effect of delta9-tetrahydrocannabinol and other cannabinoids isolated from Cannabis sativa L.* Pharmacol Biochem Behav, 2010. **95**(4): p. 434-42.
217. Lu, H.C. and K. Mackie, *An Introduction to the Endogenous Cannabinoid System.* Biol Psychiatry, 2016. **79**(7): p. 516-25.
218. Porter, A.C. and C.C. Felder, *The endocannabinoid nervous system: unique opportunities for therapeutic intervention.* Pharmacol Ther, 2001. **90**(1): p. 45-60.
219. Showalter, V.M., et al., *Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of*

cannabinoid receptor subtype selective ligands. J Pharmacol Exp Ther, 1996. **278**(3): p. 989-99.
220. Pertwee, R.G., *The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin.* Br J Pharmacol, 2008. **153**(2): p. 199-215.
221. Matsuda, L.A., et al., *Structure of a cannabinoid receptor and functional expression of the cloned cDNA.* Nature, 1990. **346**(6284): p. 561-4.
222. Howlett, A.C., et al., *International Union of Pharmacology. XXVII. Classification of cannabinoid receptors.* Pharmacol Rev, 2002. **54**(2): p. 161-202.
223. Marsicano, G., et al., *CB1 cannabinoid receptors and on-demand defense against excitotoxicity.* Science, 2003. **302**(5642): p. 84-8.
224. Tsou, K., et al., *Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system.* Neuroscience, 1998. **83**(2): p. 393-411.
225. Croxford, J.L., *Therapeutic potential of cannabinoids in CNS disease.* CNS Drugs, 2003. **17**(3): p. 179-202.
226. Galiegue, S., et al., *Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations.* Eur J Biochem, 1995. **232**(1): p. 54-61.
227. Klegeris, A., C.J. Bissonnette, and P.L. McGeer, *Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor.* Br J Pharmacol, 2003. **139**(4): p. 775-86.
228. Van Sickle, M.D., et al., *Identification and functional characterization of brainstem cannabinoid CB2 receptors.* Science, 2005. **310**(5746): p. 329-32.
229. Onaivi, E.S., et al., *Functional expression of brain neuronal CB2 cannabinoid receptors are involved in the effects of drugs of abuse and in depression.* Ann N Y Acad Sci, 2008. **1139**: p. 434-49.
230. Gonsiorek, W., et al., *Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide.* Mol Pharmacol, 2000. **57**(5): p. 1045-50.
231. Luk, T., et al., *Identification of a potent and highly efficacious, yet slowly desensitizing CB1 cannabinoid receptor agonist.* Br J Pharmacol, 2004. **142**(3): p. 495-500.
232. Zygmunt, P.M., et al., *Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide.* Nature, 1999. **400**(6743): p. 452-7.
233. Bouaboula, M., et al., *Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation.* Eur J Pharmacol, 2005. **517**(3): p. 174-81.
234. Lauckner, J.E., et al., *GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2699-704.
235. Russo, E.B., et al., *Agonistic properties of cannabidiol at 5-HT1a receptors.* Neurochem Res, 2005. **30**(8): p. 1037-43.
236. Felder, C.C., et al., *Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors.* Mol Pharmacol, 1995. **48**(3): p. 443-50.

237. Kearn, C.S., et al., *Relationships between ligand affinities for the cerebellar cannabinoid receptor CB1 and the induction of GDP/GTP exchange.* J Neurochem, 1999. **72**(6): p. 2379-87.
238. Palazuelos, J., et al., *Non-psychoactive CB2 cannabinoid agonists stimulate neural progenitor proliferation.* FASEB J, 2006. **20**(13): p. 2405-7.
239. Gertsch, J., et al., *Echinacea alkylamides modulate TNF-alpha gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways.* FEBS Lett, 2004. **577**(3): p. 563-9.
240. Turu, G. and L. Hunyady, *Signal transduction of the CB1 cannabinoid receptor.* J Mol Endocrinol, 2010. **44**(2): p. 75-85.
241. Vogel, Z., et al., *Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase.* J Neurochem, 1993. **61**(1): p. 352-5.
242. Rueda, D., et al., *The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway.* J Biol Chem, 2002. **277**(48): p. 46645-50.
243. Roche, R., et al., *Presence of the cannabinoid receptors, CB1 and CB2, in human omental and subcutaneous adipocytes.* Histochem Cell Biol, 2006. **126**(2): p. 177-87.
244. Deadwyler, S.A., et al., *Cannabinoids modulate voltage sensitive potassium A-current in hippocampal neurons via a cAMP-dependent process.* J Pharmacol Exp Ther, 1995. **273**(2): p. 734-43.
245. Poling, J.S., et al., *Anandamide, an endogenous cannabinoid, inhibits Shaker-related voltage-gated K+ channels.* Neuropharmacology, 1996. **35**(7): p. 983-91.
246. Sugiura, T., et al., *2-Arachidonoylglycerol, a putative endogenous cannabinoid receptor ligand, induces rapid, transient elevation of intracellular free Ca2+ in neuroblastoma x glioma hybrid NG108-15 cells.* Biochem Biophys Res Commun, 1996. **229**(1): p. 58-64.
247. Okada, Y., et al., *Biophysical properties of voltage-gated Na+ channels in frog parathyroid cells and their modulation by cannabinoids.* J Exp Biol, 2005. **208**(Pt 24): p. 4747-56.
248. Cohen, K., A. Weizman, and A. Weinstein, *Modulatory effects of cannabinoids on brain neurotransmission.* Eur J Neurosci, 2019. **50**(3): p. 2322-2345.
249. Wiley, J.L., J.A. Marusich, and J.W. Huffman, *Moving around the molecule: relationship between chemical structure and in vivo activity of synthetic cannabinoids.* Life Sci, 2014. **97**(1): p. 55-63.
250. Brents, L.K., et al., *Monohydroxylated metabolites of the K2 synthetic cannabinoid JWH-073 retain intermediate to high cannabinoid 1 receptor (CB1R) affinity and exhibit neutral antagonist to partial agonist activity.* Biochem Pharmacol, 2012. **83**(7): p. 952-61.
251. Brents, L.K., et al., *Phase I hydroxylated metabolites of the K2 synthetic cannabinoid JWH-018 retain in vitro and in vivo cannabinoid 1 receptor affinity and activity.* PLoS One, 2011. **6**(7): p. e21917.
252. Castaneto, M.S., et al., *Synthetic cannabinoids: epidemiology, pharmacodynamics, and clinical implications.* Drug Alcohol Depend, 2014. **144**: p. 12-41.

253. Downer, E.J., et al., *The synthetic cannabinoid R(+)WIN55,212-2 augments interferon-beta expression via peroxisome proliferator-activated receptor-alpha.* J Biol Chem, 2012. **287**(30): p. 25440-53.
254. Ruparel, N.B., et al., *Desensitization of transient receptor potential ankyrin 1 (TRPA1) by the TRP vanilloid 1-selective cannabinoid arachidonoyl-2 chloroethanolamine.* Mol Pharmacol, 2011. **80**(1): p. 117-23.
255. Martino, G., et al., *Inflammation in multiple sclerosis: the good, the bad, and the complex.* Lancet Neurol, 2002. **1**(8): p. 499-509.
256. Perry, V.H., C. Cunningham, and C. Holmes, *Systemic infections and inflammation affect chronic neurodegeneration.* Nat Rev Immunol, 2007. **7**(2): p. 161-7.
257. Freeman, L.C. and J.P. Ting, *The pathogenic role of the inflammasome in neurodegenerative diseases.* J Neurochem, 2016. **136 Suppl 1**: p. 29-38.
258. Radtke, F.A., et al., *Modulating Neuroinflammation to Treat Neuropsychiatric Disorders.* Biomed Res Int, 2017. **2017**: p. 5071786.
259. Bjorklund, G., et al., *Immune dysfunction and neuroinflammation in autism spectrum disorder.* Acta Neurobiol Exp (Wars), 2016. **76**(4): p. 257-268.
260. Panikashvili, D., et al., *The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines.* Neurobiol Dis, 2006. **22**(2): p. 257-64.
261. Vrechi, T.A., et al., *Cannabinoid Receptor Type 1 Agonist ACEA Protects Neurons from Death and Attenuates Endoplasmic Reticulum Stress-Related Apoptotic Pathway Signaling.* Neurotox Res, 2018. **33**(4): p. 846-855.
262. Lyman, W.D., et al., *Delta 9-tetrahydrocannabinol: a novel treatment for experimental autoimmune encephalomyelitis.* J Neuroimmunol, 1989. **23**(1): p. 73-81.
263. Kozela, E., et al., *Cannabidiol inhibits pathogenic T cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in C57BL/6 mice.* Br J Pharmacol, 2011. **163**(7): p. 1507-19.
264. Croxford, J.L. and S.D. Miller, *Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R+WIN55,212.* J Clin Invest, 2003. **111**(8): p. 1231-40.
265. Correa, F., et al., *The endocannabinoid anandamide downregulates IL-23 and IL-12 subunits in a viral model of multiple sclerosis: evidence for a cross-talk between IL-12p70/IL-23 axis and IL-10 in microglial cells.* Brain Behav Immun, 2011. **25**(4): p. 736-49.
266. Deutsch, D.G. and S.A. Chin, *Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist.* Biochem Pharmacol, 1993. **46**(5): p. 791-6.
267. Di Marzo, V. and M. Maccarrone, *FAAH and anandamide: is 2-AG really the odd one out?* Trends Pharmacol Sci, 2008. **29**(5): p. 229-33.
268. Webb, M., et al., *Genetic deletion of Fatty Acid Amide Hydrolase results in improved long-term outcome in chronic autoimmune encephalitis.* Neurosci Lett, 2008. **439**(1): p. 106-10.
269. Downer, E.J., *Cannabinoids and innate immunity: taking a toll on neuroinflammation.* ScientificWorldJournal, 2011. **11**: p. 855-65.
270. Palazuelos, J., et al., *The CB(2) cannabinoid receptor controls myeloid progenitor trafficking: involvement in the pathogenesis of an animal model of multiple sclerosis.* J Biol Chem, 2008. **283**(19): p. 13320-9.

271. Jackson, S.J., et al., *Cannabinoid-receptor 1 null mice are susceptible to neurofilament damage and caspase 3 activation.* Neuroscience, 2005. **134**(1): p. 261-8.
272. Centonze, D., et al., *The endocannabinoid system is dysregulated in multiple sclerosis and in experimental autoimmune encephalomyelitis.* Brain, 2007. **130**(Pt 10): p. 2543-53.
273. Di Marzo, V., *The endocannabinoid system: its general strategy of action, tools for its pharmacological manipulation and potential therapeutic exploitation.* Pharmacol Res, 2009. **60**(2): p. 77-84.
274. Fitzpatrick, J.K. and E.J. Downer, *Toll-like receptor signalling as a cannabinoid target in Multiple Sclerosis.* Neuropharmacology, 2017. **113**(Pt B): p. 618-626.
275. Coffey, R.G., et al., *Inhibition of macrophage nitric oxide production by tetrahydrocannabinol in vivo and in vitro.* Int J Immunopharmacol, 1996. **18**(12): p. 749-52.
276. Zhu, W., H. Friedman, and T.W. Klein, *Delta9-tetrahydrocannabinol induces apoptosis in macrophages and lymphocytes: involvement of Bcl-2 and caspase-1.* J Pharmacol Exp Ther, 1998. **286**(2): p. 1103-9.
277. Raborn, E.S. and G.A. Cabral, *Cannabinoid inhibition of macrophage migration to the trans-activating (Tat) protein of HIV-1 is linked to the CB(2) cannabinoid receptor.* J Pharmacol Exp Ther, 2010. **333**(1): p. 319-27.
278. Massi, P., et al., *Relative involvement of cannabinoid CB(1) and CB(2) receptors in the Delta(9)-tetrahydrocannabinol-induced inhibition of natural killer activity.* Eur J Pharmacol, 2000. **387**(3): p. 343-7.
279. Do, Y., et al., *Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation.* J Immunol, 2004. **173**(4): p. 2373-82.
280. Napimoga, M.H., et al., *Cannabidiol decreases bone resorption by inhibiting RANK/RANKL expression and pro-inflammatory cytokines during experimental periodontitis in rats.* Int Immunopharmacol, 2009. **9**(2): p. 216-22.
281. Juknat, A., et al., *miRNA expression profiles and molecular networks in resting and LPS-activated BV-2 microglia-Effect of cannabinoids.* PLoS One, 2019. **14**(2): p. e0212039.
282. Juknat, A., et al., *Microarray and pathway analysis reveal distinct mechanisms underlying cannabinoid-mediated modulation of LPS-induced activation of BV-2 microglial cells.* PLoS One, 2013. **8**(4): p. e61462.
283. Kozela, E., et al., *Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells.* J Biol Chem, 2010. **285**(3): p. 1616-26.
284. Majdi, F., et al., *Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol may be effective against methamphetamine induced mitochondrial dysfunction and inflammation by modulation of Toll-like type-4(Toll-like 4) receptors and NF-kappaB signaling.* Med Hypotheses, 2019. **133**: p. 109371.

285. Duncan, M., et al., *Cannabinoid 1 receptors are critical for the innate immune response to TLR4 stimulation.* Am J Physiol Regul Integr Comp Physiol, 2013. **305**(3): p. R224-31.
286. Wilhelmsen, K., et al., *The endocannabinoid/endovanilloid N-arachidonoyl dopamine (NADA) and synthetic cannabinoid WIN55,212-2 abate the inflammatory activation of human endothelial cells.* J Biol Chem, 2014. **289**(19): p. 13079-100.
287. Murumalla, R., et al., *Effect of the Cannabinoid Receptor-1 antagonist SR141716A on human adipocyte inflammatory profile and differentiation.* J Inflamm (Lond), 2011. **8**: p. 33.
288. Baker, D., et al., *In silico patent searching reveals a new cannabinoid receptor.* Trends Pharmacol Sci, 2006. **27**(1): p. 1-4.
289. Henriquez, J.E., R.B. Crawford, and N.E. Kaminski, *Suppression of CpG-ODN-mediated IFNalpha and TNFalpha response in human plasmacytoid dendritic cells (pDC) by cannabinoid receptor 2 (CB2)-specific agonists.* Toxicol Appl Pharmacol, 2019. **369**: p. 82-89.
290. Rizzo, M.D., et al., *Delta(9)-Tetrahydrocannabinol Suppresses Monocyte-Mediated Astrocyte Production of Monocyte Chemoattractant Protein 1 and Interleukin-6 in a Toll-Like Receptor 7-Stimulated Human Coculture.* J Pharmacol Exp Ther, 2019. **371**(1): p. 191-201.
291. Chiurchiu, V., et al., *Distinct modulation of human myeloid and plasmacytoid dendritic cells by anandamide in multiple sclerosis.* Ann Neurol, 2013. **73**(5): p. 626-36.
292. Chiurchiu, V., et al., *The differential characterization of GPR55 receptor in human peripheral blood reveals a distinctive expression in monocytes and NK cells and a proinflammatory role in these innate cells.* Int Immunol, 2015. **27**(3): p. 153-60.
293. Walter, L. and N. Stella, *Cannabinoids and neuroinflammation.* Br J Pharmacol, 2004. **141**(5): p. 775-85.
294. Puffenbarger, R.A., A.C. Boothe, and G.A. Cabral, *Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells.* Glia, 2000. **29**(1): p. 58-69.
295. Henry, R.J., et al., *FAAH-mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus.* J Neuroimmunol, 2014. **276**(1-2): p. 126-34.
296. Flannery, L.E., et al., *FAAH, but not MAGL, inhibition modulates acute TLR3-induced neuroimmune signaling in the rat, independent of sex.* J Neurosci Res, 2018. **96**(6): p. 989-1001.
297. Flannery, L.E., et al., *FAAH inhibition attenuates TLR3-mediated hyperthermia, nociceptive- and anxiety-like behaviour in female rats.* Behav Brain Res, 2018. **353**: p. 11-20.
298. Corcoran, L., et al., *Attenuation of fear-conditioned analgesia in rats by monoacylglycerol lipase inhibition in the anterior cingulate cortex: Potential role for CB2 receptors.* Br J Pharmacol, 2020. **177**(10): p. 2240-2255.
299. Marchalant, Y., S. Rosi, and G.L. Wenk, *Anti-inflammatory property of the cannabinoid agonist WIN-55212-2 in a rodent model of chronic brain inflammation.* Neuroscience, 2007. **144**(4): p. 1516-22.

300. Carlisle, S.J., et al., *Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation.* Int Immunopharmacol, 2002. **2**(1): p. 69-82.
301. Palsson-McDermott, E.M. and L.A.J. O'Neill, *Targeting immunometabolism as an anti-inflammatory strategy.* Cell Res, 2020. **30**(4): p. 300-314.
302. Auciello, F.R., et al., *Oxidative stress activates AMPK in cultured cells primarily by increasing cellular AMP and/or ADP.* FEBS Lett, 2014. **588**(18): p. 3361-6.
303. Marin, T.L., et al., *AMPK promotes mitochondrial biogenesis and function by phosphorylating the epigenetic factors DNMT1, RBBP7, and HAT1.* Sci Signal, 2017. **10**(464).
304. Saxton, R.A. and D.M. Sabatini, *mTOR Signaling in Growth, Metabolism, and Disease.* Cell, 2017. **168**(6): p. 960-976.
305. Gwinn, D.M., et al., *AMPK phosphorylation of raptor mediates a metabolic checkpoint.* Mol Cell, 2008. **30**(2): p. 214-26.
306. Ahmadian, M., et al., *Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype.* Cell Metab, 2011. **13**(6): p. 739-48.
307. Marsin, A.S., et al., *Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia.* Curr Biol, 2000. **10**(20): p. 1247-55.
308. Egan, D.F., et al., *Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy.* Science, 2011. **331**(6016): p. 456-61.
309. Dong, J., et al., *Nesfatin-1 stimulates fatty-acid oxidation by activating AMP-activated protein kinase in STZ-induced type 2 diabetic mice.* PLoS One, 2013. **8**(12): p. e83397.
310. Yu, L., et al., *Melatonin ameliorates myocardial ischemia/reperfusion injury in type 1 diabetic rats by preserving mitochondrial function: role of AMPK-PGC-1alpha-SIRT3 signaling.* Sci Rep, 2017. **7**: p. 41337.
311. O'Neill, L.A., R.J. Kishton, and J. Rathmell, *A guide to immunometabolism for immunologists.* Nat Rev Immunol, 2016. **16**(9): p. 553-65.
312. Vara, D., et al., *Anti-tumoral action of cannabinoids on hepatocellular carcinoma: role of AMPK-dependent activation of autophagy.* Cell Death Differ, 2011. **18**(7): p. 1099-111.
313. Dando, I., et al., *Cannabinoids inhibit energetic metabolism and induce AMPK-dependent autophagy in pancreatic cancer cells.* Cell Death Dis, 2013. **4**: p. e664.
314. Tedesco, L., et al., *Cannabinoid receptor stimulation impairs mitochondrial biogenesis in mouse white adipose tissue, muscle, and liver: the role of eNOS, p38 MAPK, and AMPK pathways.* Diabetes, 2010. **59**(11): p. 2826-36.
315. Chan, L.N., et al., *Metabolic gatekeeper function of B-lymphoid transcription factors.* Nature, 2017. **542**(7642): p. 479-483.
316. Ke, P., et al., *Activation of Cannabinoid Receptor 2 Ameliorates DSS-Induced Colitis through Inhibiting NLRP3 Inflammasome in Macrophages.* PLoS One, 2016. **11**(9): p. e0155076.
317. Downer, E.J., M.P. Fogarty, and V.A. Campbell, *Tetrahydrocannabinol-induced neurotoxicity depends on CB1 receptor-mediated c-Jun N-terminal*

kinase activation in cultured cortical neurons. Br J Pharmacol, 2003. **140**(3): p. 547-57.

318. Downer, E.J., A. Gowran, and V.A. Campbell, *A comparison of the apoptotic effect of Delta(9)-tetrahydrocannabinol in the neonatal and adult rat cerebral cortex.* Brain Res, 2007. **1175**: p. 39-47.
319. Schultze, N., et al., *Mitochondrial functions of THP-1 monocytes following the exposure to selected natural compounds.* Toxicology, 2017. **377**: p. 57-63.
320. Schwarz, H., F.J. Blanco, and M. Lotz, *Anadamide, an endogenous cannabinoid receptor agonist inhibits lymphocyte proliferation and induces apoptosis.* J Neuroimmunol, 1994. **55**(1): p. 107-15.
321. Sanchez, A.J., et al., *R-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzox azin-6-yl]-1-naphtalenylmethanone (WIN-2) ameliorates experimental autoimmune encephalomyelitis and induces encephalitogenic T cell apoptosis: partial involvement of the CB(2) receptor.* Biochem Pharmacol, 2006. **72**(12): p. 1697-706.
322. Pertwee, R.G., *Cannabinoids and multiple sclerosis.* Pharmacol Ther, 2002. **95**(2): p. 165-74.
323. Zajicek, J., et al., *Cannabinoids for treatment of spasticity and other symptoms related to multiple sclerosis (CAMS study): multicentre randomised placebo-controlled trial.* Lancet, 2003. **362**(9395): p. 1517-26.
324. Einhorn, L.H., et al., *Nabilone: an effective antiemetic in patients receiving cancer chemotherapy.* J Clin Pharmacol, 1981. **21**(S1): p. 64S-69S.
325. Badowski, M.E. and P.K. Yanful, *Dronabinol oral solution in the management of anorexia and weight loss in AIDS and cancer.* Ther Clin Risk Manag, 2018. **14**: p. 643-651.
326. Grotenhermen, F. and K. Muller-Vahl, *The therapeutic potential of cannabis and cannabinoids.* Dtsch Arztebl Int, 2012. **109**(29-30): p. 495-501.
327. Svendsen, K.B., T.S. Jensen, and F.W. Bach, *Does the cannabinoid dronabinol reduce central pain in multiple sclerosis? Randomised double blind placebo controlled crossover trial.* BMJ, 2004. **329**(7460): p. 253.
328. Thiele, E.A., et al., *Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial.* Lancet, 2018. **391**(10125): p. 1085-1096.
329. Rekand, T., *THC:CBD spray and MS spasticity symptoms: data from latest studies.* Eur Neurol, 2014. **71 Suppl 1**: p. 4-9.
330. Novotna, A., et al., *A randomized, double-blind, placebo-controlled, parallel-group, enriched-design study of nabiximols* (Sativex((R))), as add-on therapy, in subjects with refractory spasticity caused by multiple sclerosis.* Eur J Neurol, 2011. **18**(9): p. 1122-31.
331. Rog, D.J., et al., *Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis.* Neurology, 2005. **65**(6): p. 812-9.
332. Stott, C.G., et al., *A phase I study to assess the single and multiple dose pharmacokinetics of THC/CBD oromucosal spray.* Eur J Clin Pharmacol, 2013. **69**(5): p. 1135-47.
333. Lund, M.E., et al., *The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus.* J Immunol Methods, 2016. **430**: p. 64-70.

334. Moore, F., et al., *Two Multiple Sclerosis Quality-of-Life Measures: Comparison in a National Sample.* Can J Neurol Sci, 2015. **42**(1): p. 55-63.
335. Vickrey, B.G., et al., *Comparison of a generic to disease-targeted health-related quality-of-life measures for multiple sclerosis.* J Clin Epidemiol, 1997. **50**(5): p. 557-69.
336. Vickrey, B.G., et al., *A health-related quality of life measure for multiple sclerosis.* Qual Life Res, 1995. **4**(3): p. 187-206.
337. Rush, A.J., et al., *The 16-Item Quick Inventory of Depressive Symptomatology (QIDS), clinician rating (QIDS-C), and self-report (QIDS-SR): a psychometric evaluation in patients with chronic major depression.* Biol Psychiatry, 2003. **54**(5): p. 573-83.
338. Reinhart, W.H., *The optimum hematocrit.* Clin Hemorheol Microcirc, 2016. **64**(4): p. 575-585.
339. Maner, B.S. and L. Moosavi, *Mean Corpuscular Volume (MCV)*, in *StatPearls*. 2020: Treasure Island (FL).
340. Sarma, P.R., *Red Cell Indices*, in *Clinical Methods: The History, Physical, and Laboratory Examinations*, rd, et al., Editors. 1990: Boston.
341. Liu, G. and Y. Zhao, *Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells.* Immunology, 2007. **122**(2): p. 149-56.
342. Hanke, M.L. and T. Kielian, *Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential.* Clin Sci (Lond), 2011. **121**(9): p. 367-87.
343. Ketloy, C., et al., *Expression and function of Toll-like receptors on dendritic cells and other antigen presenting cells from non-human primates.* Vet Immunol Immunopathol, 2008. **125**(1-2): p. 18-30.
344. Randall, P.A., et al., *The Toll-Like Receptor 3 Agonist Poly(I:C) Induces Rapid and Lasting Changes in Gene Expression Related to Glutamatergic Function and Increases Ethanol Self-Administration in Rats.* Alcohol Clin Exp Res, 2019. **43**(1): p. 48-60.
345. Kunzmann, V., et al., *Polyinosinic-polycytidylic acid-mediated stimulation of human gammadelta T cells via CD11c dendritic cell-derived type I interferons.* Immunology, 2004. **112**(3): p. 369-77.
346. Kumar, A., J. Zhang, and F.S. Yu, *Toll-like receptor 3 agonist poly(I:C)-induced antiviral response in human corneal epithelial cells.* Immunology, 2006. **117**(1): p. 11-21.
347. Honda, K., A. Takaoka, and T. Taniguchi, *Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors.* Immunity, 2006. **25**(3): p. 349-60.
348. Tanaka, H. and T. Imaizumi, *Inflammatory chemokine expression via Toll-like receptor 3 signaling in normal human mesangial cells.* Clin Dev Immunol, 2013. **2013**: p. 984708.
349. Guijarro-Munoz, I., et al., *Lipopolysaccharide activates Toll-like receptor 4 (TLR4)-mediated NF-kappaB signaling pathway and proinflammatory response in human pericytes.* J Biol Chem, 2014. **289**(4): p. 2457-68.
350. Chanput, W., et al., *Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds.* Food Funct, 2010. **1**(3): p. 254-61.

351. van der Bruggen, T., et al., *Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the raf-1/MEK1-MEK2/ERK1-ERK2 pathway.* Infect Immun, 1999. **67**(8): p. 3824-9.
352. Jacobs, A.T. and L.J. Ignarro, *Lipopolysaccharide-induced expression of interferon-beta mediates the timing of inducible nitric-oxide synthase induction in RAW 264.7 macrophages.* J Biol Chem, 2001. **276**(51): p. 47950-7.
353. Vogel, D.Y., et al., *Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status.* J Neuroinflammation, 2013. **10**: p. 35.
354. Yanai, H., et al., *Revisiting the role of IRF3 in inflammation and immunity by conditional and specifically targeted gene ablation in mice.* Proc Natl Acad Sci U S A, 2018. **115**(20): p. 5253-5258.
355. Autissier, P., et al., *Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans.* Cytometry A, 2010. **77**(5): p. 410-9.
356. Haller, D., et al., *Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria in vitro: evidence of NK cells as primary targets.* Infect Immun, 2000. **68**(2): p. 752-9.
357. Green, T.L., et al., *Toll-like receptor (TLR) expression of immune system cells from metastatic breast cancer patients with circulating tumor cells.* Exp Mol Pathol, 2014. **97**(1): p. 44-8.
358. Kielian, T., *Toll-like receptors in central nervous system glial inflammation and homeostasis.* J Neurosci Res, 2006. **83**(5): p. 711-30.
359. Gooshe, M., et al., *The role of Toll-like receptors in multiple sclerosis and possible targeting for therapeutic purposes.* Rev Neurosci, 2014. **25**(5): p. 713-39.
360. Qiu, F., et al., *Activation of cytokine-producing and antitumor activities of natural killer cells and macrophages by engagement of Toll-like and NOD-like receptors.* Innate Immun, 2011. **17**(4): p. 375-87.
361. Ackerman, M.E., et al., *A robust, high-throughput assay to determine the phagocytic activity of clinical antibody samples.* J Immunol Methods, 2011. **366**(1-2): p. 8-19.
362. Zhou, Y., et al., *TLR3 activation efficiency by high or low molecular mass poly I:C.* Innate Immun, 2013. **19**(2): p. 184-92.
363. Li, Y.G., et al., *Poly (I:C), an agonist of toll-like receptor-3, inhibits replication of the Chikungunya virus in BEAS-2B cells.* Virol J, 2012. **9**: p. 114.
364. Imaizumi, T., et al., *Melanoma differentiation-associated gene 5 regulates the expression of a chemokine CXCL10 in human mesangial cells: implications for chronic inflammatory renal diseases.* Tohoku J Exp Med, 2012. **228**(1): p. 17-26.
365. Sykes, A., et al., *TLR3, TLR4 and TLRs7-9 Induced Interferons Are Not Impaired in Airway and Blood Cells in Well Controlled Asthma.* PLoS One, 2013. **8**(6): p. e65921.
366. Reimer, T., et al., *poly(I:C) and LPS induce distinct IRF3 and NF-kappaB signaling during type-I IFN and TNF responses in human macrophages.* J Leukoc Biol, 2008. **83**(5): p. 1249-57.

367. Wandinger, K.P., et al., *Diminished production of type-I interferons and interleukin-2 in patients with multiple sclerosis.* J Neurol Sci, 1997. **149**(1): p. 87-93.
368. Proost, P., et al., *Microbial Toll-like receptor ligands differentially regulate CXCL10/IP-10 expression in fibroblasts and mononuclear leukocytes in synergy with IFN-gamma and provide a mechanism for enhanced synovial chemokine levels in septic arthritis.* Eur J Immunol, 2003. **33**(11): p. 3146-53.
369. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.
370. Shahrara, S., et al., *RANTES modulates TLR4-induced cytokine secretion in human peripheral blood monocytes.* J Immunol, 2006. **177**(8): p. 5077-87.
371. Sawa, Y., et al., *LPS-induced IL-6, IL-8, VCAM-1, and ICAM-1 expression in human lymphatic endothelium.* J Histochem Cytochem, 2008. **56**(2): p. 97-109.
372. Tamassia, N., et al., *Molecular mechanisms underlying the synergistic induction of CXCL10 by LPS and IFN-gamma in human neutrophils.* Eur J Immunol, 2007. **37**(9): p. 2627-34.
373. Toshchakov, V., et al., *TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages.* Nat Immunol, 2002. **3**(4): p. 392-8.
374. Zhang, D., *Lipopolysaccharide (LPS) of Porphyromonas gingivalis induces IL-1b, TNF and IL-6 production by THP-1 cells in a way different from that of Escherichia coli LPS.* Innate Immunity, 2008(14(2)): p. 99-107.
375. Werts, C., et al., *Nod1 and Nod2 induce CCL5/RANTES through the NF-kappaB pathway.* Eur J Immunol, 2007. **37**(9): p. 2499-508.
376. Lin, R., et al., *Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription.* Mol Cell Biol, 1999. **19**(2): p. 959-66.
377. Meier, C.A., et al., *IP-10, but not RANTES, is upregulated by leptin in monocytic cells.* Cytokine, 2003. **21**(1): p. 43-7.
378. Mishra, K.P., et al., *Hypoxic Stress Induced TREM-1 and Inflammatory Chemokines in Human Peripheral Blood Mononuclear Cells.* Indian J Clin Biochem, 2014. **29**(2): p. 133-8.
379. Lu, Y.C., W.C. Yeh, and P.S. Ohashi, *LPS/TLR4 signal transduction pathway.* Cytokine, 2008. **42**(2): p. 145-151.
380. Kato, A., et al., *Lipopolysaccharide-binding protein critically regulates lipopolysaccharide-induced IFN-beta signaling pathway in human monocytes.* J Immunol, 2004. **172**(10): p. 6185-94.
381. Antonia, A.L., et al., *Pathogen Evasion of Chemokine Response Through Suppression of CXCL10.* Front Cell Infect Microbiol, 2019. **9**: p. 280.
382. Tsai, J.H., et al., *Effects of antidepressants on IP-10 production in LPS-activated THP-1 human monocytes.* Int J Mol Sci, 2014. **15**(8): p. 13223-35.
383. Shang, V.C., D.A. Kendall, and R.E. Roberts, *Delta(9)-Tetrahydrocannabinol reverses TNFalpha-induced increase in airway epithelial cell permeability through CB2 receptors.* Biochem Pharmacol, 2016. **120**: p. 63-71.

384. Vella, R.K., D.J. Jackson, and A.S. Fenning, *Delta(9)-Tetrahydrocannabinol Prevents Cardiovascular Dysfunction in STZ-Diabetic Wistar-Kyoto Rats.* Biomed Res Int, 2017. **2017**: p. 7974149.
385. Gilbert, G.L., et al., *Delta9-tetrahydrocannabinol protects hippocampal neurons from excitotoxicity.* Brain Res, 2007. **1128**(1): p. 61-9.
386. Iversen, L., *The science of marijuana.* 2000. **New York: Oxford University Press.**
387. Iwamura, H., et al., *In vitro and in vivo pharmacological characterization of JTE-907, a novel selective ligand for cannabinoid CB2 receptor.* J Pharmacol Exp Ther, 2001. **296**(2): p. 420-5.
388. Rinaldi-Carmona, M., et al., *SR141716A, a potent and selective antagonist of the brain cannabinoid receptor.* FEBS Lett, 1994. **350**(2-3): p. 240-4.
389. Ibeas Bih, C., et al., *Molecular Targets of Cannabidiol in Neurological Disorders.* Neurotherapeutics, 2015. **12**(4): p. 699-730.
390. Demuth, D.G. and A. Molleman, *Cannabinoid signalling.* Life Sci, 2006. **78**(6): p. 549-63.
391. Giacoppo, S., et al., *Target regulation of PI3K/Akt/mTOR pathway by cannabidiol in treatment of experimental multiple sclerosis.* Fitoterapia, 2017. **116**: p. 77-84.
392. Chung, E.S., et al., *Cannabinoids prevent lipopolysaccharide-induced neurodegeneration in the rat substantia nigra in vivo through inhibition of microglial activation and NADPH oxidase.* Brain Res, 2012. **1451**: p. 110-6.
393. Molina-Holgado, F., A. Lledo, and C. Guaza, *Anandamide suppresses nitric oxide and TNF-alpha responses to Theiler's virus or endotoxin in astrocytes.* Neuroreport, 1997. **8**(8): p. 1929-33.
394. Molina-Holgado, F., et al., *Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures.* J Neurosci Res, 2002. **67**(6): p. 829-36.
395. Facchinetti, F., et al., *Cannabinoids ablate release of TNFalpha in rat microglial cells stimulated with lypopolysaccharide.* Glia, 2003. **41**(2): p. 161-8.
396. Howlett, A.C., *Cannabinoid receptor signaling.* Handb Exp Pharmacol, 2005(168): p. 53-79.
397. Jiang, S., et al., *Expression and function of cannabinoid receptors CB1 and CB2 and their cognate cannabinoid ligands in murine embryonic stem cells.* PLoS One, 2007. **2**(7): p. e641.
398. Zygmunt, P.M., D.A. Andersson, and E.D. Hogestatt, *Delta 9-tetrahydrocannabinol and cannabinol activate capsaicin-sensitive sensory nerves via a CB1 and CB2 cannabinoid receptor-independent mechanism.* J Neurosci, 2002. **22**(11): p. 4720-7.
399. Kaplan, B.L., C.E. Rockwell, and N.E. Kaminski, *Evidence for cannabinoid receptor-dependent and -independent mechanisms of action in leukocytes.* J Pharmacol Exp Ther, 2003. **306**(3): p. 1077-85.
400. Esposito, G., et al., *Cannabidiol reduces Abeta-induced neuroinflammation and promotes hippocampal neurogenesis through PPARgamma involvement.* PLoS One, 2011. **6**(12): p. e28668.
401. O'Sullivan, S.E., *An update on PPAR activation by cannabinoids.* Br J Pharmacol, 2016. **173**(12): p. 1899-910.

402. Williams, J.C., et al., *Delta(9)-Tetrahydrocannabinol (THC) enhances lipopolysaccharide-stimulated tissue factor in human monocytes and monocyte-derived microvesicles.* J Inflamm (Lond), 2015. **12**: p. 39.
403. Yamaori, S., et al., *Delta-Tetrahydrocannabinol induces cytotoxicity in macrophage J774-1 cells: involvement of cannabinoid receptor 2 and p38 MAPK.* Toxicology, 2013. **314**(2-3): p. 254-61.
404. Mato, S., M. Victoria Sanchez-Gomez, and C. Matute, *Cannabidiol induces intracellular calcium elevation and cytotoxicity in oligodendrocytes.* Glia, 2010. **58**(14): p. 1739-47.
405. Feliu, A., et al., *A Sativex((R)) -like combination of phytocannabinoids as a disease-modifying therapy in a viral model of multiple sclerosis.* Br J Pharmacol, 2015. **172**(14): p. 3579-95.
406. Osborne, A.L., et al., *Improved Social Interaction, Recognition and Working Memory with Cannabidiol Treatment in a Prenatal Infection (poly I:C) Rat Model.* Neuropsychopharmacology, 2017. **42**(7): p. 1447-1457.
407. Peres, F.F., et al., *Peripubertal treatment with cannabidiol prevents the emergence of psychosis in an animal model of schizophrenia.* Schizophr Res, 2016. **172**(1-3): p. 220-1.
408. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nature immunology, 2010. **11**(5): p. 373-84.
409. Ngaotepprutaram, T., B.L. Kaplan, and N.E. Kaminski, *Impaired NFAT and NFkappaB activation are involved in suppression of CD40 ligand expression by Delta(9)-tetrahydrocannabinol in human CD4(+) T cells.* Toxicol Appl Pharmacol, 2013. **273**(1): p. 209-18.
410. Hu, K., et al., *Alpinetin inhibits LPS-induced inflammatory mediator response by activating PPAR-gamma in THP-1-derived macrophages.* Eur J Pharmacol, 2013. **721**(1-3): p. 96-102.
411. Roy, R., et al., *Toll-like receptor 6 mediated inflammatory and functional responses of zinc oxide nanoparticles primed macrophages.* Immunology, 2014. **142**(3): p. 453-64.
412. Pandolfo, P., et al., *Cannabinoids inhibit the synaptic uptake of adenosine and dopamine in the rat and mouse striatum.* Eur J Pharmacol, 2011. **655**(1-3): p. 38-45.
413. Di Marzo, V., et al., *Formation and inactivation of endogenous cannabinoid anandamide in central neurons.* Nature, 1994. **372**(6507): p. 686-91.
414. Li, K., et al., *A role for O-1602 and G protein-coupled receptor GPR55 in the control of colonic motility in mice.* Neuropharmacology, 2013. **71**: p. 255-63.
415. Ramer, R., et al., *COX-2 and PPAR-gamma confer cannabidiol-induced apoptosis of human lung cancer cells.* Mol Cancer Ther, 2013. **12**(1): p. 69-82.
416. Kaplan, J.S., et al., *Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome.* Proc Natl Acad Sci U S A, 2017. **114**(42): p. 11229-11234.
417. Zeissler, M.L., et al., *Delta-9-tetrahydrocannabinol protects against MPP+ toxicity in SH-SY5Y cells by restoring proteins involved in mitochondrial biogenesis.* Oncotarget, 2016. **7**(29): p. 46603-46614.
418. Grotenhermen, F., *Pharmacokinetics and pharmacodynamics of cannabinoids.* Clin Pharmacokinet, 2003. **42**(4): p. 327-60.

419. Lehnardt, S., *Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury.* Glia, 2010. **58**(3): p. 253-63.
420. De Keyser, J., E. Zeinstra, and E. Frohman, *Are astrocytes central players in the pathophysiology of multiple sclerosis?* Arch Neurol, 2003. **60**(1): p. 132-6.
421. Elliott, D.M., et al., *Cannabidiol Attenuates Experimental Autoimmune Encephalomyelitis Model of Multiple Sclerosis Through Induction of Myeloid-Derived Suppressor Cells.* Front Immunol, 2018. **9**: p. 1782.
422. Al-Ghezi, Z.Z., et al., *Combination of Cannabinoids, Delta9-Tetrahydrocannabinol and Cannabidiol, Ameliorates Experimental Multiple Sclerosis by Suppressing Neuroinflammation Through Regulation of miRNA-Mediated Signaling Pathways.* Front Immunol, 2019. **10**: p. 1921.
423. Lamoureux, B.E., et al., *Using the QIDS-SR16 to identify major depressive disorder in primary care medical patients.* Behav Ther, 2010. **41**(3): p. 423-31.
424. Barry, A., et al., *Impact of short-term cycle ergometer training on quality of life, cognition and depressive symptomatology in multiple sclerosis patients: a pilot study.* Neurol Sci, 2018. **39**(3): p. 461-469.
425. Peng, Y.F., et al., *Assessment of the Relationship Between Red Cell Distribution Width and Multiple Sclerosis.* Medicine (Baltimore), 2015. **94**(29): p. e1182.
426. Sheremata, W.A., et al., *Evidence of platelet activation in multiple sclerosis.* J Neuroinflammation, 2008. **5**: p. 27.
427. Markova, J., et al., *Sativex((R)) as add-on therapy vs. further optimized first-line ANTispastics (SAVANT) in resistant multiple sclerosis spasticity: a double-blind, placebo-controlled randomised clinical trial.* Int J Neurosci, 2019. **129**(2): p. 119-128.
428. Fox, E.J., et al., *Lymphopenia and DMTs for relapsing forms of MS: Considerations for the treating neurologist.* Neurol Clin Pract, 2019. **9**(1): p. 53-63.
429. Tembe, N., et al., *Reference values for clinical laboratory parameters in young adults in Maputo, Mozambique.* PLoS One, 2014. **9**(5): p. e97391.
430. Solari, A., *Role of health-related quality of life measures in the routine care of people with multiple sclerosis.* Health Qual Life Outcomes, 2005. **3**: p. 16.
431. Mitchell, A.J., et al., *Quality of life and its assessment in multiple sclerosis: integrating physical and psychological components of wellbeing.* Lancet Neurol, 2005. **4**(9): p. 556-66.
432. Lehnardt, S., et al., *Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia.* J Neuroimmunol, 2007. **190**(1-2): p. 28-33.
433. Lampropoulou, V., et al., *TLR-activated B cells suppress T cell-mediated autoimmunity.* J Immunol, 2008. **180**(7): p. 4763-73.
434. Richard, K.L., et al., *Toll-like receptor 2 acts as a natural innate immune receptor to clear amyloid beta 1-42 and delay the cognitive decline in a mouse model of Alzheimer's disease.* J Neurosci, 2008. **28**(22): p. 5784-93.
435. Guo, B., E.Y. Chang, and G. Cheng, *The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice.* J Clin Invest, 2008. **118**(5): p. 1680-90.

436. Fujiwara, M., et al., *Enhanced TLR2 responses in multiple sclerosis.* Clin Exp Immunol, 2018. **193**(3): p. 313-326.
437. Baker, D., et al., *Cannabinoids control spasticity and tremor in a multiple sclerosis model.* Nature, 2000. **404**(6773): p. 84-7.
438. Giacoppo, S., P. Bramanti, and E. Mazzon, *Sativex in the management of multiple sclerosis-related spasticity: An overview of the last decade of clinical evaluation.* Mult Scler Relat Disord, 2017. **17**: p. 22-31.
439. ElSohly, M.A., et al., *Phytochemistry of Cannabis sativa L.* Prog Chem Org Nat Prod, 2017. **103**: p. 1-36.
440. Klein, T.W. and G.A. Cabral, *Cannabinoid-induced immune suppression and modulation of antigen-presenting cells.* J Neuroimmune Pharmacol, 2006. **1**(1): p. 50-64.
441. Croxford, J.L. and T. Yamamura, *Cannabinoids and the immune system: potential for the treatment of inflammatory diseases?* J Neuroimmunol, 2005. **166**(1-2): p. 3-18.
442. Rieder, S.A., et al., *Cannabinoid-induced apoptosis in immune cells as a pathway to immunosuppression.* Immunobiology, 2010. **215**(8): p. 598-605.
443. Lee, C.Y., et al., *A comparative study on cannabidiol-induced apoptosis in murine thymocytes and EL-4 thymoma cells.* Int Immunopharmacol, 2008. **8**(5): p. 732-40.
444. George, K.L., et al., *Ajulemic acid, a nonpsychoactive cannabinoid acid, suppresses osteoclastogenesis in mononuclear precursor cells and induces apoptosis in mature osteoclast-like cells.* J Cell Physiol, 2008. **214**(3): p. 714-20.
445. Derocq, J.M., et al., *Cannabinoids enhance human B-cell growth at low nanomolar concentrations.* FEBS Lett, 1995. **369**(2-3): p. 177-82.
446. Hardie, D.G., F.A. Ross, and S.A. Hawley, *AMPK: a nutrient and energy sensor that maintains energy homeostasis.* Nat Rev Mol Cell Biol, 2012. **13**(4): p. 251-62.
447. Inoki, K., J. Kim, and K.L. Guan, *AMPK and mTOR in cellular energy homeostasis and drug targets.* Annu Rev Pharmacol Toxicol, 2012. **52**: p. 381-400.
448. Lu, L., et al., *Exocytosis of MTT formazan could exacerbate cell injury.* Toxicol In Vitro, 2012. **26**(4): p. 636-44.
449. Bruni, N., et al., *Cannabinoid Delivery Systems for Pain and Inflammation Treatment.* Molecules, 2018. **23**(10).
450. Galvao, J., et al., *Unexpected low-dose toxicity of the universal solvent DMSO.* FASEB J, 2014. **28**(3): p. 1317-30.
451. Chen, J., et al., *Protective effects of Delta(9)-tetrahydrocannabinol against N-methyl-d-aspartate-induced AF5 cell death.* Brain Res Mol Brain Res, 2005. **134**(2): p. 215-25.
452. Tzavara, E.T., M. Wade, and G.G. Nomikos, *Biphasic effects of cannabinoids on acetylcholine release in the hippocampus: site and mechanism of action.* J Neurosci, 2003. **23**(28): p. 9374-84.
453. Oreja-Guevara, C., *Clinical efficacy and effectiveness of Sativex, a combined cannabinoid medicine, in multiple sclerosis-related spasticity.* Expert Rev Neurother, 2012. **12**(4 Suppl): p. 3-8.
454. Nabissi, M., et al., *Cannabinoids synergize with carfilzomib, reducing multiple myeloma cells viability and migration.* Oncotarget, 2016. **7**(47): p. 77543-77557.

455. Tubaro, A., et al., *Comparative topical anti-inflammatory activity of cannabinoids and cannabivarins.* Fitoterapia, 2010. **81**(7): p. 816-9.
456. Hill, A.J., et al., *Cannabidivarin is anticonvulsant in mouse and rat.* Br J Pharmacol, 2012. **167**(8): p. 1629-42.
457. Russo, C., et al., *Low doses of widely consumed cannabinoids (cannabidiol and cannabidivarin) cause DNA damage and chromosomal aberrations in human-derived cells.* Arch Toxicol, 2019. **93**(1): p. 179-188.
458. Fellous, T., et al., *Phytocannabinoids promote viability and functional adipogenesis of bone marrow-derived mesenchymal stem cells through different molecular targets.* Biochem Pharmacol, 2020. **175**: p. 113859.
459. Wang, M., et al., *Decarboxylation Study of Acidic Cannabinoids: A Novel Approach Using Ultra-High-Performance Supercritical Fluid Chromatography/Photodiode Array-Mass Spectrometry.* Cannabis Cannabinoid Res, 2016. **1**(1): p. 262-271.
460. Giacoppo, S., et al., *Cannabinoid CB2 receptors are involved in the protection of RAW264.7 macrophages against the oxidative stress: an in vitro study.* Eur J Histochem, 2017. **61**(1): p. 2749.
461. Granja, A.G., et al., *A cannabigerol quinone alleviates neuroinflammation in a chronic model of multiple sclerosis.* J Neuroimmune Pharmacol, 2012. **7**(4): p. 1002-16.
462. Carrillo-Salinas, F.J., et al., *A cannabigerol derivative suppresses immune responses and protects mice from experimental autoimmune encephalomyelitis.* PLoS One, 2014. **9**(4): p. e94733.
463. Shinjyo, N. and V. Di Marzo, *The effect of cannabichromene on adult neural stem/progenitor cells.* Neurochem Int, 2013. **63**(5): p. 432-7.
464. van Meerloo, J., G.J. Kaspers, and J. Cloos, *Cell sensitivity assays: the MTT assay.* Methods Mol Biol, 2011. **731**: p. 237-45.
465. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nat Cell Biol, 2011. **13**(2): p. 132-41.
466. Miranda-Hernandez, S. and A.G. Baxter, *Role of toll-like receptors in multiple sclerosis.* Am J Clin Exp Immunol, 2013. **2**(1): p. 75-93.
467. Fitzpatrick, J.M., et al., *MyD88-dependent and -independent signalling via TLR3 and TLR4 are differentially modulated by Delta(9)-tetrahydrocannabinol and cannabidiol in human macrophages.* J Neuroimmunol, 2020. **343**: p. 577217.
468. Barann, M., et al., *Direct inhibition by cannabinoids of human 5-HT3A receptors: probable involvement of an allosteric modulatory site.* Br J Pharmacol, 2002. **137**(5): p. 589-96.
469. De Petrocellis, L., et al., *Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes.* Br J Pharmacol, 2011. **163**(7): p. 1479-94.
470. De Petrocellis, L. and V. Di Marzo, *Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels.* J Neuroimmune Pharmacol, 2010. **5**(1): p. 103-21.
471. Pertwee, R.G., *Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists.* Curr Med Chem, 2010. **17**(14): p. 1360-81.
472. Kuhlmann, T., et al., *An updated histological classification system for multiple sclerosis lesions.* Acta Neuropathol, 2017. **133**(1): p. 13-24.

473. Rawji, K.S. and V.W. Yong, *The benefits and detriments of macrophages/microglia in models of multiple sclerosis.* Clin Dev Immunol, 2013. **2013**: p. 948976.
474. Almolda, B., B. Gonzalez, and B. Castellano, *Antigen presentation in EAE: role of microglia, macrophages and dendritic cells.* Front Biosci (Landmark Ed), 2011. **16**: p. 1157-71.
475. Jiang, H.R., et al., *IL-33 attenuates EAE by suppressing IL-17 and IFN-gamma production and inducing alternatively activated macrophages.* Eur J Immunol, 2012. **42**(7): p. 1804-14.
476. Gholamzad, M., et al., *A comprehensive review on the treatment approaches of multiple sclerosis: currently and in the future.* Inflamm Res, 2019. **68**(1): p. 25-38.
477. Fischer-Stenger, K., D.A. Dove Pettit, and G.A. Cabral, *Delta 9-tetrahydrocannabinol inhibition of tumor necrosis factor-alpha: suppression of post-translational events.* J Pharmacol Exp Ther, 1993. **267**(3): p. 1558-65.
478. Srivastava, M.D., B.I. Srivastava, and B. Brouhard, *Delta9 tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells.* Immunopharmacology, 1998. **40**(3): p. 179-85.
479. Berdyshev, E., et al., *Effects of cannabinoid receptor ligands on LPS-induced pulmonary inflammation in mice.* Life Sci, 1998. **63**(8): p. PL125-9.
480. Jamontt, J.M., et al., *The effects of Delta-tetrahydrocannabinol and cannabidiol alone and in combination on damage, inflammation and in vitro motility disturbances in rat colitis.* Br J Pharmacol, 2010. **160**(3): p. 712-23.

Appendices

Appendix 1

Peer-reviewed publications

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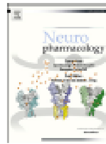

Invited review

Toll-like receptor signalling as a cannabinoid target in Multiple Sclerosis

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Toll-like receptors (TLRs) are the sensors of pathogen-associated molecules that trigger tailored innate immune intracellular signalling responses to initiate innate immune reactions. Data from the experimental autoimmune encephalomyelitis (EAE) model indicates that TLR signalling machinery is a pivotal player in the development of murine EAE. To compound this, data from human studies indicate that complex interplay exists between TLR signalling and Multiple Sclerosis (MS) pathogenesis. Cannabis-based therapies are in clinical development for the management of a variety of medical conditions, including MS. In particular Sativex®, a combination of plant-derived cannabinoids, is an oromucosal spray with efficacy in MS patients, particularly those with neuropathic pain and spasticity. Despite this, the precise cellular and molecular mechanisms of action of Sativex® in MS patients remains unclear. This review will highlight evidence that novel interplay exists between the TLR and cannabinoid systems, both centrally and peripherally, with relevance to the pathogenesis of MS.

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Contents

1. Introduction 618
2. Multiple Sclerosis 619
 2.1. MS pathogenesis 620
 2.2. Current treatments of MS 620
3. Innate immunity 620
 3.1. TLR signalling events 620
 3.2. Role of TLRs in MS 620
4. Cannabinoids 621
 4.1. Role of cannabinoids and MS 621
 4.2. Cross-talk between cannabinoid and TLR signalling: relevance to MS 622
5. Outlook 624
 Acknowledgements: 624
 References 624

1. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory autoimmune condition of the central nervous system (CNS) characterised by inflammatory episodes (relapses) that damage CNS myelin leading to neuronal dysfunction and a broad spectrum of neurological symptoms (Weiner, 2008). Hallmarks of the disease include demyelination, axonal loss, inflammation and gliosis (Bruck, 2005). Both the grey and white matter of the CNS are affected with concomitant cell death of neurons and axons (Vosoughi and Freedman, 2010). Common symptomology in the disease are diverse, and include spasticity (often associated with hyper-reflexia, muscle weakness and loss of dexterity), diplopia, bowel/

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bladder dysfunction, fatigue and cognitive disturbances (Gaby, 2013). MS is more common in women than men with a ratio of 2:1 observed, and the illness usually manifests in patients between the ages of 20 and 40 years, but cases have been reported at all stages of life (Popescu, 2014). It is estimated that more than 2 million people worldwide suffer from MS, and currently it is the primary cause of neurological disability in young adults (O'Connell et al., 2014).

Autoimmunity drives the development of MS, and both innate and adaptive arms of the immune system are pivotal in the progression of disease. The innate immune system is intricately controlled by a number of cell types including macrophages, dendritic cells (DC), natural killer (NK) cells and CNS glia, and a body of evidence continues to identify the role of innate immune cells in MS progression (Gandhi et al., 2010). Toll-Like Receptors (TLRs) are a major orchestrator of innate immunity, enabling the recognition of conserved microbial motifs by innate immune cells to tailor the cellular response to pathogens (Moynagh, 2005). Indeed, TLR signalling events continue to emerge as an important player in pro- and anti-inflammatory signalling events associated with the development of MS (Marta, 2009).

Cannabinoids incorporate the components of the cannabis plant, the endogenous cannabinoids and the synthetic cannabinoid ligands (Iversen, 2000). Cannabinoids elicit diverse effects by activating G protein–coupled cannabinoid receptors CB_1 and CB_2, the expression of which has been localised on glia, immune cells and neurons (Iversen, 2000). Indeed cannabinoid receptors have been identified on the major glial cells, in addition to virtually all immune cells associated with MS (Galiegue et al., 1995). Cannabinoids attenuate the development of disease in murine models of MS (Palazuelos et al., 2008), and furthermore, endocannabinoid concentration is altered in the cerebrospinal fluid and lymphocytes of MS patients, suggesting that the endocannabinoid system is altered in the disease (Centonze et al., 2007). In 2005 Canada became the first country to approve Sativex®, a combination of plant-derived cannabinoids tetrahydrocannabinol (THC) and cannabidiol (CBD), to alleviate MS-associated symptoms. Sativex® has efficacy in patients with central pain and spasticity, with no evidence of intoxication-like symptoms (Serpell et al., 2013).

This review is an update of the literature examining the treatment options of MS with regard to cannabinoids, and highlights potential avenues for cross-talk between the cannabinoid and TLR systems that may represent future therapeutic targets for investigation. Targeting TLR signalling cascades with cannabinoids in the CNS may indeed represent a novel avenue on the road to developing improved therapies for MS, and possibly other neuroinflammatory conditions.

2. Multiple Sclerosis

MS is associated with myelin degradation, axonal loss and reactive changes in glia (Compston and Coles, 2002) (MS pathophysiology summarised in Fig. 1). Research has highlighted the role of the adaptive, and more recently innate, immune systems in MS. Indeed, interferon (IFN)-γ secreting T helper 1 (Th1) cells, and/or IL-17 secreting "Th17" cells, infiltrate the CNS in MS, initiating an immune response that culminates in myelin destruction (Cua et al., 2003). Evidence also suggests that DC, microglia and astrocytes

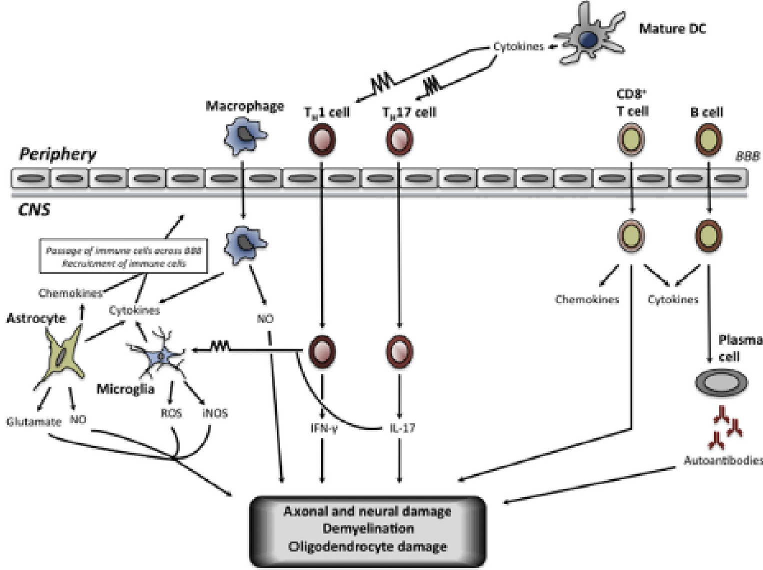

Fig. 1. A simplified view of the pathophysiology of MS. The infiltration of activated adaptive immune cells (T_H1 cells, T_H17 cells, $CD8^+$ T cells, B cells), alongside the activation of innate immune cells (DC, macrophages, microglia), play key roles in MS pathogenesis. BBB dysfunction is considered a central hallmark of MS pathogenesis, and is responsible for the invasion of the CNS by lymphocytes. The activation of autoimmune cells, resident microglia, astrocytes and macrophages, results in an immunological storm that involves abundant secretion of reactive species, cytokines, chemokines, autoantibody production and enhanced excitotoxicity. This results in damage to oligodendrocytes, demyelination and axonal damage and dysfunction.

display activated phenotypes in MS and regulate T cell activation, in addition to acting as producers of pro-inflammatory cytokines (Gandhi et al., 2010). Plasmacytoid DC (pDC), a subset of DC that act as a major producer of type-I IFNs, display impaired maturation in MS patients, which may impact the development of disease (Stasiolek et al., 2006). Hence, both innate and adaptive arms of the immune system are linked with disease progression, and are targets for therapeutic regulation.

2.1. MS pathogenesis

MS has been classified into four clinically distinct types: relapsing-remitting MS (RRMS), secondary-progressive MS (SPMS), primary-progressive MS (PPMS) and progressive-relapsing MS (PRMS). RRMS is the most common form of MS, representing approximately 80–85% of all cases (Compston and Coles, 2008). RRMS begins with a uni- or multi-focal demyelinating attack known as a clinically isolated syndrome (CIS) (Miller et al., 2012). Of the four types of clinical MS, most patients are first diagnosed with RRMS (Mowry, 2011). SPMS is a chronic phase characterised by attacks without recovery, leading to neurological impairments and consequently progressive physical deterioration of the patient (Trapp and Nave, 2008). Approximately 65% of RRMS patients will eventually transition to SPMS. PPMS is diagnosed in approximately 10–15% of patients at onset (Compston and Coles, 2008). PRMS is the least common subtype of MS, with approximately 5% of all cases of MS being categorised by this subtype. It is characterised by progressive neurological deterioration from the onset coupled by clear acute relapses with or without recovery (Thompson et al., 1997).

Despite much research the exact cellular and molecular mechanisms driving MS progression remains unclear (cellular mechanisms summarised in Fig. 1). However it is accepted that clinically observed hallmarks of MS are a consequence of three neural tissue injury mechanisms combining synergistically: inflammation, demyelination, and axonal damage (Compston and Coles, 2008; Trapp and Nave, 2008). The inflammatory lesions associated with MS contain T and B cells, macrophages and microglia, along with an extensive repertoire of cytokines, chemokines, antibodies and complement (Lucchinetti et al., 2011). Autoreactive T-lymphocytes, which are myelin-specific, are thought to underlie nervous system attack and commencement of disease progression (Compston and Coles, 2008). Microglia and astrocytes also contribute to inflammation observed in MS by releasing proteolytic enzymes, cytokines, oxidative products and free radicals, promoting toxicity towards oligodendrocytes and myelin (Block and Hong, 2005; Correale and Farez, 2015).

2.2. Current treatments of MS

There is no cure for MS, however a number of disease modifying agents have been approved which reduce disease activity, the frequency of relapses and long-term accrual of disability. Currently available immunomodulatory therapies include injectable medications such as interferon (IFN)-β-1a (Avonex® and Rebif®), IFN-β-1b (Betaseron®) and glatiramer acetate (copaxone), oral medications including fingolimod (gilenya®), dimethyl fumarate (tecfidera®) and teriflunomide (aubagio®), and infused medications such as alemtuzumab (lemtrada™) and natalizumab (tysabri®). Although the exact mechanism of action of many MS disease modifying agents is not fully elucidated, these medications are thought to act by several therapeutic mechanisms, via immunomodulation, restoration of the blood brain barrier (BBB) dysfunction and neuroprotection, and have been shown to reduce the rate of relapse and accrual of disability (Minagar, 2013). In terms of injectable therapy, IFN-β-1a and -1b exert effects on BBB permeability and the activation of lymphocytes, while glatiramer acetate binds major histocompatibility complex (MHC) molecules and competes with endogenous myelin antigens for T cell recognition. Oral medications are thought to act via several mechanisms, including acting as inhibitors of lymphocyte egress from lymph nodes thus reducing CNS infiltration (fingolimod), reducing oxidative stress, pro-inflammatory cytokine production and BBB dysfunction (dimethyl fumarate), and also acting as immunosuppressant's (teriflunomide). Evidence indicates that natalizumab inhibits T cell infiltration into the CNS while alemtuzumab is thought to deplete and repopulate T/B cells (Gold et al., 2012; Minagar, 2013).

Overall, IFN-β-1a and -1b (Avonex®, Rebif® and Betaseron®) are the most commonly used therapies for symptom management in MS, and recognised as first-line disease modifying agents (Harrison and Calabresi, 2009). Importantly, endogenous IFN-β insufficiency has been shown in MS patients (Croze et al., 2013). Again, a comprehensive mechanism(s) of therapeutic action of IFN-β is incompletely understood, but it is known that IFN-β has anti-inflammatory properties as well as effects on the BBB permeability (Minagar et al., 2012). Overall, much effort continues to be placed on the development of new medications for the treatment of MS symptoms and progression.

3. Innate immunity

Originally, the innate immune system was thought of as an inelegant precursor to the more sophisticated adaptive immune system. Immunologists regarded the innate immune system as the initiation event that took place to enable the mature adaptive immune response to confer its protective effect on the organism. The innate immune system is now recognised as the first line of host defence against pathogens, detecting the presence of infection, and regulating the initiation of the adaptive immune responses (Medzhitov, 2001). The discovery of DC, complement, and in particular TLRs, has sparked huge research interest in this field.

3.1. TLR signalling events

TLRs are pathogen-recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) from microorganisms or danger-associated molecular patterns (DAMPs) from damaged tissue. TLRs are expressed in immune cells and cells of the CNS. To date 10 functional TLR genes have been discovered in humans and 12 in mice, and receptor expression is localised on endosomal compartments and the cell membrane (Kawai and Akira, 2009). TLRs are categorised as a family of type I transmembrane receptors, and contain an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain (Rock et al., 1998). TLRs recognise both DAMPs and PAMPs and signal via several adaptor molecules (Medzhitov, 2001) to activate nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinases to induce target genes that function in host defence (Thoma-Uszynski et al., 2001). TLRs (apart from TLR3) recruit the adaptor molecule myeloid differentiation factor 88 (MyD88), while TLR3 promotes MyD88-independent signalling to regulate NF-κB via the TIR-domain–containing adaptor-inducing IFN-β (TRIF) adaptor protein (Medzhitov et al., 1998). TRIF-induced cell signalling constitutes the MyD88-independent pathway, and this cascade promotes the phosphorylation of IFN regulatory factors (IRFs) to promote induction of type-I IFNs (Fitzgerald et al., 2003).

3.2. Role of TLRs in MS

TLRs are continually emerging as important players in CNS

diseases, and a body of evidence indicates that TLR signalling is a key player in the pathogenesis of MS. Data indicate that TLRs have specific functions in neuroinflammatory disease (Hoffmann et al., 2007; O'Brien et al., 2008), and the expression of TLRs, and their related signalling proteins, has been characterised in CNS glia and neurons, in addition to immune cells (Nishimura and Naito, 2005). Indeed, TLR expression has been determined on glial cells in human CNS tissue derived from patients suffering from MS, and interestingly, changes in the TLR expression profiles has been determined in the neuroinflammed brain (Bsibsi et al., 2002; Guo et al., 2010) and immune cells in disease models (Andersson et al., 2008; Chearwae and Bright, 2008). Furthermore, TLR2 expression is upregulated in peripheral blood mononuclear cells (PBMCs) from MS patients, and in support of this, recent data from our laboratory demonstrate that PBMCs from RRMS patients are hypersensitive to TLR4 activation in terms of TNF-α production (Crowley et al., 2015).

Knockout studies using the murine model of MS, experimental autoimmune encephalomyelitis (EAE), have further elucidated the complex role of TLRs, and TLR signalling proteins, in neuroinflammatory events associated with MS. Indeed, TLR2 (Lehnardt et al., 2007), TLR9 (Prinz et al., 2006), MyD88 (Prinz et al., 2006; Lampropoulou et al., 2008; Marta et al., 2008) and IRF-3 (Fitzgerald et al., 2014) deficiency is protective in models of neuroinflammation, while TLR4 (Marta et al., 2008), TLR2 (Richard et al., 2008) and TRIF (Guo et al., 2008) deficiency has been shown to exacerbate disease, indicating the complex role of TLR pathways in inflammatory changes associated with the development of EAE. In addition, loss of TLR2 in $CD4^+$ T cells ameliorates EAE (Reynolds et al., 2010), while TLR7 (Hayashi et al., 2012) and TLR8 (Li et al., 2013) ligand treatment reduces the clinical development of EAE. Recently, data from Mellanby et al. (2012) indicates that TLR4-induced activation on DC promotes the function of pathogenic T cells in EAE (Mellanby et al., 2012), further supporting the intricate role of TLRs in EAE development.

Exogenous IFN-β is a current front-line therapy for MS, displaying beneficial effects on disability progression and relapse rate. Efficient production of IFN-β is orchestrated by TLRs, specifically, TLRs 3, 7, 8 and 9 localised on endosomal compartments where they recognise double stranded (ds)RNA, single stranded (ss)RNA and dsDNA (Jefferies and Fitzgerald, 2005). Importantly, data from the EAE model indicates that the signalling machinery regulating IFN-β expression is a pivotal player in the development of murine EAE. Indeed, knockout of TRIF, a receptor proximal adaptor for TLR3, exacerbates EAE (Guo et al., 2008), indicating that TRIF is protective in the model. To compound this, $IFN\text{-}\beta^{-/-}$ mice exhibit an earlier onset and a more rapid progression of EAE (Galligan et al., 2010), demonstrating that IFN-β suppresses the development of disease. Furthermore, treatment with recombinant IFN-β (Liu et al., 2010), the TLR3 agonist poly(I:C) (Touil et al., 2006) and the TLR7 agonist imiquimod (O'Brien et al., 2010), is protective in EAE, indicating that promoting IFN-β expression is a neuroprotective strategy in murine MS. In terms of human studies, complex interplay exists between IFN-β signalling and MS pathogenesis. However, few studies have clearly characterised the role of endogenous IFN-β in MS. This is important as data suggests an IFN-β insufficiency in MS, and endogenous IFN-β tone may predict clinical response to therapy (Croze et al., 2013). Indeed, lower expression of IFN-stimulated genes (ISGs) has been shown in patients with active MS (Feng et al., 2002).

4. Cannabinoids

Cannabis is a derivative of the Indian hemp plant *Cannabis Sativa* with a long history of use recreationally and medicinally in human populations (Mailleux and Vanderhaeghen, 1992). Structurally, cannabinoids are a set of over 60 oxygen-containing aromatic hydrocarbons (Killestein et al., 2004), and all parts of the male and female plant contain psychoactive cannabinoids. The most well known plant-derived cannabinoid is THC (the psychoactive component of cannabis). Furthermore, CBD (a non-psychoactive cannabinoid) is a major plant-derived cannabinoid of therapeutic potential (Devinsky et al., 2014). To date the two best understood endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Iversen, 2000). Cannabinoids act via G-protein-coupled cannabinoid receptors, and currently two cannabinoid receptors, CB_1 and CB_2, have been cloned and characterised (Porter and Felder, 2001). CB_1 is predominately expressed in the CNS (Matsuda et al., 1990) and has also been detected on cells of the immune system, testis, vascular endothelium, small intestine and peripheral nerve pre-synapses (Croxford, 2003). Conversely, the CB_2 receptor is found almost exclusively on immune cells (macrophages, mast cells, B and T lymphocytes) and immune organs (spleen, thymus, lymph nodes) (Howlett et al., 2002). However, some evidence exists that demonstrates that CB_2 is expressed on microglia of the CNS (Klegeris et al., 2003). In addition, there is some evidence, albeit limited, that CB_2 is expressed on neurons (Van Sickle et al., 2005). Following cannabinoid receptor activation, cannabinoids modulate multiple signal transduction pathways, with the biological outcome depending on both the cell type and the state of activation of other signalling mechanisms in the cell (Demuth and Molleman, 2006).

4.1. *Role of cannabinoids and MS*

Cannabinoids have distinct anti-inflammatory potential in neuroinflammatory conditions, particularly in models of inflammation: for reviews see (Downer, 2011; Hernangomez et al., 2014; Henry et al., 2016). Furthermore, a large body of data indicate that cannabinoids target the development of MS, particularly the development of EAE induced by myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), myelin proteolipid protein (PLP) or mouse spinal cord homogenate emulsified in Freund's complete adjuvant. For example, an early study by Lyman et al. (1989) assessed the effect of daily administration of THC on EAE progression in rats, starting several days prior to inoculation and continuing after EAE induction. Their findings indicate that the development of EAE was ameliorated, indicating that THC suppressed the development of disease (Lyman et al., 1989). Extensive research from Baker et al. (2000) has further elucidated the role of cannabinoids in EAE, demonstrating that THC, in addition to the synthetic cannabinoid *R*(+)WIN55,212, the CB_2 agonist JWH-133, and methanandamide (a stable AEA analogue) ameliorates tremor and spasticity in EAE mice (Baker et al., 2000). The protective role of the synthetic cannabinoid *R*(+)WIN55,212 has been supported elsewhere by several other laboratories (Downer et al., 2011; Hasseldam and Johansen, 2011; de Lago et al., 2012). Recent evidence also indicates that treatment with the CB_2 agonists HU-308 (Shao et al., 2014) and Gp1a (Kong et al., 2014) ameliorates the clinical development of EAE. Furthermore, exogenous administration of the endogenous cannabinoids AEA and 2-AG, the *N*-acylethanolamine palmitoylethanolamide (PEA) (Baker et al., 2001), and the non-psychotropic cannabinoid dexanabinol (HU-211) (Achiron et al., 2000), ameliorates the development of EAE, while inhibitors of AEA re-uptake are potent inhibitors of spasticity in EAE (Ligresti et al., 2006). In terms of plant cannabinoids, Kozela et al. (2011) have shown that CBD reduces the severity of EAE, and this was accompanied by diminished axonal damage and inflammation as well as microglial activation and T-cell recruitment in the spinal cord (Kozela et al., 2011). Interestingly, recent evidence indicates that co-administration of PEA with CBD in EAE was not as effective

as treatment with each drug alone, indicating that these non-psychoactive cannabinoids demonstrate antagonistic interactions in EAE (Rahimi et al., 2015).

Much data indicates that cannabinoids can also target the development of progressive forms of MS using murine models of disease. Indeed, using the Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) model, Ortega-Gutierrez et al. (2005) showed that UCM707, a selective AEA re-uptake inhibitor, improves motor function and reduced glial activation (Ortega-Gutierrez et al., 2005). In support of this, exogenous delivery of PEA reduces motor deficits in animals subjected to TMEV-IDD (Loria et al., 2008). More recent evidence also indicates that plant-derived cannabinoids cannabigerol (CBG) quinone (a CBG derivative) (Granja et al., 2012), CBD (Mecha et al., 2013), and a combination of CBD and THC (Feliu et al., 2015), improves motor activity while reducing microglial activation in TMEV-IDD. Furthermore, administration of cannabinoid synthetics including WIN55,212-2 (Arevalo-Martin et al., 2003; Croxford and Miller, 2003; Mestre et al., 2009), ACEA and JWH-015 (Arevalo-Martin et al., 2003) also improves motor behaviour in TMEV-IDD mice. Overall, these findings indicate that endogenous, plant-derived and synthetic cannabinoids can also target the development of progressive forms of MS using the TMEV-IDD model of disease.

Several knockout studies have further clarified the role of the endocannabinoid system in the development of EAE. Indeed, a body of research has demonstrated that CB_1 (Pryce et al., 2003; Pryce and Baker, 2007; Rossi et al., 2011) and CB_2 (Palazuelos et al., 2008) knock-out animals display enhanced clinical score and inflammation, indicating that the endogenous cannabinoid system plays a protective role in EAE. In support of this, mice lacking fatty acid amide hydrolase (FAAH), and hence expressing higher levels of AEA, develop less severe EAE (Rossi et al., 2011). Furthermore, Lou et al. (2016) have recently shown that over-expression of CB_1 in the lumbar spine, using a lentiviral vector, delayed the onset, and ameliorated the severity, of EAE (Lou et al., 2016), indicating that the endogenous cannabinoid system plays a neuroprotective role in EAE. Evidence that CB_1 is downregulated in motor-related brain regions during acute and chronic phases of EAE development (Cabranes et al., 2006) compounds this.

Overall, data from EAE studies indicates that the activation of the endocannabinoid system is a novel protective mechanism that reduces both neurodegenerative and neuroinflammatory aspects of MS. These findings in animal models translate to the clinic, as intricate findings from Centonze et al. (2007) indicate that the levels of AEA in cerebrospinal fluid and peripheral lymphocytes is enhanced in individuals with MS (Centonze et al., 2007). Furthermore, Sanchez Lopez et al. (2015) recently demonstrated that CB_1, CB_2 and AEA are enhanced in immune cells isolated from individuals with RRMS (Sanchez Lopez et al., 2015). In support of this, AEA levels are elevated in plasma of individuals with RRMS, SPMS and PPMS (Jean-Gilles et al., 2009), and in inflammatory lesions of patients with MS (Eljaschewitsch et al., 2006). In addition, post-mortem analysis indicates that CB_2 is abundantly expressed in endothelia in chronic inactive plaques in the MS brain (Zhang et al., 2011). The result of the combination of these studies on cannabinoids and MS, strongly links the endocannabinoid system with the pathophysiological mechanisms underlying MS progression, and offers potential for therapeutic interventions in the disease (Pertwee, 2002). Indeed, it is widely accepted that some MS patients self-medicate with cannabis and clinical trial evidence indicates that cannabis extracts can control and alleviate symptoms (Zajicek et al., 2003). Currently several cannabinoid-based therapies are in the clinic, including Cesamet® (nabilone; a synthetic derivative of THC), Marinol® (dronabinol; synthetic THC), and Sativex® (a combination of THC and CBD). Nabilone is prescribed for the treatment of nausea in patients undergoing cancer treatment, while dronabinol is used as a treatment of nausea for patients receiving chemotherapy and as an appetite stimulant for AIDs patients (Takeda, 2014). Sativex® is an important cannabinoid-based medication developed to target the neuroinflammatory events associated with MS. Sativex® contains a 1:1 mixture of THC and CBD and is prescribed for MS patients with moderate to severe spasticity (Rekand, 2014). Despite this, the precise cellular and molecular mechanisms of action of Sativex® in MS remain unclear.

4.2. Cross-talk between cannabinoid and TLR signalling: relevance to MS

TLRs are key players in the development of MS, while cannabinoids also have the proclivity to regulate the development of MS pathogenesis. Given the role of both TLR and cannabinoid signalling systems in MS development, several studies have begun to identify clear interplay between both systems that may have relevance to inflammation and the pathogenesis of disease (Table 1).

Data indicates that cross-talk exists between TLR and cannabinoid signalling, both in the CNS and in the periphery. Firstly, CB_1 has been shown to mediate the LPS-induced fever response (including LPS-induced hyperthermia, hyperalgesia and pro-inflammatory cytokine production in macrophages), indicating that this receptor is pivotal in mediating TLR4-induced febrile responses (Duncan et al., 2013). Furthermore, recent data demonstrating that LPS inhibits 2-AG hydrolysis in the liver/spleen indicates that the endocannabinoid system is switched on in response to bacterial stimuli to ameliorate inflammation (Szafran et al., 2015). Cross-talk also exists between TLR and cannabinoid signalling in endothelia and in adipocytes. Indeed, the synthetic cannabinoid *R*(+)WIN55,212-2 and endocannabinoid *N*-arachidonoyl dopamine (NADA), have been shown to ablate both LPS- and FSL-1 (TLR2/6 ligand)-induced pro-inflammatory cytokine expression in endothelia (Wilhelmsen et al., 2014), while Murumalla et al. (2011) have shown that TLR4-induced pro-inflammatory cytokine production in adipocytes is blocked by the CB_1 antagonist SR141716A, indicating the CB_1 regulates LPS-induced inflammation in adipocytes (Murumalla et al., 2011).

Secondly, a body of data has demonstrated cross-talk between cannabinoids and immune cell signalling and function. For example, THC inhibits TLR4-induced nitric oxide (NO) production (Coffey et al., 1996) and induces apoptosis (when co-cultured with LPS) (Zhu et al., 1998) in macrophages. The synthetic cannabinoid *R*(+)WIN55,212-2 has also been shown to prevent TLR3-induced inflammatory cytokine production in PBMCs isolated from healthy subjects and MS patients (Downer et al., 2011). Furthermore, exposure to *R*(+)WIN55,212-2 enhances IFN-β expression in MS patient, but not healthy subject, PBMCs, indicating the PBMCs isolated from MS patients are uniquely sensitive to *R*(+)WIN55,212-2 in terms of IFN-β expression (Downer et al., 2011). In a similar set of experiments, Chiurchiu et al. (2013) demonstrated that TLR7/8 activation promotes cytokine expression in myeloid DC isolated from healthy individuals and MS patients, and these effects are attenuated by both AEA and JWH-015 in a CB_2-dependent manner, thus suggesting the involvement of CB_2 in the modulation of inflammatory signalling induced by TLR7/8 (Chiurchiu et al., 2013). Recently, Chiurchiu et al. (2015) have also demonstrated that the novel cannabinoid receptor G protein-coupled receptor 55 (GPR55) potentiates LPS-induced pro-inflammatory cytokine expression in human monocytes (Chiurchiu et al., 2015), suggesting that GPR55-induced signalling modulates TLR4 signalling in immune cells.

Finally, several important findings indicate that cross-talk

Table 1
Literature assessing cross-talk between cannabinoid ligands, endocannabinoid signalling and TLR signalling.

Cannabinoid ligand/ endocannabinoid signalling	TLR	Human/Animal study	Observation	Reference
2-AG	TLR4	Animal	LPS inhibits 2-AG hydrolysis in liver/spleen	Szafran et al., 2015
CB_1	TLR4	Animal	Inhibits LPS-induced, hyperthermia, hyperalgesia and proinflammatory cytokine production in macrophages	Duncan et al., 2013
WIN/NADA	TLR2/ 4/6	Human	Inhibit LPS and FSL-1 inflammatory signalling in endothelia	Wilhelmsen et al., 2014
SR141716A	TLR4	Human	Reduced pro-inflammatory cytokines in adipocytes	Murumalla et al., 2011
THC	TLR4	Animal	Inhibits LPS-induced NO production in macrophages	Coffey et al., 1996
THC	TLR4	Animal	Promotes apoptosis in macrophages when co-cultured with LPS	Zhu et al., 1998
WIN	TLR3	Animal	Inhibits poly (I:C)-induced RANTES in astrocytes	Downer et al., 2012
W1N/HU-210	TLR4	Animal	Neuroprotective and anti-inflammatory in substantia nigra following LPS challenge	Chung et al., 2012
WIN	TLR3	Human	Inhibits poly (I:C)-induced inflammatory cytokine in PBMCs	Downer, 2011
AEA/JWH-015	TLR7/ 8	Human	Reduces RX4X-induced cytokine expression in myeloid DC	Chiurchiu et al., 2013
GPR55	TLR4	Human	Potentiates LPS-induced cytokine expression in monocytes	Chiurchiu et al., 2015
CBD	TLR4	Animal	Protects oligodendrocyte progenitors against LPS/IFN-γ-induced apoptosis	Mecha et al., 2013
FAAH	TLR3	Animal	URB597 regulates poly (I:C)-induced inflammatory genes in hippocampus	Henry et al., 2014
WIN	TLR4	Animal	Reduces LPS-induced microglial activation in hippocampus	Marchalant et al., 2007
MDA7	TLR2	Animal	Reduces TLR2 expression in spine	Xu et al., 2014
THC/AEA/methanandamide/CP	TLR4	Animal	Inhibits LPS-induced pro-inflammatory cytokines in microglia	Puffenbarger et al., 2000
AEA/WIN/ACPA/AM1214	TLR4	Animal	Inhibits LPS-induced activation of BV-2 microglia	Ribeiro et al., 2013
THC/CBD	TLR4	Animal	Inhibits LPS-induced pro-inflammatory cytokines in BV-2 cells	Kozela et al., 2010
2-AG	TLR2	Human	Inhibits PGN-induced inflammatory signalling in glioma cells	Echigo et al., 2012
WIN	TLR2	Human	Reduces PGN-induced cell growth in gliomas	Echigo et al., 2012
AM1214	TLR4	Animal	Reduces LPS/IFN-γ-induced microglial activation	Ma et al., 2015
THC/CBD	TLR4	Animal	Differentially regulate LPS-induced inflammatory signalling in BV-2 cells	Juknat et al., 2013
CD-101	TLR4	Animal	Reduces LPS-induced BV-2 activation	More et al., 2013
AEA/HU-210/CP	TLR4	Animal	Inhibit LPS-induced NO in astrocytes	Molina-Holgado et al., 2002 Molina-Holgado et al., 1997
AEA/2-AG/WIN/CP/HU-210	TLR4	Animal	Ablate LPS-induced TNF-a expressionin microglia	Facchinetti et al., 2003
CB2	TLR4	Animal	LPS reduces CB_2 expression in macrophages/microglia	Carlisle et al., 2002

CD-101, Novel cannabinoid derivative: CP, CP55,940; NADA, A-arachidonoyl dopamine: NO, nitric oxide: WIN, WIN55,212-2.

exists between cannabinoid and TLR signalling in the CNS. Indeed, recent data from Henry et al. (2014) demonstrates that TLR3 promotes the expression of neuroinflammatory mediators in the hippocampus, and these effects are regulated by the FAAH inhibitor URB597 (Henry et al., 2014). This indicates that cross-talk exists between TLR3 and endocannabinoid signalling with relevance to neuroinflammation in the rat *in vivo* (Henry et al., 2014). Data elsewhere indicates that administration of WIN55,212-2 attenuates the number of LPS-activated microglia in the rat hippocampus *in vivo* (Marchalant et al., 2007). Similarly, neuroprotective and anti-inflammatory properties of WIN55,212-2 and HU-210 have been shown following intranigral injection of LPS in nigral dopaminergic (DA) neurons and microglia, respectively (Chung et al., 2012). Using a model of peripheral neuropathy, Xu et al. (2014) have also shown that the CB_2 agonist MDA7 reduces TLR2 expression in the rat CNS, and the authors suggest that these findings indicate that the neuroprotective effects of CB_2 are linked to regulating the expression of innate immune genes in the spine (Xu et al., 2014). Indeed, LPS also reduces CB_2 expression on macrophages and microglia, indicating that CB_2 expression undergoes modulatory changes due to cell activation (Carlisle et al., 2002).

Cannabinoid receptors are expressed by the major glial cells (Walter and Stella, 2004) and there is much evidence that suggests that cannabinoids regulate TLR-induced inflammation in glia, with the outcome dependent on the cannabinoid tested (plant-derived, synthetic, endogenous), cannabinoid dose and the duration of cannabinoid administration. Studies in astrocytes indicate that HU-210, AEA and CP55,940 inhibits LPS-induced NO release from primary mouse astrocytes (Molina-Holgado et al., 1997; 2002), while *R*(+)WIN55,212-2 blunts TLR3-induced RANTES expression in murine astrocytes (Downer et al., 2012). A body of literature supports these findings in microglia. Indeed, THC, AEA, methanandamide and CP55,940 down-regulate pro-inflammatory gene expression induced by LPS in microglia (Puffenbarger et al., 2000). These findings are supported by Facchinetti et al. (2003), indicating that AEA, 2-AG, WIN55,212-2, CP55,940 and HU-210 inhibit LPS-induced TNF-α expression in primary rat microglia (Facchinetti et al., 2003). Elsewhere, AEA, WIN55,212-2, the CB_1 agonist ACPA, the CB_2 agonist AM1214 (Ribeiro et al., 2013), and the novel cannabinoid derivative CD-101, ablate LPS-induced BV-2 activation (More et al., 2013), while recent data also indicate that the CB_2 agonist AM1214 reduces microglial cell activation in the presence of LPS and IFN-γ (Ma et al., 2015). Similarly, plant-derived cannabinoids THC and CBD inhibit TLR4-induced inflammatory signalling in BV-2 microglia (Kozela et al., 2010), and more recent microarray analysis in microglia by the same group indicates that CBD and THC intricately regulate inflammatory signalling targets in response to LPS (Juknat et al., 2013). Using oligodendrocyte progenitor cells, Mecha et al. (2013) have also shown neuroprotective effects of CBD against LPS/IFN-γ-induced apoptosis (Mecha et al., 2013). Finally, 2-AG has been shown to inhibit TLR2-induced inflammatory signalling in human glioma cells, while R(+)WIN55,212-2 can reduce TLR2-activated cell growth in glioma cells, indicating that cannabinoids suppress inflammation and cell growth also in gliomas (Echigo et al., 2012).

5. Outlook

MS is a neurodegenerative disease affecting millions worldwide, yet there is no cure and the management of symptoms remains a clinical challenge. Current therapies are costly, are associated with side effects and are partly efficacious. Much research is needed to elucidate the clear mechanism(s) of action of currently approved treatment options, alongside the pathogenesis of the disease itself. Over the last 10–15 years, cannabinoids have emerged as therapeutic targets for MS, and are in the clinic for MS symptom management. This review has highlighted evidence that TLRs, the innate immune receptors, are key players in the development of MS. Given the clear role of both TLR and cannabinoid signalling mechanism in the development of MS pathogenesis, alongside evidence that cross-talk exists between both systems, targeting the TLR system with cannabinoids in MS may have therapeutic relevance to the pathogenesis of disease.

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References

Achiron, A., Miron, S., Lavie, V., Margalit, R., Biegon, A., 2000. Dexanabinol (HU-211) effect on experimental autoimmune encephalomyelitis: implications for the treatment of acute relapses of multiple sclerosis. J. Neuroimmunol. 102, 26–31.
Andersson, A., Covacu, R., Sunnemark, D., Danilov, A.I., Dal Bianco, A., Khademi, M., Wallstrom, E., Lobell, A., Brundin, L., Lassmann, H., Harris, R.A., 2008. Pivotal advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis. J. Leukoc. Biol. 84, 1248–1255.
Arevalo-Martin, A., Vela, J.M., Molina-Holgado, E., Borrell, J., Guaza, C., 2003. Therapeutic action of cannabinoids in a murine model of multiple sclerosis. J. Neurosci. 23, 2511–2516.
Baker, D., Pryce, G., Croxford, J.L., Brown, P., Pertwee, R.G., Huffman, J.W., Layward, L., 2000. Cannabinoids control spasticity and tremor in a multiple sclerosis model. Nature 404, 84–87.
Baker, D., Pryce, G., Croxford, J.L., Brown, P., Pertwee, R.G., Makriyannis, A., Khanolkar, A., Layward, L., Fezza, F., Bisogno, T., Di Marzo, V., 2001. Endocannabinoids control spasticity in a multiple sclerosis model. Faseb J. 15, 300–302.
Block, M.L., Hong, J.S., 2005. Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. Prog. Neurobiol. 76, 77–98.
Bruck, W., 2005. The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage. J. Neurol. 252 (Suppl. 5), v3–9.
Bsibsi, M., Ravid, R., Gveric, D., van Noort, J.M., 2002. Broad expression of Toll-like receptors in the human central nervous system. J. Neuropathol. Exp. Neurol. 61, 1013–1021.
Cabranes, A., Pryce, G., Baker, D., Fernandez-Ruiz, J., 2006. Changes in CB1 receptors in motor-related brain structures of chronic relapsing experimental allergic encephalomyelitis mice. Brain Res. 1107, 199–205.
Carlisle, S.J., Marciano-Cabral, F., Staab, A., Ludwick, C., Cabral, G.A., 2002. Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. Int. Immunopharmacol. 2, 69–82.
Centonze, D., Bari, M., Rossi, S., Prosperetti, C., Furlan, R., Fezza, F., De Chiara, V., Battistini, L., Bernardi, G., Bernardini, S., Martino, G., Maccarrone, M., 2007. The endocannabinoid system is dysregulated in multiple sclerosis and in experimental autoimmune encephalomyelitis. Brain 130, 2543–2553.
Chearwae, W., Bright, J.J., 2008. 15-deoxy-Delta(12,14)-prostaglandin J(2) and curcumin modulate the expression of toll-like receptors 4 and 9 in autoimmune T lymphocyte. J. Clin. Immunol. 28, 558–570.
Chiurchiu, V., Cencioni, M.T., Bisicchia, E., De Bardi, M., Gasperini, C., Borsellino, G., Centonze, D., Battistini, L., Maccarrone, M., 2013. Distinct modulation of human myeloid and plasmacytoid dendritic cells by anandamide in multiple sclerosis. Ann. Neurol. 73, 626–636.
Chiurchiu, V., Lanuti, M., De Bardi, M., Battistini, L., Maccarrone, M., 2015. The differential characterization of GPR55 receptor in human peripheral blood reveals a distinctive expression in monocytes and NK cells and a proinflammatory role in these innate cells. Int. Immunol. 27, 153–160.
Chung, E.S., Bok, E., Chung, Y.C., Baik, H.H., Jin, B.K., 2012. Cannabinoids prevent lipopolysaccharide-induced neurodegeneration in the rat substantia nigra in vivo through inhibition of microglial activation and NADPH oxidase. Brain Res. 1451, 110–116.
Coffey, R.G., Snella, E., Johnson, K., Pross, S., 1996. Inhibition of macrophage nitric oxide production by tetrahydrocannabinol in vivo and in vitro. Int. J. Immunopharmacol. 18, 749–752.
Compston, A., Coles, A., 2002. Multiple sclerosis. Lancet 359, 1221–1231.
Compston, A., Coles, A., 2008. Multiple sclerosis. Lancet 372, 1502–1517.
Correale, J., Farez, M.F., 2015. The role of astrocytes in multiple sclerosis progression. Front. Neurol. 6, 180.
Crowley, T., Fitzpatrick, J.M., Kuijper, T., Cryan, J.F., O'Toole, O., O'Leary, O.F., Downer, E.J., 2015. Modulation of TLR3/TLR4 inflammatory signaling by the GABAB receptor agonist baclofen in glia and immune cells: relevance to therapeutic effects in multiple sclerosis. Front. Cell Neurosci. 9, 284.
Croxford, J.L., 2003. Therapeutic potential of cannabinoids in CNS disease. CNS Drugs 17, 179–202.
Croxford, J.L., Miller, S.D., 2003. Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R+WIN55,212. J. Clin. Invest. 111, 1231–1240.
Croze, E., Yamaguchi, K.D., Knappertz, V., Reder, A.T., Salamon, H., 2013. Interferon-beta-1b-induced short- and long-term signatures of treatment activity in multiple sclerosis. Pharmacogenomics J. 13, 443–451.
Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S.A., Gorman, D., Kastelein, R.A., Sedgwick, J.D., 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421, 744–748.
de Lago, E., Moreno-Martet, M., Cabranes, A., Ramos, J.A., Fernandez-Ruiz, J., 2012. Cannabinoids ameliorate disease progression in a model of multiple sclerosis in mice, acting preferentially through CB1 receptor-mediated anti-inflammatory effects. Neuropharmacology 62, 2299–2308.
Demuth, D.G., Molleman, A., 2006. Cannabinoid signalling. Life Sci. 78, 549–563.
Devinsky, O., Cilio, M.R., Cross, H., Fernandez-Ruiz, J., French, J., Hill, C., Katz, R., Di Marzo, V., Jutras-Aswad, D., Notcutt, W.G., Martinez-Orgado, J., Robson, P.J., Rohrback, B.G., Thiele, E., Whalley, B., Friedman, D., 2014. Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. Epilepsia 55, 791–802.
Downer, E.J., 2011. Cannabinoids and innate immunity: taking a toll on neuroinflammation. ScientificWorldJournal 11, 855–865.
Downer, E.J., Clifford, E., Amu, S., Fallon, P.G., Moynagh, P.N., 2012. The synthetic cannabinoid R(+)WIN55,212-2 augments interferon-beta expression via peroxisome proliferator-activated receptor-alpha. J. Biol. Chem. 287, 25440–25453.
Downer, E.J., Clifford, E., Gran, B., Nel, H.J., Fallon, P.G., Moynagh, P.N., 2011. Identification of the synthetic cannabinoid R(+)WIN55,212-2 as a novel regulator of IFN regulatory factor 3 activation and IFN-beta expression: relevance to therapeutic effects in models of multiple sclerosis. J. Biol. Chem. 286, 10316–10328.
Duncan, M., Galic, M.A., Wang, A., Chambers, A.P., McCafferty, D.M., McKay, D.M., Sharkey, K.A., Pittman, Q.J., 2013. Cannabinoid 1 receptors are critical for the innate immune response to TLR4 stimulation. Am. J. Physiol. Regul. Integr. Comp. Physiol. 305, R224–R231.
Echigo, R., Sugimoto, N., Yachie, A., Ohno-Shosaku, T., 2012. Cannabinoids inhibit peptidoglycan-induced phosphorylation of NF-kappaB and cell growth in U87MG human malignant glioma cells. Oncol. Rep. 28, 1176–1180.
Eljaschewitsch, E., Witting, A., Mawrin, C., Lee, T., Schmidt, P.M., Wolf, S., Hoertnagl, H., Raine, C.S., Schneider-Stock, R., Nitsch, R., Ullrich, O., 2006. The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. Neuron 49, 67–79.
Facchinetti, F., Del Giudice, E., Furegato, S., Passarotto, M., Leon, A., 2003. Cannabinoids ablate release of TNFalpha in rat microglial cells stimulated with lypopolysaccharide. Glia 41, 161–168.
Feliu, A., Moreno-Martet, M., Mecha, M., Carrillo-Salinas, F.J., de Lago, E., Fernandez-Ruiz, J., Guaza, C., 2015. A Sativex((R)) -like combination of phytocannabinoids as a disease-modifying therapy in a viral model of multiple sclerosis. Br. J. Pharmacol. 172, 3579–3595.
Feng, X., Petraglia, A.L., Chen, M., Byskosh, P.V., Boos, M.D., Reder, A.T., 2002. Low expression of interferon-stimulated genes in active multiple sclerosis is linked to subnormal phosphorylation of STAT1. J. Neuroimmunol. 129, 205–215.
Fitzgerald, D.C., O'Brien, K., Young, A., Fonseca-Kelly, Z., Rostami, A., Gran, B., 2014. Interferon regulatory factor (IRF) 3 is critical for the development of experimental autoimmune encephalomyelitis. J. Neuroinflammation 11, 130.
Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., Coyle, A.J., Liao, S.M., Maniatis, T., 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat. Immunol. 4, 491–496.
Gaby, A., 2013. Multiple sclerosis. Glob. Adv. Health Med. 2, 50–56.
Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., Casellas, P., 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. Eur. J. Biochem. 232, 54–61.
Galligan, C.L., Pennell, L.M., Murooka, T.T., Baig, E., Majchrzak-Kita, B., Rahbar, R., Fish, E.N., 2010. Interferon-beta is a key regulator of proinflammatory events in experimental autoimmune encephalomyelitis. Mult. Scler. 16, 1458–1473.
Gandhi, R., Laroni, A., Weiner, H.L., 2010. Role of the innate immune system in the pathogenesis of multiple sclerosis. J. Neuroimmunol. 221, 7–14.
Gold, R., Kappos, L., Arnold, D.L., Bar-Or, A., Giovannoni, G., Selmaj, K., Tornatore, C.,

Sweetser, M.T., Yang, M., Sheikh, S.I., Dawson, K.T., Investigators, D.S., 2012. Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. N. Engl. J. Med. 367, 1098–1107.
Granja, A.G., Carrillo-Salinas, F., Pagani, A., Gomez-Canas, M., Negri, R., Navarrete, C., Mecha, M., Mestre, L., Fiebich, B.L., Cantarero, I., Calzado, M.A., Bellido, M.L., Fernandez-Ruiz, J., Appendino, G., Guaza, C., Munoz, E., 2012. A cannabigerol quinone alleviates neuroinflammation in a chronic model of multiple sclerosis. J. Neuroimmune Pharmacol. 7, 1002–1016.
Guo, B., Chang, E.Y., Cheng, G., 2008. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. J. Clin. Invest. 118, 1680–1690.
Guo, X., Harada, C., Namekata, K., Matsuzawa, A., Camps, M., Ji, H., Swinnen, D., Jorand-Lebrun, C., Muzerelle, M., Vitte, P.A., Ruckle, T., Kimura, A., Kohyama, K., Matsumoto, Y., Ichijo, H., Harada, T., 2010. Regulation of the severity of neuroinflammation and demyelination by TLR-ASK1-p38 pathway. EMBO Mol. Med. 2, 504–515.
Harrison, D.M., Calabresi, P.A., 2009. Promising treatments of tomorrow for multiple sclerosis. Ann. Indian Acad. Neurology 12, 283–290.
Hasseldam, H., Johansen, F.F., 2011. Cannabinoid treatment renders neurons less vulnerable than oligodendrocytes in experimental autoimmune encephalomyelitis. Int. J. Neurosci. 121, 510–520.
Hayashi, T., Yao, S., Crain, B., Chan, M., Tawatao, R.I., Gray, C., Vuong, L., Lao, F., Cottam, H.B., Carson, D.A., Corr, M., 2012. Treatment of autoimmune inflammation by a TLR7 ligand regulating the innate immune system. PLoS One 7, e45860.
Henry, R.J., Kerr, D.M., Finn, D.P., Roche, M., 2014. FAAH-mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus. J. Neuroimmunol. 276, 126–134.
Henry, R.J., Kerr, D.M., Finn, D.P., Roche, M., 2016. For whom the endocannabinoid tolls: modulation of innate immune function and implications for psychiatric disorders. Prog. Neuropsychopharmacol. Biol. Psychiatry 64, 167–180.
Hernangomez, M., Carrillo-Salinas, F.J., Mecha, M., Correa, F., Mestre, L., Loria, F., Feliu, A., Docagne, F., Guaza, C., 2014. Brain innate immunity in the regulation of neuroinflammation: therapeutic strategies by modulating CD200-CD200R interaction involve the cannabinoid system. Curr. Pharm. Des. 20, 4707–4722.
Hoffmann, O., Braun, J.S., Becker, D., Halle, A., Freyer, D., Dagand, E., Lehnardt, S., Weber, J.R., 2007. TLR2 mediates neuroinflammation and neuronal damage. J. Immunol. 178, 6476–6481.
Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R., Pertwee, R.G., 2002. International union of pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol. Rev. 54, 161–202.
Iversen, L., 2000. The Science of Marijuana. Oxford University Press, New York.
Jean-Gilles, L., Feng, S., Tench, C.R., Chapman, V., Kendall, D.A., Barrett, D.A., Constantinescu, C.S., 2009. Plasma endocannabinoid levels in multiple sclerosis. J. Neurol. Sci. 287, 212–215.
Jefferies, C.A., Fitzgerald, K.A., 2005. Interferon gene regulation: not all roads lead to tolls. Trends Mol. Med. 11, 403–411.
Juknat, A., Pietr, M., Kozela, E., Rimmerman, N., Levy, R., Gao, F., Coppola, G., Geschwind, D., Vogel, Z., 2013. Microarray and pathway analysis reveal distinct mechanisms underlying cannabinoid-mediated modulation of LPS-induced activation of BV-2 microglial cells. PLoS One 8, e61462.
Kawai, T., Akira, S., 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int. Immunol. 21, 317–337.
Killestein, J., Uitdehaag, B.M., Polman, C.H., 2004. Cannabinoids in multiple sclerosis: do they have a therapeutic role? Drugs 64, 1–11.
Klegeris, A., Bissonnette, C.J., McGeer, P.L., 2003. Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor. Br. J. Pharmacol. 139, 775–786.
Kong, W., Li, H., Tuma, R.F., Ganea, D., 2014. Selective CB2 receptor activation ameliorates EAE by reducing Th17 differentiation and immune cell accumulation in the CNS. Cell Immunol. 287, 1–17.
Kozela, E., Lev, N., Kaushansky, N., Eilam, R., Rimmerman, N., Levy, R., Ben-Nun, A., Juknat, A., Vogel, Z., 2011. Cannabidiol inhibits pathogenic T cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in C57BL/6 mice. Br. J. Pharmacol. 163, 1507–1519.
Kozela, E., Pietr, M., Juknat, A., Rimmerman, N., Levy, R., Vogel, Z., 2010. Cannabinoids delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells. J. Biol. Chem. 285, 1616–1626.
Lampropoulou, V., Hoehlig, K., Roch, T., Neves, P., Calderon Gomez, E., Sweenie, C.H., Hao, Y., Freitas, A.A., Steinhoff, U., Anderton, S.M., Fillatreau, S., 2008. TLR-activated B cells suppress T cell-mediated autoimmunity. J. Immunol. 180, 4763–4773.
Lehnardt, S., Lehmann, S., Kaul, D., Tschimmel, K., Hoffmann, O., Cho, S., Krueger, C., Nitsch, R., Meisel, A., Weber, J.R., 2007. Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia. J. Neuroimmunol. 190, 28–33.
Li, B., Baylink, D.J., Deb, C., Zannetti, C., Rajaallah, F., Xing, W., Walter, M.H., Lau, K.H., Qin, X., 2013. 1,25-Dihydroxyvitamin D3 suppresses TLR8 expression and TLR8-mediated inflammatory responses in monocytes in vitro and experimental autoimmune encephalomyelitis in vivo. PLoS One 8, e58808.
Ligresti, A., Cascio, M.G., Pryce, G., Kulasegram, S., Beletskaya, I., De Petrocellis, L., Saha, B., Mahadevan, A., Visintin, C., Wiley, J.L., Baker, D., Martin, B.R., Razdan, R.K., Di Marzo, V., 2006. New potent and selective inhibitors of anandamide reuptake with antispastic activity in a mouse model of multiple sclerosis. Br. J. Pharmacol. 147, 83–91.
Liu, Y., Teige, I., Ericsson, I., Navikas, V., Issazadeh-Navikas, S., 2010. Suppression of EAE by oral tolerance is independent of endogenous IFN-beta whereas treatment with recombinant IFN-beta ameliorates EAE. Immunol. Cell Biol. 88, 468–476.
Loria, F., Petrosino, S., Mestre, L., Spagnolo, A., Correa, F., Hernangomez, M., Guaza, C., Di Marzo, V., Docagne, F., 2008. Study of the regulation of the endocannabinoid system in a virus model of multiple sclerosis reveals a therapeutic effect of palmitoylethanolamide. Eur. J. Neurosci. 28, 633–641.
Lou, Z.Y., Yu, W.B., Chen, J., Li, L., Jiang, L.S., Xiao, B.G., Liu, Z.G., 2016. Neuroprotective effect is driven through the upregulation of CB1 receptor in experimental autoimmune encephalomyelitis. J. Mol. Neurosci. 58 (2), 193–200.
Lucchinetti, C.F., Popescu, B.F., Bunyan, R.F., Moll, N.M., Roemer, S.F., Lassmann, H., Bruck, W., Parisi, J.E., Scheithauer, B.W., Giannini, C., Weigand, S.D., Mandrekar, J., Ransohoff, R.M., 2011. Inflammatory cortical demyelination in early multiple sclerosis. N. Engl. J. Med. 365, 2188–2197.
Lyman, W.D., Sonett, J.R., Brosnan, C.F., Elkin, R., Bornstein, M.B., 1989. Delta 9-tetrahydrocannabinol: a novel treatment for experimental autoimmune encephalomyelitis. J. Neuroimmunol. 23, 73–81.
Ma, L., Jia, J., Liu, X., Bai, F., Wang, Q., Xiong, L., 2015. Activation of murine microglial N9 cells is attenuated through cannabinoid receptor CB2 signaling. Biochem. Biophys. Res. Commun. 458, 92–97.
Mailleux, P., Vanderhaeghen, J.J., 1992. Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and in situ hybridization histochemistry. Neuroscience 48, 655–668.
Marchalant, Y., Rosi, S., Wenk, G.L., 2007. Anti-inflammatory property of the cannabinoid agonist WIN-55212-2 in a rodent model of chronic brain inflammation. Neuroscience 144, 1516–1522.
Marta, M., 2009. Toll-like receptors in multiple sclerosis mouse experimental models. Ann. N. Y. Acad. Sci. 1173, 458–462.
Marta, M., Andersson, A., Isaksson, M., Kampe, O., Lobell, A., 2008. Unexpected regulatory roles of TLR4 and TLR9 in experimental autoimmune encephalomyelitis. Eur. J. Immunol. 38, 565–575.
Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.
Mecha, M., Feliu, A., Inigo, P.M., Mestre, L., Carrillo-Salinas, F.J., Guaza, C., 2013. Cannabidiol provides long-lasting protection against the deleterious effects of inflammation in a viral model of multiple sclerosis: a role for A2A receptors. Neurobiol. Dis. 59, 141–150.
Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nat. Rev. Immunol. 1, 135–145.
Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., Janeway Jr., C.A., 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol. Cell 2, 253–258.
Mellanby, R.J., Cambrook, H., Turner, D.G., O'Connor, R.A., Leech, M.D., Kurschus, F.C., MacDonald, A.S., Arnold, B., Anderton, S.M., 2012. TLR-4 ligation of dendritic cells is sufficient to drive pathogenic T cell function in experimental autoimmune encephalomyelitis. J. Neuroinflammation 9, 248.
Mestre, L., Docagne, F., Correa, F., Loria, F., Hernangomez, M., Borrell, J., Guaza, C., 2009. A cannabinoid agonist interferes with the progression of a chronic model of multiple sclerosis by downregulating adhesion molecules. Mol. Cell Neurosci. 40, 258–266.
Miller, D.H., Chard, D.T., Ciccarelli, O., 2012. Clinically isolated syndromes. Lancet Neurol. 11, 157–169.
Minagar, A., 2013. Current and future therapies for multiple sclerosis. Scientifica 2013, 249101.
Minagar, A., Maghzi, A.H., McGee, J.C., Alexander, J.S., 2012. Emerging roles of endothelial cells in multiple sclerosis pathophysiology and therapy. Neurological Res. 34, 738–745.
Molina-Holgado, F., Lledo, A., Guaza, C., 1997. Anandamide suppresses nitric oxide and TNF-alpha responses to Theiler's virus or endotoxin in astrocytes. Neuroreport 8, 1929–1933.
Molina-Holgado, F., Molina-Holgado, E., Guaza, C., Rothwell, N.J., 2002. Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures. J. Neurosci. Res. 67, 829–836.
More, S.V., Park, J.Y., Kim, B.W., Kumar, H., Lim, H.W., Kang, S.M., Koppula, S., Yoon, S.H., Choi, D.K., 2013. Anti-neuroinflammatory activity of a novel cannabinoid derivative by inhibiting the NF-kappaB signaling pathway in lipopolysaccharide-induced BV-2 microglial cells. J. Pharmacol. Sci. 121, 119–130.
Mowry, E.M., 2011. Natural history of multiple sclerosis: early prognostic factors. Neurol. Clin. 29, 279–292.
Moynagh, P.N., 2005. TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. Trends Immunol. 26, 469–476.
Murumalla, R., Bencharif, K., Gence, L., Bhattacharya, A., Tallet, F., Gonthier, M.P., Petrosino, S., di Marzo, V., Cesari, M., Hoareau, L., Roche, R., 2011. Effect of the cannabinoid receptor-1 antagonist SR141716A on human adipocyte inflammatory profile and differentiation. J. Inflamm. (Lond.) 8, 33.
Nishimura, M., Naito, S., 2005. Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. Biol. Pharm. Bull. 28, 886–892.
O'Brien, K., Fitzgerald, D., Rostami, A., Gran, B., 2010. The TLR7 agonist, imiquimod, increases IFN-beta production and reduces the severity of experimental autoimmune encephalomyelitis. J. Neuroimmunol. 221, 107–111.

O'Brien, K., Fitzgerald, D.C., Naiken, K., Alugupalli, K.R., Rostami, A.M., Gran, B., 2008. Role of the innate immune system in autoimmune inflammatory demyelination. Curr. Med. Chem. 15, 1105–1115.

O'Connell, K., Kelly, S.B., Fogarty, E., Duggan, M., Buckley, L., Hutchinson, M., McGuigan, C., Tubridy, N., 2014. Economic costs associated with an MS relapse. Multiple Scler. Relat. Disord. 3, 678–683.

Ortega-Gutierrez, S., Molina-Holgado, E., Arevalo-Martin, A., Correa, F., Viso, A., Lopez-Rodriguez, M.L., Di Marzo, V., Guaza, C., 2005. Activation of the endocannabinoid system as therapeutic approach in a murine model of multiple sclerosis. FASEB J. 19, 1338–1340.

Palazuelos, J., Davoust, N., Julien, B., Hatterer, E., Aguado, T., Mechoulam, R., Benito, C., Romero, J., Silva, A., Guzman, M., Nataf, S., Galve-Roperh, I., 2008. The CB(2) cannabinoid receptor controls myeloid progenitor trafficking: involvement in the pathogenesis of an animal model of multiple sclerosis. J. Biol. Chem. 283, 13320–13329.

Pertwee, R.G., 2002. Cannabinoids and multiple sclerosis. Pharmacol. Ther. 95, 165–174.

Popescu, C.D., 2014. Multiple sclerosis and pregnancy. Rev. medico-chirurgicala Soc. Medici si Nat. din Iasi 118, 28–32.

Porter, A.C., Felder, C.C., 2001. The endocannabinoid nervous system: unique opportunities for therapeutic intervention. Pharmacol. Ther. 90, 45–60.

Prinz, M., Garbe, F., Schmidt, H., Mildner, A., Gutcher, I., Wolter, K., Piesche, M., Schroers, R., Weiss, E., Kirschning, C.J., Rochford, C.D., Bruck, W., Becher, B., 2006. Innate immunity mediated by TLR9 modulates pathogenicity in an animal model of multiple sclerosis. J. Clin. Invest. 116, 456–464.

Pryce, G., Ahmed, Z., Hankey, D.J., Jackson, S.J., Croxford, J.L., Pocock, J.M., Ledent, C., Petzold, A., Thompson, A.J., Giovannoni, G., Cuzner, M.L., Baker, D., 2003. Cannabinoids inhibit neurodegeneration in models of multiple sclerosis. Brain 126, 2191–2202.

Pryce, G., Baker, D., 2007. Control of spasticity in a multiple sclerosis model is mediated by CB1, not CB2, cannabinoid receptors. Br. J. Pharmacol. 150, 519–525.

Puffenbarger, R.A., Boothe, A.C., Cabral, G.A., 2000. Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells. Glia 29, 58–69.

Rahimi, A., Faizi, M., Talebi, F., Noorbakhsh, F., Kahrizi, F., Naderi, N., 2015. Interaction between the protective effects of cannabidiol and palmitoylethanolamide in experimental model of multiple sclerosis in C57BL/6 mice. Neuroscience 290, 279–287.

Rekand, T., 2014. THC: CBD spray and MS spasticity symptoms: data from latest studies. Eur. Neurol. 71 (Suppl. 1), 4–9.

Reynolds, J.M., Pappu, B.P., Peng, J., Martinez, G.J., Zhang, Y., Chung, Y., Ma, L., Yang, X.O., Nurieva, R.I., Tian, Q., Dong, C., 2010. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. Immunity 32, 692–702.

Ribeiro, R., Wen, J., Li, S., Zhang, Y., 2013. Involvement of ERK1/2, cPLA2 and NF-kappaB in microglia suppression by cannabinoid receptor agonists and antagonists. Prostagl. Other Lipid Mediat. 100–101, 1–14.

Richard, K.L., Filali, M., Prefontaine, P., Rivest, S., 2008. Toll-like receptor 2 acts as a natural innate immune receptor to clear amyloid beta 1-42 and delay the cognitive decline in a mouse model of Alzheimer's disease. J. Neurosci. Official J. Soc. Neurosci. 28, 5784–5793.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A., Bazan, J.F., 1998. A family of human receptors structurally related to drosophila toll. Proc. Natl. Acad. Sci. U. S. A. 95, 588–593.

Rossi, S., Furlan, R., De Chiara, V., Muzio, L., Musella, A., Motta, C., Studer, V., Cavasinni, F., Bernardi, G., Martino, G., Cravatt, B.F., Lutz, B., Maccarrone, M., Centonze, D., 2011. Cannabinoid CB1 receptors regulate neuronal TNF-alpha effects in experimental autoimmune encephalomyelitis. Brain Behav. Immun. 25, 1242–1248.

Sanchez Lopez, A.J., Roman-Vega, L., Ramil Tojeiro, E., Giuffrida, A., Garcia-Merino, A., 2015. Regulation of cannabinoid receptor gene expression and endocannabinoid levels in lymphocyte subsets by interferon-beta: a longitudinal study in multiple sclerosis patients. Clin. Exp. Immunol. 179, 119–127.

Serpell, M.G., Notcutt, W., Collin, C., 2013. Sativex long-term use: an open-label trial in patients with spasticity due to multiple sclerosis. J. Neurol. 260 (1), 285–295.

Shao, B.Z., Wei, W., Ke, P., Xu, Z.Q., Zhou, J.X., Liu, C., 2014. Activating cannabinoid receptor 2 alleviates pathogenesis of experimental autoimmune encephalomyelitis via activation of autophagy and inhibiting NLRP3 inflammasome. CNS Neurosci. Ther. 20, 1021–1028.

Stasiolek, M., Bayas, A., Kruse, N., Wieczarkowiecz, A., Toyka, K.V., Gold, R., Selmaj, K., 2006. Impaired maturation and altered regulatory function of plasmacytoid dendritic cells in multiple sclerosis. Brain 129, 1293–1305.

Szafran, B., Borazjani, A., Lee, J.H., Ross, M.K., Kaplan, B.L., 2015. Lipopolysaccharide suppresses carboxylesterase 2g activity and 2-arachidonoylglycerol hydrolysis: a possible mechanism to regulate inflammation. Prostagl. Other Lipid Mediat. 121 (Pt B), 199–206.

Takeda, S., 2014. Delta(9)-tetrahydrocannabinol targeting estrogen receptor signaling: the possible mechanism of action coupled with endocrine disruption. Biol. Pharm. Bull. 37, 1435–1438.

Thoma-Uszynski, S., Stenger, S., Takeuchi, O., Ochoa, M.T., Engele, M., Sieling, P.A., Barnes, P.F., Rollinghoff, M., Bolcskei, P.L., Wagner, M., Akira, S., Norgard, M.V., Belisle, J.T., Godowski, P.J., Bloom, B.R., Modlin, R.L., 2001. Induction of direct antimicrobial activity through mammalian toll-like receptors. Science 291, 1544–1547.

Thompson, A.J., Polman, C.H., Miller, D.H., McDonald, W.I., Brochet, B., Filippi, M.M.X., De Sa, J., 1997. Primary progressive multiple sclerosis. Brain 120 (Pt 6), 1085–1096.

Touil, T., Fitzgerald, D., Zhang, G.X., Rostami, A., Gran, B., 2006. Cutting edge: TLR3 stimulation suppresses experimental autoimmune encephalomyelitis by inducing endogenous IFN-beta. J. Immunol. 177, 7505–7509.

Trapp, B.D., Nave, K.A., 2008. Multiple sclerosis: an immune or neurodegenerative disorder? Annu. Rev. Neurosci. 31, 247–269.

Van Sickle, M.D., Duncan, M., Kingsley, P.J., Mouihate, A., Urbani, P., Mackie, K., Stella, N., Makriyannis, A., Piomelli, D., Davison, J.S., Marnett, L.J., Di Marzo, V., Pittman, Q.J., Patel, K.D., Sharkey, K.A., 2005. Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 310, 329–332.

Vosoughi, R., Freedman, M.S., 2010. Therapy of MS. Clin. Neurol. Neurosurg. 112, 365–385.

Walter, L., Stella, N., 2004. Cannabinoids and neuroinflammation. Br. J. Pharmacol. 141, 775–785.

Weiner, H.L., 2008. A shift from adaptive to innate immunity: a potential mechanism of disease progression in multiple sclerosis. J. Neurol. 255 (Suppl. 1), 3–11.

Wilhelmsen, K., Khakpour, S., Tran, A., Sheehan, K., Schumacher, M., Xu, F., Hellman, J., 2014. The endocannabinoid/endovanilloid N-arachidonoyl dopamine (NADA) and synthetic cannabinoid WIN55,212-2 abate the inflammatory activation of human endothelial cells. J. Biol. Chem. 289, 13079–13100.

Xu, J.J., Diaz, P., Bie, B., Astruc-Diaz, F., Wu, J., Yang, H., Brown, D.L., Naguib, M., 2014. Spinal gene expression profiling and pathways analysis of a CB2 agonist (MDA7)-targeted prevention of paclitaxel-induced neuropathy. Neuroscience 280, 185–194.

Zajicek, J., Fox, P., Sanders, H., Wright, D., Vickery, J., Nunn, A., Thompson, A., Group, U.M.R., 2003. Cannabinoids for treatment of spasticity and other symptoms related to multiple sclerosis (CAMS study): multicentre randomised placebo-controlled trial. Lancet 362, 1517–1526.

Zhang, H., Hilton, D.A., Hanemann, C.O., Zajicek, J., 2011. Cannabinoid receptor and N-acyl phosphatidylethanolamine phospholipase D–evidence for altered expression in multiple sclerosis. Brain Pathol. 21, 544–557.

Zhu, W., Friedman, H., Klein, T.W., 1998. Delta9-tetrahydrocannabinol induces apoptosis in macrophages and lymphocytes: involvement of Bcl-2 and caspase-1. J. Pharmacol. Exp. Ther. 286, 1103–1109.

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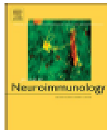

MyD88-dependent and -independent signalling via TLR3 and TLR4 are differentially modulated by Δ^9-tetrahydrocannabinol and cannabidiol in human macrophages

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ABSTRACT

Toll-like receptors (TLRs) are sensors of pathogen-associated molecules that trigger inflammatory signalling in innate immune cells including macrophages. All TLRs, with the exception of TLR3, promote intracellular signalling via recruitment of the myeloid differentiation factor 88 (MyD88) adaptor, while TLR3 signals via Toll-Interleukin-1 Receptor (TIR)-domain-containing adaptor-inducing interferon (IFN)-β (TRIF) adaptor to induce MyD88-independent signalling. Furthermore, TLR4 can activate both MyD88-dependent and –independent signalling (via TRIF). The study aim was to decipher the impact of the highly purified plant-derived (phyto) cannabinoids Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), when delivered in isolation and in combination (1:1), on MyD88-dependent and -independent signalling in macrophages. We employed the use of the viral dsRNA mimetic poly(I:C) and endotoxin lipopolysaccharide (LPS), to induce viral TLR3 and bacterial TLR4 signalling in human Tamm-Horsfall protein-1 (THP-1)-derived macrophages, respectively. TLR3/TLR4 stimulation promoted the activation of interferon (IFN) regulatory factor 3 (IRF3) and TLR4 promoted the activation of nuclear factor (NF)-κB signalling, with downstream production of the type I IFN-β, the chemokines CXCL10 and CXCL8, and cytokine TNF-α. THC and CBD (both at 10 μM) attenuated TLR3/4-induced IRF3 activation and induction of CXCL10/IFN-β, while both phytocannabinoids failed to impact TLR4-induced IκB-α degradation and TNF-α/CXCL8 expression. The role of CB_1, CB_2 and PPARγ receptors in mediating the effect of THC and CBD on MyD88-independent signalling was investigated. TLRs are attractive therapeutic targets given their role in inflammation and initiation of adaptive immunity, and data herein indicate that both CBD and THC preferentially modulate TLR3 and TLR4 signalling via MyD88-independent mechanisms in macrophages. This offers mechanistic insight into the role of phytocannabinoids in modulating cellular inflammation.

1. Introduction

Macrophages are innate immune cells associated with inflammation and infection, and contribute to the pathogenesis of various disorders including multiple sclerosis (MS) (Bogie et al., 2013), epilepsy (Varvel et al., 2016; Zattoni et al., 2011) and Alzheimer's disease (Park et al., 2017). A significant body of research indicates that macrophages infiltrate the CNS in neurodegenerative disease (Biju et al., 2010), and promote increased levels of inflammation in distinct brain regions (Hendriks et al., 2005). Macrophages express an array of pattern recognition receptors (PRRs), including the toll-like receptors (TLRs), consisting in humans as a family of 10 members (Kenny and O'Neill, 2008). TLRs can intricately distinguish between bacterial and viral molecules to initiate innate immune responses via nuclear factor (NF)-κB and interferon (IFN) regulatory factor (IRF) transcription factors, to orchestrate the cellular production of cytokines, chemokines and type I IFNs. TLRs are localized in cellular membranes (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10) or endosomal compartments (TLR3, TLR7, TLR8, TLR9) (O'Neill, 2004b), and the specificity in cellular response is dependent on the TLR adaptor proteins that are engaged by the receptor (O'Neill, 2004a). All TLRs signal via the adaptor myeloid differentiation factor 88 (MyD88), while TLR3 is an exception, signalling via Toll-Interleukin-1 Receptor (TIR)-domain-containing adaptor-inducing IFN-β (TRIF) adaptor to induce MyD88-independent signalling. TLR4 can also

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signal via the MyD88-independent pathway via association with TRIF-related adaptor molecule (TRAM) (Kawai and Akira, 2010). TLRs are linked with CNS disorders, with data from human and animal studies (in TLR/adaptor-deficient mice) identifying TLRs as central players in the pathogenesis of MS (Fitzpatrick and Downer, 2017; Miranda-Hernandez and Baxter, 2013). Indeed, uncontrolled and atypical activation of TLR signalling can result in neuroinflammation and neurodegeneration (Owens, 2009), and in macrophages, innate immune responses to bacterial (via TLR4) (Zuniga et al., 2017) and viral (via TLR3) (Lai et al., 2017) signalling is key in mediating cellular inflammation.

The *Cannabis sativa* plant contains more than 400 compounds, including a large group (over 100) of phytocannabinoids (ElSohly et al., 2017). Δ^9-tetrahydrocannabinol (THC; a euphoric component of *Cannabis sativa*) and cannabidiol (CBD; a non-euphoric cannabinoid) are the most abundant phytocannabinoids derived from *Cannabis sativa* extracts, and preclinical research has focused on THC and CBD to demonstrate their anti-inflammatory (Petrosino et al., 2018; Shang et al., 2016), antioxidant (Sun et al., 2017; Vella et al., 2017) and anti-excitotoxic efficacy (Gilbert et al., 2007; Khaksar and Bigdeli, 2017). Some phytocannabinoids can act via G-protein–coupled cannabinoid receptors CB_1 and CB_2, the expression of which has been localized on cells of the nervous and immune systems (Iversen, 2000). THC is a CB_1 and CB_2 receptor partial agonist, with in vitro evidence indicating that THC binds to CB_1 and CB_2 with K_i values in the low nanomolar range (Iwamura et al., 2001; Rinaldi-Carmona et al., 1994). Unlike THC, CBD demonstrates minimal agonist activity (and very low affinity) for both CB_1 and CB_2, and commonly occurs in in vitro studies at supraphysiological concentrations (Ibeas Bih et al., 2015; Pertwee, 2008). $CB_{1/2}$-independent mechanisms of action for CBD have also been extensively studied and have identified several receptor targets for this cannabinoid, including peroxisome proliferator-activated receptor (PPAR)-γ (0.1–10 μM range) (Esposito et al., 2011; Hind et al., 2016; Ramer et al., 2013; Scuderi et al., 2014), transient receptor potential vanilloid 1 (TRPV1) receptor (EC_{50} = −1.0 μM) (De Petrocellis et al., 2011), G protein-coupled receptor 55 (GPR55) (IC_{50} = 0.45 μM) (Ryberg et al., 2007), serotonin (5-HT) receptors (10–16 μM range) (Alharris et al., 2019; Hind et al., 2016; Ledgerwood et al., 2011; Russo et al., 2005) and both μ- and δ-opioid receptors (IC_{50} = −10.0 μM) (Kathmann et al., 2006). Cannabinoids also modulate multiple intracellular signal transduction pathways involving adenylyl cyclase, mitogen-activated protein (MAP) kinases, phosphoinositide 3-kinase/protein kinase B, mammalian target of rapamycin (mTOR), caspases, NF-κB, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and voltage-dependent ion channels (K^+, Ca^{2+}, Na^+) (Demuth and Molleman, 2006; Downer et al., 2003; Giacoppo et al., 2017; Kozela et al., 2010).

Various studies have demonstrated that cannabinoids, including phytocannabinoids, the endogenous (endo) cannabinoids and synthetic cannabinoid compounds, modify innate immune responses via TLR-mediated signalling in various cell and tissue types. Indeed, TLR2, TLR3, TLR4, TLR7 and TLR8 signalling is sensitive to cannabinoid ligands and endocannabinoid signalling (reviewed by (Fitzpatrick and Downer, 2017), identifying that TLR signalling mechanisms can be modulated by cannabinoids. Specifically in terms of TLR4, the phytocannabinoids (THC, CBD), synthetic cannabinoids (*R*(+)WIN55212, HU-210, CP55,940), and endogenous cannabinoids (anandamide, N-arachidonoyl dopamine), inhibit TLR4-induced signalling in various cell types including endothelia, astrocytes and microglia (Chung et al., 2012; Facchinetti et al., 2003; Kozela et al., 2010; Marchalant et al., 2007; Molina-Holgado et al., 1997; Molina-Holgado et al., 2002; Wilhelmsen et al., 2014). In terms of TLR3, the synthetic cannabinoid *R* (+)WIN55212 (5–50 μM dose range) has been shown to regulate TLR3-induced signalling in immune cells and astrocytes (Downer et al., 2011), while a range of phytocannabinoids, including CBD, cannabigerol (CBG), cannabichromene (CBC), tetrahydrocannabivarin (THCV), and cannabigevarin (CBGV) (all in the 5–20 μM dose range) inhibit TLR3 signalling in keratinocytes (Petrosino et al., 2018). Furthermore, systemic and central administration of a fatty acid amide hydrolase (FAAH) inhibitor has been shown to regulate TLR3 signalling in hippocampal tissue (Henry et al., 2014).

Given reports linking TLR3 and TLR4 with the pathogenesis of disease, particularly MS (Guo et al., 2008; Marta et al., 2008), in addition to the role of macrophages in MS (Bogie et al., 2013), we were interested to explore the role of phytocannabinoids in mediating TLR3/4-induced inflammatory signalling in macrophages, with the aim of this study being twofold. Firstly, given that there is limited evidence to indicate that phytocannabinoids regulate TLR3 signalling induced by poly(I:C), we determined the impact of THC and CBD on the TLR3-TRIF-IRF3 signalling axis, in addition to assessing the effect of THC/CBD on TLR4 signalling via the MyD88-independent pathway. Secondly, we determined whether the proclivity of THC and CBD to impact TLR3 and TLR4 signalling in macrophages was influenced by the administration of each cannabinoid alone or in a 1:1 combination. Data herein demonstrate that both the viral TLR3-TRIF-IRF and bacterial TLR4-MyD88-NF-κB signalling axes are operative in THP-1 macrophages. We present evidence that THC and CBD inhibited MyD88-independent signalling via IRF3 following TLR3 and TLR4 activation. In contrast, both phytocannabinoids had no effect on TLR4 signalling via MyD88 to control IκB-α degradation, and downstream pro-inflammatory TNF-α expression. Both THC and CBD were not cytotoxic and the effects of the combination of THC and CBD on certain MyD88-independent signalling events were independent of $CB_{1/2}$ and PPARγ. Overall these findings demonstrate that THC and CBD differentially modulate MyD88-dependent and –independent signalling via TLR3 and TLR4 in macrophages, and offers mechanistic insight by which phytocannabinoids regulate inflammatory signalling pathways at a cellular level.

2. Materials and methods

2.1. Cell culture

The human monocytic cell line (THP-1) were from American Type Culture Collection (ATTC) or were kind gifts from Prof. Andrew Bowie and Prof. Marina Lynch, Trinity College Dublin. THP-1 monocytes were maintained in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% (*v/v*) FBS (Sigma-Aldrich) and penicillin streptomycin (100 μg/ml) solution (Gibco) in a humidified environment at 37 °C with 5% CO_2. Cells were passaged using dissociation every 2–3 days. To induce THP-1 monocyte differentiation, THP-1 cells were resuspended and seeded in RPMI 1640 medium containing phorbol myristate acetate (PMA; 10 ng/ml) (Sigma-Aldrich), and maintained in 5% CO_2 at 37 °C for 48 h to allow differentiation.

2.2. Cytokine analysis in culture supernatants

THP-1 cells (1 × 10^6 cells/ml) were seeded in 24-well plates and differentiated for 48 h in RPMI 1640 medium containing PMA (10 ng/ml). Macrophages were incubated with the TLR4 agonist LPS (100 ng/ml) (Enzo Life Sciences, Inc.) or the TLR3 agonist poly(I:C) (10 μg/ml) (Invivogen) for 4 h. Cells were also pre-exposed (45 min) to purified phytocannabinoids; plant-derived highly purified THC (10 μM), CBD (10 μM) or their 1:1 combination (THC 10 μM: CBD 10 μM) (GW Research Ltd., Cambridge, UK) prior to LPS (100 ng/ml; 4 h) or poly(I:C) (10 μg/ml; 4 h) exposure. The concentration of both THC and CBD used are in line with those used in various inflammatory studies elsewhere (Kozela et al., 2010; Petrosino et al., 2018; Rajan et al., 2016; Rao et al., 2015). Furthermore, treatment (4 h) of THP-1 macrophages with both THC and CBD at final concentrations of 10 μM was not cytotoxic in cells (see Fig. 5). In some experiments, cells were pre-treated with the CB_1 receptor antagonist SR141716 (N-[piperidin-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-

4-methyl-1-H-pyrazole-3-carboxamide], NIMH Chemical Synthesis Programme Batch 12,446–49-1; 1 μM for 1 h), the CB_2 receptor antagonist SR144528 ([N-[(1 s)-endo-1,3,3-timethylbicyclo[2.2.1]heptan-2-yl]5-(4-choro-3-methylpanyl)-1-(4-methlbenzyl)pyrazole-3-carboxamide] Chemical Synthesis Programme: Batch No. 11183–173-2; 1 μM for 1 h), or the PPARγ receptor antagonist T0070907 (Tocris Bioscience; 1 μM; 1 h) prior to treatment with the phytocannabinoids and LPS or poly(I:C). Control wells were incubated with RPMI media or RPMI media containing sterile ethanol (0.1%). Supernatants were assayed for TNF-α, CXCL10, IFN-β and CXCL8 by ELISA according to manufacturer's instructions (R&D Systems).

2.3. *Quantitative real-time PCR*

THP-1 cells (1 × 10^6 cells/ml) were seeded in 24-well plates and differentiated for 48 h using PMA (10 ng/ml). Macrophages were incubated with LPS (100 ng/ml) or poly(I:C) (10 μg/ml) for 4 h. Cells were also pre-exposed (45 min) to THC (10 μM), CBD (10 μM) or a combination of both (1:1 ratio; each cannabinoid at 10 μM) prior to LPS (100 ng/ml; 4 h) or poly(I:C) (10 μg/ml; 4 h) exposure. Control wells were incubated with RPMI media or RPMI media containing ethanol (0.1%). RNA was extracted from macrophages using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc.). The concentration of RNA was determined using a UV/Vis spectrophotometer. cDNA synthesis was performed on 1 μg RNA using a High Capacity cDNA RT Kit (Applied Biosystems) according to the manufacturer's instructions. Equal amounts of cDNA were used for PCR amplification. Real-time PCR primers were delivered as "Taqman® Gene Expression Assays" containing forward and reverse primers, and a FAM-labeled MGB Taqman probe for each gene (Applied Biosystems). Primers used were as follows: TLR3, TLR4, CXCL10, IFN-β, CB_1, CB_2 and PPARγ (Taqman® Gene Expression Assay no. Hs00152933_m1, Hs00152939_m1, Hs00171042_m1, Hs00171042_m1, Hs00275634_m1, Hs00361490_m1 and Hs01115513_m1, respectively). cDNA (1:4 dilution) was prepared and real-time PCR performed using Applied Biosystems 7300 Real-time PCR System. cDNA was mixed with qPCR™ Mastermix Plus (Applied Biosystems) and the respective gene assay in a 25 μl volume (10 μl of diluted cDNA, 12.5 μl Taqman® Universal PCR Mastermix, 1.25 μl target primer and 1.25 μl 18S rRNA). Eukaryotic 18S rRNA was used as an endogenous control and expression was conducted using a gene expression assay containing forward and reverse primers and a VIC-labeled MGB Taqman probe (#4319413E; Applied Biosystems). Samples were run in duplicate and 40 cycles were run as follows: 10 min at 95 °C and for each cycle, 15 s at 95 °C and 1 min at 60 °C. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. In all experiments, no change in relative 18S rRNA expression between treatment groups was observed.

2.4. *Immunocytochemistry*

THP-1 cells (0.5–1 × 10^6 cells/ml) were seeded on 13-mm diameter coverslips coated with poly-L-lysine (Sigma-Aldrich) and differentiated for 48 h in RPMI 1640 medium containing PMA (10 ng/ml). THP-1 macrophages were incubated with LPS (100 ng/ml) or poly(I:C) (10 μg/ml) for timepoints ranging from 10 min - 4 h. Macrophages were also pre-exposed (45 min) to THC, CBD or THC:CBD (1:1 ratio) (GW Research Ltd., Cambridge, UK) (10 μM) prior to LPS (100 ng/ml; 30 min) or poly(I:C) (10 μg/ml; 60 min). Control wells were incubated with RPMI media or RPMI media containing ethanol (0.1%). Cells were then fixed in ice-cold methanol for 10 min, permeabilised with 0.2% Triton X-100 (Thermo Fisher Scientific) in PBS for 10 min at room temperature and blocked with 10% normal goat serum (Vector Laboratories) for 2 h. Cells were incubated overnight at 4 °C with rabbit polyclonal NF-κB p65 or IRF3 antibody (1:200 in 5% goat serum; Santa Cruz Biotechnology). Cells were then washed and incubated with goat anti-rabbit Alexa488 secondary antibody (1:1000 in 5% normal goat serum; Invitrogen) and 4′,6-diamidino-2-phenylindole (DAPI; 1.5 μg/ml) in PBS, washed and mounted (Vectashield; Vector Laboratories). Cells were imaged using an Olympus BX51P fluorescent microscope. The fluorescence intensity in the nucleus of individual cells stained for NF-κB p65 and IRF3 was measured using the Image J analysis software. The relative fluorescence intensity was calculated as the intensity after subtraction of the background noise. For each treatment, 4–5 fields of view were captured per coverslip. Negative control experiments were performed by replacing the primary antibody with PBS and using equal gain settings during acquisition and analysis.

2.5. *Western immunoblotting*

THP-1 cells (0.5 × 10^6 cells/ml) were seeded in 6-well plates and differentiated for 48 h using PMA (10 ng/ml). Macrophages were incubated with LPS (100 ng/ml) for timepoints ranging from 15 to 60 min. Cells were also pre-exposed (45 min) to THC (10 μM), CBD (10 μM) or a combination of both (1:1 ratio; each cannabinoid at 10 μM) (GW Research Ltd., Cambridge, UK) prior to LPS (100 ng/ml; 30 min) exposure. Following treatment, cells were washed (x3) in ice-cold PBS before being lysed on ice for 5 min in 70 μl of cytoplasmic lysis buffer (10 mM Tris-HCL, pH 7.5, containing 3 mM $MgCl_2$, 10 mM NaCl, 0.5% Igepal, phosphatase inhibitor cocktail 2 and 3 (Sigma), protease inhibitor cocktail (Sigma)). Cells were scraped in cytoplasmic lysis buffer and maintained on ice for 5 min. Cell lysates were centrifuged (5000 rpm for 5 min at 4 °C). The supernatants were carefully removed and stored at −80 °C for future analysis. The remaining pellets were resuspended in nuclear extraction buffer (10 μl) (20 mM HEPES, pH 7.5, containing 5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 1 mM DTT, glycerol (20%), phosphatase inhibitor cocktail 2 and 3 (Sigma), protease inhibitor cocktail (Sigma)), and subjected to liquid nitrogen – warm water, freeze-thaw to aid in nuclear membrane lysis. The resuspended pellet was centrifuged (14,000 rpm for 20 min at 4 °C). The supernatants (nuclear fractions) were stored at −80 °C for future analysis. Protein concentration was determined using the BCA method, with unknown protein concentrations interpolated from a bovine serum albumin (BSA) standard curve. Lysate protein concentration was equalized and mixed with denaturing buffer (0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 12.5% β-mercaptoethanol, and 0.0025% (w/v) bromophenol blue). Lysates were subjected to 95 °C for 5 min to aid complete protein denaturing prior to electrophoresis. Lysates in denaturing buffer were loaded onto 10% acrylamide gels, submerged in running buffer (25 mM Tris base, 190 mM Glycine, 0.1% SDS, pH 8.3) and a current of 150 V applied for approximately 90 min, or until the proteins had run off the end of the gel. Proteins were transferred to activated PVDF (Merck Millipore, Ireland) membranes (5 s in methanol, 5 min in dH_2O, and 15 min in transfer buffer; 25 mM Tris-base, 190 mM glycine, 20% methanol, pH 8.3) for 2 h at 250 mA current passing through the membrane. Proteins transferred onto PVDF were blocked for 2 h in 5% BSA in TBS-T. Membranes were incubated overnight at 4 °C with rabbit monoclonal phospho-IRF3 antibody (1:2000 in TBS-T, Cell Signalling Technology, USA) or mouse monoclonal IκB-α (1:1000 in TBS-T, Cell Signalling Technology, USA)). Membranes were incubated with mouse monoclonal anti-β-actin antibody (1:20,000; 1 h, Sigma, UK) as a loading control. Membranes were washed and incubated with anti-mouse or anti-rabbit IRDye Infrared secondary antibody (1:10,000 in TBS-T; Licor Biosciences, Lincoln, NE) for 1 h in the dark at room temperature. The membranes were washed and immunoreactive bands were detected using the Odyssey Infrared Imaging System (Licor Biosciences). Molecular weight markers were used to calculate molecular weights of proteins represented by immunoreactive bands. Densitometry was performed using ImageStudioLite software, and values were normalized for protein loading relative to levels of β-actin.

2.6. *Cell viability assay*

THP-1 cells (2 × 10^4 cells/well) were seeded in 96-well plates and

differentiated for 48 h using PMA (10 ng/ml). Macrophages were incubated with sterile ethanol (0.1%), THC (10 μM), CBD (10 μM) or a combination of both phytocannabinoids (1:1 ratio; 10 μM final concentration for each compound) (GW Research Ltd., Cambridge, UK) for 4 h, and the impact of phytocannabinoid treatment on the proliferation of macrophages was quantified using MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, whereby MTT (5 mg/ml; Sigma) was added to each well, followed by an incubation period for a further 3.5 h at 37 °C. At the end of the incubation period the media and MTT were removed and isopropanol was then added to each well for solubilisation of the formazan products. Positive control experiments were performed by incubating cultures with Triton X-100 (Sigma) solution (0.2%) for 10 min prior to the addition of MTT. Absorbance was measured at 540–560 nm with a reference wavelength of 690 nm.

2.7. Statistical analysts

All data were analysed using GraphPad Prism (version 8). All data were tested for normality using the Shapiro-Wilk test, and were analysed using Student's *t*-test for independent means, or one-way analysis of variance (ANOVA) as appropriate. When analysis by ANOVA indicated significance ($p < .05$), the post hoc Dunnett's multiple comparison test was used. Data are expressed as means ± standard errors of the mean (SEM) from at least three separate experimental cell passages treated and harvested on separate days for each passage. Within each experimental passage, duplicate/triplicate determinations were performed for each condition/drug treatment.

3. Results

3.1. TLR3 activation in THP-1 macrophages promotes IRF-3 activation and the downstream expression of CXCL10 and IFN-β

TLR3 signalling was targeted given previous evidence from our laboratory indicating that TLR3 signalling is desensitized in immune cells isolated from healthy donors and people with MS (pwMS) (Crowley et al., 2015), and that TLR3 plays a role in EAE pathogenesis (Touil et al., 2006). In addition, there is limited evidence to indicate that phytocannabinoids regulate TLR3 signalling induced by poly(I:C). We initially determined that THP-1 differentiated macrophages express TLR3 by PCR, and that poly(I:C) treatment markedly increased *TLR3* mRNA ($p < .001$; Fig. 1A). To characterize the impact of poly(I:C) stimulation on TLR3 signalling, we temporally assessed the impact of poly(I:C) on the activation of IRF3 transcription factor, and downstream production of both the CXCL10 chemokine and the type I IFN, IFN-β. THP-1 macrophages were stimulated with poly(I:C) for various timepoints ranging from 10 to 60 min, and the cellular localization of endogenous IRF3 was assessed by immunofluorescence (Fig. 1B). In control cells, IRF3 was predominantly cytoplasmic, as evidenced by the detection of 488-conjugated immunocomplexes outside the nuclear regions (Fig. 1B). Stimulation of macrophages with poly(I:C) time-dependently promoted the accumulation of IRF3 in the nucleus ($p < .05$, $p < .001$; Fig. 1B).

TLR3 can induce MyD88-independent signalling to couple to NF-κB and downstream pro-inflammatory cytokine/chemokine expression (Matsumoto et al., 2004); however, TLR3 activation in macrophages failed to promote the expression of the pro-inflammatory cytokine, TNF-α (Supplemental Fig. 1), a finding we have also determined in THP-1 monocytes and peripheral blood mononuclear cells (data not shown). We next characterised the impact of TLR3 activation on the downstream production of CXCL10 and IFN-β in THP-1 macrophages, at both mRNA and protein levels. Poly(I:C) treatment significantly enhanced *CXCL10* mRNA ($p < .001$; Fig. 1C) and protein ($p < .01$; Fig. 1D) expression. Similarly, TLR3 activation promoted the induction of *IFN-β* mRNA ($p < .01$; Fig. 1E) and protein ($p < .001$; Fig. 1F) expression. This indicates that the viral dsRNA mimetic poly(I:C) promotes the TLR3 signalling machinery in THP-1 differentiated macrophages.

3.2. CBD, THC and THC:CBD (1:1) inhibit TLR3-induced IRF3 activation and induction of CXCL10/IFN-β in THP-1 macrophages

We next examined the impact of the phytocannabinoids, THC and CBD, alone and in a 1:1 combination, on TLR3-induced signalling in THP-1 macrophages. Macrophages were pre-treated with THC (10 μM), CBD (10 μM) and THC:CBD (1:1 both phytocannabinoids at a final concentration of 10 μM) prior to poly(I:C) exposure, and nuclear expression of IRF3 measured by fluorescence microscopy. Poly(I:C) promoted the accumulation of IRF3 in the nucleus ($p < .001$; Fig. 2A). Pre-exposure to THC, CBD and THC:CBD (each cannabinoid at a final concentration of 10 μM) attenuated TLR3-induced IRF3 activation, returning nuclear IRF3 expression to basal levels ($p < .001$; Fig. 2A).

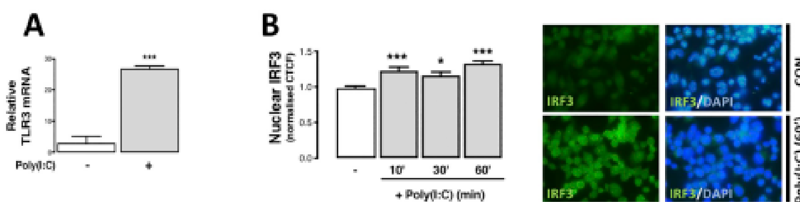
As IRF3 transcription factor can induce the downstream expression of type I IFNs (Honda et al., 2006) and CXCL10 (Brownell et al., 2014), the sensitivity of CXCL10/IFN-β to THC and CBD in response to poly (I:C) was next evaluated. Pre-exposure to THC and CBD (at final concentrations of 10 μM) significantly attenuated TLR3-induced *CXCL10* mRNA ($p < .01$; Fig. 2B) and protein ($p < .05$, $p < .01$; Fig. 2C) expression. Furthermore, Fig. 2D demonstrates that THC, CBD and THC:CBD attenuated poly(I:C)-induced *IFN-β* mRNA ($p < .01$, $p < .001$), but not IFN-β protein expression (Fig. 2E), in macrophages at concentrations of 10 μM. These findings indicate that both THC and CBD can negatively regulate TLR3 signalling to IRF3, CXCL10 and IFN-β (mRNA) in macrophages.

3.3. TLR4 activation increases NF-κB and IRF3 activation, while promoting the downstream expression of TNF-α, IFN-β and CXCL10 in THP-1 macrophages

TLR4 is well characterised for its role in systemic inflammation, and is a player in neurodegenerative and neuroinflammatory disorders (Buchanan et al., 2010; Marta, 2009). The expression of TLR4 in THP-1 differentiated macrophages was determined by real time PCR, and data herein indicate that LPS treatment had no significant effect on *TLR4* mRNA (Fig. 3A). To assess the impact of TLR4 stimulation on MyD88-dependent and -independent signalling events in human macrophages, we assessed the effect of LPS exposure on intracellular signalling via NF-κB and IRF3 transcription factors, respectively.

NF-κB is sequestered in the cytoplasm by inhibitory IκB proteins, and TLR4 activation via LPS classically promotes IκB phosphorylation and ubiquitin-dependent degradation of IκB, facilitating NF-κB p65 nuclear sequestration (Rothwarf and Karin, 1999). To confirm that TLR4-induced NF-κB signalling is operative, THP-1 macrophages were stimulated with LPS for timepoints ranging from 10 to 240 min, and the localization of endogenous NF-κB p65 subunit (Fig. 3B) was assessed by immunofluorescence. In control cells, p65 was predominantly cytoplasmic, and stimulation with LPS promoted the accumulation of p65 in the nucleus at 10–120 min ($p < .001$; Fig. 3B). Given that LPS promoted NF-κB nuclear sequestration, we next stimulated macrophages with LPS and determined that TLR4 activation significantly promoted IκB-α degradation ($p < .001$; Fig. 3C) in cytoplasmic fractions, following a 30 min incubation. We also characterised the impact of TLR4 activation on downstream production of *TNF-α* mRNA and protein expression. LPS enhanced *TNF-α* mRNA ($p < .05$; Fig. 3D) and protein ($p < .001$; Fig. 3E) expression following a 4 h treatment.

TLR4 can also induce MyD88-independent signalling to couple to IRF3 via the TRIF adaptor protein and downstream production of type I IFNs and CXCL10 (Sakaguchi et al., 2003). To assess MyD88-independent signalling via TLR4, THP-1 macrophages were stimulated with LPS for timepoints ranging from 15 to 60 min and the phosphorylation of IRF3 determined in nuclear fractions via immunoblotting (Fig. 3F). LPS enhanced IRF3 phosphorylation in nuclear fractions in a



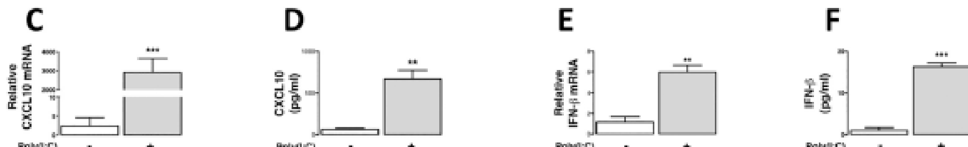


Fig. 1. Characterisation of TLR3 signalling in THP-1-derived macrophages. Poly(I:C) (10 μg/ml; 4 h) treatment induced the expression of (A) *TLR3* mRNA ($p < .001$; Student's *t*-test), (B) nuclear sequestration of IRF3 (10–60 min) ($F(3,539) = 17.33$; $p < .05$; ANOVA), (C) *CXCL10* mRNA ($p < .001$; Student's *t*-test), (D) CXCL10 protein ($p < .01$; Student's *t*-test), (E) *IFN-β* mRNA ($p < .01$; Student's *t*-test) and (F) IFN-β protein ($p < .001$; Student's *t*-test). (B) Immunofluorescent images demonstrating the subcellular localization of IRF3 in macrophages following exposure to poly(I:C). Nuclei were stained with DAPI, and images obtained using an Olympus BX51P fluorescent microscope equipped with the appropriate filter sets. Data are expressed as means ± S.E.M from 3 to 4 independent passages. *$p < .05$, **$p < .01$ and ***$p < .001$ versus control groups.

time-dependent manner ($p < .05$; Fig. 3F). These findings were confirmed by immunofluorescence, with LPS promoting the time-dependent nuclear localization of endogenous IRF3 ($p < .001$; Fig. 3G). Data presented in Fig. 3H, I indicate that LPS promoted the induction of *IFN-β* mRNA ($p < .01$; Fig. 3H) and protein ($p < .001$; Fig. 3I), while TLR4 activation also promoted the induction of *CXCL10* mRNA ($p < .01$; Fig. 3J) and protein ($p < .001$; Fig. 3K). This indicates that signalling induced by the endotoxin LPS promoted TLR4 signalling via both MyD88-dependent and -independent pathways in THP-1 differentiated macrophages.

3.4. Effect of CBD and THC on TLR4-induced IκB-α degradation, IRF3 activation, CXCL10 and IFN-β expression in THP-1 macrophages

Given that both THC and CBD can negatively regulate TLR3 signalling to IRF3, CXCL10 and IFN-β (Fig. 2), we next examined the proclivity of phytocannabinoids to impact TLR4-induced signalling via the MyD88 adaptor, and independently of MyD88. Firstly, macrophages were pre-treated with THC (10 μM), CBD (10 μM) and THC:CBD (both at a final concentration of 10 μM) prior to LPS treatment (30 min), and cytoplasmic fractions assessed for IκB-α degradation via immunoblotting (Fig. 4A). Interestingly, THC (10 μM), CBD (10 μM) and THC:CBD (both at a final concentration of 10 μM) failed to inhibit LPS-induced IκB-α degradation in cytoplasmic fractions ($p < .001$; Fig. 4A). Furthermore, pre-exposure to THC, CBD and THC:CBD (at 10 μM) failed to impact TLR4-induced TNF-α (Fig. 4B) and CXCL8 (Fig. 4C) expression. These findings suggest that both THC and CBD do not regulate the TLR4 signalling pathways controlling IκB proteins and the production of pro-inflammatory TNF-α and CXCL8 in THP-1 macrophages.

Next, the ability of THC/CBD to regulate TLR4 signalling independent of the MyD88 adaptor was assessed. Macrophages were pre-treated with THC (10 μM), CBD (10 μM) and THC:CBD (both at 10 μM) prior to LPS, and the phosphorylation of IRF3 determined in nuclear (Fig. 4D) fractions via immunoblotting. LPS significantly induced IRF3 phosphorylation in nuclear fractions ($p < .05$, $p < .01$; Fig. 4D). Pre-treatment with THC/CBD had no significant effect on LPS-induced p-IRF3 (Fig. 4D). However, to investigate this finding further, nuclear expression of endogenous IRF3 was measured by fluorescence microscopy (Fig. 4E). LPS promoted the accumulation of IRF3 in the nucleus ($p < .001$), and pre-treatment to THC, CBD and THC:CBD (at 10 μM) attenuated the TLR4-induced nuclear sequestration of IRF3 ($p < .001$; Fig. 4E). Next, the effect of THC/CBD (at 10 μM) on TLR4-induced CXCL10 mRNA (Fig. 4F) and protein (Fig. 4G) expression was determined, and data in Fig. 4G demonstrate that pre-exposure to THC, CBD and THC:CBD (each cannabinoid at a final concentration of 10 μM) attenuated TLR4-induced CXCL10 expression ($p < .001$; Fig. 4G). Similarly, an investigation of the effect of THC/CBD on IFN-β mRNA (Fig. 4H) and protein (Fig. 4I) indicates that CBD and THC:CBD (each cannabinoid at a final concentration of 10 μM) significantly attenuated TLR4-induced IFN-β protein ($p < .01$; Fig. 4I), but not mRNA, expression. These findings suggest that THC and CBD can negatively regulate the MyD88-independent pathways induced by TLR4 to control the production of CXCL10 and IFN-β in THP-1 macrophages.

3.5. CBD and THC are not cytotoxic to THP-1 macrophages

Both THC and CBD, when delivered at a final concentration of 10 μM, have anti-inflammatory efficacy in vitro (Kozela et al., 2010; Petrosino et al., 2018; Rajan et al., 2016; Rao et al., 2015). However, given that both THC and CBD can also exert antiproliferative effects in vitro (Liu et al., 2008; Scott et al., 2015), and cannabinoids in the μM range (including THC, anandamide and HU210) have the ability to regulate mitochondrial oxygen consumption (Athanasiou et al., 2007), the effect of THC and CBD on THP-1 macrophage viability was determined using MTT assays. No cytotoxicity was determined following treatment of THP-1 macrophages for 4 h with CBD and THC (when delivered alone and as a 1:1 ratio) at the final concentration of 10 μM (Fig. 5). Triton X-100 (0.1% for 10 min) treatment was used as a

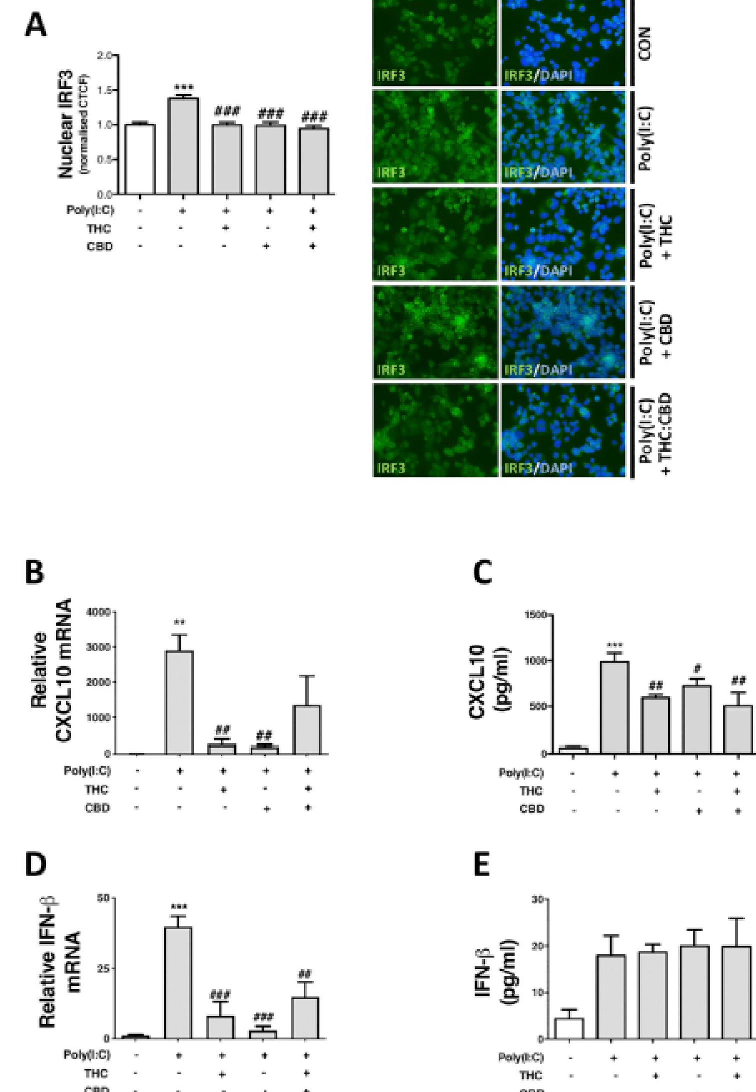


Fig. 2. THC, CBD and THC:CBD (1:1 combination) inhibit MyD88-independent signalling via TLR3 in THP-1-derived macrophages. (A) THC, CBD and the combination (1:1) of phytocannabinoids (final concentration of 10 μM for each cannabinoid; 45 min pre-treatment) inhibited poly(I:C)-induced (10 μg/ml; 60 min) IRF3 translocation to the nucleus ($F(4,714) = 17.73$; $p < .001$; ANOVA). Immunofluorescent images demonstrating the subcellular localization of IRF3 in macrophages following exposure to poly(I:C), THC and CBD. Nuclei were stained with DAPI, and images obtained using an Olympus BX51P fluorescent microscope equipped with the appropriate filter sets. THC and CBD (all at 10 μM; 45 min pre-treatment) attenuated poly(I:C)-induced (10 μg/ml; 4 h) (B) *CXCL10* mRNA ($F(4,12) = 6.15$; $p < .01$; ANOVA) and (C) CXCL10 protein expression ($F(4,24) = 20.81$; $p < .001$; ANOVA), in addition to (D) *IFN-β* mRNA expression ($F(4,11) = 18.13$; $p < .001$; ANOVA). (E) THC and CBD did not impact the effect of poly(I:C) on IFN-β protein expression. Data are expressed as means ± S.E.M from 3 to 8 independent passages. $^{**}p < .01$, $^{***}p < .001$ versus control groups and $^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ versus poly(I:C)-treated groups.

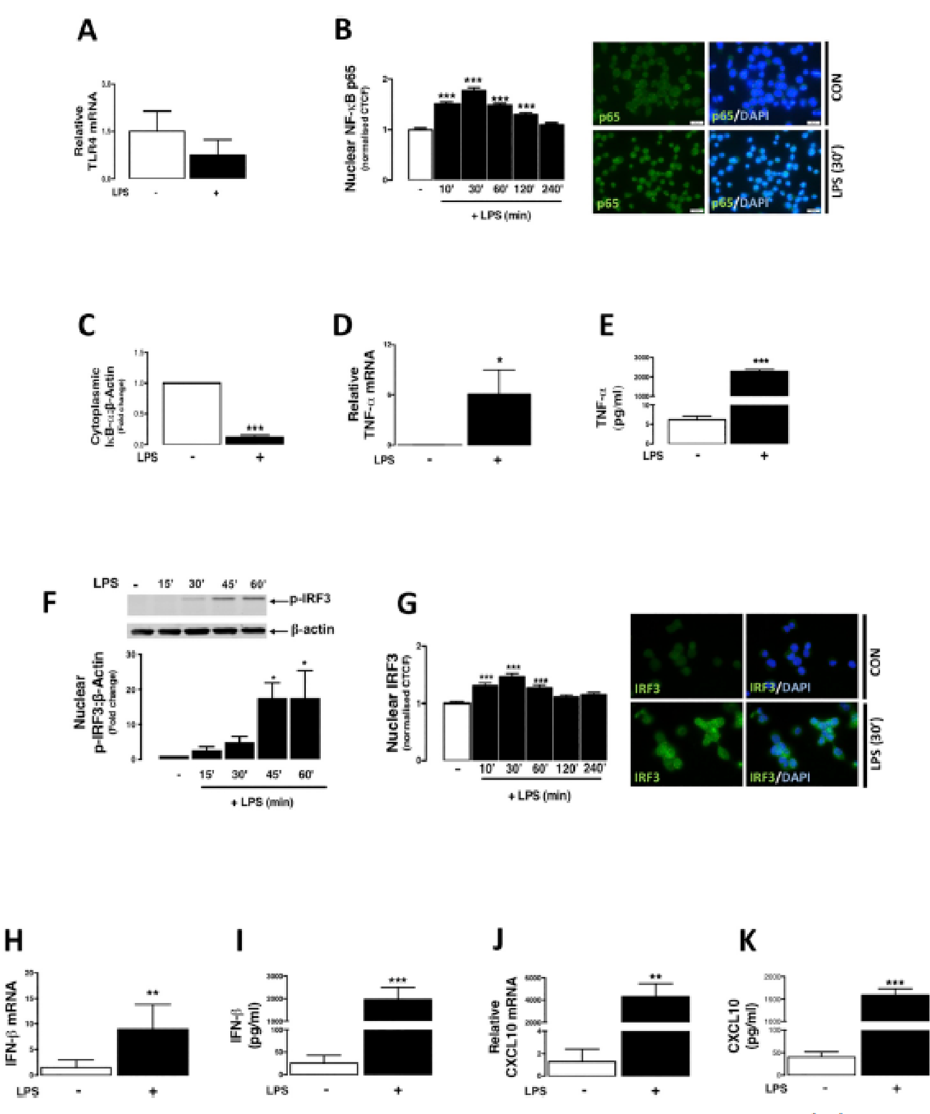


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Fig. 3. Characterisation of TLR4 signalling in THP-1-derived macrophages. (A) The effect of LPS (100 ng/ml; 4 h) treatment on *TLR4* mRNA expression. (B) LPS (100 ng/ml) time-dependently (10–120 min) promoted the translocation of NF-κB p65 subunit to the nucleus ($F(5,954) = 48.54$; $p < .001$; ANOVA). Immunofluorescent images demonstrating the subcellular localization of NF-κB p65 in macrophages following exposure to LPS. Nuclei were stained with DAPI, and images obtained using an Olympus BX51P fluorescent microscope equipped with the appropriate filter sets. (C) Effect of LPS (100 ng/ml; 30 min) on the degradation of IκB-α in cytoplasmic fractions ($p < .001$; Student's *t*-test). LPS (100 ng/ml; 4 h) induced the expression of (D) *TNF-α* mRNA ($p < .05$; Student's *t*-test) and (E) TNF-α protein ($p < .001$; Student's *t*-test). (F) Time-dependent (15–60 min) effect of LPS (100 ng/ml) treatment on the phosphorylation of IRF3 in nuclear fractions ($F(4,15) = 4.01$; $p < .05$; ANOVA). (G) LPS (100 ng/ml) treatment promoted the time-dependent (10–60 min) nuclear sequestration of IRF3 ($F(5,707) = 12.98$; $p < .001$; ANOVA). Immunofluorescent images demonstrating the subcellular localization of IRF3 in macrophages following exposure to LPS. Nuclei were stained with DAPI, and images obtained using an Olympus BX51P fluorescent microscope equipped with the appropriate filter sets. LPS (100 ng/ml; 4 h) induced the expression of (H) *IFN-β* mRNA ($p < .01$; Student's *t*-test), (I) IFN-β protein ($p < .001$; Student's *t*-test), (J) *CXCL10* mRNA ($p < .01$; Student's *t*-test) and (K) CXCL10 protein ($p < .001$; Student's *t*-test). Data are expressed as means ± S.E.M from 3 to 6 independent passages. *$p < .05$, **$p < .01$ and ***$p < .001$ versus control.

positive control, and reduced cell viability by approximately 85–90% ($p < .001$; Fig. 5). This suggests that the proclivity of both CBD and THC to reduce TLR3/4-induced CXCL10 and IFN-β is due to their pharmacological effects on TLR-induced cellular signalling events that regulate chemokine/type I IFN expression.

3.6. The role of CB_1 and CB_2 cannabinoid receptors, and the PPARγ receptor, in mediating the effects of CBD and THC on TLR4-induced CXCL10 and IFN-β expression

The cannabinoid pharmacology underlying the above effects was next assessed. CB_1 and CB_2 cannabinoid receptor expression was confirmed on THP-1 macrophages by PCR (Table. 1), and receptor involvement was addressed using the CB_1 and CB_2 antagonists, SR141716 and SR144528, respectively. Pre-exposure to SR141716 or SR144528 (both at 1 μM) did not impact the proclivity of THC and THC:CBD (at 10 μM) to reduce LPS-induced IFN-β ($p < .001$; Fig. 6A). Data in Fig. 6B also indicates the effect of LPS treatment on CXCL10 protein expression following pre-treatment with CB_1/CB_2 antagonists and THC/CBD (Fig. 6B). Both CB_1 and CB_2 antagonists had no effect on IFN-β production when delivered independently (Supplemental Fig. 2). This indicates that THC and THC:CBD impacts the signalling pathways leading from TLR4 to IFN-β independently of CB_1/CB_2 receptors.

Both CB_1- and CB_2-independent effects of THC (Kozela et al., 2010; Zygmunt et al., 2002) and CBD (Kaplan et al., 2003; Kozela et al., 2010) have also been demonstrated, with evidence that phytocannabinoids can act via PPARs in the μM range (Ramer et al., 2013). PPAR-γ was also detected on THP-1 macrophages (Table. 1), and pre-exposure to the PPAR-γ antagonist T0070907 (at 1 μM) did not reverse the inhibitory effect of CBD and THC:CBD (10 μM) on TLR3-induced CXCL10 expression ($p < .001$; Fig. 6C).

4. Discussion

This study set out to determine if the phytocannabinoids THC and CBD modulate viral TLR3 and bacterial TLR4 signalling in human macrophages, and has identified that both THC and CBD, when delivered alone and in a 1:1 combination, have the proclivity to differentially modulate TLR3 and TLR4 inflammatory signalling events in THP-1 macrophages. The significant finding is that both phytocannabinoids, when administered at a single concentration of 10 μM, preferentially modulated MyD88-independent signalling via TLR3 and TLR4, to inhibit poly(I:C)- and LPS-induced IRF3 activation and the expression of CXCL10. Interestingly, both phytocannabinoids do not appear to impact MyD88-dependent signalling via TLR4 controlling the degradation of IκB-α, and the downstream production of TNF-α and CXCL8. The inhibitory effects of THC and THC:CBD on TLR4-induced CXCL10 expression were independent of the CB_1/CB_2, while CBD and THC:CBD inhibited TLR3-induced CXCL10 independent of the PPAR-γ receptors.

Macrophages are innate immune cells that elicit the synthesis of appropriate chemokines and cytokines upon recognition of highly conserved pathogen-associated molecular patterns (PAMPs) via their PRRs, including TLRs (Kielian, 2006). Macrophages have clear roles in neuroinflammatory and neurodegenerative disorders, maintaining tissue homeostasis whilst acting as phagocytic clearers of pathogens (Mammana et al., 2018; Ricardo et al., 2008). 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 (Auwerx, 1991), and these cells are an appropriate in vitro model in which to study TLR signalling (Maess et al., 2014; Pan et al., 2011). Indeed, our findings indicate that both the TLR3-IRF3-IFN-β/CXCL10 and TLR4-NF-κB-TNF-α signalling axes are operative in THP-1-differentiated macrophages. Our data also indicate that incubation of macrophages with poly(I:C) enhanced the expression of TLR3, indicating that TLR3 stimulation can modulate TLR expression itself, which is consistent with findings elsewhere (McCartney et al., 2009; Zarember and Godowski, 2002). However, we found no effect of LPS on TLR4 expression, which is in contrast to data in THP-1 cells (Wan et al., 2016). The transcriptional regulation of TLRs following such treatments is unclear.

TLRs signal via the adaptor molecule MyD88, or conversely through recruitment of the TRIF adaptor protein in the case of TLR3. Indeed, TLR3 signals in a MyD88-independent manner via recruitment of TRIF to promote the dimerization of IRF3 and translocation of this transcription factor to the nucleus to regulate the transcription of type I IFNs and other inflammatory mediators including CXCL10 (Kawai and Akira, 2008; Tanaka and Imaizumi, 2013). Indeed, CXCL10 is an IFN inducible protein, and the regulation of IFN-β and CXCL10 has diverse immunomodulatory effects on cytokine expression, antigen presentation and chemotaxis (Blank and Prinz, 2017; Liu et al., 2011). MyD88-dependent and -independent signalling is also triggered by the TLR4 receptor agonist LPS to signal via MyD88 to regulate NF-κB, and also independently of MyD88 (via TRIF-TRAM) to regulate IRFs and IFNs (Vaure and Liu, 2014). Importantly, using the EAE model of murine MS, data indicate that both TLR3 and TLR4 signalling mechanisms play a central role in disease pathogenesis. Indeed, TLR3 stimulation suppresses demyelination in EAE via induction of IFN-β (Touil et al., 2006), while IRF3 and TRIF deficiency improves the clinical signs of disease (Fitzgerald et al., 2014; Wang et al., 2015). In terms of the role of TLR4 in EAE, TLR4 knockout in $CD4^+$ T cells diminishes disease symptoms (Reynolds et al., 2012), whereas findings elsewhere suggest that MyD88 deficient mice are resistant to EAE, highlighting a complex role for adaptor proteins in EAE progression (Marta et al., 2008).

A number of cannabinoid-based therapies are approved as medicinal products, including Marinol® (dronabinol; synthetic THC) and Cesamet® (nabilone; a synthetic derivative of THC), which are indicated as anti-emetics in patients undergoing cancer treatment (Takeda, 2014). Sativex® (a 1:1 mixture of THC and CBD) and Epidiolex® (plant-derived highly purified cannabidiol oral solution) are indicated for spasticity associated with MS (Rekand, 2014) and the treatment of seizures associated with Lennox-Gastaut or Dravet syndromes (Devinsky et al., 2017; Devinsky et al., 2018; Thiele et al., 2018), respectively. Cannabinoids exert their cellular effects via an array of molecular targets including ion channels (including K^+, Ca^{2+}, Na^+ and TRPV) (Demuth and Molleman, 2006; Ibeas Bih et al., 2015; Ligresti et al., 2006), transporters (including neurotransmitter transporters, anandamide membrane transporters) (Di Marzo et al., 1994; Pandolfo

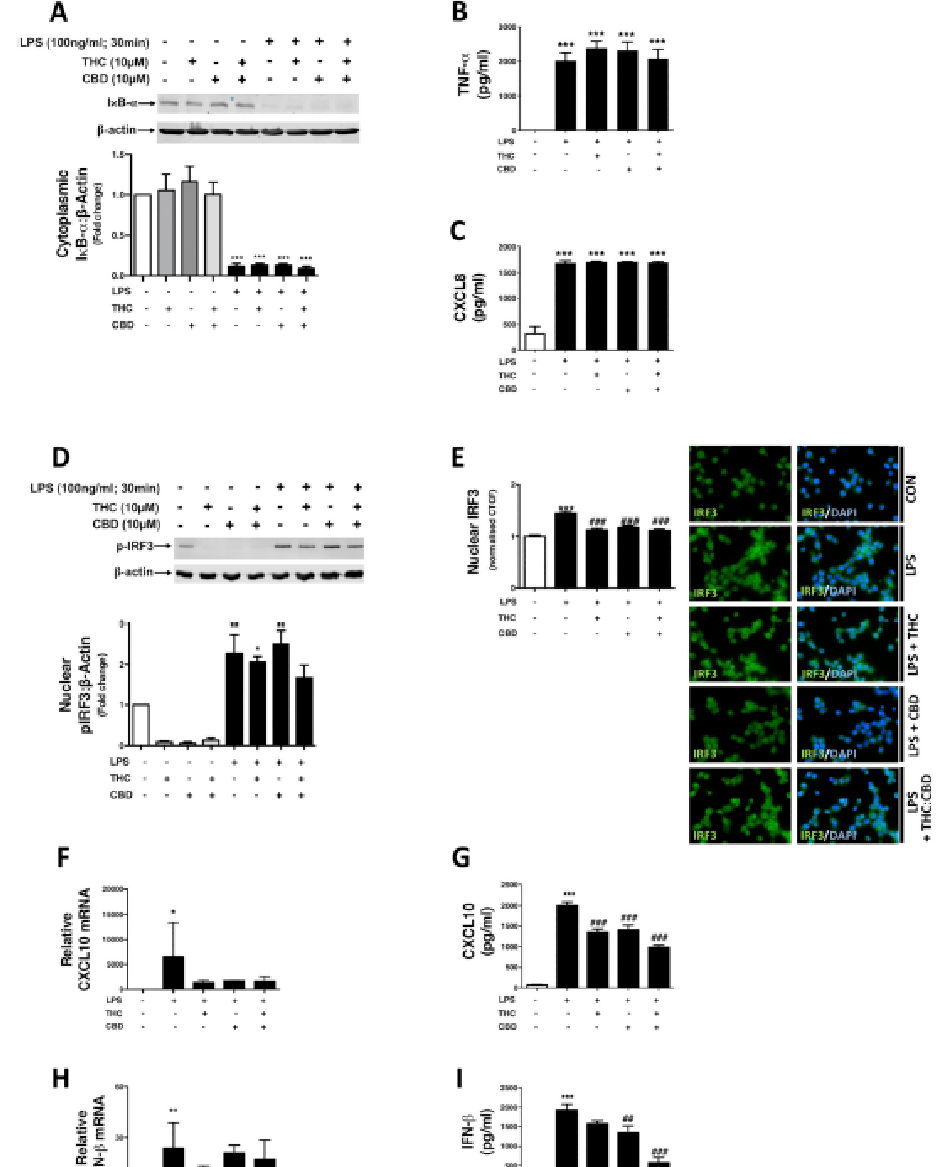

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Fig. 4. Effect of THC, CBD and a 1:1 combination of both phytocannabinoids on TLR4-induced signalling events in THP-1-derived macrophages. (A) Treatment with THC and CBD, alone and in a 1:1 combination (all at a final concentration of 10 μM; 45 min pre-treatment), had no impact on the LPS- (100 ng/ml; 30 min) induced IκB-α degradation ($F(7,24) = 20.49$; $p < .001$; ANOVA) in cytoplasmic fractions. THC and CBD (10 μM; 45 min pre-treatment) did not impact LPS-induced (100 ng/ml; 4 h) (B) TNF-α expression ($F(4,14) = 17.30$; $p < .001$; ANOVA) or (C) CXCL8 expression ($F(4,15) = 111.4$; $p < .001$; ANOVA). (D) LPS (100 ng/ml; 30 min) treatment promoted the phosphorylation of IRF3 in nuclear fractions $F(7,16) = 19.39$; $p < .01$; ANOVA), and THC and CBD (all at 10 μM; 45 min pre-treatment) had no significant effect on LPS-induced IRF3 phosphorylation. (E) Treatment with THC and CBD, alone and in a 1:1 combination (all at a final concentration of 10 μM; 45 min pre-treatment) inhibited LPS-induced (100 ng/ml; 30 min) IRF3 translocation to the nucleus ($F(4,1185) = 26.41$; $p < .001$; ANOVA). Immunofluorescent images demonstrating the subcellular localization of IRF3 in macrophages following exposure to LPS, THC and CBD. Nuclei were stained with DAPI, and images obtained using an Olympus BX51P fluorescent microscope equipped with the appropriate filter sets. Effect of THC/CBD (all at 10 μM; 45 min pre-treatment) on LPS-induced (100 ng/ml; 4 h) CXCL10 (F) mRNA ($F(4,17) = 2.77$; $p < .05$; ANOVA) and (G) protein expression ($F(4,30) = 72.98$; $p < .001$; ANOVA). Effect of THC/CBD on LPS-induced (100 ng/ml; 4 h) (H) *IFN-β* mRNA ($F(4,16) = 5.97$; $p < .01$; ANOVA) and (I) IFN-β protein expression ($F(4,24) = 31.88$; $p < .001$; ANOVA). Data are expressed as means ± S.E.M from 3 to 8 independent passages. *$p < .05$, **$p < .01$, ***$p < .001$ versus control groups and ##$p < .01$ and ###$p < .001$ versus LPS-treated cells.

et al., 2011) and intracellular signal transduction pathways (including MAP kinases, JAK/STATs) (Demuth and Molleman, 2006; Kozela et al., 2010), and can act via cannabinoid receptor-dependent- and -independent mechanisms involving PPAR-γ, GPR55 and 5-HT receptors (Ibeas Bih et al., 2015; Iwamura et al., 2001; Kaplan et al., 2017; Li et al., 2013; Pertwee, 2008; Ramer et al., 2013; Rinaldi-Carmona et al., 1994; Russo et al., 2005). CBD and THC differ in their pharmacology at the classic cannabinoid receptors, CB_1 and CB_2; THC is a CB_1/CB_2 receptor partial agonist, and binds to CB_1 and CB_2 with K_i values in the low nanomolar range (Iwamura et al., 2001; Rinaldi-Carmona et al., 1994). In contrast to THC, CBD demonstrates very low affinity and minimal agonist activity for both CB_1 and CB_2, and commonly occurs in in vitro studies at supraphysiological concentrations (Ibeas Bih et al., 2015; Pertwee, 2008). To determine the pharmacological targets for THC/CBD in mediating their effects on TLR signalling, we confirmed the expression of CB_1/CB_2 on THP-1 macrophages, and then employed the use of selective CB_1 and CB_2 receptor antagonists. Our findings indicate that neither antagonist reversed the effect of THC and THC:CBD on TLR4-induced CXCL10 expression, suggesting that CB_1 and CB_2 receptors do not mediate the anti-inflammatory propensity of phytocannabinoids in this cellular model of macrophage inflammation. It is important to note that both CB_1- and CB_2-independent effects of THC (Kozela et al., 2010; Zygmunt et al., 2002) and CBD (Kaplan et al., 2003; Kozela et al., 2010) have been demonstrated, with evidence that phytocannabinoids can act via PPARs (Zeissler et al., 2016). In this study PPAR-γ was detected on THP-1 macrophages, and furthermore the PPAR-γ antagonist T0070907 failed to reverse the inhibitory effect of CBD and THC:CBD on TLR3-induced signalling events. This indicates that CB_1, CB_2 and PPAR-γ do not mediate the effects of THC/CBD on certain TLR signalling events in this macrophage cell line. Both THC and CBD are lipid soluble phytocannabinoids (Grotenhermen, 2003), and hence their cellular effect on TLR signalling in macrophages may be attributed to their lipophilicity due to their direct partitioning into cellular membranes. Further research is required to pinpoint the pharmacological target(s) of THC and CBD in modulating signalling induced by TLR3 and TLR4 activation.

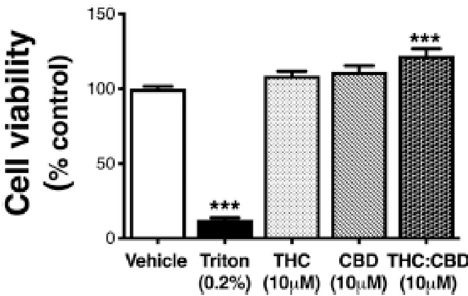


Fig. 5. THC and CBD are not cytotoxic in THP-1-derived macrophages. MTT assay of THP-1 macrophages treated (for 4 h) with THC and CBD, alone and in a 1:1 combination, at a final concentration of 10 μM. The number of control cells, i.e. viable cells exposed to vehicle (0.1% ethanol), was defined as 100%. Triton X-100 (0.2% for 10 min) treatment was used as a positive control. Treatment with Triton X-100 reduced cell viability ($F(4,40) = 188.4$; $p < .001$; ANOVA). Data are expressed as means ± S.E.M from 3 independent passages performed in triplicate. ***$p < .001$ versus vehicle control group.

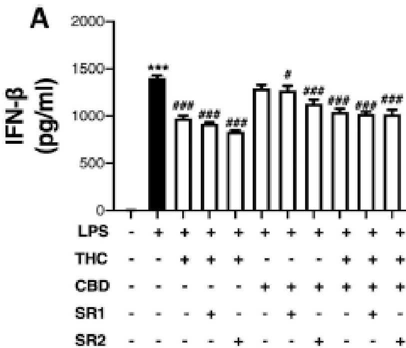
Table 1
Constitutive expression of CB_1, CB_2 and PPAR-γ in THP-1 macrophages.

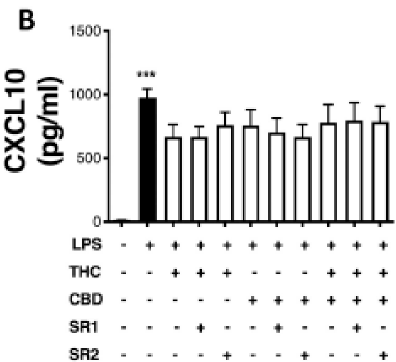
	Target genes		
	CB_1	CB_2	PPAR-γ
Average delta Ct[a] values ($n = 3$)	17.69 ± 0.23	27.98 ± 0.45	12.55 ± 0.67

[a] delta Ct = Ct of Target gene – Ct of housekeeping gene.

A key objective of this study was to determine if the cellular actions of THC and CBD on TLR signalling differed when cannabinoids were delivered independently or in combination. Overall, data herein indicate that a similar degree of anti-inflammatory efficacy was seen following treatment with THC and CBD alone when compared to their combination, with the exception of TLR4-induced CXCL10. Indeed, both THC and CBD inhibited LPS-induced CXCL10 protein expression, although an exaggerated inhibition was determined when THC and CBD were delivered in a 1:1 combination. The mechanistic basis to this remains to be elucidated. However, in support of this, it was recently shown that a Sativex®-like combination of CBD and THC was more effective in the restoration of motor function in the Theiler's murine encephalomyelitis virus (TMEV)-induced demyelination model, when compared to the administration of botanical extracts of CBD, and THC, alone (Feliu et al., 2015). Indeed, a botanical extract containing 1:1 combination of CBD and THC (in addition to CBG, CBC and other phytocannabinoids) was more effective at improving motor deficits in the chronic phase of TMEV infection than administration of CBD or THC botanical extract alone.

Caution must be applied when extrapolating the effects of phytocannabinoid administration in vivo in animal models, and in cells in a culture system in vitro, to clinical application of cannabinoid-based therapies in humans. Each 100 μl Sativex® actuation delivers 2.5 mg CBD and 2.7 mg THC alongside other constituents (Stott et al., 2013), and in terms of Epidiolex®, both 10 mg/kg and 20 mg/kg doses (delivered in two doses/day for 14 weeks) have been shown to reduce drop seizures in patients with Lennox-Gastaut syndrome when compared to placebo (Devinsky et al., 2018). However, consideration must be given to the dosage, absorption, duration of exposure and cannabinoid oxidation/metabolism when considering THC and CBD bioavailability in vivo. For example, administration of a single dose of Sativex® via oral capsules (containing 10 mg THC + 10 mg CBD) produces mean peak plasma concentration (C_{max}) of 2.50 ng/ml CBD and 5.54 ng/ml THC





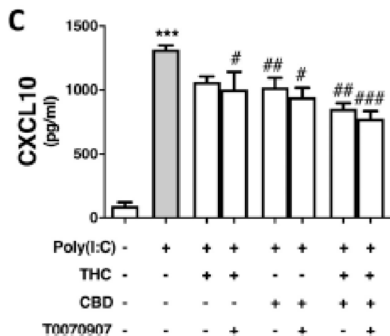


Fig. 6. CB_1, CB_2 and PPARγ receptor-independent effects of THC:CBD on TLR-induced CXCL10 expression. THP-1 macrophages were pre-treated with SR141716 (SR1), SR144528 (SR2) or T0070907 (all 1 μM; 1 h), followed by treatment with phytocannabinoids (all at 10 μM for 45 min) and stimulation with LPS (100 ng/ml) or poly(I:C) (10 μg/ml) for 4 h. (A) Pre-exposure to SR141716 and SR144528 failed to impact the inhibitory effect of THC and THC:CBD on LPS-induced IFN-β ($F(10,46) = 265.1$; $p < .001$; ANOVA). (B) The effect of THC and CBD on LPS-induced CXCL10 protein expression ($F(10,49) = 15.60$; $p < .001$; ANOVA). (C) Pre-treatment with T0070907 failed to impact the proclivity of CBD and THC:CBD to inhibit poly(I:C)-induced CXCL10 ($F(7,16) = 26.25$; $p < .001$; ANOVA). Data are expressed as means ± S.E.M from 3 to 4 independent passages. ***$p < .001$ versus control groups. #$p < .05$, ##$p < .01$ and ###$p < .001$ versus LPS- or poly(I:C)-treated groups.

(Guy and Robson, 2004), with 9 days administration of 8 oromucosal sprays once daily of Sativex® (containing 21.6 mg THC and 20 mg CBD) producing a C_{max} of 5.40 ng/ml for THC and 3.22 ng/ml for CBD (Stott et al., 2013). Elsewhere, a C_{max} of 0.93 ng/ml CBD has been reported following oral capsule administration of 5.4 mg CBD administration (Nadulski et al., 2005), while sublingual drops of CBD (20 mg) produces a C_{max} of 2.05 ng/ml (Guy and Flint, 2004). Overall, cannabinoids are commonly tested in vitro at supraphysiological concentrations that do not reflect what is observed in vivo. It is noteworthy, however, that whilst the concentration and treatment regimen employed with THC and CBD in the present study are supraphysiological, they are in line with those used in in vitro inflammatory studies elsewhere (Kozela et al., 2010; Ngaotepprutaram et al., 2013). Furthermore, the effects of both phytocannabinoids in our study cannot be explained by the potential toxic characteristics of THC (Yamaori et al., 2013) or CBD (Mato et al., 2010), as both THC and CBD when delivered alone and in a 1:1 combination at a concentration of 10 μM did not negatively impact macrophage viability in our studies.

We report that THC, CBD and a 1:1 combination of both cannabinoids impacted MyD88-independent signalling via both TLR3 and TLR4, in terms of IRF3 activation and production of CXCL10/IFN-β. In vitro data elsewhere has elucidated a role for synthetic cannabinoids in regulating poly(I:C)-induced IRF3 activation and induction of IFN-β (Downer et al., 2011), and an array of phytocannabinoids (including CBD, CBC, THCV, CBG) have been shown to inhibit poly(I:C)-induced monocyte chemotactic protein-2 production in human keratinocytes (Petrosino et al., 2018). THC has also been shown to inhibit TLR7 signalling to TBK1 and IRF7 in primary human plasmacytoid dendritic cells (Henriquez et al., 2019). Furthermore, intraperitoneal administration of CBD (10 mg/kg for 3 weeks, twice a day, at 12 h intervals) attenuates cognitive and social interaction deficits induced by pre-natal poly(I:C) exposure in rats (Osborne et al., 2017), while Peres et al. (2016) have shown that peripubertal treatment with CBD (1 mg/kg; intraperitoneal administration) attenuates hyperlocomotion induced by prenatal exposure to poly(I:C) (Peres et al., 2016). However, to our knowledge, our findings represent the first evidence to indicate that the phytocannabinoids THC and CBD can modulate viral signalling induced by activation of TLR3 in macrophages, and identifies cell signalling mechanisms that are altered following administration of THC and CBD.

TLR4 can initiate MyD88-independent signalling via TRIF by employing the bridging adaptor TRAM (Kawai and Akira, 2010). In terms of TLR4-induced MyD88-independent signalling, data herein also indicate that both THC and CBD have the proclivity to inhibit TLR4 signalling to IRF3, IFN-β and CXCL10. This is supported by in vitro evidence in the BV-2 microglial cell line indicating that both THC and CBD (at 10 μM) inhibit LPS-induced IFN-β expression (Kozela et al., 2010), which further suggests that phytocannabinoids preferentially modulate TRIF-dependent signalling independently of MyD88.

TLR4 is expressed on monocytes, macrophages, granulocytes and mature DCs (Hornung et al., 2002). Indeed, TLR4 was detected in THP-1-derived macrophages, which is consistent with data elsewhere (Wan et al., 2016). LPS activates NF-κB and IRF3 (Vaure and Liu, 2014), which promotes the expression of a range of inflammatory mediators including IFN-β (Toshchakov et al., 2002), CXCL10 (Hansen et al., 2015) and TNF-α (Zhang, 2008). Cannabinoids have well-characterised anti-inflammatory propensity by modulating TLR4 signalling, and it has been reported that the endogenous cannabinoid anandamide (2.5 μM) can attenuate TLR4-induced pro-inflammatory signalling in vitro in monocytes isolated from healthy subjects (Chiurchiu et al., 2016), while THC (10 μM) and CBD (5 μM, 10 μM) inhibit TLR4-induced IL-1β production in the BV-2 microglial cell line in vitro (Kozela et al., 2010). Surprisingly, our findings indicate that TLR4-induced signalling via MyD88 to the degradation of IκB-α, and production of TNFα and CXCL8, was refractory to phytocannabinoids. This is in contrast with evidence indicating that CBD inhibits TLR4-induced NF-κB activation in BV2 microglia (Juknat et al., 2013; Kozela et al., 2010), and that THC inhibits NF-κB activation in human T cells (Ngaotepprutaram et al., 2013). Our findings indicating that THC/CBD failed to inhibit LPS-induced degradation of IκB-α, and LPS-induced TNF-α and CXCL8 expression, may reflect differential roles of cannabinoids, or indeed MyD88 and TRIF adaptor molecules, in various cell types (T cells versus microglia versus macrophages). Furthermore, LPS has a role in the activation of the MAPK family members in THP-1 macrophages (Hu et al., 2013), and given that MAPK signalling cascades play key roles in the inflammatory responses in macrophages (Roy et al., 2014), further experiments are required to determine the role of MAPKs in determining the effect of both THC and CBD on inflammatory cytokine expression in THP-1 cells.

Efficient and robust induction of IFN-β is central in host anti-viral and anti-bacterial responses (Goubau and Deddouche, 2013), and IFN-β transcription is classically induced following upstream phosphorylation of IRF3 and its nuclear sequestration (Uematsu and Akira, 2007). This initial induction of IFN-β is referred to as the "early phase". However, the induction of IFN-β expression can act as its own positive-feedback loop, whereby induced IFN-β engages the IFN receptors (IFNAR1 and IFNAR2); referred to as the "late phase". This receptor-ligand interaction promotes the activation of the JAKs and tyrosine kinase 2 (TYK2), which phosphorylates STATs to control IFN-inducible genes such as CXCL10 (Stark and Darnell Jr., 2012). Our results indicate that THC and CBD target both TLR4- and TLR3-induced IRF3 nuclear sequestration, and the expression of CXCL10 and IFN-β; however, whether phytocannabinoids target early or late phase induction of IFN-β and IFN-inducible genes in our study is unknown. It is noteworthy however that both THC and CBD inhibit LPS-induced STAT1 phosphorylation (Kozela et al., 2010) and induction of JAK/STAT regulatory molecules (Juknat et al., 2013), which may indicate the mechanism by which phytocannabinoids regulate IFN-β-dependent pro-inflammatory processes in macrophages.

5. Conclusion

Elucidating the mechanisms by which phytocannabinoids elicit their effects in immune cells may contribute to the development of novel cannabinoid-based therapeutics to target inflammatory disorders. In addition, targeting TLR signalling is significant given the pivotal role of TLRs in innate immunity and inflammation. This study gives mechanistic insight for the immunomodulatory effects of both THC and CBD, that is dependent on the regulation of MyD88-independent signal transduction in macrophages. Data presented herein demonstrate the proclivity of THC and CBD, when delivered alone and in a 1:1 combination, to regulate the innate branch of the immune system. We identify that THC and CBD preferentially modulate MyD88-independent signalling to inhibit TLR3/4-induced CXCL10 production, in addition to reporting evidence that both phytocannabinoids modulate the upstream transcription factor IRF3. Overall, data presented herein identify CBD and THC as regulators of TLR signalling and offer mechanistic insight into the role of phytocannabinoids in modulating TLR signalling in macrophages, and hence in the treatment of inflammatory disorders.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2020.577217.

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References

Alharris, E., Singh, N.P., Nagarkatti, P.S., Nagarkatti, M., 2019. Role of miRNA in the regulation of cannabidiol-mediated apoptosis in neuroblastoma cells. Oncotarget. 10, 45–59.

Athanasiou, A., Clarke, A.B., Turner, A.E., Kumaran, N.M., Vakilpour, S., Smith, P.A., et al., 2007. Cannabinoid receptor agonists are mitochondrial inhibitors: a unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death. Biochem. Biophys. Res. Commun. 364, 131–137.

Auwerx, J., 1991. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia. 47, 22–31.

Biju, K., Zhou, Q., Li, G., Imam, S.Z., Roberts, J.L., Morgan, W.W., et al., 2010. Macrophage-mediated GDNF delivery protects against dopaminergic neurodegeneration: a therapeutic strategy for Parkinson's disease. Mol. Ther. 18, 1536–1544.

Blank, T., Prinz, M., 2017. Type I interferon pathway in CNS homeostasis and neurological disorders. Glia. 65, 1397–1406.

Bogie, J.F., Jorissen, W., Mailleux, J., Nijland, P.G., Zelcer, N., Vanmierlo, T., et al., 2013. Myelin alters the inflammatory phenotype of macrophages by activating PPARs. Acta Neuropathol Commun. 1, 43.

Brownell, J., Bruckner, J., Wagoner, J., Thomas, E., Loo, Y.M., Gale Jr., M., et al., 2014. Direct, interferon-independent activation of the CXCL10 promoter by NF-kappaB and interferon regulatory factor 3 during hepatitis C virus infection. J. Virol. 88, 1582–1590.

Buchanan, M.M., Hutchinson, M., Watkins, L.R., Yin, H., 2010. Toll-like receptor 4 in CNS pathologies. J. Neurochem. 114, 13–27.

Chiurchiu, V., Leuti, A., Cencioni, M.T., Albanese, M., De Bardi, M., Bisogno, T., et al., 2016. Modulation of monocytes by bioactive lipid anandamide in multiple sclerosis involves distinct toll-like receptors. Pharmacol. Res. 113, 313–319.

Chung, E.S., Bok, E., Chung, Y.C., Baik, H.H., Jin, B.K., 2012. Cannabinoids prevent lipopolysaccharide-induced neurodegeneration in the rat substantia nigra in vivo through inhibition of microglial activation and NADPH oxidase. Brain Res. 1451, 110–116.

Crowley, T., Fitzpatrick, J.M., Kuijper, T., Cryan, J.F., O'Toole, O., O'Leary OF, et al., 2015. Modulation of TLR3/TLR4 inflammatory signaling by the GABAB receptor agonist baclofen in glia and immune cells: relevance to therapeutic effects in multiple sclerosis. Front. Cell. Neurosci. 9, 284.

De Petrocellis, L., Ligresti, A., Moriello, A.S., Allara, M., Bisogno, T., Petrosino, S., et al., 2011. Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. Br. J. Pharmacol. 163, 1479–1494.

Demuth, D.G., Molleman, A., 2006. Cannabinoid signalling. Life Sci. 78, 549–563.

Devinsky, O., Cross, J.H., Laux, L., Marsh, E., Miller, I., Nabbout, R., et al., 2017. Trial of Cannabidiol for drug-resistant seizures in the Dravet syndrome. N. Engl. J. Med. 376, 2011–2020.

Devinsky, O., Patel, A.D., Cross, J.H., Villanueva, V., Wirrell, E.C., Privitera, M., et al., 2018. Effect of Cannabidiol on drop seizures in the Lennox-Gastaut syndrome. N. Engl. J. Med. 378, 1888–1897.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C., et al., 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature. 372, 686–691.

Downer, E.J., Fogarty, M.P., Campbell, V.A., 2003. Tetrahydrocannabinol-induced neurotoxicity depends on CB1 receptor-mediated c-Jun N-terminal kinase activation in cultured cortical neurons. Br. J. Pharmacol. 140, 547–557.

Downer, E.J., Clifford, E., Gran, B., Nel, H.J., Fallon, P.G., Moynagh, P.N., 2011. Identification of the synthetic cannabinoid R(+)WIN55,212-2 as a novel regulator of IFN regulatory factor 3 activation and IFN-beta expression: relevance to therapeutic effects in models of multiple sclerosis. J. Biol. Chem. 286, 10316–10328.

ElSohly, M.A., Radwan, M.M., Gul, W., Chandra, S., Galal, A., 2017. Phytochemistry of Cannabis sativa L. Prog. Chem. Org. Nat. Prod. 103, 1–36.

Esposito, G., Scuderi, C., Valenza, M., Togna, G.I., Latina, V., De Filippis, D., et al., 2011.

Cannabidiol reduces Abeta-induced neuroinflammation and promotes hippocampal neurogenesis through PPARgamma involvement. PLoS One 6, e28668.
Facchinetti, F., Del Giudice, E., Furegato, S., Passarotto, M., Leon, A., 2003. Cannabinoids ablate release of TNFalpha in rat microglial cells stimulated with lypopolysaccharide. Glia. 41, 161–168.
Feliu, A., Moreno-Martet, M., Mecha, M., Carrillo-Salinas, F.J., de Lago, E., Fernandez-Ruiz, J., et al., 2015. A Sativex((R)) -like combination of phytocannabinoids as a disease-modifying therapy in a viral model of multiple sclerosis. Br. J. Pharmacol. 172, 3579–3595.
Fitzgerald, D.C., O'Brien, K., Young, A., Fonseca-Kelly, Z., Rostami, A., Gran, B., 2014. Interferon regulatory factor (IRF) 3 is critical for the development of experimental autoimmune encephalomyelitis. J. Neuroinflammation 11, 130.
Fitzpatrick, J.K., Downer, E.J., 2017. Toll-like receptor signalling as a cannabinoid target in multiple sclerosis. Neuropharmacology. 113, 618–626.
Giacoppo, S., Pollastro, F., Grassi, G., Bramanti, P., Mazzon, E., 2017. Target regulation of PI3K/Akt/mTOR pathway by cannabidiol in treatment of experimental multiple sclerosis. Fitoterapia. 116, 77–84.
Gilbert, G.L., Kim, H.J., Waataja, J.J., Thayer, S.A., 2007. Delta9-tetrahydrocannabinol protects hippocampal neurons from excitotoxicity. Brain Res. 1128, 61–69.
Goubau, D., Deddouche, S., 2013. Reis e Sousa C. cytosolic sensing of viruses. Immunity. 38, 855–869.
Grotenhermen, F., 2003. Pharmacokinetics and pharmacodynamics of cannabinoids. Clin. Pharmacokinet. 42, 327–360.
Guo, B., Chang, E.Y., Cheng, G., 2008. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. J. Clin. Invest. 118, 1680–1690.
Guy, G.W., Flint, M.E., 2004. A single centre, placebo-controlled, four period, crossover, tolerability study assessing, pharmacodynamic effects, pharmacokinetic characteristics and cognitive profiles of a single dose of three formulations of Cannabis Based Medicine Extracts (CBMEs) (GWPD9901), plus a two period tolerability study comparing pharmacodynamic effects and pharmacokinetic characteristics of a single dose of a cannabis based medicine extract given via two administration routes (GWPD9901 EXT). J Cannabis Ther. 3, 35–77.
Guy, G.W., Robson, P.J., 2004. A phase I, open label, four-way crossover study to compare the pharmacokinetic profiles of a single dose of 20 mg of a Cannabis based medicine extract (CBME) administered on 3 different areas of the buccal mucosa and to investigate the pharmacokinetics of CBME per Oral in healthy male and female volunteers (GWPK0112). J Cannabis Ther. 3, 79–120.
Hansen, F.C., Kalle-Brune, M., van der Plas, M.J., Stromdahl, A.C., Malmsten, M., Morgelin, M., et al., 2015. The thrombin-derived host defense peptide GKY25 inhibits endotoxin-induced responses through interactions with lipopolysaccharide and macrophages/monocytes. J. Immunol. 194, 5397–5406.
Hendriks, J.J., Teunissen, C.E., de Vries, H.E., Dijkstra, C.D., 2005. Macrophages and neurodegeneration. Brain Res. Brain Res. Rev. 48, 185–195.
Henriquez, J.E., Crawford, R.B., Kaminski, N.E., 2019. Suppression of CpG-ODN-mediated IFNalpha and TNFalpha response in human plasmacytoid dendritic cells (pDC) by cannabinoid receptor 2 (CB2)-specific agonists. Toxicol. Appl. Pharmacol. 369, 82–89.
Henry, R.J., Kerr, D.M., Finn, D.P., Roche, M., 2014. FAAH-mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus. J. Neuroimmunol. 276, 126–134.
Hind, W.H., England, T.J., O'Sullivan, S.E., 2016. Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARgamma and 5-HT1A receptors. Br. J. Pharmacol. 173, 815–825.
Honda, K., Takaoka, A., Taniguchi, T., 2006. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. Immunity. 25, 349–360.
Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., et al., 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168, 4531–4537.
Hu, K., Yang, Y., Tu, Q., Luo, Y., Ma, R., 2013. Alpinetin inhibits LPS-induced inflammatory mediator response by activating PPAR-gamma in THP-1-derived macrophages. Eur. J. Pharmacol. 721, 96–102.
Ibeas Bih, C., Chen, T., Nunn, A.V., Bazelot, M., Dallas, M., Whalley, B.J., 2015. Molecular targets of cannabidiol in neurological disorders. Neurotherapeutics. 12, 699–730.
Iversen, L., 2000. The Science of Marijuana. Oxford University Press, New York.
Iwamura, H., Suzuki, H., Ueda, Y., Kaya, T., Inaba, T., 2001. In vitro and in vivo pharmacological characterization of JTE-907, a novel selective ligand for cannabinoid CB2 receptor. J. Pharmacol. Exp. Ther. 296, 420–425.
Juknat, A., Pietr, M., Kozela, E., Rimmerman, N., Levy, R., Gao, F., et al., 2013. Microarray and pathway analysis reveal distinct mechanisms underlying cannabinoid-mediated modulation of LPS-induced activation of BV-2 microglial cells. PLoS One 8, e61462.
Kaplan, B.L., Rockwell, C.E., Kaminski, N.E., 2003. Evidence for cannabinoid receptor-dependent and -independent mechanisms of action in leukocytes. J. Pharmacol. Exp. Ther. 306, 1077–1085.
Kaplan, J.S., Stella, N., Catterall, W.A., Westenbroek, R.E., 2017. Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome. Proc. Natl. Acad. Sci. U. S. A. 114, 11229–11234.
Kathmann, M., Flau, K., Redmer, A., Trankle, C., Schlicker, E., 2006. Cannabidiol is an allosteric modulator at mu- and delta-opioid receptors. Naunyn Schmiedeberg's Arch. Pharmacol. 372, 354–361.
Kawai, T., Akira, S., 2008. Toll-like receptor and RIG-I-like receptor signaling. Ann. N. Y. Acad. Sci. 1143, 1–20.
Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat. Immunol. 11, 373–384.
Kenny, E.F., O'Neill, L.A., 2008. Signalling adaptors used by toll-like receptors: an update. Cytokine. 43, 342–349.
Khaksar, S., Bigdeli, M.R., 2017. Anti-excitotoxic effects of cannabidiol are partly mediated by enhancement of NCX2 and NCX3 expression in animal model of cerebral ischemia. Eur. J. Pharmacol. 794, 270–279.
Kielian, T., 2006. Toll-like receptors in central nervous system glial inflammation and homeostasis. J. Neurosci. Res. 83, 711–730.
Kozela, E., Pietr, M., Juknat, A., Rimmerman, N., Levy, R., Vogel, Z., 2010. Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells. J. Biol. Chem. 285, 1616–1626.
Lai, R., Gu, M., Jiang, W., Lin, W., Xu, P., Liu, Z., et al., 2017. Raf kinase inhibitor protein preferentially promotes TLR3-triggered signaling and inflammation. J. Immunol. 198, 4086–4095.
Ledgerwood, C.J., Greenwood, S.M., Brett, R.R., Pratt, J.A., Bushell, T.J., 2011. Cannabidiol inhibits synaptic transmission in rat hippocampal cultures and slices via multiple receptor pathways. Br. J. Pharmacol. 162, 286–294.
Li, K., Fichna, J., Schicho, R., Saur, D., Bashashati, M., Mackie, K., et al., 2013. A role for O-1602 and G protein-coupled receptor GPR55 in the control of colonic motility in mice. Neuropharmacology. 71, 255–263.
Ligresti, A., Moriello, A.S., Starowicz, K., Matias, I., Pisanti, S., De Petrocellis, L., et al., 2006. Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. J. Pharmacol. Exp. Ther. 318, 1375–1387.
Liu, W.M., Scott, K.A., Shamash, J., Joel, S., Powles, T.B., 2008. Enhancing the in vitro cytotoxic activity of Delta9-tetrahydrocannabinol in leukemic cells through a combinatorial approach. Leuk Lymphoma. 49, 1800–1809.
Liu, M., Guo, S., Hibbert, J.M., Jain, V., Singh, N., Wilson, N.O., et al., 2011. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. Cytokine Growth Factor Rev. 22, 121–130.
Maess, M.B., Wittig, B., Cignarella, A., Lorkowski, S., 2014. Reduced PMA enhances the responsiveness of transfected THP-1 macrophages to polarizing stimuli. J. Immunol. Methods 402, 76–81.
Mammana, S., Fagone, P., Cavalli, E., Basile, M.S., Petralia, M.C., Nicoletti, F., et al., 2018. The role of macrophages in neuroinflammatory and neurodegenerative pathways of Alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis: pathogenetic cellular effectors and potential therapeutic targets. Int. J. Mol. Sci. 19.
Marchalant, Y., Rosi, S., Wenk, G.L., 2007. Anti-inflammatory property of the cannabinoid agonist WIN-55212-2 in a rodent model of chronic brain inflammation. Neuroscience. 144, 1516–1522.
Marta, M., 2009. Toll-like receptors in multiple sclerosis mouse experimental models. Ann. N. Y. Acad. Sci. 1173, 458–462.
Marta, M., Andersson, A., Isaksson, M., Kampe, O., Lobell, A., 2008. Unexpected regulatory roles of TLR4 and TLR9 in experimental autoimmune encephalomyelitis. Eur. J. Immunol. 38, 565–575.
Mato, S., Victoria Sanchez-Gomez, M., Matute, C., 2010. Cannabidiol induces intracellular calcium elevation and cytotoxicity in oligodendrocytes. Glia. 58, 1739–1747.
Matsumoto, M., Funami, K., Oshiumi, H., Seya, T., 2004. Toll-like receptor 3: a link between toll-like receptor, interferon and viruses. Microbiol. Immunol. 48, 147–154.
McCartney, S., Vermi, W., Gilfillan, S., Cella, M., Murphy, T.L., Schreiber, R.D., et al., 2009. Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. J. Exp. Med. 206, 2967–2976.
Miranda-Hernandez, S., Baxter, A.G., 2013. Role of toll-like receptors in multiple sclerosis. Am J Clin Exp Immunol. 2, 75–93.
Molina-Holgado, F., Lledo, A., Guaza, C., 1997. Anandamide suppresses nitric oxide and TNF-alpha responses to Theiler's virus or endotoxin in astrocytes. Neuroreport. 8, 1929–1933.
Molina-Holgado, F., Molina-Holgado, E., Guaza, C., Rothwell, N.J., 2002. Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures. J. Neurosci. Res. 67, 829–836.
Nadulski, T., Pragst, F., Weinberg, G., Roser, P., Schnelle, M., Fronk, E.M., et al., 2005. Randomized, double-blind, placebo-controlled study about the effects of cannabidiol (CBD) on the pharmacokinetics of Delta9-tetrahydrocannabinol (THC) after oral application of THC verses standardized cannabis extract. Ther. Drug Monit. 27, 799–810.
Ngaotepprutaram, T., Kaplan, B.L., Kaminski, N.E., 2013. Impaired NFAT and NFkappaB activation are involved in suppression of CD40 ligand expression by Delta(9)-tetrahydrocannabinol in human CD4(+) T cells. Toxicol. Appl. Pharmacol. 273, 209–218.
O'Neill, L.A., 2004a. TLRs: professor Mechnikov, sit on your hat. Trends Immunol. 25, 687–693.
O'Neill, L.A.J., 2004b. TLRs: professor Mechnikov, sit on your hat. Trends Immunol. 25, 687–693.
Osborne, A.L., Solowij, N., Babic, I., Huang, X.F., Weston-Green, K., 2017. Improved social interaction, recognition and working memory with cannabidiol treatment in a prenatal infection (poly I:C) rat model. Neuropsychopharmacology. 42, 1447–1457.
Owens, T., 2009. Toll-like receptors in neurodegeneration. Curr. Top. Microbiol. Immunol. 336, 105–120.
Pan, Z.K., Fisher, C., Li, J.D., Jiang, Y., Huang, S., Chen, L.Y., 2011. Bacterial LPS up-regulated TLR3 expression is critical for antiviral response in human monocytes: evidence for negative regulation by CYLD. Int. Immunol. 23, 357–364.
Pandolfo, P., Silveirinha, V., dos Santos-Rodrigues, A., Venance, L., Ledent, C., Takahashi, R.N., et al., 2011. Cannabinoids inhibit the synaptic uptake of adenosine and dopamine in the rat and mouse striatum. Eur. J. Pharmacol. 655, 38–45.
Park, L., Uekawa, K., Garcia-Bonilla, L., Koizumi, K., Murphy, M., Pistik, R., et al., 2017. Brain perivascular macrophages initiate the neurovascular dysfunction of Alzheimer Abeta peptides. Circ. Res. 121, 258–269.

Peres, F.F., Diana, M.C., Suiama, M.A., Justi, V., Almeida, V., Bressan, R.A., et al., 2016. Peripubertal treatment with cannabidiol prevents the emergence of psychosis in an animal model of schizophrenia. Schizophr. Res. 172, 220–221.

Pertwee, R.G., 2008. The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. Br. J. Pharmacol. 153, 199–215.

Petrosino, S., Verde, R., Vaia, M., Allara, M., Iuvone, T., Di Marzo, V., 2018. Anti-inflammatory properties of Cannabidiol, a nonpsychotropic cannabinoid, in experimental allergic contact dermatitis. J. Pharmacol. Exp. Ther. 365, 652–663.

Rajan, T.S., Giacoppo, S., Iori, R., De Nicola, G.R., Grassi, G., Pollastro, F., et al., 2016. Anti-inflammatory and antioxidant effects of a combination of cannabidiol and moringin in LPS-stimulated macrophages. Fitoterapia. 112, 104–115.

Ramer, R., Heinemann, K., Merkord, J., Rohde, H., Salamon, A., Linnebacher, M., et al., 2013. COX-2 and PPAR-gamma confer cannabidiol-induced apoptosis of human lung cancer cells. Mol. Cancer Ther. 12, 69–82.

Rao, R., Nagarkatti, P.S., Nagarkatti, M., 2015. Delta(9) tetrahydrocannabinol attenuates staphylococcal enterotoxin B-induced inflammatory lung injury and prevents mortality in mice by modulation of miR-17-92 cluster and induction of T-regulatory cells. Br. J. Pharmacol. 172, 1792–1806.

Rekand, T., 2014. THC:CBD spray and MS spasticity symptoms: data from latest studies. Eur. Neurol. 71 (Suppl. 1), 4–9.

Reynolds, J.M., Martinez, G.J., Chung, Y., Dong, C., 2012. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. Proc. Natl. Acad. Sci. U. S. A. 109, 13064–13069.

Ricardo, S.D., van Goor, H., Eddy, A.A., 2008. Macrophage diversity in renal injury and repair. J. Clin. Invest. 118, 3522–3530.

Rinaldi-Carmona, M., Barth, F., Heaulme, M., Shire, D., Calandra, B., Congy, C., et al., 1994. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. 350, 240–244.

Rothwarf, D.M., Karin, M., 1999. The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. Sci. STKE 1999, RE1.

Roy, R., Singh, S.K., Das, M., Tripathi, A., Dwivedi, P.D., 2014. Toll-like receptor 6 mediated inflammatory and functional responses of zinc oxide nanoparticles primed macrophages. Immunology. 142, 453–464.

Russo, E.B., Burnett, A., Hall, B., Parker, K.K., 2005. Agonistic properties of cannabidiol at 5-HT1a receptors. Neurochem. Res. 30, 1037–1043.

Ryberg, E., Larsson, N., Sjogren, S., Hjorth, S., Hermansson, N.O., Leonova, J., et al., 2007. The orphan receptor GPR55 is a novel cannabinoid receptor. Br. J. Pharmacol. 152, 1092–1101.

Sakaguchi, S., Negishi, H., Asagiri, M., Nakajima, C., Mizutani, T., Takaoka, A., et al., 2003. Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. Biochem. Biophys. Res. Commun. 306, 860–866.

Scott, K.A., Dennis, J.L., Dalgleish, A.G., Liu, W.M., 2015. Inhibiting heat shock proteins can potentiate the cytotoxic effect of Cannabidiol in human glioma cells. Anticancer Res. 35, 5827–5837.

Scuderi, C., Steardo, L., Esposito, G., 2014. Cannabidiol promotes amyloid precursor protein ubiquitination and reduction of beta amyloid expression in SHSY5YAPP+ cells through PPARgamma involvement. Phytother. Res. 28, 1007–1013.

Shang, V.C., Kendall, D.A., Roberts, R.E., 2016. Delta(9)-tetrahydrocannabinol reverses TNFalpha-induced increase in airway epithelial cell permeability through CB2 receptors. Biochem. Pharmacol. 120, 63–71.

Stark, G.R., Darnell Jr., J.E., 2012. The JAK-STAT pathway at twenty. Immunity. 36, 503–514.

Stott, C.G., White, L., Wright, S., Wilbraham, D., Guy, G.W., 2013. A phase I study to assess the single and multiple dose pharmacokinetics of THC/CBD oromucosal spray. Eur. J. Clin. Pharmacol. 69, 1135–1147.

Sun, S., Hu, F., Wu, J., Zhang, S., 2017. Cannabidiol attenuates OGD/R-induced damage by enhancing mitochondrial bioenergetics and modulating glucose metabolism via pentose-phosphate pathway in hippocampal neurons. Redox Biol. 11, 577–585.

Takeda, S., 2014. Delta(9)-tetrahydrocannabinol targeting estrogen receptor signaling: the possible mechanism of action coupled with endocrine disruption. Biol. Pharm. Bull. 37, 1435–1438.

Tanaka, H., Imaizumi, T., 2013. Inflammatory chemokine expression via toll-like receptor 3 signaling in normal human mesangial cells. Clin Dev Immunol. 2013, 984708.

Thiele, E.A., Marsh, E.D., French, J.A., Mazurkiewicz-Beldzinska, M., Benbadis, S.R., Joshi, C., et al., 2018. Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. Lancet. 391, 1085–1096.

Toshchakov, V., Jones, B.W., Perera, P.Y., Thomas, K., Cody, M.J., Zhang, S., et al., 2002. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat. Immunol. 3, 392–398.

Touil, T., Fitzgerald, D., Zhang, G.X., Rostami, A., Gran, B., 2006. Cutting edge: TLR3 stimulation suppresses experimental autoimmune encephalomyelitis by inducing endogenous IFN-beta. J. Immunol. 177, 7505–7509.

Uematsu, S., Akira, S., 2007. Toll-like receptors and type I interferons. J. Biol. Chem. 282, 15319–15323.

Varvel, N.H., Neher, J.J., Bosch, A., Wang, W., Ransohoff, R.M., Miller, R.J., et al., 2016. Infiltrating monocytes promote brain inflammation and exacerbate neuronal damage after status epilepticus. Proc. Natl. Acad. Sci. U. S. A. 113, E5665–E5674.

Vaure, C., Liu, Y., 2014. A comparative review of toll-like receptor 4 expression and functionality in different animal species. Front. Immunol. 5, 316.

Vella, R.K., Jackson, D.J., Fenning, A.S., 2017. Delta(9)-tetrahydrocannabinol prevents cardiovascular dysfunction in STZ-diabetic Wistar-Kyoto rats. Biomed. Res. Int. 2017, 7974149.

Wan, J., Shan, Y., Fan, Y., Fan, C., Chen, S., Sun, J., et al., 2016. NF-kappaB inhibition attenuates LPS-induced TLR4 activation in monocyte cells. Mol. Med. Rep. 14, 4505–4510.

Wang, X., Zheng, X., Ma, C., Zhao, L., 2015. Role of TRIF small interference RNA (siRNA) in chronic experimental allergic encephalomyelitis (EAE). Med. Sci. Monit. 21, 2583–2587.

Wilhelmsen, K., Khakpour, S., Tran, A., Sheehan, K., Schumacher, M., Xu, F., et al., 2014. The endocannabinoid/endovanilloid N-arachidonoyl dopamine (NADA) and synthetic cannabinoid WIN55,212-2 abate the inflammatory activation of human endothelial cells. J. Biol. Chem. 289, 13079–13100.

Yamaori, S., Ishii, H., Chiba, K., Yamamoto, I., Watanabe, K., 2013. Delta-tetrahydrocannabinol induces cytotoxicity in macrophage J774-1 cells: involvement of cannabinoid receptor 2 and p38 MAPK. Toxicology. 314, 254–261.

Zarember, K.A., Godowski, P.J., 2002. Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J. Immunol. 168, 554–561.

Zattoni, M., Mura, M.L., Deprez, F., Schwendener, R.A., Engelhardt, B., Frei, K., et al., 2011. Brain infiltration of leukocytes contributes to the pathophysiology of temporal lobe epilepsy. J. Neurosci. 31, 4037–4050.

Zeissler, M.L., Eastwood, J., McCorry, K., Hanemann, C.O., Zajicek, J.P., Carroll, C.B., 2016. Delta-9-tetrahydrocannabinol protects against MPP+ toxicity in SH-SY5Y cells by restoring proteins involved in mitochondrial biogenesis. Oncotarget. 7, 46603–46614.

Zhang, D., 2008. Lipopolysaccharide (LPS) of Porphyromonas gingivalis induces IL-1b, TNF and IL-6 production by THP-1 cells in a way different from that of Escherichia coli LPS. Innate Immunity. 99–107.

Zuniga, M.C., Raghuraman, G., Hitchner, E., Weyand, C., Robinson, W., Zhou, W., 2017. PKC-epsilon and TLR4 synergistically regulate resistin-mediated inflammation in human macrophages. Atherosclerosis. 259, 51–59.

Zygmunt, P.M., Andersson, D.A., Hogestatt, E.D., 2002. Delta 9-tetrahydrocannabinol and cannabinol activate capsaicin-sensitive sensory nerves via a CB1 and CB2 cannabinoid receptor-independent mechanism. J. Neurosci. 22, 4720–4727.

Appendix 2

Ethical approval letters

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,

pp [signature]
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 Costelloe - Recruitment)

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: gvalc@rcsi.ie www.beaumontethics.ie

Beaumont Hospital
Ethics (Medical Research) Committee

Chairperson: Professor Gerry McElvaney
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

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,
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To: edowner@tcd.ie

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Dr. Peter Branagan
Convenor
Ethics (Medical Research) Committee

c.c.

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- 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 May2016

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-SR_{16})
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

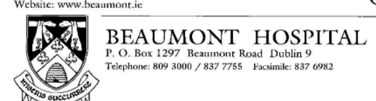
Yours sincerely

Phil Oglesby
Acting Administrator
Ethics (Medical Research) Committee

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

Appendix 3

Study Questionnaires (MSQOL-54, QUIDS-SR$_{16}$, background information), 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:

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 related studies or other studies in the future.	***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 future	**Yes** ☐	**No** ☐

academic research **related** *to the current study in, and outside, the EU, but only if the research is approved by a Research Ethics Committee.*		
I agree that some future research projects may be carried out by researchers working for ***commercial/pharmaceutical companies.***	**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 biological material be destroyed but *I give permission for my* data *derived from my biological material to be* stored *for* possible future research **related** *to the current study* **without further consent** *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.

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: Secretarys 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 situation's 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. Downers 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.

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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This survey asks about your health and daily activities.

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. , 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. , such as running, lifting heavy objects, participating in strenuous sports	1	2	3
4. , 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 seyncal flights of stairs	1	2	3
7. Climbing oos flight of stairs	1	2	3
8. Bending, kneeling, or stooping	1	2	3
9. Walking	1	2	3
10. Walking	1	2	3
11. Walking one block	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

(Circle one number on each line)

	YES	NO
13. Cut down on the yo u could spend on work or other activities	1	2
14. than you would like	1	2
15. Were limited in the kiad of work or other activities	1	2
16. Had difficulty 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
prnblems (such as feeling depressed or anxious).

(Circle one number on each line)

	YES	NO
17. Cut down on the you could spend on work or other activities	1	2
18. than you would like	1	2
19. Didn't do work or other activities as carefully 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?

(

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 ....523-32.

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 last 4 weeks...

Circle one number on each line)

	All of the Time	Most Of the Time	A Good Bit of the Time	Some ofthe Time	A Little of the Time	None ofthe 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 calmand 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 ofthe Time	A Little ofthe Time	None ofthe 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 ofthe Time	A Little ofthe Time	None ofthe 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, suchas 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 keepingan 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 last 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:

10 9 8 7 6 S 4 3 2
1 0

Best Possible

Worst Possible
Quality-of-Life

Quality-of-Life

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-SR_{16})

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.

Appendix 4

8.1 Effect of $CB_{1/2}$ antagonists, THC and CBD on TLR4-induced *IFN-β* or *CXCL10* mRNA expression in THP-1-derived macrophages

The cannabinoid pharmacology underlying the effect of THC and CBD on LPS-induced MyD88-independent regulation of *IFN-β* and *CXCL10* mRNA in THP-1-derived macrophages was assessed. Receptor involvement was addressed by employing the use of the CB_1 and CB_2 antagonists, SR141716 and SR144528, respectively. Pre-exposure to SR141716 or SR144528 (both at 1 μM for 1 h), failed to impact the proclivity of THC (10 μM) and CBD (10 μM) to regulate LPS-induced *IFN-β* (Fig. 8.1A, B) and *CXCL10* (Fig. 8.1C, D) mRNA expression. However, it is important to note that THC (Fig. 8.1A, C) and CBD (Fig. 8.1B, D) failed to significantly inhibit TLR4-induced *IFN-β* and *CXCL10* mRNA in this experiment. This is in contradiction to data outlined in Figure 4.5 where THC and CBD significantly inhibited TLR4-induced IFN-β and CXCL10 protein, with a trend towards inhibiting *IFN-β* and *CXCL10* mRNA observed.

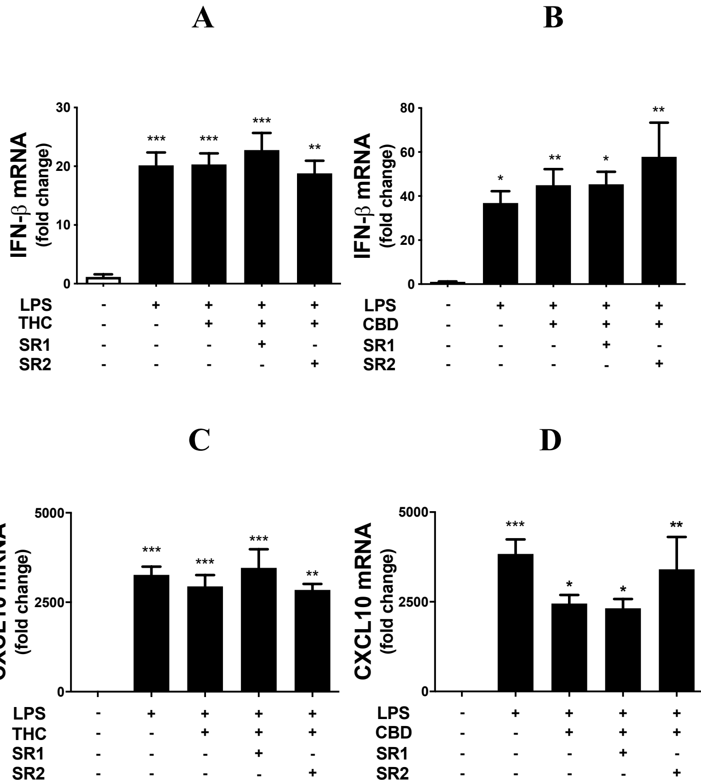


Figure 8.1. Effect of SR141716, SR144528, THC and CBD on LPS-induced IFN-β and CXCL10 expression in macrophages. THP-1 macrophages were pre-treated with SR141716 (SR1) or SR144528 (SR2) (all 1 μM; 1 h), followed by treatment with phytocannabinoids (all at 10 μM for 45 min) and stimulation with LPS (100 ng/ml) for 4 h. Effect of SR141716, SR144528, THC and CBD to LPS-induced **(A, B)** *IFN-β* and **(C, D)** *CXCL10* mRNA expression. Data are expressed as mean ± S.E.M from 3-4 independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used for statistical assessment. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *versus* control groups.

8.2 Inhibition of $CB_{1/2}$ does not alter the effect of THC and CBD on TLR3-induced *IFN-β* or *CXCL10* expression in THP-1-derived macrophages

The cannabinoid pharmacology underlying the effect of THC and CBD on poly(I:C)-induced regulation of *IFN-β* and *CXCL10* mRNA expression in THP-1-derived macrophages was assessed. Receptor involvement was addressed by employing the use of the CB_1 and CB_2 antagonists, SR141716 and SR144528, respectively. Pre-exposure to SR141716 or SR144528 (both at 1 μM for 1 h), failed to impact the proclivity of THC (10 μM), CBD (10 μM) and THC:CBD (10 μM) to regulate poly(I:C)-induced *IFN-β* (Fig. 8.2A, B, C) and *CXCL10* (Fig. 8.2D, E, F) mRNA expression. However, it is important to note that THC (Fig. 8.1A, D), CBD (Fig. 8.2B, E) and THC:CBD (Fig. 8.2C, F) failed to significantly impact TLR3-induced *IFN-β* and *CXCL10* mRNA in this experiment. This is in contradiction to data outlined in Figure 4.3, where THC, CBD and THC:CBD significantly inhibited TLR3-induced *IFN-β* and *CXCL10* mRNA expression.

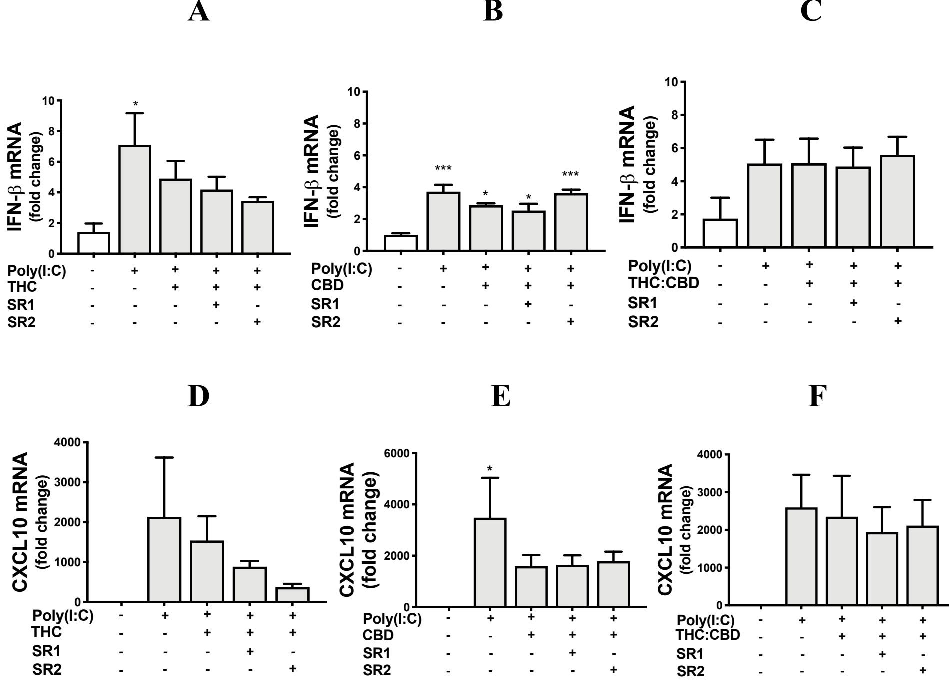


Figure 8.2. THC and CBD do not act via CB_1 or CB_2 to modulate TLR signalling. THP-1 macrophages were pre-treated with SR141716 (SR1) or SR144528 (SR2) (all 1 μM; 1 h), followed by treatment with phytocannabinoids (all at 10 μM for 45 min) and stimulation with poly(I:C) (10 μg/ml) for 4 h. Effect of SR141716, SR144528, THC and CBD on poly(I:C)-induced **(A, B, C)** *IFN-β* and **(D, E, F)** *CXCL10* mRNA expression. Data are expressed as mean ± S.E.M from 3-4 independent passages. One-way ANOVA followed by Dunnett's post-hoc test was used for statistical assessment. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *versus* control groups.

Appendix 5

9.1 Poly(I:C) does not induce IL-6 or IL-8 protein expression over a range of doses in primary human PBMCs

PBMCs from HC subjects were cultured with poly(I:C) to optimise the dose/timepoints required to induce cytokine and chemokine production in this cell type. PBMCs were cultured at a cell density of 0.5 x 10^6 cells/well, with or without poly(I:C) (0.5 - 50 μg/ml), for 8 h and 24 h, and supernatants analysed for IL-6 (Fig. 9.1A, B) and IL-8 (Fig. 9.1C, D) protein expression via ELISA. Poly(I:C) did not promote IL-6 expression after treatment for 8 h (Fig. 9.1A) or 24 h (Fig. 9.1B). Furthermore, poly(I:C) had no effect on IL-8 protein expression at both timepoints (8 and 24 h) assessed (Fig. 9.1C, D). These data suggest that poly(I:C) does not induce IL-6 and IL-8 expression in primary human PBMCs.

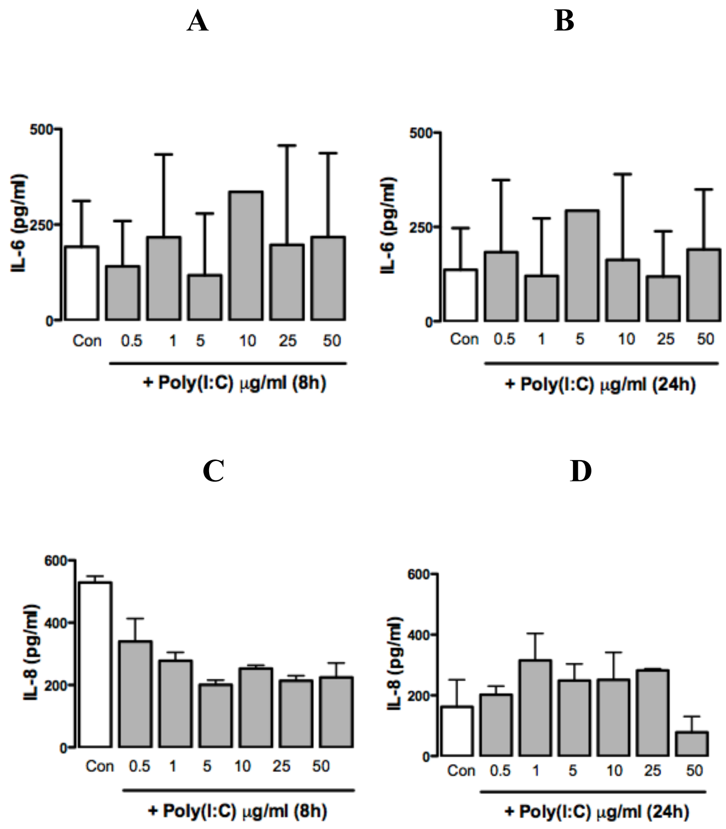
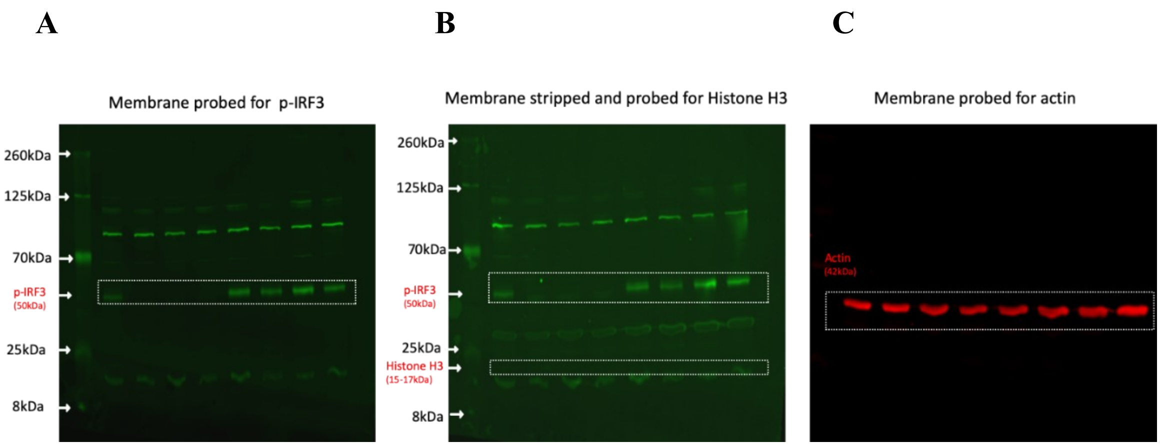


Figure 9.1. TLR3 activation does not promote IL-6 or IL-8 protein expression in PBMCs from healthy volunteers. The concentration of **(A, B)** IL-6 and **(C, D)** IL-8 protein was determined in primary human PBMCs cultured at a cell density of 0.5 x 10^6 PBMCs/well following exposure to poly(I:C) (0.5 - 50 μg/ml) for either 8 h or 24 h. Data are represented as the mean ± S.E.M from 2 HC donors.

Appendix 6

Histone H3 nuclear marker western blot data in THP-1-derived macrophages

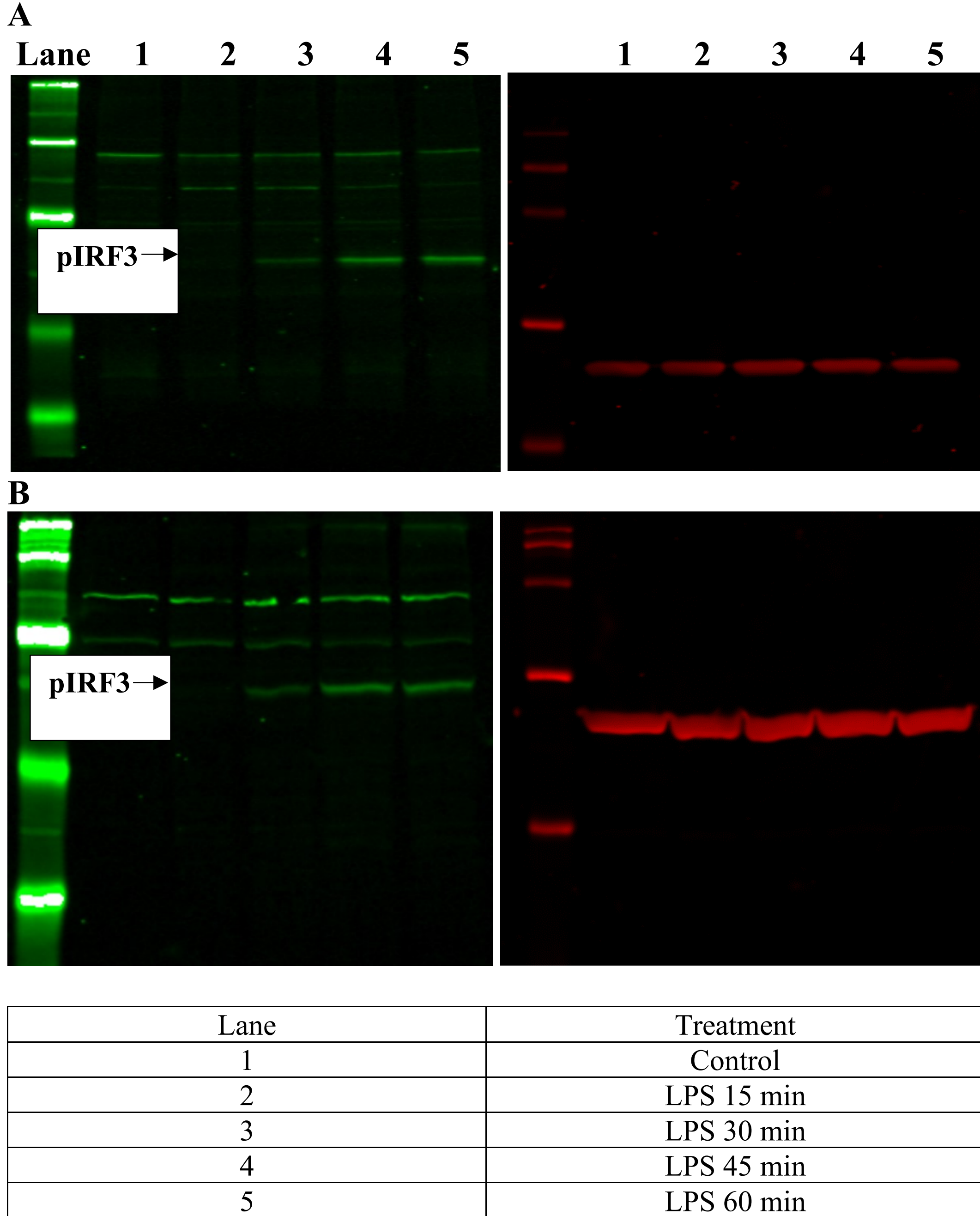
Western blot data in figure 4.4 demonstrated β-actin as a housekeeper nuclear marker. In this series of experiments selected nuclear extract membranes were probed for p-IRF3 and β-actin, and were then stripped and re-probed for the nuclear specific marker histone H3. Unfortunately, histone H3 could not be detected on any re-probed membrane.



Images showing (A) original p-IRF3 (50 kDa) probe detected (highlighted in red and inside box), (B) stripped membrane and stain for H3 (15-17 kDa) (highlighted in red and inside box), and (C) original β-actin probe in red.

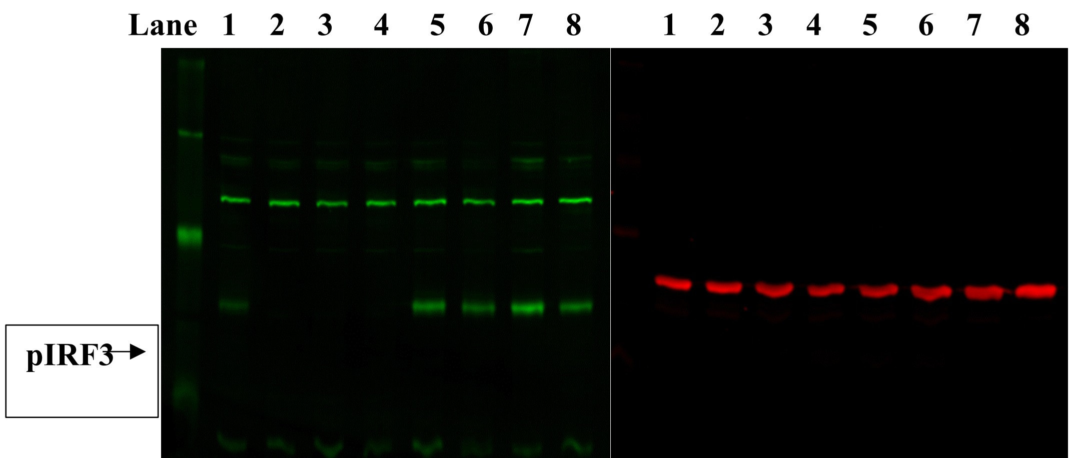
Original blots for pIRF3, pIκBα, and IκBα

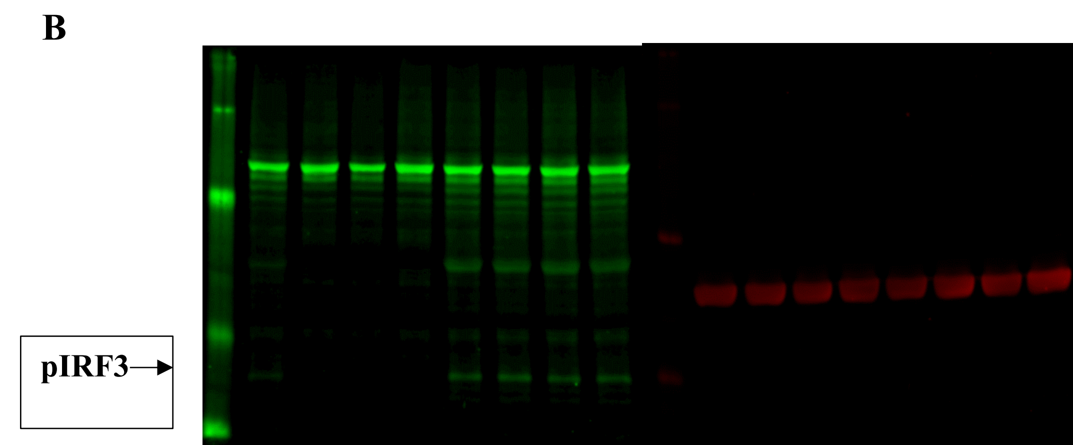
1. (A)Nuclear and (B) cytoplasmic expression of pIRF3 (green) timecourse (0-60 min) after LPS (100 ng/ml) treatment in THP-1 macrophages (Fig. 3.11), β-actin (red) used as an endogenous control.



Lane	Treatment
1	Control
2	LPS 15 min
3	LPS 30 min
4	LPS 45 min
5	LPS 60 min

2. (A)Nuclear and (B) cytoplasmic expression of pIRF3 after LPS (100 ng/ml) ± THC, CBD and THC:CBD (including cannabinoid alone) treatment in THP-1 macrophages (Fig. 4.5), β-actin used as an endogenous control.





Lane	Treatment
1	Control
2	THC
3	CBD
4	THC:CBD
5	LPS
6	LPS+THC
7	LPS+CBD
8	LPS+THC:CBD

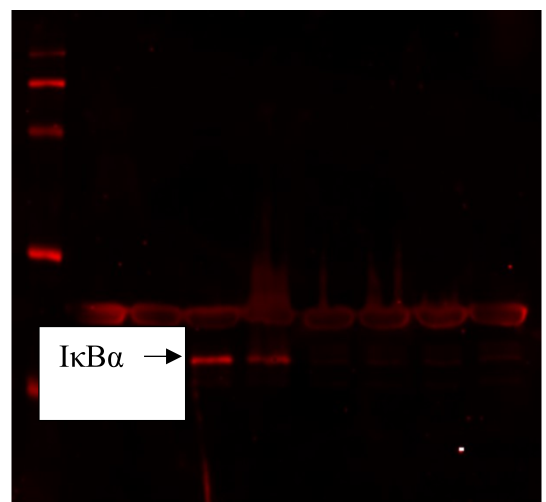
1. Cytoplasmic expression of (A) pIκBα and (B) IκBα after LPS (100 ng/ml) ± THC, CBD and THC:CBD (including cannabinoid alone) treatment in THP-1 macrophages (Fig. 4.4), β-actin used as an endogenous control. Both targets detected from the same membrane.

A

Lane 1 2 3 4 5 6 7 8 **1 2 3 4 5 6 7 8**

pIκBα →

B



Lane	Treatment
1	Control
2	THC
3	CBD
4	THC:CBD
5	LPS
6	LPS+THC
7	LPS+CBD
8	LPS+THC:CBD