EXPLORING THE MOLECULAR BASIS TO THE REGULATORY EFFECTS OF CANNABINOIDS ON TOLL-LIKE RECEPTOR SIGNALLING

by

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Table of Contents

Abstract

Toll like receptor (TLR) signalling is central in controlling innate immune responses, with dysregulation of TLR pathways associated in both autoimmune and inflammatory diseases. Numerous reports suggest that cannabinoids, known to exhibit general immunosuppressive properties, have potential therapeutic value in the treatment of inflammatory conditions. In relation to multiple sclerosis (M.S.), cannabinoids palliate M.S. patient symptoms and the severity of clinical signs associated with experimental autoimmune encephalomyelitis (EAE), an animal model of M.S. However, the precise molecular mechanism for these effects is not understood. While the synthetic cannabinoid R(+)WIN55,212-2 exerts established anti-inflammatory effects *in vitro* and *in vivo*, the role of cannabinoid compounds in modulating TLR signalling events is unknown. Here, using a variety of cell model systems, evidence is provided that R(+)WIN55,212-2, in the presence of the TLR3 ligand, Poly(I:C), targets interferon regulatory factor 3 (IRF3), a transcription factor essential for regulating type I interferon (IFN) expression. These findings also demonstrate that protective effects of R(+)WIN55,212-2 in EAE mice exist, as evidenced by reduced clinical scores, demyelination and inflammation. Furthermore, the anti-inflammatory effects of R(+)WIN55,212-2 in this model are IFN-β dependent. In addition to blunting pro-inflammatory signaling induced by Poly(I:C) in PBMCs from both healthy donors and M.S. patients, R(+)WIN55,212-2 robustly enhanced IFN-β production in these cells, an event restricted to M.S. patients.

This study also highlights the anti-inflammatory potential of R(+)WIN55,212-2 by virtue of its inhibitory effects on the NFκB pathway. R(+)WIN55,212-2 can inhibit TLR3/4-induced activation of NFκB. This likely makes a major contribution to the inhibitory effects of R(+)WIN55,212-2 on pro-inflammatory gene expression. Indeed, it is also demonstrated that R(+)WIN55,212-2 blunts TLR3/4 induction of TNF-α. The regulatory effects of R(+)WIN55,212-2 on TLR3/TLR4-induced activation of IRF3, IFN-β and NFκB are independent of the cannabinoid receptors and evidenced is presented suggesting a potential role for peroxisome proliferator activated receptors in mediating the regulatory effects R(+)WIN55,212-2.

Overall these findings highlight that R(+)WIN55,212-2, by targeting IRF3 and IFN-β downstream of TLR3 stimulation, may exert anti-inflammatory properties and

provide evidence that cannabinoid administration may offer therapeutic potential in the treatment of M.S.

Abbreviations

AC:	Adenylyl cyclase
AEA:	Anandamide
AF:	Activation function
AMV RT:	Avian myeloblastosis virus reverse transcriptase
AP-1:	Activating protein-1
APC:	Antigen presenting cells
APS:	Ammonium persulfate
Asp:	Aspartic acid
ATF:	Activated transcription factor
βTrCP:	β-transducing repeat-containing protein
B:	Bursa
BAFF:	B cell activating factor
BBB:	Blood brain barrier
BJAB:	Burkitt lymphoma B
BMDM:	Bone marrow derived macrophages
bp:	Base pair
BSA:	Bovine serum albumin
Btk:	Bruton's tyrosine residue
C terminal:	Carboxyl terminal
cAMP:	Cyclic adenosine 3', 5'- monophosphate
CARD:	Caspase-recruitment domain
CB:	Cannabinoid
CBP:	CREB-binding protein
CD:	Cluster of differentiation
cDNA:	Complementary DNA
CHO:	Chinese hamster ovary
cIAPs:	Cellular inhibitor of apoptosis proteins
CNS:	Central nervous system
COX:	Cyclo-oxygenase
CpG:	2' deoxyribocytidine-phosphate-guanosine

CREAE:	chronic relapsing experimental allergic encephalomyelitis
CREB:	Cyclic AMP-response element binding protein
CSF:	Cerebrospinal fluid
DAG:	Diacyglycerol
DAPI:	4’,6-diamidino-2-phenylindole
DC:	Dendritic cell
DD:	Death domain
δ-9-THC:	delta-9-tetrahydrocannabinol
DEPC:	Diethylpyrocarbonate
DMD:	Disease modifying drugs
DMEM:	Dulbecco’s Modified Eagle’s Medium
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic acid
dNTP:	Deoxyribonucleotide triphosphate
dsRNA:	Double-stranded RNA
dToll:	Drosophila Toll
DTT:	Dithiothreitol
E. coli:	Escherichia coli
EAE:	Experimental autoimmune encephalomyelitis
EBV:	Epstein Bar Virus
EBVA1:	EBV nuclear antigen 1
ED:	Extracellular domain
EDSS:	Expanded Disability Status Scale
EDTA:	Ethylenediaminetetraacetic acid
EGFP:	Enhanced GFP
EGTA:	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EV:	Empty vector
FAAH:	Fatty acid amide hydrolase
FADD:	FAS-associated via death domain
FBS:	Foetal bovine serum
FOXP3:	Forkhead box P3
F protein:	Fusion protein

GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GFP:	Green fluorescence protein
GPCR:	G protein coupled receptor
GPI:	Glycosylphosphatidylinositol
GPP:	Geranyl diphosphate
h:	Hours
HBSS:	Hanks Balanced Salt Solution
HEK:	Human embryonic kidney
HLA:	Human leukocyte antigen
HRP:	Horseradish peroxidase
HSP:	Heat shock protein
HSV:	Herpes simplex virus
IAD:	IRF association domain
ICAM-1:	Intercellular adhesion molecule 1
ICE:	IL-1-converting enzyme
ID:	Intermediate domain
IFN:	Interferon
IFNAR:	Type 1 IFN receptor
Ig:	Immunoglobulin
IκB:	Inhibitor of NFκB
IKK:	IκB kinase
IKK:	Inducible IKK
IL-1:	Interleukin-1
IL-1R:	Interleukin-1 receptor
IL-1Ra:	IL-1 receptor antagonist
IL-1RAcP:	IL-1R accessory protein
iNOS:	Inducible nitric oxide synthase
IP-10:	IFN-γ- inducible protein 10
IPAF:	ICE-protease activating factor
IPC:	IFN-producing cell
IPS1:	IFN-β promoter stimulator 1
IRAK:	IL-1 receptor-associated kinase
IRF:	Interferon regulatory factor
ISGF3:	IFN-stimulated gene factor 3

ISRE:	Interferon-stimulated response element
JAK:	Janus-activated kinase
JC:	Jacob–Creutzfeldt
JNK:	c-Jun N-terminal kinase
KD:	Kinase-dead
kDa:	Kilo Daltons
KO:	Knockout
LBD:	Ligand binding domain
LBP:	LPS-binding protein
Leu:	Leucine
LPS:	Lipopolysaccharide
LRR:	Leucine-rich repeat
LTA:	Lipoteichoic acid
MAL:	MyD88-adaptor-like
MALP:	Mycoplasma lipoprotein
MAP:	Mitogen-activated protein
MAPK:	MAP kinase
MAPKAP:	MAPK activated protein kinase
MBP:	Myelin basic protein
MCMV:	Murine cytomegalovirus
MCP-1:	Monocyte-chemoattractant protein 1
MD-2:	Myeloid differentiation protein 2
MDA: :	Melanoma differentiation-associated gene
mDC:	Myeloid DC
MDP:	Muramyl dipeptide
MHC:	Major histocompatibility complex
min:	Minutes
MIP:	Macrophage inflammatory protein
MMTV:	Mouse mammary tumour virus
MOG:	Myelin oligodendrocyte glycoprotein
MRI:	Magnetic resonance imaging
mRNA:	Messenger RNA
M.S.:	Multiple Sclerosis
MSK:	Mitogen- and stress-activated kinase

MyD88:	Myeloid differentiation factor 88
N terminal:	Amino terminal
NACHT:	Domain present in NAIP, CIITA, HET-E and TP1
NADA:	*N*-arachidonoyl-dopamine
NALP:	NACHT-LRR- and pyrin-domain containing proteins
NArPE:	N-Arachidonoyl-phosphatidylethanolamine
NAT:	N-acyltransferase
NCoR:	Nuclear receptor co-repressor
NEMO:	NFκB essential modulator
NES:	Nuclear export sequence
NK:	Natural killer
NFκB:	Nuclear factor κB
NIK:	NFκB-inducing kinase
NLR:	NACHT-LRR
NLS:	Nuclear localisation sequence
NOD:	Nucleotide-binding oligomerization domain
PAMP:	Pathogen-associated molecular pattern
PBMC:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
pDC:	Plasmacytoid DC
PDK1:	3-phosphoinositidedependent protein kinase 1
PG:	Peptidoglycan
PI3K:	Phosphatidylinositol-3 kinase
PIP:	phosphatidylinositol 4,5-bisphosphate
PKC:	Protein kinase C
PLP:	Proteolipid protein
PML:	Progressive multifocal leukoencephalopathy
PMSF:	Phenylmethylsulfonyl fluoride
Poly:(I:C):	Polyriboinosinic:polyribocytidylic acid
PPAR:	Peroxisome proliferator activated receptors
PP-M.S.:	Primary-progressive M.S.
PRD:	Positive regulatory domain
PRD-LE:	Positive regulatory domain-like elements

PPRE:	Peroxisome proliferator response element
PR-M.S.:	Progressive-relapsing M.S
PRR:	Pathogen recognition receptor
PTX:	Pertussis toxin
PYD:	Pyrin domain
RANTES:	Regulated on activation normal T cell expressed and secreted
RD:	Regulatory domain
RHD:	Rel homology domain
RHIM:	RIP homotypic interaction motif
RIG-I:	Retinoic acid-inducible protein
RING:	Really interesting new gene
RIP:	Receptor-interacting protein
RLR:	RIG-I like receptor
RNA:	Ribonucleic acid
RR-M.S.:	Relapsing-remitting M.S.
RSV:	Respiratory syncytial virus
RXR:	Retinoid X receptor
S1P:	Sphingosine 1-phosphate
SARM:	sterile α- and armadillo-motif-containing protein
SCF:	SKp1-Cullin-F-box
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
Ser:	Serine
SFM:	Serum-free medium
SH:	*Src* homology
siRNA:	Small interfering RNA
SMRT:	Silencing mediator for retinoid and thyroid hormone receptors
SOCS:	Suppressor of cytokine signalling
SP-M.S.:	Secondary progressive M.S.
SNP:	Single-nucleotide polymorphism
SRC:	Steroid receptor co-activator
ssRNA:	Single-stranded RNA

sTLR:	Soluble TLR
STAT:	Signal transducer and activator of transcription
SV:	Sendai virus
T:	Thymus
Ta:	Annealing temperature
TAD:	Transcription activation domain
TAB:	TAK-1 binding protein
TAE:	Tris-acetate-EDTA
TAK-1:	TGF-β-activated protein kinase 1
TANK:	TRAF family-member-associated NFκB activator
TBK-1:	TANK binding kinase 1
TCR:	T cell receptor
TE:	Tris-EDTA
TGF-β:	Transforming growth factor β
Th:	T helper
TICAM:	Toll/IL-1 receptor domain-containing adaptor molecule
TIR:	Toll/IL-1 receptor
TIRAP:	TIR-associated protein
TLR:	Toll like receptor
Tm:	Melting temperature
TMEV:	Theiler's murine encephalomyelitis virus
TMEV-IDD:	TMEV-induced demyelinating disease
TNF:	Tumour necrosis factor
TNFR:	TNF receptor
TRADD:	TNFR1-associated death domain protein
TRAF:	TNF receptor associated factor
TRAM:	TIR domain-containing adaptor inducing IFN-β -related adaptor molecule
Tregs:	T regulatory
TRIF:	TIR domain-containing adaptor inducing IFN-β
TTBS:	Tris buffered saline containing Tween 20
TZD:	Thiazolidinediones
VCAM-1:	Vascular cell adhesion molecule-1

WT:	Wild-type

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This thesis is dedicated to my mother who was diagnosed with multiple sclerosis in 1999. Her ability to smile and laugh during the tough times is a true testament to what a great mum she is and has been. I thank my supervisor for giving me the opportunity to further extend my knowledge of this neurological condition, and I wish both present and future researchers the determination, skill and commitment to work together in finding the cause and hopefully one day, a cure for multiple sclerosis.

Declaration

I, Eileen Clifford, declare that this thesis is my own work and has not been submitted in any form for another qualification at any university or Institute of Education. Information derived from the work of others has been acknowledged and cited in this text.

Signed: _____ Date: _____

Chapter 1

General Introduction

1.1 The mammalian immune system

As mammals are continually at risk from the pathogenic influences of a highly diverse range of microorganisms in the environment, this has resulted in the evolution of an extremely effective and efficient mammalian immune system. The mammalian immune system is typically characterised by its ability to distinguish between the self and non-self recognition of various pathogenic molecules and can be divided into two interactive systems: the innate immune system and the adaptive immune system. The phylogenetically older innate immune system surfaced approximately one billion years ago, while the adaptive immune system appeared around 450 million years ago with the emergence of the gnathostomes or jawed vertebrates (Andersson and Matsunaga, 1996).

The innate immune system represents the hosts' first line of defense against a wide array of pathogens and does not require prior exposure to foreign antigens in order to be triggered. Its antigen-nonspecific defense mechanism is fast acting and short lived. Elements of the innate immune system include anatomical barriers e.g. skin and mucosal membranes, cellular components e.g. macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, resident central nervous system (CNS) microglia and perivascular macrophages and soluble proteins e.g. complement. Its ability to recognise such a broad spectrum of microorganisms is accomplished by a set of germline-encoded pathogen recognition receptors (PRRs) which detect conserved microbial sequence patterns or products of microbial metabolism known as pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). The initial stimulation of PPRs by microbial targets culminates in the subsequent induction and control of the adaptive immune system.

The adaptive immune system, also known as the acquired immune system, is found only in vertebrates. This system is involved in the elimination of pathogens during the late phase of infection and is both specific and customised for each pathogen. The adaptive immune system is mediated by the activation of lymphocytes e.g. T (Thymus) cells and B (Bursa) cells. Lymphocytes express a diverse range of recombinant receptors which are randomly generated by somatic gene rearrangement. Lymphocyte activation in the adaptive immune system is governed by the type of immune response e.g. a cell-mediated immune response is responsible for T cell activation while a humoral immune response

chiefly involves B cells and is responsible for triggering clonal selection (Burgio and Ugazio, 1975).

In recent years, PPRs e.g. toll like receptors (TLRs) have been subject to intensive characterisation as key recognition receptors of the innate immune system. TLR stimulation by microbial components can result in the maturation of DCs, where DCs function in the presentation of pathogen-derived antigens to naïve T cells. This presentation subsequently triggers the activation of the adaptive immune system and therefore highlights the important role TLRs play in linking innate and adaptive immunity.

1.2 Pathogen and pathogen-associated molecular patterns

Different PRRs react with specific PAMPs which determines the fate of specific signalling pathways (Janeway and Medzhitov, 2002). PAMPs are identifiable microbial motifs that include lipids and nucleic acids. PAMPs are not found in host cells, are highly conserved and essential for microbial viability. PPRs can often form homodimers or heterodimers with each other thus allowing two separate PPRs to activate the same PAMP. Bacteria, viruses, fungi and protozoa all contain structures that can act as PAMPs (Fig 1.1) and hence elicit an innate immune response.

1.3 Pathogen Recognition Receptors

A number of PRRs have been identified, including receptors expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. These PRRs include members of nucleotide-binding oligomerization domain (NOD) proteins containing leucine-rich repeats (NLRs), retinoic acid-inducible gene (RIG-I)-like receptors (RLRs) and TLRs (Fig 1.2). To date, TLRs have been the most intensively studied PRRs of the innate immune system.

1.3.1 Toll-Like Receptors

The Toll protein was first discovered in *Drosophila Melanogaster* as a gene required for the establishment of dorso-ventral polarity in the developing embryo (Lohs-Schardin et al., 1979). Subsequently, it was shown to play a critical role in the antifungal immune response in flies against *Aspergillus fumigatus* (Lemaitre et al., 1996). This finding prompted a search for orthologous receptors which led to the discovery of the human homolog of the *Drosophila* Toll (dToll), namely human Toll (Medzhitov et al., 1997). Human Toll has since been named TLR4. Sequence alignment of human and Drosophila Toll proteins display a high degree of homology, with particular similarity between their cytoplasmic domains.

TLRs are type I transmembrane proteins of the interleukin-1 receptor (IL-1R) family. TLRs are horseshoe shape in structure and possess an extracellular N (amino) terminal domain, a multiple leucine-rich repeats (LRR) domain and a C (carboxyl) terminal cytoplasmic domain. The LRR domain comprises 24 amino acids and functions in the recognition of PAMPs (West et al., 2006). This is followed by a short single transmembrane region and finally a conserved C terminal cytoplasmic domain. The C terminal domain is highly homologous among individual TLRs and also to the intracellular cytoplasmic domain of the IL-1R, therefore terming this domain Toll/IL-1R (TIR). While the extracellular N terminal domain of TLR's consists of multiple LRR, the extracellular domain of IL-1R contains immunoglobulin (Ig)-like domains.

Since 1997, 13 members of the mammalian TLR family have been identified, 12 of which are expressed in humans. TLRs 1–9 are conserved among humans and mice, yet TLR10 is present only in humans and TLR11 is only functional in mice (West et al., 2006). TLRs are widely expressed in many cell types including B cells, certain subsets of T cells, nonhematopoietic epithelial and endothelial cells and hematopoietically derived sentinel cells, such as macrophages, neutrophils, and DCs (West et al., 2006). DCs primarily have the highest expression of TLRs, however variations do exist among their different subsets, for example, between conventional DCs and plasmacytoid DCs (pDCs).

TLRs can be divided into subfamilies based on their sequence similarity, subcellular localisation, and the nature of the recognised PAMP. While certain TLRs (TLR 1, 2, 4, 5,

and 6) are expressed on the cell surface, other TLRs (TLR 3, 7, 8 and 9) are found almost exclusively in intracellular components such as endosomes (Akira et al., 2006). Despite the conservation among their extracellular domain, different TLRs can recognise structurally unrelated ligands, enabling this family of receptors to detect microorganisms ranging from bacteria and protozoans, to fungi and viruses (Fig 1.1).

At the cell surface most TLRs appear to function as homodimers. However certain TLRs can form heterodimers. It is speculated that before ligand binding, TLR dimers are pre-assembled in a low-affinity complex (O'Neill and Bowie, 2007). Upon ligand binding, a conformational change occurs, which brings the two TIR domains into close proximity, thus creating a platform for adaptor recruitment and subsequent signalling induction.

1.3.1.1 TLR1, TLR2 and TLR6

Bacteria are generally classified as either Gram-positive or Gram-negative based on their staining characteristics (Manafi and Kneifel, 1990). Lipoproteins and lipopeptides are abundant on the outer membrane of bacteria and are potent stimuli for certain TLRs. TLR1, TLR2 and TLR6 are all expressed on the plasma membrane (West et al., 2006). TLR1 can form homodimeric complexes and interact with the bacterial PAMP; triacylated lipoproteins. TLR1 can also form heterodimers with TLR2 and also interact with triacylated lipoproteins including the 19 kDa mycobacterial lipoprotein, meningococcal lipoproteins (Wyllie et al., 2000) and the synthetic lipoprotein structure PAM3CSK4 (Takeuchi et al., 2001). Homodimerisation of TLR2 generates specificity to various ligands including lipoproteins and cell wall components including peptidoglycan (PG) and lipoteichoic acid (LTA) which are present in both gram positive and gram negative bacteria (Akira et al., 2006). In accordance with the above, TLR2 deficient mice were found to be highly susceptible to infection by Gram-positive bacteria such as *Staphylococcus aureus* (Takeuchi et al., 2000). The interaction of PG with cluster of differentiation 14 (CD14), an LRR-containing glycosylphosphatidylinositol (GPI)-linked molecule, not only causes TLR2 activation but several groups have postulated that CD14 also acts to transfer PG to TLR2 for subsequent signalling (Sellati et al., 1998, Vasselon et al., 2004).

While triacylated lipoproteins are preferentially recognised by the TLR1/TLR2 complex, diacylated lipoproteins including mycoplasma lipoproteins (MALPs) are recognised by the TLR2/TLR6 complex (Takeuchi et al., 2002). This discrimination between TLR complexes is associated with cysteine residues present in lipoproteins i.e. all lipoproteins contain a lipolyated cysteine at their N terminus which is responsible for bacterial-associated immunostimulatory activity (Akira, 2003). PAM3CSK4 contains a triacylated cysteine residue at its N terminus while the cysteine residue present in MALP-2 is diacylated. Takeuchi and colleagues established that TLR6 deficient mice failed to produce tumour necrosis factor alpha (TNF-α) in response to diacylated lipoprotein stimulation but displayed a normal response to triacylated lipoproteins (Takeuchi et al., 2002). Furthermore, TLR1 knockout mice had impaired TNF-α production to triacylated lipoproteins but not to diacylated lipoproteins. In parallel, CD36 is involved in the recognition of diacylated lipoproteins by TLR2/TLR6 heterodimers (Hoebe et al., 2005).

Zymosan, a mixture of cell membrane components from *Saccharomyces cerevisiae* can also induce signalling through interaction with the TLR2/TLR6 complex (Kataoka et al., 2002). In addition, a C-type lectin; Dectin-1 can collaborate with the TLR2/TLR6 complex to mediate independent but cooperative signalling in response to zymosan. Lipopolysaccharide (LPS) has also been proposed to act as a TLR2 ligand, however this erroneous conclusion was later shown to be caused by contamination of 'pure' LPS with traces of biologically active lipopeptides (Hirschfeld et al., 2000).

In comparison to other TLRs, TLR2 appears to respond to the largest repertoire of PAMPs. This is believed to be, in part, due to its ability to form heterodimeric complexes with other TLRs. Previous crystallographic analyses of the TIR domain of human TLR1, TLR2 and TLR10 revealed that the TIR domain of each TLR is composed of five β-strands alternated with five α-helices connected by eight loops (Xu et al., 2000). Moreover, this domain contains three highly conserved motifs. One such conserved motif is the BB-loop which is situated between the second β-strand and second α-helix. This loop is key for TLR signalling, as single residue substitutions abolish its ability to recognise PAMPs without changing the overall structure of the TIR domain (Xu et al., 2000).

1.3.1.2 TLR3

TLR3 is composed of twenty-three LRRs and recognises the viral PAMP; double-stranded RNA (dsRNA) which is the form of genetic information carried by many viruses (West et al., 2006). TLR3 also binds to polyriboinosinic:polyribocytidylic acid (Poly(I:C)), a synthetic version of dsRNA. TLR3 is found mainly in endosomal compartments. This strategic location enables TLR3 to recognise nucleic acids released by viruses that are internalised for delivery to the endosome (Barton and Kagan, 2009).

Much of the TLR3 protein surface is covered with carbohydrate sugar molecules, where one face is glycosylation free, suggesting that this is where TLR3 may bind to its respective PAMP (Choe et al., 2005). This surface also contains two distinct patches that are rich in positively-charged residues, which may be a possible binding site for negatively charged dsRNA.

TLR3 expression is rapidly and dramatically upregulated via ligand binding interaction and culminates in the activation of interferon (IFN) regulatory factor- (IRF)-3 and late phase nuclear factor κ B (NFκB) activation. TLR3 is expressed in DCs, in a variety of epithelial cells, including airway, uterine, corneal and intestinal epithelial cells, which function as efficient barriers to infection (Akira et al., 2006). Furthermore, TLR3 is strongly expressed in the brain, specifically in astrocytes and glioblastoma cell lines which highlights its potential involvement in multiple sclerosis (M.S.) (Bsibsi et al., 2006). Excessive TLR3 activation has also been associated with a number of other inflammatory diseases including lupus nephritis and West Nile virus-driven CNS inflammation (Wang et al., 2004).

Recent studies have reported on two cytoplasmic PRRs that recognise dsRNA independently of TLR3; RIG-I and (melanoma-differentiation-associated gene 5) MDA5. RIG-I and MDA5 are abundantly expressed in multiple cell types (Andrejeva et al., 2004, Yoneyama et al., 2004) and overcome the pathogen-detection limitations posed on TLRs confined to the endosomes by surveying the cytoplasm for viral particles.

1.3.1.3 TLR4

TLR4 was the first mammalian TLR to be discovered in 1997 (Medzhitov et al., 1997). TLR4 is expressed in cells including monocytes/macrophages, myeloid DCs (mDCs), mast cells and the intestinal epithelium (Sallusto and Lanzavecchia, 2002). TLR4 recognises a diverse range of structurally unrelated PAMPs including LPS, the plant diterpene paclitaxel, the fusion protein from respiratory syncytial virus (RSV), fibronectin, and heat-shock proteins (HSP) e.g. HSP60 (Akira et al., 2006).

LPS is the most thoroughly studied TLR4 ligand. LPS, also known as endotoxin, is a glycolipid component of the outer membranes of Gram-negative bacteria. However, LPS has also been found in one species of Gram-positive bacteria; *Listeria monocytogenes* (Oppenheim et al., 1981). LPS is composed of a core oligosaccharide with polysaccharide lipid portion and a Lipid A part. Lipid A is responsible for most of the pathogenic phenomena associated with LPS and it is therefore unsurprising that it proves to be the portion of LPS recognised by TLR4 (Akira et al., 2006). Lipid A is a unique and distinctive phosphoglycolipid, the structure of which is highly conserved among species (Tsubery et al., 2002). However, variations in Lipid A can arise from the degree of phosphorylation, the presence of phosphate substituents, and the number and position of the acyl groups.

Prior to TLR4 ligand stimulation, LPS leaves gram-negative bacteria and associates with the LPS-binding protein (LBP) (Schumann et al., 1994). LBP is an acutephase protein present in the bloodstream. Upon association with LBP, the LPS/LBP complex is delivered to CD14, where CD14 serves as a receptor for the LPS/LBP complex. As CD14 has no intracellular signalling domain, CD14 therefore associates with TLR4 and myeloid differentiation protein 2 (MD-2), a small accessory protein. MD-2 belongs to the MD-2 related lipid-recognition family and is found associated with the extracellular domain of TLR4 on the cell surface (Shimazu et al., 1999). The importance of MD-2 in TLR4 ligand recognition has previously been demonstrated (Schromm et al., 2001). In Chinese hamster ovary (CHO) cells with mutant MD-2, TLR4 signalling is abolished, whereas, wild-type MD-2 cells are capable of restoring LPS-induced signalling to normal.

Although it is widely accepted that LPS is the dominant ligand for TLR4, variations do exist in the structure of LPS that can have both a drastic and negative impact on TLR4-induced signalling. For instance, *Yersina pestis*, the cause of the plague, augments TLR4 signalling when grown at 25°C (Montminy et al., 2006). However, at 37°C, the bacterium changes its hexa-acylated LPS into a tetra-acylated form which acts as a TLR4 antagonist and prevents the transcription of various pro-inflammatory cytokines. Similarly in *Escherichia coli* (*E. coli*) LPS, the best known example of a potent agonist for TLR4, the tetra-acylated lipid A precursor of *E. coli* LPS, lipid IVa, exhibits potent antagonist activity to *E. coli* LPS in human monocytes (Golenbock et al., 1991). Furthermore, the penta-acylated form of *E. coli* LPS that is derived from a mutant *E. coli* strain can efficiently antagonise the ability of hexa-acylated *E. coli* LPS to activate human endothelial cells (Somerville et al., 1996). While LPS variations can negatively impact on TLR4 signalling, their ability to behave as LPS antagonists has received significant attention as potential therapeutic agents. For example, the synthetic penta-acylated lipid A-like compound, E5531, is an antagonist for LPS-dependent cell activation which has been considered as a potential therapeutic agent for bacterial induced septic shock (Christ et al., 1995, Kawata et al., 1999).

In addition to its bacterial recognition ability, TLR4 has also been reported to recognise viral and fungal pathogens. TLR4 is implicated in the recognition of *Candida albicans* (Netea et al., 2004), pertussis toxin from *Bordetella pertussis* (Kerfoot et al., 2004) and mannan, a fungal constituent of *Saccharomyces cerevisiae*. As aforementioned, TLR4 recognises the fusion protein from RSV. In TLR4 knockout mice, RSV can persist for longer periods in the lungs in comparison to wild type mice (Kurt-Jones et al., 2000). The envelope glycoprotein of mouse mammary tumor virus (MMTV) can also activate B cells through TLR4 stimulation (Rassa et al., 2002). Taxol, an anti-tumour agent purified from the bark of the pacific yew, is capable of mimicking the effects of LPS signalling in mice but not in humans (Kawasaki et al., 2001).

1.3.1.4 TLR5

Flagellin is a protein component of gram negative bacterial flagella; the mobility apparatus used by many pathogenic microorganisms and constitutes as the main ligand for TLR5 (Hayashi et al., 2001). The structure of flagellin is highly conserved. Recent analysis of the crystal structure of a *Salmonella* flagellin revealed that the flagellin domains are composed of N- and C-terminal α-helix chains (D0), central α-helix chains (D1), and a hypervariable central region with β-sheets (D2 and D3) (Yonekura et al., 2003). TLR5 recognises the highly conserved central D1 domain in flagellin that is essential for protofilament assembly and bacterial mobility (Hayashi et al., 2001). More specifically, flagellin appears to bind directly to TLR5 at residues 386–407 of the extracellular domain (ED), as TLR5-ED knockouts lacking this domain are unable to interact with flagellin (Mizel et al., 2003).

Previous analysis of flagellin from various species has revealed that *Campylobacter jejuni* and *Helicobacter pylori* flagellin each fail to activate TLR5 (Andersen-Nissen et al., 2005). Likewise, the hypervariable D2 and D3 domain of flagellins from *E. coli* and *Salmonella* species is not required for TLR5 signalling (Smith et al., 2003). Smith and colleagues also demonstrated that monomers of flagellin induce TLR5 signalling whereas filamentous flagella do not.

TLR5 is expressed on a number of immune cells including monocytes, DCs, T cells and NK cells. TLR5 expression is also found in epithelial cells which provide the receptor with the best strategy to respond to flagellated bacteria i.e. TLR5 is expressed on the basolateral side of the intestinal epithelium and can thereby only sense flagellin once the bacteria have crossed the epithelium (Akira, 2003).

1.3.1.5 TLR7 and TLR8

TLR7 and TLR8 are highly homologous and are located on the X chromosome (Wang et al., 2006). While both TLR7 and TLR8 are expressed in mice, many reports suggest that mouse TLR8 appears to be non-functional (Zhu et al., 2008, Chi and Flavell, 2008). However, a recent study demonstrates that TLR8 deficient DC's over express TLR7

and induce greater NFκB activation upon stimulation with the synthetic antiviral imidazoquinoline compound; R848 (Demaria et al., 2010). Both TLR7/8 are also expressed in the endosomal or phagosomal compartments of cells including monocytes and neutraphils and recognise guanosine or uridine-rich single stranded RNA (ssRNA) from viruses such as influenza, Sendai and Coxsackie B. TLR7/8 also recognise Imiquimod, an additional synthetic antiviral imidazoquinoline compound (Jurk et al., 2002) which is consistent with reports where TLR7 knockout mice fail to induce inflammatory cytokines and type I IFN in response to Imiquimod (Hemmi et al., 2002).

Viral ssRNA is rich in the nucleosides; uridine or guanosine. Since these nucleosides are not specific to viruses, TLR7 does not distinguish between self or viral RNA. Instead, it triggers an inflammatory response to any ssRNA encountered in the endosome (Diebold et al., 2006). Host nucleic acids are not usually found in endosomal compartments but can trigger TLR signalling. For example, inefficient removal of apoptotic debris may result in host nucleic acids being available to activate TLRs and therefore break self-tolerance, thus leading to autoimmunity (Leadbetter et al., 2002, Midwood et al., 2009). Systemic lupus erythematosus (SLE), a systemic autoimmune disease, is characterised by the production of autoantibodies directed against self nuclear antigens such as chromatin or small nuclear ribonuceoproteins (snRNPs) (Rahman and Eisenberg, 2006, Somarelli et al., 2011). B cells normally produce autoantibodies in response to engagement with self-antigens when activated by T helper cells (Linterman and Vinuesa, 2010). However, a recent study has demonstrated the ability of B cells to produce autoantibodies independently of T helper cells (Herlands et al., 2008). The activation, expansion, and differentiation of AM14 B lymphocytes depended on the TLR7/9 activation of MyD88, as mice deficient in either TLR displayed partial defects in B lymphocyte development. TLR7 and TLR9 signalling pathways can also result in the transcription of IFN-α via IRF7. An increase in IFN-α has been associated in the pathology of SLE (Plotz, 2003, Liu et al., 2011).

1.3.1.6 TLR9

Bacterial genomic DNA, which contains unmethylated 2'-deoxyribocytidine-phosphate-guanosine (CpG) motifs, has potent immunostimulatory activity. Mutation studies have reported that a single nucleotide mutation or methylation of a cytosine residue within a CpG motif, results in the loss of the immunostimulatory property of bacterial DNA (Krieg et al., 1995). Mammalian DNA differs from that of bacterial DNA in that it contains relatively few CpG motifs and is highly methylated. TLR9 recognises bacterial DNA containing unmethylated CpG motifs, as TLR9-deficient mice are unresponsive to CpG DNA ligand stimulation (Hemmi et al., 2000).

TLR9 is believed to be localised in the endoplasmic reticulum and is recruited to late endosomes or lysosomes upon recognition of its respective ligand (Leifer et al., 2004). This endosomal restriction of TLR9 is critical for discriminating between self and non-self DNA because host DNA, unlike microbial DNA, do not usually enter the endosomal compartments. Activation of TLR9 also requires acidification of endosomes and lysosomes, as agents that block endosomal acidification completely abrogate CpG-induced signalling (Häcker et al., 1998).

Studies have demonstrated that TLR5 and TLR9 co-operate in their signalling events (Merlo et al., 2007). Likewise, human TLR8 can dimerise with both TLR7 and TLR9 (Wang et al., 2006). Wang and colleagues also demonstrated that TLR9 can interact with TLR7 and antagonise its signalling. Overall, the interactions within this family subset reveal their complexity of TLR signalling networks. To date, pDCs are the only immune cells to selectively express TLR7 and TLR9 (Bekeredjian-Ding et al., 2005).

1.3.1.7 TLR10 and TLR11

TLR10 was first identified in 2001 and was shown to be expressed in lymphoid tissues e.g. spleen, lymph nodes, thymus and tonsil (Chuang and Ulevitch, 2001). In addition, the transcription factor forkhead box P3 (FOXP3) has been shown to regulate the expression of TLR10 in human T regulatory (Tregs) cells (Bell et al., 2007). TLR10 contains 811 amino acid residues and is most closely related to TLR1 and TLR6 in regard

to the overall amino acid identity (Chuang and Ulevitch, 2001). TLR10 appears to be only functional in humans and is absent in rodents which has limited the discovery of its specific ligand. However, TLR10 is thought to have the ability to not only homodimerise but also to form heterodimers with TLR1 and TLR2 (Hasan et al., 2005). This suggests that lipoproteins may be possible targets for TLR10 stimulation e.g. PAM3CSK4 (Govindaraj et al., 2010). As previously mentioned, crystallographic analyses of the TIR domain of human TLR10 has revealed that it is composed of five β-strands alternated with five α-helices connected by eight loops (Xu et al., 2000).

TLR11 was initially identified in mice (Zhang et al., 2004) and is similar in structure to TLR5. It is abundantly expressed in the bladder and kidneys and recognises components of uropathogenic bacteria as TLR11-deficient mice were found to be susceptible to infections caused by these bacteria (Zhang et al., 2004). However, the exact nature of these ligands has yet to be identified. Human TLR11 is non-functional due to the presence of a premature stop codon in the gene and may explain why humans are predisposed to urinary tract infections. Mouse TLR11 can recognise a class of prolin-like molecules expressed by protozoans such as *Toxoplasma gondii* (Plattner et al., 2008). TLR11 ligand interaction with protozoans induces the transcription of IL-12, which may be mediated by NFκB, as this transcription factor is activated in a dose-dependent manner by mouse TLR11 in response to *Toxoplasma gondii* (Yarovinsky et al., 2005).

1.3.1.8 TLR12 and TLR13

Minor information is available on the ligand recognition by and cellular expression of TLR12 and TLR13. However, a previous study has reported on the detection of each TLR in macrophages and in liver, kidney and bladder epithelial cells (Tabeta et al., 2004) which is consistent with the potential role of TLR12 in preventing infection of internal organs of the urogenital system. Murine TLR12 and TLR13 are expressed in the brain (Mishra et al., 2008) where this expression is upregulated in response murine neurocysticercosis infection. Mishra and colleagues also demonstrated the expression of TLR13 in ependymal cells, endothelial cells of pial blood vessels and astrocytes.

1.4 The IL-1 Receptor

The IL-1 receptor (IL-1R) was first described in 1988 (Sims et al., 1988), but it was not until several years later that the IL-1R accessory protein (IL-1RAcP) was characterised as the signal transducing subunit for the IL-1R complex (Fig 1.3). The IL-1R was the first mammalian protein shown to have homology to the Toll protein (Gay and Keith, 1991) and belong to the TIR superfamily (Martin and Wesche, 2002). Numerous studies have reported on the role of IL-1 in inflammation, in the pathogenesis of various inflammatory disorders and as a crucial cytokine in mediating the link between the innate and adaptive immune responses (Rothwell and Luheshi, 2000, O'Neill, 2008).

The IL-1 family (IL-1F) comprises of 11 members including IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and IL-18 (Taylor et al., 2002). Each member is highly conserved. Aside from IL-18 and IL-33, all genes encoding the IL-1F members are clustered on human chromosome 2. With the recent characterisation of newly cloned IL-1F members, it is now proposed that each member be assigned an individual interleukin designation (Dinarello et al., 2010). Previously, the IL-1F members were renamed e.g. IL-6 (IL-1F6), IL-7 (IL-1F7) etc, where IL-1α, IL-1β and IL-1RA were renamed IL-1F1, IL-1F2 and IL-1F3, respectively (Sims et al., 2001, Dunn et al., 2001). Dinarello and colleagues now propose that IL-1F6, IL-1F8, and IL-1F9 be designated IL-36α, IL-36β and IL-36γ, respectively, due to the fact that each member signals through the same receptor complex and initiates similar signalling cascades. IL-1F7 is to be renamed IL-37 and its various splice forms (Smith et al., 2000) be designated IL-37a, IL-37b etc.

IL-1 is produced by a wide variety of cell types including immune cells, endothelial cells and astrocytes, and functions as a ubiquitous mediator of inflammation due to the widespread expression of the IL-1R (Dinarello, 1991). As previously stated, the IL-1R contains extracellular Ig-like domains responsible for ligand binding and a TIR domain in its cytoplasmic portion which interacts with other TIR-domain containing proteins. Upon ligand binding to the IL-1R, recruitment of the IL-1RAcP occurs and forms a heterodimeric complex with IL-1R. Formation of the receptor heterodimer induces signalling as the juxtaposition of the two TIR domains enables the recruitment of the adaptor protein, myeloid differentiation factor 88 (MyD88) via its TIR domain. The

ensuing phosphorylation cascade typically follows that of the MyD88-dependent pathway which is discussed below and culminates in the activation of transcription factors including NFκB and activator protein-1 (AP-1) (O'Neill, 2008).

1.5 TLR signal transduction

TLRs mediate a vital part of the induction of the innate immune system. Ligand binding to respective TLRs leads to their conformational change and facilitates the interaction of their cytoplasmic TIR domains with downstream TIR domain-containing adaptors proteins. There are five such adaptor proteins characterised to date; MyD88, MyD88 adaptor-like (Mal) also known as TIR-associated protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF) also known as TIR-domain-containing molecule 1 (TICAM-1), TRIF-related adaptor molecule (TRAM) also known as TICAM-2 and sterile α- and armadillo-motif-containing protein (SARM) (O'Neill et al., 2003). Selective use of the adaptor molecules partly explains the differential signalling responses that are observed in response to TLR ligand stimulation (Fig 1.3). Furthermore, these responses occur through two distinct signalling pathways, broadly characterised as MyD88-dependent or MyD88-independent.

1.5.1 TIR domain-containing adaptor proteins

The TIR domain is present in all TLRs, the IL-1 receptor family and in the five intracellular TLR adaptor proteins as mentioned above. The TIR domain is typically composed of 135–160 residues, with sequence conservation ranging from 20 to 30% across the five adaptor proteins (Ohnishi et al., 2009). Within the TIR domain, three conserved, highly homologous boxes are crucial for signalling (Akira and Takeda, 2004). While the hydrophobic core residues are conserved, the surface exposed residues vary greatly between each TIR domain. Indeed, MyD88, Mal, TRIF and TRAM were all uncovered by groups searching the human genome for TIR domain-containing proteins. The interest in these proteins derives from the intrigue of how different TLRs can induce individual gene expression profiles.

1.5.1.1 MyD88

MyD88 transduces signals for all TLRs, except TLR3. It is also a key mediator for the IL-1 and IL-18 receptors (Adachi et al., 1998, Janssens and Beyaert, 2002). MyD88 was isolated as a myeloid differentiation response gene that was induced when M1 myeloleukaemic cells were differentiated into macrophages upon IL-6 stimulation (Lord et al., 1990). MyD88 is 296 amino acids in length and contains two domains. It has a modular structure, comprising of a C terminal TIR domain, through which it interacts with the TIR domain of TLRs, and an N terminal death domain (DD), which facilitates its interaction with other DD-containing proteins. These domains are separated by a small intermediate domain (ID) (Janssens et al., 2002).

Previous analysis of the MyD88 TIR domain revealed a mutation in an isoleucine residue at position 179, which abolished MyD88-mediated NFκB activation (Jiang et al., 2006). This is called the Poc site after the mutant phenotype Pococurante, and demonstrates the importance of MyD88 in NFκB signalling. Recently, three functional surface sites (Sites I–III) of the MyD88 TIR domain that are important for the LPS-induced activation of the TLR4 signalling pathway have been identified (Ohnishi et al., 2009). Sites II and III serve as binding sites for the TIR domain of Mal. Site I is unlikely to be involved with Mal or TLRs and therefore is hypothesised to serve as a binding site for a yet unidentified MyD88 binding protein or specific membrane protein. The TLR2 TIR domain has been demonstrated to directly interact with MyD88, while the TIR domain of the closely related TLR1 and TLR6 failed to mimic this interaction with MyD88 (Brown et al., 2006). TLR5 and TLR10, like TLR4, can also interact with MyD88 (Hasan et al., 2005).

1.5.1.2 Mal

Mal, also named TIRAP, was the second TIR domain-containing adaptor to be identified in 2001 by two independent groups (Fitzgerald et al., 2001, Horng et al., 2001). Mal is 256 amino acids long and contains many domains and phosphorylation sites. At its N terminus is a phosphatidylinositol 4,5-bisphosphate (PIP)-2-binding domain (Kagan and Medzhitov, 2006) which functions to recruit Mal specifically to PIP2-rich regions in the

plasma membrane. This is followed by the TIR domain and a TNF receptor associated factor (TRAF)-6-binding domain. There are two separate phosphorylation sites for Bruton's tyrosine kinase present on Mal (Watters et al., 2007). Mal also contains a single-nucleotide polymorphism (SNP) representing a serine (Ser) to leucine (Leu) mutation located at position 180 and this has been linked to several diseases including tuberculosis and malaria (Sheedy and O'Neill, 2007). At the C terminus is an aspartic acid (Asp) residue which is cleaved by caspase-1, therefore indicating the novel ability of Mal to interact with caspase-1 (Miggin et al., 2007).

TLR1, TLR2, TLR4 and TLR6 are expressed in the plasma membrane. Since PIP-2 is enriched here, Mal may mediate the signalling events induced by ligand activation of these TLRs. Studies utilising Mal-deficient mice revealed that it lies on the MyD88-dependent pathway and functions as a bridging adaptor for MyD88 by recruiting it to the cytoplasmic domain of TLR4 and TLR2 (Horng et al., 2002). The importance of Mal in TLR2 signalling is emphasised in studies where NFκB and p38 activation is abolished in Mal-deficient cells in response to TLR2 ligands (Yamamoto et al., 2002). However, Mal is not utilised by TLR5 or the IL-1R, which are also found on the plasma membrane as Mal-deficient mice show normal signalling in response to these receptors.

Studies also hypothesised that Mal may be the elusive adaptor for the MyD88-independent pathway. However, it was soon discovered that this was not the case as MyD88 and Mal double knockout mice display a similar phenotype to single knockouts of each, revealing that these two adaptors could not compensate for one another (Yamamoto et al., 2002). Furthermore, IRF3 transcriptional activation and subsequent IFN-β induction remained intact in double knockout mice, thus excluding a role for Mal in the MyD88-independent pathway.

In relation to both TLR2 and TLR4 signalling events, it is well recognised that Mal acts as a bridging adaptor for MyD88, helping to recruit MyD88 to the plasma membrane via its PIP-2 domain. In TLR2 signalling, the TIR domain of MyD88 specifically interacts with the TIR domain of TLR2 and activates NFκB and the mitogen-activated protein (MAP) kinases via a phosphorylation cascade. Interestingly, a recent study has confirmed that the TIR domain of MyD88 does not exclusively interact with TLR4 TIR domain (Ohnishi et al., 2009) and instead Mal may interact directly with TLR4 TIR domain and

recruit MyD88 to the complex, where it can activate downstream signalling components required to bring about the TLR4-induced response. Ohnishi and colleagues also revealed that the distal location of the Mal binding sites on the MyD88 TIR surface suggests that the TIR domain of MyD88 simultaneously interacts with two Mal TIR molecules, which may provide a highly efficient scaffold for signal transduction. The importance of the PIP-2 binding domain in TLR4 activation has also been revealed as blocking this localisation abolishes TLR4-MyD88-dependent signalling (Sheedy and O'Neill, 2007).

Mal may function as more than a bridging adaptor. Mal contains a TRAF6 binding domain and unlike MyD88, can interact directly with TRAF6 in response to TLR2 and TLR4 stimulation (Verstak et al., 2009). Moreover, a Mal mutant lacking the TRAF6-binding motif fails to initiate the proinflammatory response to TLR2 and TLR4 ligand activation.

Mal also contains two separate phosphorylation sites for Bruton's tyrosine kinase. Interestingly, these phosphorylation sites allow the suppressor of cytokine signalling 1 (SOCS-1) protein to bind to Mal via its *Src* homology (SH)2 domain and orchestrate its degradation by mediating the ubiquitation of Mal and subsequent degradation by the 26S proteasome (Mansell et al., 2006). Furthermore, SOCS-1-deficient cells are particularly sensitive to LPS as Mal is not targeted for degradation (Kinjyo et al., 2002, Nakagawa et al., 2002).

1.5.1.3 TRIF

Previous analysis of MyD88- and Mal-deficient cells, which lead to late phase NFκB activation following TLR4 and TLR3 ligand activation (Yamamoto et al., 2003a), suggested the possible presence of additional adaptor proteins. Searches of the human genome for TIR domain-containing proteins unveiled the TIR adaptor; TRIF, also known as TICAM-1. Yeast two-hybrid screens performed on TLR3, also identified the TRIF molecule, which was of particular interest because no known adaptor for TLR3 had been identified prior to this discovery (Oshiumi et al., 2003a). Overexpression studies revealed that TRIF induces the IFN-β promoter while in TRIF-deficient macrophages, cytokine production was decreased in response to TLR4 signalling but normal for TLR2, TLR7 and

TLR9 (Yamamoto et al., 2003a). Furthermore, LPS induction of NFκB was completely abolished in MyD88 and TRIF double knockouts macrophages (Hirotani et al., 2005).

TRIF is the longest adaptor protein characterised to date and is composed of 712 amino acids (Han et al., 2004). TRIF contains three TRAF6-binding sites in its N terminal domain, a TIR domain and a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) in its C terminal domain. Upon ligand activation of TLR3 (and also TLR4), a MyD88-independent signalling response is induced to regulate IRF3 and late phase NFκB transcriptional activity. This signalling response occurs via interaction with TRIF. In relation to type I IFN induction, TRIF interacts with TLR3 through its TIR domain while its N terminal domain forms a complex with TRAF family-member-associated NFκB activator (TANK) binding kinase 1 (TBK1) and inhibitor of NFκB (IκB) kinase (IKK)ε (Fitzgerald et al., 2003). This leads to direct phosphorylation of IRF3 at C-terminal serine residues which culminates in nuclear translocation of IRF3 and increased transcription of type I interferons.

TLR4 appears to utilise both the MyD88-dependent and MyD88-independent pathways to induce the activation of NFκB (Kawai et al., 1999). As aforementioned, TRIF contains three TRAF6-binding sites in its N terminal region. Mutations of these sites results in the partial loss of TRIF induction of NFκB including the failure to associate with TRAF6, implying that TRIF directly binds to TRAF6 to mediate NFκB activation. However, the TRAF6-binding motif mutants remain capable of activating the IFN-β promoter (Jiang et al., 2004) as TLR3 signalling via activation of TRIF is not affected by TRAF6 deletion in macrophages (Häcker et al., 2000).

A recent study has elaborated on the TRAF6 binding sites where TRAF1, 2 and 6 are capable of interacting with the N-terminus of TRIF (Sasai et al., 2010). While a single mutation in the TRAF2-binding motif of TRIF only marginally affects TRIF induction of the IFN-β promoter, mutations in both the TRAF2- and TRAF6-binding sites of TRIF abrogate the TRIF signalling pathway. Furthermore, mutations in the TRAF2 and TRAF6-binding domains of TRIF reduce N terminal polyubiquitination of the adaptor molecule as TRAF2 and TRAF6 both function as E3 ligases to mediate the K63 ubiquitination of TRIF. TRAF1 is also capable of directly interacting with TRIF via binding to the TRIF TIR domain. However, overexpression of TRAF1 negatively regulates the TRIF pathway and

the TRIF mediated activation of NFκB, IFN-stimulated response element (ISRE) and the IFN-β promoter. Numerous groups have also reported on TRIFs recruitment of TRAF3 in the activation of IRF3 and subsequent IFN-β activation (Hacker, Redecke et al. 2006; Colonna 2007).

The RHIM motif present in the C terminal domain of TRIF is responsible for direction association with RIP1 and RIP3. In RIP1-deficient mice embryonic fibroblasts, NFκB activation is normal in response to LPS induction but is lost in response to Poly(I:C) stimulation (Meylan et al., 2004), therefore suggesting that TLR3 and TLR4 utilise TRIF in a dissimilar manner. RIP3 can also bind to the RHIM motif and acts as an inhibitor of TRIF-RIP1 signalling.

TRIFs direct binding with RIP1 can also induce apoptosis via recruitment of Fas-associated death domain (FADD) by DD interactions and subsequent caspase-8 activation (Han et al., 2004). The ability to elicit TRIF-dependent apoptosis may be due to its role as protector against viruses in the TLR3 pathway as TRIF is targeted for immune evasion by the vaccinia virus protein, A46R (Stack et al., 2005) and also by the hepatitis C virus protease, NS3-4A (Li et al., 2005). While TRIF is a positive regulator of TLR3 induced signalling, TRIF has shown to play an inhibitory role in TLR5 elicited responses by inducing proteolytic degradation of TLR5 (Choi et al., 2010).

1.5.1.4 TRAM

TRAM /TICAM-2 was also initially discovered by the use of bioinformatics, on the basis that TRAM possessed strong homology to the TIR domain of TRIF (Yamamoto et al., 2003b). While Mal functions as a bridging adaptor by recruiting MyD88 to TLR2 and TLR4, TRAM also functions as a bridging adaptor by recruiting TRIF to TLR4 to mediate the activation of the MyD88-independent pathway (Oshiumi et al., 2003b). However, TLR3 does not require TRAM for IFN-β production (Yamamoto et al., 2003b).

TRAM is the smallest adaptor protein at 235 amino acids long (Yamamoto et al., 2003b). It contains a putative myristoylation site at its N terminus followed by serine at position 16 which is phosphorylated by protein kinase Cε (PKCε) and a TIR domain in its C terminus.

Analogous to Mal, TRAM is also targeted to the plasma membrane, albeit in a diverse manner. Studies have demonstrated that a mutation in the N-terminal myristoylation site causes the dissociation of TRAM from the plasma membrane (Sheedy and O'Neill, 2007) and relocates TRAM to the cytoplasm where this mutant is unable to restore LPS-induced signalling in macrophages deficient in wild-type TRAM (Rowe et al., 2006), indicating that this site serves to target TRAM to the plasma membrane. The membrane localisation of TRAM may serve to recruit TRIF to the membrane as the only known function attributed to TRAM is that of a bridging adaptor between TLR4 and TRIF. Small interfering RNA (siRNA) and dominant negative studies also reveal that although TLR3 and TLR4 both utilise TRIF, TLR4 requires initial binding to TRAM before recruiting TRIF (Fitzgerald et al., 2003). TLR4 activation of IRF3 and subsequent induction of the type I interferons is dependent on TRAM.

TRAM is also subject to regulation by phosphorylation. After LPS stimulation, TRAM leaves the membrane and this is dependent on PKCε-mediated phosphorylation of TRAM on Ser16 (McGettrick et al., 2006). This phosphorylation event is necessary for TRAM signalling as a mutant containing a substituted alanine for Ser16; TRAM S16A, abolished the ability of the adaptor protein to activate IRF3 or NFκB.

1.5.1.5 SARM

SARM was first discovered in 2001 as an ortholog of the *Drosophila melanogaster* protein, CG7915, which is highly conserved in mice that contained sterile alpha and HEAT-Armadillo motifs (SAM and ARM motifs) (Mink et al., 2001). SARM was also described to be a human orthologue of the *Caenorhabditis elegans* protein TIR-1, which is involved in the induction of the antifungal peptides; NLP-29 and NLP-31 and is required for antibacterial responses occurring upstream of PMK-1, the ortholog of human p38 (Couillault et al., 2004, Liberati et al., 2004). While the SARM ortholog of *C. elegans* has a positive function both in development and in immunity, the role of SARM in humans is quite different.

SARM is 690 amino acids in length and is made up of several domains all located near the N terminus with two SAM motifs followed by the TIR domain (Watters et al.,

2007). SARM has been reported to be closely related to bacterial proteins with TIR domains (Zhang et al., 2011) but is functionally distinct from TIR domain containing adaptor proteins in that it fails to activate NFκB or IRF3 (Liberati et al., 2004) and negatively regulates TRIF-mediated signalling (Carty et al., 2006). Although SARM fails to activate NFκB, LPS induced activation of TLR4 in primary human peripheral blood mononuclear cells (PBMC) strongly enhances endogenous SARM expression (Carty et al., 2006) indicating a potential auto-regulatory feedback system.

In resting cells, SARM and TRIF weakly associate (O'Neill, 2006). However, upon ligand activation of TLR4 and possibly TLR3, the SARM and TRIF complex stabilises via interaction with the SAM motifs. The TIR domains of SARM and TRIF possibly also interact by stabilising the SARM-TRIF complex and also by preventing TRIF interacting with other TIR domain containing adaptors, as a SARM construct lacking the TIR domain fails inhibit TRIF (Carty et al., 2006). However, the exact mechanism by which SARM targets TRIF has yet to be discovered. O'Neill and colleagues speculate that because SAM domains are necessary for the interaction with TRIF, it is possible that other SAM domain containing proteins may be recruited to limit TRIF-mediated signalling (O'Neill et al., 2003). CrSARM, a functional SARM from the horseshoe crab, downregulates the TRIF-dependent TLR signalling pathway culminating in the inhibition of NFκB, suggesting the conservation of SARM function from horseshoe crab to human (Belinda et al., 2008). While CrSARM targets TRIF-dependent signalling, it has no effect on MyD88 or TNF-α induced activation of NFκB (Belinda et al., 2008). However, in contrast, amphioxus SARM (*Branchiostoma belcheri tsingtauense* SARM) can suppress TLR signalling by targeting amphioxus MyD88 and TRAF6 (Yuan et al., 2010).

1.5.2 TLR signalling and activation of transcription factors

TLR signalling pathways were intensively studied after the discovery of MyD88. The subsequent identification of additional TIR domain–containing adaptor proteins has demonstrated that individual TLRs selectively recruit distinct adaptor molecules, thus initiating various signalling transduction cascades. This ultimately culminates in the

activation of various transcription factors including NFκB and the IRFs that, can act alone or in concert to regulate gene expression.

1.5.2.1 NFκB

As a central regulator of cell survival and a key mediator in the activation of various inflammatory signalling pathways, NFκB has been subject to intense research since its discovery over 20 years ago (Sen and Baltimore, 1986). NFκB-binding sites, consisting of the consensus sequence GGGRNNYYCC (where R = purine, Y = pyrimidine, N = any base), have been identified in the promoter regions of genes encoding a plethora of cytokines, chemokines, adhesion molecules and including mediators of the adaptive immune response and proteins involved in antigen presentation (Phal, HL, 1999). NFκB was first identified as a nuclear transcription-enhancing, DNA-binding complex governing the Ig light chain gene in mature B cells (Sen and Baltimore, 1986). As NFκB binding sites were present in the promoters of genes that were not B cell specific, it became clear that NFκB was ubiquitously expressed and played a key role in regulating the expression of many inflammatory related genes. Sen and Baltimore also demonstrated that in order to induce NFκB activation, an external stimulus for example LPS or phorbol ester treatment was required in certain cell types.

NFκB is evolutionarily and structurally conserved and has representative members in a wide range of species. Mammalian NFκB consists of five subunits; p100/52, p105/p50, p65 (RelA), c-Rel and RelB, which exist as homo- and heterodimers in the cytoplasm of resting cells, except for RelB which only forms heterodimers. Each of the NFκB subunits contains a Rel homology domain (RHD) at its N terminus of roughly 300 amino acids long. The RHD facilitates dimerisation, DNA-binding, nuclear localisation, and cytoplasmic retention by members of the inhibitor of NFκB (IκB) family. The dimerisation domain is located in the C terminal region of the RHD, whereas the N terminal part of the RHD contains the DNA-binding domain. In contrast, the transcription activation domain (TAD), necessary for target gene expression, is present only in the C terminus of p65, c-Rel, and RelB subunits (Ghosh et al., 1998).

p50 and p52 are made from immature IκB precursors, p105 and p100 respectively. The partial proteolysis of p105 and p100 is mediated by the ubiquitin/proteasome pathway. Both p105 and p100 have long C terminal domains that contain multiple copies of ankyrin repeats which are degraded upon maturation to p50 and p52, respectively. Ankyrin repeats can mediate the repressive function of p50 and p52 in transcription (Moynagh, 2005) by binding to the RHD of NFκB subunits thus masking its nuclear localisation sequence (NLS) and retaining NFκB in the cytoplasm. As a result, these subunits are generally termed repressors of transcription, unless when they form heterodimers with p65, RelB and c-Rel which is evident in the noncanonical NFκB pathway (discussed below). Mice deficient in certain NFκB subunits, p50, RelA, c-Rel or RelB are highly susceptible to microbial infections from *Streptococcus pneumoniae* and *Toxoplasma gondii* infections (Ghosh et al., 1998, Sha et al., 1995, Caamano et al., 1999).

NFκB subunits are sequestered in the cytoplasm in an inactive form and impeded from entering into the nucleus by virtue of their association with members IκB family, homologs of Drosophila Cactus (Quivy and Van Lint, 2004). IκBs, which bind to NFκB, mask its NLS, thereby preventing nuclear uptake of the transcription factor. Upon IL-1R or TLR ligand stimulation, downstream activation of the IKK signalosome occurs and results in the phosphorylation and subsequent traffic of the inhibitory IκBs to the 26S proteasome for degradation (Hacker and Karin, 2006). This cytoplasmic "switch" liberates the NFκB complex from the cytoplasm and targets it for subsequent nuclear translocation and target gene transcription. The IKK signalosome complex comprises of two catalytic subunits; IKKα, IKKβ and a regulatory subunit; NFκB essential modulator (NEMO) or IKKγ (Karin and Ben-Neriah, 2000). Analysis of IKKβ and IKKα deficient cells has demonstrated that the IKKβ subunit performs a significant contribution to canonical NFκB activation more so than IKKα. Furthermore, NEMO also appears to be essential for NFκB activation, as NEMO-deficient cells fail to activate NFκB in response to the ligand stimulation by IL-1β, TNF-α, and LPS (Israel, 2006).

The IκBs consist of three main subunits, IκBα, -β and -ε. Although these proteins retain NFκB in the cytoplasm, IκBα can be potently upregulated by NFκB and enter the nucleus and interfere with NFκB binding to DNA. IκBα can then transport NFκB back to

the cytoplasm leading to the resequestration of NFκB thus forming an overall negative feedback loop (Hoffmann and Baltimore, 2006).

NFκB is also targeted by stimulation of the IL-1R. Together with stimulation of TLRs and TNF receptor (TNFR), these pathways comprise the canonical NFκB pathway and converge on the IKK complex. In noncanonical NFκB activation, stimulation of a subset of TNFR superfamily members, including the B cell activating factor (BAFF) receptor, induces NFκB activation via activation of NFκB-inducing kinase (NIK) and the IKKα subunit. The noncanonical pathway is particularly important in the regulation, survival and maturation of B cells (Pomerantz and Baltimore, 2002) as B cells deficient in NIK or IKKα display defects in their ability to mature or survive. In resting cells, the BAFF receptor recruits TRAF3 and TRAF2 to the plasma membrane where interactions occur via their TRAF-binding motif (Morrison et al., 2005). BAFF induces proteolysis of TRAF3, leading to stabilisation of NIK. NIK is consequently ubiquitinated by an E3 complex consisting of TRAF3, TRAF2, and cellular inhibitor of apoptosis proteins (cIAPs). This polyubiquitination targets NIK for degradation by the proteasome and therefore it fails to engage with IKKα and mediate the subsequent activation of NFκB. However, in stimulated cells, the formation of the TRAF3-TRAF2-cIAP complex causes TRAF2 to catalyse the K63 polyubiquitination of cIAPs, which promotes the ability of cIAPs to catalyse K48 polyubiquitination of TRAF3 and target it for degradation by the proteasome. NIK then phosphorylates IKKα, which in turn phosphorylates the p100 subunit, leading to its ubiquitination by the SKp1-Cullin-F-box (SCF)-protein β-transducing repeat-containing protein (βTrCP) (SCF-βTrCP) complex and subsequent proteasomal processing to p52. p52 forms a complex with RelB where the active heterodimer translocates to the nucleus and induces gene expression. In contrast, p105 undergoes constitutive cleavage to produce p50. Proteasomal processing of p105 occurs cotranslationally and posttranslationally, and can result in the complete degradation or formation of p50.

1.5.2.2 The interferon regulatory factors

The initial discovery of ‘the interferon’ inhibition of viral replication occurred in 1954 (Nagano and Kojima, 1954) where wider recognition was attained by Isaacs and

Lindenmann who coined the word "interferon" in 1957 (Lindenmann et al., 1957). Since the 1950s, a vast body of evidence has now accumulated to demonstrate the central involvement of the IRFs and their associated transcription of a diverse range of interferons in various phases of host defense, including antiviral and anticancer immune responses. The type I IFNs are a multigene family evolutionarily conserved in vertebrates and are expressed following exposure to a wide variety of infectious agents. They are characteristic of an antiviral response and are so called because of their ability to interfere with virus replication (Samuel, 2001). The type I IFNs are the most diverse of all the cytokine families. IFNs and their receptors are a subset of the class 2 α-helical cytokines and comprise of multiple subtypes including IFN-α, -β and -γ. Thirty type I IFN gene clusters have been found on the human chromosome 9p:13, while 24 type I IFN genes are clustered on chromosome 4p:13 (Hardy et al., 2004). Many cell types induce type I IFN secretion; however, the main IFN-producing cells (IPCs) are DCs (primarily pDC's), macrophages and other haemopoietic cells (Taniguchi and Takaoka, 2001). These cells constitutively produce low levels of IFN-α/β which renders the cells 'ready-to-go' for the enhancement of cellular responses to external stimuli.

Viral infection induces the transcription of multiple type I IFN genes, a response that is in part mediated by the activation of the IRFs. The initially characterised members; IRF1 and IRF2 are now part of a growing family of transcriptional regulators that has expanded to nine members (Taniguchi et al., 2001). Although IRF1, IRF3, IRF5, and IRF7 function as positive-feedback regulators of type I IFN genes, only IRF3, IRF5 and IRF7 play a crucial role in the expression of type I IFN genes (Tamura et al., 2008).

Each IRF family member contains an N-terminal DNA-binding domain of approximately 120 amino acid residues and a C-terminal IRF association domain (IAD) (also known as the regulatory domain (RD)) that is responsible for signal transduction (Mamane et al., 1999, Taniguchi et al., 2001). The well conserved DNA-binding domain forms a helix-turn-helix motif with a conserved tryptophan cluster and mediates specific binding to 5′-GAAA-3′ and 5′-AANNGAAA-3′ sequences termed ISRE, that are found in promoters of all genes activated by IRFs (Taniguchi et al., 2001). The C terminal IAD domain also includes the cyclic AMP-response element binding protein (CREB)-binding protein (CBP)/p300 interaction surface, an autoinhibitory domain and various

phosphorylation sites. Transient transfection assays utilising IRF3, IRF5 and IRF7 have revealed that the autoinhibitory domain can suppress the transcriptional activity of these transcription factors (Barnes et al., 2002), a mechanism that is more likely used in resting cells. Autoinhibition in IRFs is released upon phosphorylation which induces conformational changes, dimerisation and nuclear translocation. Only one autoinhibitory domain has been found in the N terminus of IRF5 and IRF7 (Marie, Smith et al. 2000; Barnes, Kellum et al. 2002) while two autoinhibitory domains have been identified in both the C and N terminus of IRF3 (Lin et al., 1999). A NLS is also present in the N terminus of IRF3, IRF5 and IRF7, which functions in nuclear translocation and cell retention (Chen and Royer Jr, 2010).

IRF3 and IRF7 are key regulators of type I IFN gene expression in response to viral infection. IRF7 was first described to bind to and repress the Epstein Bar Virus (EBV) Qp promoter, which regulates expression of the EBV nuclear antigen 1 (EBNA1) (Zhang and Pagano, 1997). Virus-mediated phosphorylation of IRF7 occurs on Ser483 and Ser484 which induces a conformational change in the transcription factor (Yang et al., 2003). This is critical for its dimerisation, nuclear accumulation, DNA-binding, and transcriptional transactivation. Although clusters of serine residues located in the C terminal regulatory domain of IRF7 are putative targets of virus-activated kinases, the critical residues for phosphorylation-induced activation have yet to be established. However, similar to IRF3, TBK1 and IKKε have been reported to phosphorylate and activate IRF7 (Sharma et al., 2003). While IRF3 phosphorylation prominently induces IFN-β, IRF7 has the ability to preferentially activate IFN-α (Noppert et al., 2007). Virus-mediated phosphorylation of IRF3 is discussed in Chapter 5.

Although IRF3 is constitutively and ubiquitously expressed in many cell types, IRF7 expression is dependent on the cell type and in general, expression is much lower than IRF3 (Izaguirre et al., 2003). However, pDCs constitutively express IRF7; therefore activation of TLR7 and TLR9 in these cells rapidly induces the production of type I IFN via the MyD88-dependent pathway. Furthermore, IRF3 mediated IFN-β and IFNα4 activation can subsequently signal via the type I IFN receptor (IFNAR) to activate the Janus activated kinase (JAK)–signal transducer and activator of transcription (JAK–STAT) pathway (Noppert et al., 2007). This in turn activates the IFN-stimulated gene factor 3 (ISGF3)

transcriptional regulator, which induces IRF7 expression. In a similar manner to IRF3, phosphorylated IRF7 translocates to the nucleus and binds to positive regulatory domain (PRD) on the IFN-α promoter. Thus, the induction of type I IFN initiates a positive feedback loop, which enables cells to produce large amounts of IFN-α/β, ensuring the efficient production of type I IFN during viral infection.

1.5.2.3 MyD88 dependent pathway

The MyD88-dependent pathway (Fig 1.3) transduces signals for all TLRs, except TLR3. MyD88 is also a key mediator for the IL-1 and IL-18 receptors (Adachi et al., 1998, Janssens and Beyaert, 2002). Following ligand interaction with TLR1, TLR2, TLR4 and TLR6, activated Mal recruits MyD88 to the plasma membrane via its PIP-2 binding domain. Whether MyD88 interacts directly with the TIR domain of TLR4 following recruitment to the plasma membrane is controversial, as a recent study suggests that the TIR domain of MyD88 does not exclusively interact with TLR4 TIR domain (Ohnishi et al., 2009). However, in relation to the other TLRs (except TLR3), MyD88 interacts with the TIR domain of these TLRs via MyD88 C terminal domain. MyD88 subsequently recruits the IL-1R–associated kinase (IRAK); IRAK4, IRAK1, and IRAK2 through homotypic interactions between their respective DDs (Ohnishi et al., 2009). Once IRAK4 binds to MyD88, it is initially activated, whereby it recruits and phosphorylates IRAK1 and IRAK2 in a sequential manner. IRAK1 then autophosphorylates, dissociates from the MyD88 complex and interacts with the E3 ligase, TRAF6. TRAF6 catalyses the K63 polyubiquitination on target proteins including itself and IRAK1. This is in conjunction with the dimeric E2 ubiquitin conjugating enzymes Ubc13 and Uev1A. The K63-linked polyubiquitin chains bind to the novel zinc finger–type ubiquitin-binding domain of TGF-β-activated protein kinase (TAK-1) binding protein (TAB)-1 and -2 which leads to the activation of TAK1. TRAF6, K63-linked polyubiquitin chains also bind to the ubiquitin-binding domain of NEMO and they may also be responsible for the recruitment of TAK1 to the TAB1/2 complex (Kawai and Akira, 2010). As previously mentioned, NFκB subunits are sequestered in the cytoplasm in an inactive form and impeded from entering the nucleus by virtue of their association with members of the IκB family. Once TAK1 is activated, it

interacts with the IKK signalsome complex and induces phosphorylation of IKKβ (Kawai and Akira, 2010). IKKβ subsequently induces the phosphorylation of IκB where IκB becomes polyubiquitinylates and trafficked to the 26S proteasome for degradation (Hacker and Karin, 2006). This cytoplasmic "switch" liberates the NFκB complex from the cytoplasm for subsequent nuclear translocation and target gene transcription.

IRF7, which is constitutively expressed by pDCs, is also targeted by the MyD88 dependent pathway. Following the engagement of MyD88 with TLR7, TLR8 or TLR9, a multiprotein signalling complex involving IRAK4, TRAF6, TRAF3, IRAK1 and IKKα occurs. IRF7 becomes phosphorylated by IRAK1 and/or IKKα, on its C terminus where it dissociates from this complex, dimerises and translocates into the nucleus to induce transcription of IFNα/β. IRAK1 plays an important role in IRF7 activation. Not only does the kinase phosphorylate IRF7, but IRAK1 deficient pDC's display defects in IRF7 translocation and ensuing IFNα expression (Uematsu et al., 2005). In relation to IRF3, NRDP1, a RING-containing E3 ligase has reported to positively regulate TBK1 activation of IRF3 (Wang et al., 2009). NRDP1 directly targets and polyubiquitinated MyD88 and TBK1, leading to degradation of MyD88 but activation of TBK1.

1.5.2.4 MyD88 independent pathway

The MyD88 independent pathway (Fig 1.3) is a key inducer of IRF3. As previously mentioned, TRIF is the sole adaptor protein utilised by TLR3 in response to IRF3 activation. Upon engagement with the TIR domain of TLR3, TRIF recruits a signalling complex involving TRAF3-TBK1-IKKε which target the N terminal domain of IRF3, ultimately resulting in the phosphorylation of the latter on its C terminal domain. Phosphorylated IRF3 dimerises and translocates to the nucleus where it associates with CBP/p300 on its hydrophobic surface and binds to the cognate-DNA PRD in the promoter region of IRF3 target genes, including type I IFN.

TRIF is also involved in mediating MyD88 independent signalling events in the TLR4 pathway. TRIF and TRAM act cooperatively in the activation of IRF3 and late phase induction of NFκB. Upon TLR4 activation, TRAM is recruited to the plasma membrane and subsequently interacts with TRIF. TRIF forms a multi-protein signalling

complex with TRAF6 and RIP1. The interaction between TRIF and RIP1 occurs through the distinct RIP homotypic interaction motif which leads to the K63-linked polyubiquitination of RIP1. The adaptor TNFR1-associated death domain protein (TRADD) also binds RIP1 (Natoli and Austenaa, 2008). Studies have revealed that TRADD-deficient cells show impaired RIP1 ubiquitination with concomitant loss of NFκB activation (Pobezinskaya et al., 2008), thus demonstrating the importance of the TRADD/RIP1 complex in NFκB activation. The TRADD/RIP1 complex consequently leads to the activation of TAK1, which in turn activates the NFκB pathway.

Whilst TLRs and the above signalling pathways play key protective roles in the innate immune system, their dysregulation can often lead to chronic inflammatory diseases. One such disease of particular relevance to the present thesis is M.S.

1.6 Multiple Sclerosis

M.S. is a chronic, inflammatory, demyelinating disease of the CNS characterised by demyelinated plaques with relative axonal sparing and glial scar formation (Lassmann, 1983). The target of the chronic inflammatory response, predominantly by autoreactive T cells, is the myelin sheath. The myelin sheath is a protective phosolipid covering that surrounds the axon of nerves and facilities the rapid and effective transmission of nerve impulses. There are over 350,000 people in Europe with M.S.(Pozzilli et al., 2002), with approximately 2.5 million patients worldwide (Baker and Tickle-Degnen, 2001).

Although the earliest suggestive description of M.S. dates back to the late fourteenth century, it was not until 1868 that the condition was recognised as a distinct disease by French neurologist, Jean-Martin Charcot (Murray, 2009). In drawings from brain autopsies in suspected M.S. patients, Charcot described the characteristic scars or "plaques" of M.S., thus naming the disease; *sclerose en plaques*.

While the aetiology of M.S. remains unknown, data gathered from extensive studies on experimental autoimmune encephalomyelitis (EAE), an animal model of M.S., suggest that it is an immune-mediated disease consisting primarily of autoreactive $CD4^+$ T cells, where also a small number of autoreactive B cells (Harp et al., 2008), plasma cells, and

extensive macrophage/glial activation act cooperatively in the destructive process of demyelination.

The major focus on the role of the adaptive immune system in autoimmune disease has increased our understanding in the pathogenesis of M.S. In the peripheral immune system, antigen presenting cells (APC) e.g. DCs, B cells and macrophages present myelin specific antigens to autoreactive $CD4^+$ T cells via major histocompatibility complex (MHC) class II-T cell receptor (TCR) interactions. MHC is a highly polymorphic gene region found in most vertebrates which function in the defence against viral infection. In humans, MHC is also called human leukocyte antigen (HLA) and is located on chromosome 6p21. Due to the nature of multiple variants at this locus, MHC is one of the most extensively studied regions in the human genome and in its role in autoimmune disease.

The upregulation of various adhesion molecules specifically intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) also facilitate the interaction between APCs and T cells. This cellular interaction is crucial in determining T cell differentiation into either Th cells (Th1, Th2 and Th17 cells) (Ivanov et al., 2007, Mendoza and Pardo, 2010) or Treg cells (natural Tregs and induced Tr1 cells) (Gilliet and Liu, 2002, McClymont et al., 2011) depending on the cytokines produced. For example, IL-12 induces a Th1 cell response while IL-23 induces a Th17 cell response (O'Brien et al., 2008). Transforming growth factor-β (TGF-β) and IL-6 can also act in concert to induce the polarization of Th17 cells. Th1 and Th17 are the proposed pathogenic cells in M.S. (Lovett-Racke et al., 2011) In the peripheral immune system, these cells reside and along with macrophages, cross the blood brain barrier (BBB) and interact with resident CNS APCs. This leads to the reactivation of the IFN-γ secreting Th1 cells (Bettelli et al., 2004) and IL-17 producing Th17 cells ((Bettelli et al., 2008)) within the CNS. Th1 and Th17 act in concert with other cytokines including TNF-α to activate resident CNS cells which target myelin specific antigens including myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and myelin basic protein (MBP). This attack on myelin antigens results in demyelination, axonal loss and subsequent neurological disability (Sospedra and Martin, 2005).

Recent studies have suggested that the innate immune system also plays an important function in the initiation and progression of M.S. (Weiner, 2008, Gandhi et al.,

2010). Cytokines secreted from innate immune cells including microglial, mast and NK cells, not only function in T cell differentiation but the cells can also cross the BBB and mediate the direct cytotoxicity against myelin or oligodendrocytes thus resulting in demyelination.

Epidemiological data implicates both genetic and environmental factors in the aetiology of M.S., with various factors interacting with one another. Due to the high polymorphic nature of the MHC and its viral deference mechanisms, it is not surprising that the MHC has been implicated in M.S. The precise role of the various associated HLA alleles in particular the HLA class II antigens e.g. HLA-DP, -DM, -DOA, -DQ, -DOB and –DR is largely unresolved. Nevertheless, the recent availability of dense SNP marker sets that span the HLA class II antigens in individuals reveals that each locus has several genetically determined alleles where many of these are associated with certain autoimmune diseases. The association of the HLA-DR2 haplotype among M.S. patients was first noted around 1972 (Bertrams et al., 1974) and remains one of the most reproduced findings concerning MHC genetics. The geographical spread of M.S. suggests a relationship between latitude and disease prevalence, where M.S. is predominately a disease of temperate latitudes and of the western hemisphere. The low rate of M.S. in tropical climates may be due to a person's long-term exposure to sunlight, as vitamin D has previously demonstrated to be protective in M.S. patients (Munger et al., 2004).

Any part of the brain is susceptible to demyelination during an M.S. immune mediated-attack. Consequently, M.S. is associated with a broad range of symptoms, including muscular weakness, tremor, disturbances of coordination and motor function and visual complications. Depending on the intensity of the inflammatory response and the subsequent severity of demyelination, M.S. patients suffer from relapses which determine a patient's progression through the four clinical types of M.S (Confavreux and Vukusic, 2008). The most frequent is relapsing-remitting M.S. (RR-M.S.) which affects around 80% of patients and is characterised by acute attacks (relapses) which can last from 24 hours to several days/weeks, followed by partial or full recovery (remission) occurring at variable intervals (Weiner, 2008). Many patients with RR-M.S. may later progress to the second clinical stage, secondary progressive M.S. (SP-M.S.), where gradual worsening of the disease between relapses occurs with no real recovery. The third form of M.S., primary-

progressive M.S. (PP-M.S.), affects 10%-15% of patients, whereas the fourth and most rare form of M.S. is progressive-relapsing M.S. (PR-M.S.). In PR-M.S., gradual worsening of symptoms is observed from the onset of disease, with superimposed relapses and remissions.

M.S. diagnosis is based on the confirmation of neurological deficits, the development of the clinical course and the use magnetic resonance imaging (MRI), a powerful tool which scans the brain and spine to detect the presence of plaques caused by M.S. Depending on the instance of diagnosis and with currently available treatments, many M.S. patients remain in stage one or two for the majority of their life.

1.6.1 Current Multiple Sclerosis Therapeutics

The start of the therapeutic era in M.S. was heralded by the introduction of recombinant IFN-β in the USA, the first therapy proven effective in altering the natural course of RR-M.S. and SP-M.S. patients (Jacobs et al., 1996). To date, there are three formulations of beta-interferon disease-modifying drugs (DMD); intramuscular IFNβ-1a (Avonex®), subcutaneous IFNβ-1a (Rebif®) and subcutaneous IFNβ-1b (Betaseron or Betaferon). Each DMD differs in method of production, chemical structure, method of injection and dosage. Recently a Novartis branded version of IFNβ-1b, Extavia, has been approved by FDA for treatment of M.S. However, there are no published studies on the efficacy of this new brand.

IFN-β is widely recognised for its ability to interfere with viral infection. In the 1980's, studies profiling the inflammatory response in the cerebrospinal fluid (CSF) of M.S. patients determined that the antibody response observed was due to viral infection (Tourtellotte et al., 1984). Although no causative virus was identified, it was accepted that endogenous lymphocyte IFN production was deficient in M.S. patients (Neighbour et al., 1981). This lead to the idea that exogenously delivered IFN may correct this deficit. While it was unsure which IFN may be an attractive agent in M.S. clinical trials, further studies revealed that IFN-γ induced exacerbations in M.S. patients which correlated with an increase in NK cell activity (Panitch et al., 1987). However since this finding, numerous studies now provide evidence suggesting both detrimental and protective effects of IFN-γ in

M.S. and EAE (Furlan et al., 2001, Petereit et al., 2000, Franciotta et al., 2003). IFN-α has also been investigated as a possible therapeutic in M.S. While the cytokine may reduce relapse rate, it is not as significant as IFN-β (Jacobs and Johnson, 1994). Furthermore, the reduction in relapse rate is temporary and restricted to the period of IFN-α administration (Durelli et al., 1996). As a result of the comparative interferon studies, IFN-β was targeted and clinically assessed.

Although the precise mechanisms by which IFN-β exerts its therapeutic effects in M.S. remain unresolved, various studies have published on its immunomodulatory effects. Generally, IFN-β inhibits the proliferation of T cells and reduces the production of proinflammatory cytokines including IFN-γ, thus shifting the cytokine response from an inflammatory Th1 response to a anti-inflammatory Th2 type (Noronha et al., 1993). IFN-β is also associated with the ability to enhance immunoglobulin class switching (Meyer, 2009) and reduce leukocyte migration across the BBB (Stone et al., 1995), an effect that is likely dependent on the downregulation of adhesion molecules, chemokines and matrix metalloproteinases.

Jacobs and colleagues were among the first to report on IFN-β (delivered intrathecally) reducing relapse rates in patients with active RR-M.S. (Jacobs et al., 1981). Clinical trials of systemically administered IFN-β followed and established the efficacy of this cytokine in reducing relapse rate and slowing disease progression. The Expanded Disability Status Scale (EDSS) gauges the extent of a person's disability by measuring the level of neurologic impairment (Kurtzke, 1983). EDSS scores range from 0 to 10 where higher scores indicate a more severe form of disability. Most patients have an EDSS of 1-6. The time interval for development of disability varies with every patient. This scale is frequently used to compare the effectiveness of currently available IFN-β treatments on patient relapse, where on average relapse rate is reduced by 30% (Bermel and Rudick, 2007).

Comparative analysis studies of the three currently approved IFN-β treatments report various dissimilarities among each. Although Limmroth and collegeues showed similar effectiveness among the IFN-β products (Limmroth et al., 2007), others have demonstrated the superiority of Betaseron to Rebif and Rebif to Avonex in decreasing relapse rate and EDSS (Etemadifar et al., 2006). Likewise, Rebif (Panitch et al., 2002) and

Betaseron (Khan et al., 2001) have also been shown to be more effective than Avonex. Each treatment also displays markedly different side effects. Avonex is injected intramuscularly, where the agent is slowly released into the bloodstream. As effective amounts of Avonex stay longer in the body, the injection does not have to be repeated as frequently at its counterpart agents. Betaseron and Rebif are injected subcutaneously. As both agents remain in the body for shorter periods, they are administered up to three times a week or every other day. This frequent occurrence of injections causes patients to experience injection site reactions including discomfort, swelling and pain. The incidence of flu-like symptoms occurs approximately 50% in patients taking Avonex and Rebif while is higher in patients (~75%) taking Betaseron (Bermel and Rudick, 2007). Furthermore, universal side effects associated with all three treatments include muscle aches, spasms, fever, chills, headache, and back pain. Avonex, Rebif and Betaseron are generally given to RR-M.S. and SP-M.S. patients. Current randomised double or single blind placebo-controlled trials in patients with PP-M.S. receiving IFN-β report no effect on the primary outcome measure of EDSS progression (Rojas et al., 2009, Leary et al., 2003). However, the numbers of active lesions on brain MRI scans are significantly lower than placebo groups.

Several other DMD are now available that specifically target RR-M.S. and SP-M.S. Administration of the synthetic polypeptide (that resembles myelin protein), glatiramer acetate (Copaxone®) is comparably effective as IFN-β given subcutaneously in RR-M.S. patients (O'Connor et al., 2009). To date, no direct comparisons of Copaxone with IFN-β given intramuscularly have been undertaken. The precise mechanism of action of Copaxone is not completely understood. It is felt that the drug acts in the periphery by inducing Copaxone-specific T cells that secrete anti-inflammatory cytokines at the site of inflammation (Brandes, 2010). Copaxone may shift the inflammatory response from a pro-inflammatory Th1 type to an anti-inflammatory Th2 response, incorporating the induction of regulatory CD8+ and CD4+ CD25+ T-cells. Furthermore, given its resemblance to myelin protein, Copaxone may also act as a sort of decoy, diverting an autoimmune response against myelin. Interestingly, a recent pilot study demonstrated that M.S. patients receiving Copaxone experienced less spasticity than those taking IFN-β (Meca-Lallana et al., 2010).

Natalizumab (Tysabri®) is also licensed for treatment of RR-M.S. Tysabri is an α4-integrin antagonist. Its proposed mechanism is the ability to interfere with leukocyte migration across the BBB by binding to the α4 subunit of α4β1-integrin and preventing leukocyte adhesion to VCAM-1 (Coyle, 2010). While the efficacy of Tysabri among RR-M.S. patients has been demonstrated (Polman et al., 2006), Tysabri is generally considered a second-line therapy for patients who are refractory to other treatments (Ransohoff, 2007). This is mainly due to cases of progressive multifocal leukoencephalopathy (PML). PML is a rapidly progressive and often fatal human brain infection by the Jacob–Creutzfeldt (JC) virus which has been associated in M.S. patients taking Tysabri.

Fingolimod is a novel oral treatment that has recently been submitted for FDA approval (Vasiliou, 2010). Fingolimod is a synthetic analogue of the fungal sphingosine 1-phosphate (S1P) receptor agonist myriocin. Fingolimod is proposed to prevent lymphocyte egress from lymph nodes thus reducing lymphocyte infiltration into the CNS. In a recent proof-of-concept study, daily use of oral fingolimod in patients with RR-M.S. showed a statistically significant benefit on time to first relapse, annual relapse rate and a reduction in enhancing lesions on MRI compared with placebo (Cohen et al., 2010). In 2005, Canadian authorities approved the marketing of Sativex, an oromucosal spray containing a combination of two natural cannabis plant extracts for the treatment of neuropathic pain and spasticity associated with M.S. In 2010, Sativex was licensed as a prescription only medicine in the U.K. (Kmietowicz, 2010). While there is currently no approved disease modifying treatment for PP-M.S, natalizumab, IFN-β and fingolimod are currently under investigation.

1.7 Cannabinoids

Cannabinoids are a group of lipid soluble chemical messengers derived from the cannabis plant. The cannabis plant is one of the oldest plant-derived recreational and therapeutic drugs in human history and contains over 70 cannabinoid compounds. Cannabis has been exploited in the past in the treatment of migraine, convulsions, neuralgia, nausea, asthma and anorexia (Di Marzo et al., 1998, Russo, 1998). The term “cannabinoid” incorporates the active components (phytocannabinoids) of the cannabis

plant including delta-9-tetrahydrocannabinol (δ-9-THC) and cannabinol, endogenous cannabinoids (endocannabinoids) e.g. anandamide and 2-Arachidonoylglycerol and synthetic cannabinoid ligands e.g. WIN55,212-2, HU-210 and JWH-133. Cannabinoids elicit their immunosuppressive properties by activating the cannabinoid receptors (see Chapter 4).

Cannabinoids can be further classified into two types, neutral cannabinoids and cannabinoid acids, based on whether they contain a carboxyl group or not (Taura et al., 2007). It is now known that in fresh cannabis plants the concentrations of neutral cannabinoids are much lower than those of cannabinoid acids. The isolation and synthesis of δ-9-THC in 1964 served to further our understanding of the molecular basis of action of this class of drug (Gaoni and Mechoulam, 1964). In the δ-9-THC biosynthetic pathway, δ-9-THC initially occurs as δ-9-tetrahydrocannabinolic acid (δ-9-THCA). Initial precursors, geranyl diphosphate (GPP) and olivetolic acid, are condensed by the prenylase geranyl diphosphate: olivetolate geranyltransferase (Fellermeier and Zenk, 1998) to yield cannabigerolic acid (CBGA). CBGA is further oxido-cyclized into cannabidiolic acid (CBDA) by the enzyme; cannabidiolic acid synthase (Futoshi et al., 2007). δ-9-THCA and cannabichromenic acid (CBCA) (Morimoto et al., 1999) are also further oxido-cyclized by the enzymes; δ-9-tetrahydrocannabinolic acid synthase and cannabichromenic acid synthase (Sirikantaramas et al., 2004), respectively. Over time, or when heated, δ-9-THCA is decarboxylated to produce δ-9-THC.

Arachidonylethanolamine, which is commonly known as anandamide (AEA), is an arachidonic acid derivative and was the first endocannabinoid to be discovered in porcine brain (Devane et al., 1992). The endocannabinoid was termed "anandamide" based on "'ananda", the Sanskrit word for bliss. AEA is a lipid neurotransmitter and has also been proposed to function as a neuromodulator (Di Marzo et al., 1999). It acts as a retrograde messenger and exerts a modulatory effect on memory, cognition and pain perception (Wilson and Nicoll, 2002). The generation of AEA in stimulated cells was first described in 1994 (Di Marzo et al., 1994). AEA is not stored in cell vesicles, but rather is synthesised by neurons on demand from lipid precursors such as N-Arachidonoyl-phosphatidylethanolamine (NAPE) and diacyglycerols (DAGs) (Rossi et al., 2010, Fowler et al., 2005) (Fig 1.4). The synthesis of AEA is dependent on two distinct pathways; a

calcium-dependent pathway and a G-protein coupled receptor (GPCR)-dependent pathway which control N-acyltransferase (NAT) activity; an enzyme responsible for the production of NArPE (Cadas et al., 1996). A specific phospholipase D (NAPE-PLD) catalyses the release of AEA (Rossi et al., 2010). The release of AEA is either by diffusion through the postsynaptic cell membrane or by transport out of the cell where AEA can gain access to cannabinoid receptors concentrated on the nerve terminals of either GABAergic interneuron's such as those found in the hippocampus, neocortex and amygdale or excitatory glutamergic axons located in the cerebellum (Alger, 2002). AEA is subsequently taken up into the post- or pre-synaptic cells via the AEA-transporter where once within the cell, AEA is hydrolysed to arachidonic acid and ethanolamine by the integral membrane enzyme, fatty acid amide hydrolase (FAAH) (Di Marzo, 1998). AEA has been detected in immune cells including DCs, macrophages and microglia (Felder et al., 1996). The activation of certain types of these cells can causes a rapid and robust increase in AEA levels, thus further supporting a role for AEA in immune regulation (Maccarrone et al., 2001).

Until the early 1990s, all compounds known to display cannabimimetic properties were structurally related to δ-9-THC. This situation changed when Sterling Winthrop researchers reported a new family of aminoalkylindoles possessing cannabimimetic properties that were structurally unrelated to δ-9-THC. This discovery resulted from the development of structurally constrained analogs of pravadoline (Pacheco et al., 1991, Bell et al., 1991). One such analog was WIN55,212-2 and it is now the most highly studied, commercially available compound of the series. Although WIN55,212-2 is structurally diverse from δ-9-THC, it was found to exhibit a pharmacological profile similar to the phytocannabinoid. The existence of chiral centres in classical cannabinoids such as δ-9-THC and WIN55,212-2 has made it possible to develop enantiomers of this class of cannabinoids (Howlett et al., 2002). These stereo-chemical changes have shown that the pharmacological activity of the (-) enantiomer is greater than that of the (+) enantiomer in terms of receptor affinity and potency at both cannabinoid receptor subtypes.

Many reports suggest that cannabinoids, known to exhibit general immunosuppressive properties, have potential therapeutic value in the treatment of neuroinflammatory conditions (Ungerleider et al., 1987, Consroe et al., 1997). Most of

these immunosuppressive properties occur by interfering with both humoral and cell-mediated immunity (Correa et al., 2009) e.g. inhibition of T cell mitogenesis, IL-2 production (Kaplan et al., 2003, Börner et al., 2009) with consequent inhibition of T cell, NK cell, and B cell proliferation.. Furthermore, cannabinoid inhibition of cell proliferation, induction of apoptosis and inhibition of bone-marrow-derived myeloid cell recruitment also play key roles to down-regulate the immune system (Guzmán et al., 2002, Wu et al., 2008). Cannabinoids have also been demonstrated to target various transcription factors involved in inflammation including NFκB (Downer et al., 2011) and CREB (Ofek et al., 2011).

Cannabinoids elicit their effects via the cannabinoid receptors (CB1 and CB2) (Cencioni et al., 2010, Jean-Gilles et al., 2010). Cannabinoid receptors are localized throughout the CNS (Walter and Stella, 2004) and on immune cells associated with neuroinflammation (Galiègue et al., 1995). CB1 expression on neurons can suppress CNS inflammation (Maresz et al., 2007) while CB2 activation on immune cells has been shown to shift immune responses from a Th1 (IFN-γ, TNF-α, and IL-2 production) to a Th2 phenotype (with production of IL-4 and IL-5) (Yuan et al., 2002). Certain studies have also demonstrated the regulation of the cannabinoid receptor by cytokines (Halttunen and Mäki, 1999, Gardner et al., 2002). TGF-β regulates CB2 expression on lymphocytes (Gardner et al., 2002) while splenocytes cultures stimulated with anti-CD40 antibody show a increase in CB1 mRNA expression (Noe et al., 2000, Noe et al., 2002). However, some cannabinoid-induced effects are mediated independently of the receptors (Mestre et al., 2009, O'Sullivan and Kendall, 2009). This is particularly relevant as cannabinoids therapeutically impact diseases associated with a dysregulation of the immune and nervous systems (Walter and Stella, 2004). Indeed in EAE cannabinoids attenuate the development of disease (Lyman et al., 1989). The role of CB1/2 in mediating these effects varies depending on the pharmacological profile of the cannabinoid (Palazuelos et al., 2008). Furthermore, much interest has also been generated by reports of the therapeutic effects of cannabis in alleviating the symptoms of M.S. (Consroe et al., 1997) where patients with spasticity claim relief from using the drug (Iversen, 2003). The introduction of Sativex further demonstrates the therapeutic action of cannabinoids in M.S. (Kmietowicz, 2010). In relation to R(+)WIN55,212-2, many groups have published on the protective effects of this drug in various animal models of M.S. (Hasseldam and Johansen, 2010, Arevalo-Martin et

al., 2003, Croxford and Miller, 2003, Baker et al., 2000). As a result, this project hoped to define the molecular basis to its mechanism of therapeutic action.

1.8 Specific aims of this project

The specific objectives of this project were to:

- Define the regulatory effects of WIN55,212-2 on TLR3 and TLR4 signalling
- Identify the pharmacological mechanism of the regulatory effects of WIN55,212-2 on TLR3 and TLR4 signalling
- Define the molecular target for the action of WIN55,212-2 on TLR3 signalling

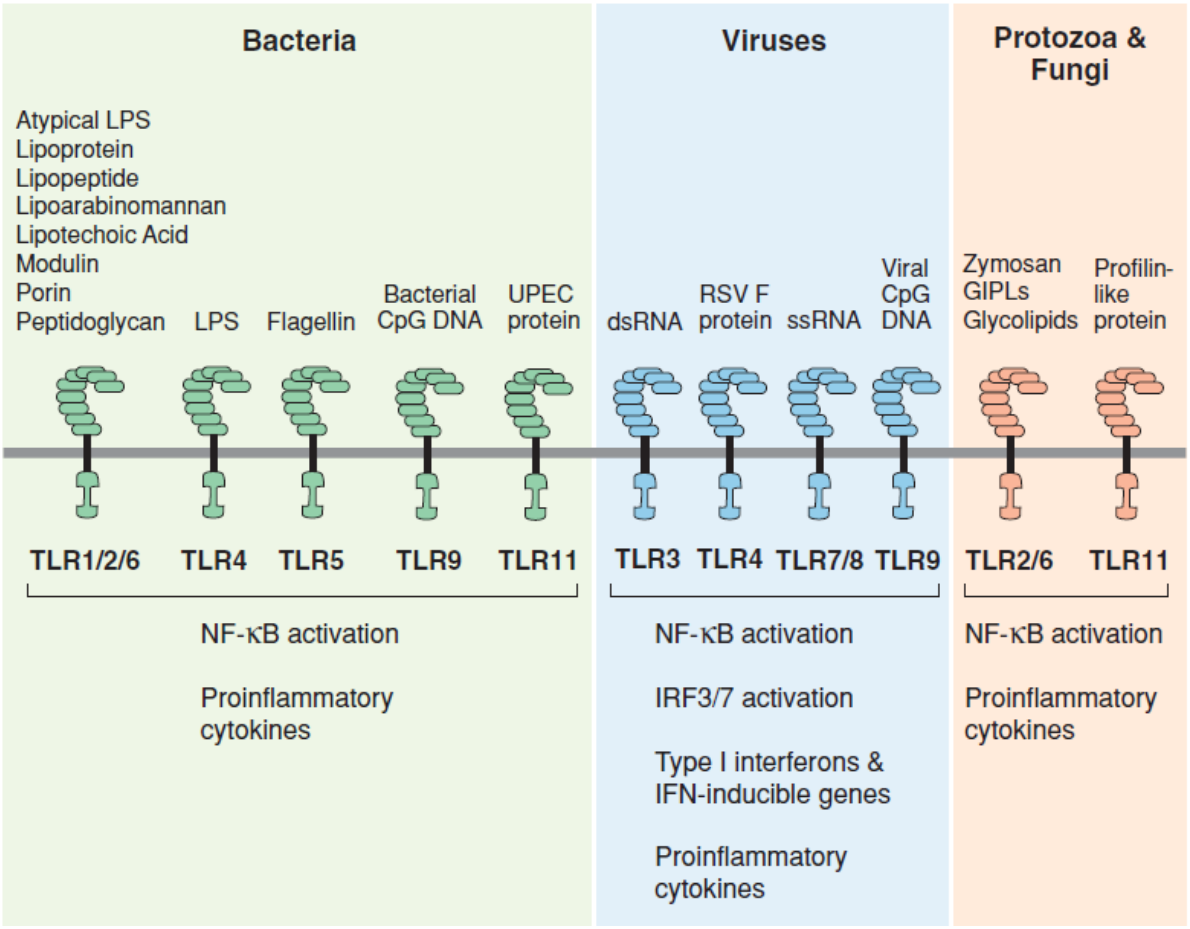


Fig. 1.1 TLR ligand specificities.

TLRs recognise a diverse array of PAMPs from bacteria, viruses, protozoa, and fungi. In the detection of bacteria, heterodimeric TLR2/1 bind triacyl lipopeptides, whereas TLR2/6 dimers bind diacyl lipopeptides and lipoteichoic acid. Homodimeric TLR2 binds peptidoglycan, atypical LPS, phenol-soluble modulin from *Staphylococcus epidermidis*, and porin proteins from *Neisseria*. In addition, TLR4 binds LPS, TLR5 binds flagellin, and TLR9 binds bacterial CpG DNA. TLR11 detects an unidentified protein(s) from uropathogenic *Escherichia coli*. Viral dsRNA, RSV F protein, ssRNA, and unmethylated CpG DNA are sensed by TLRs 3, 4, 7/8, and 9, respectively. Finally, heterodimeric TLR2/6 binds fungal-derived zymosan and *Trypanosoma cruzi* GIPLs, whereas TLR11 also senses a profilin-like protein from *Toxoplasma gondii*.

(Adapted from West, A. P., A. A. Koblansky, et al. (2006). "Recognition and Signaling by Toll-Like Receptors." Annual Review of Cell and Developmental Biology 22(1): 409-437.)

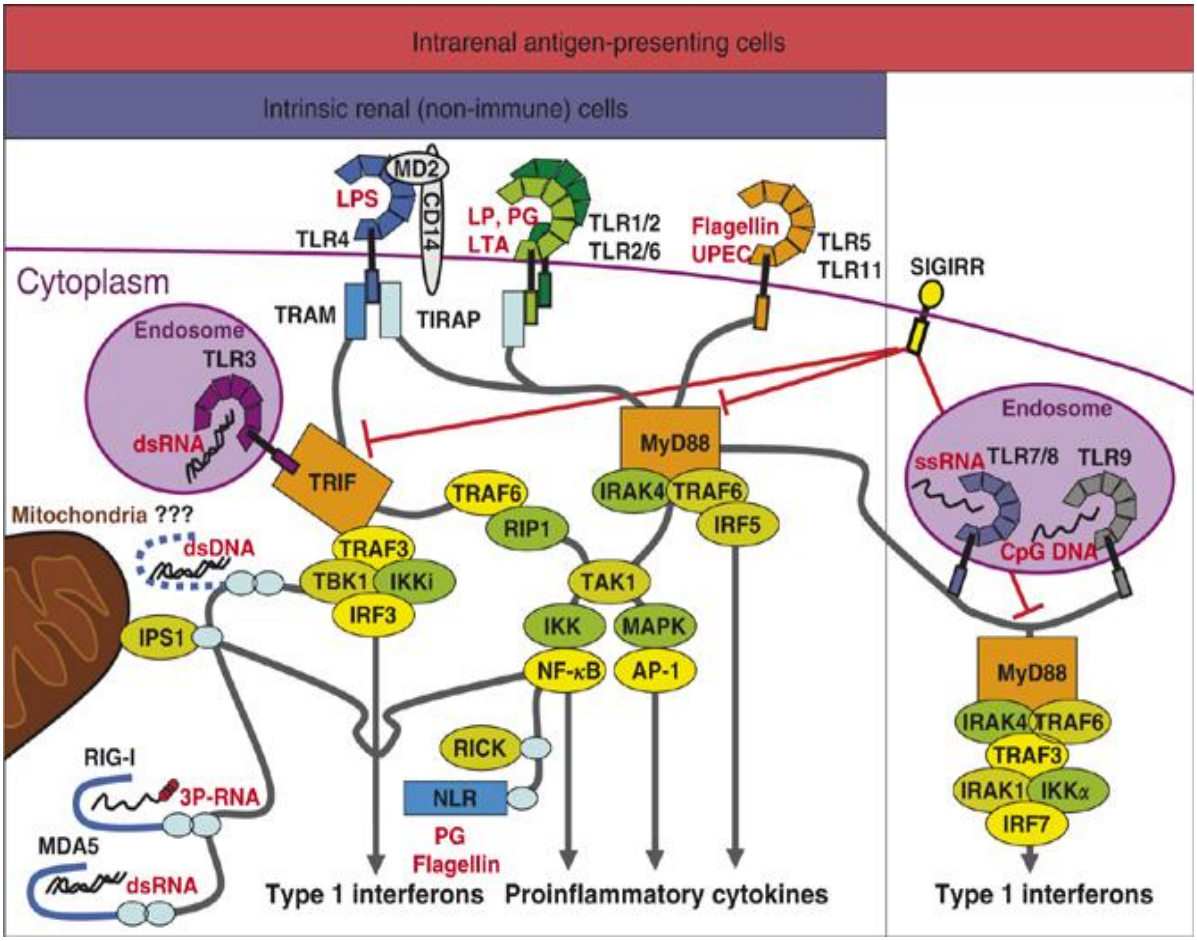


Figure 1.2 TLRs, RLHs, and NLRs in renal cells.

TLRs signal through the MyD88 and/or TIR domain-containing adaptor proteins. The RLHs signal through mitochondrial IPS1 and NLRs interact with RICK/RIP2. The respective ligands to these receptors are indicated in red; LPS, PG, LTA, LP and 3P-RNA.

(Adapted from Anders, H. J. (2007). "Innate pathogen recognition in the kidney: Toll-like receptors, NOD-like receptors, and RIG-like helicases." Kidney Int 72(9): 1051-1056.)

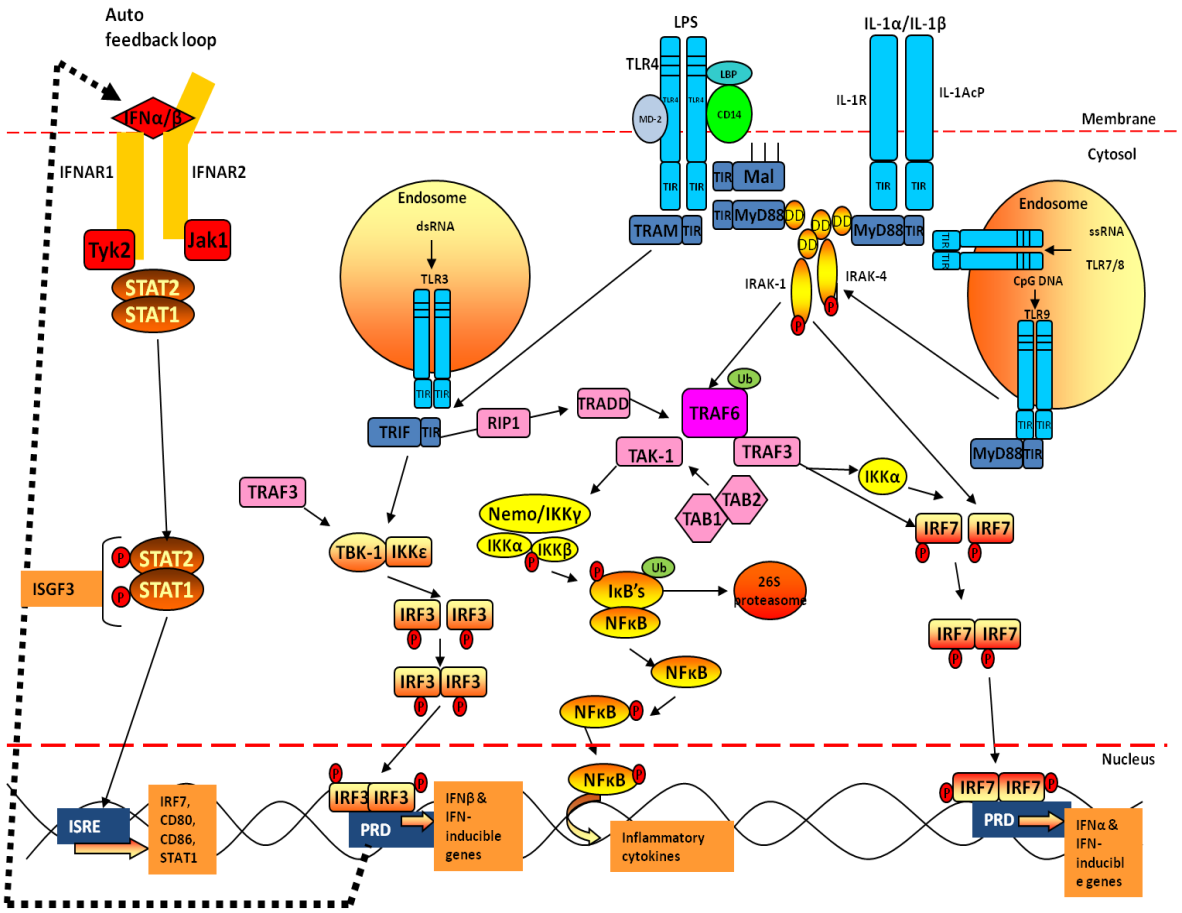


Fig. 1.3 The MyD88-dependent, -independent and interferon signaling pathways.

TLR signalling consists of two main pathways that are governed by the principle TIR domain-containing adaptors, MyD88 and TRIF. All TLRs, except TLR3, induce NFκB activation and subsequent production of pro-inflammatory cytokines via the MyD88-dependent pathway. IL-1 also signals through the MyD88-dependent pathway to activate NFκB. In pDCs, TLR7, TLR8 and TLR9 induce IRF7 activation via the MyD88-dependent pathway. TRIF mediates TLR3 and TLR4 signalling to induce IRF3 and late phase NFκB activation, respectively, via the MyD88-independent pathway. Type I IFN signalling is mediated by the IFNAR1 and IFNAR2 receptor components, which activate multiple STATs, particularly the ISGF3 transcriptional regulator.

(P) = phosphorylation, (Ub) = ubiquitination.

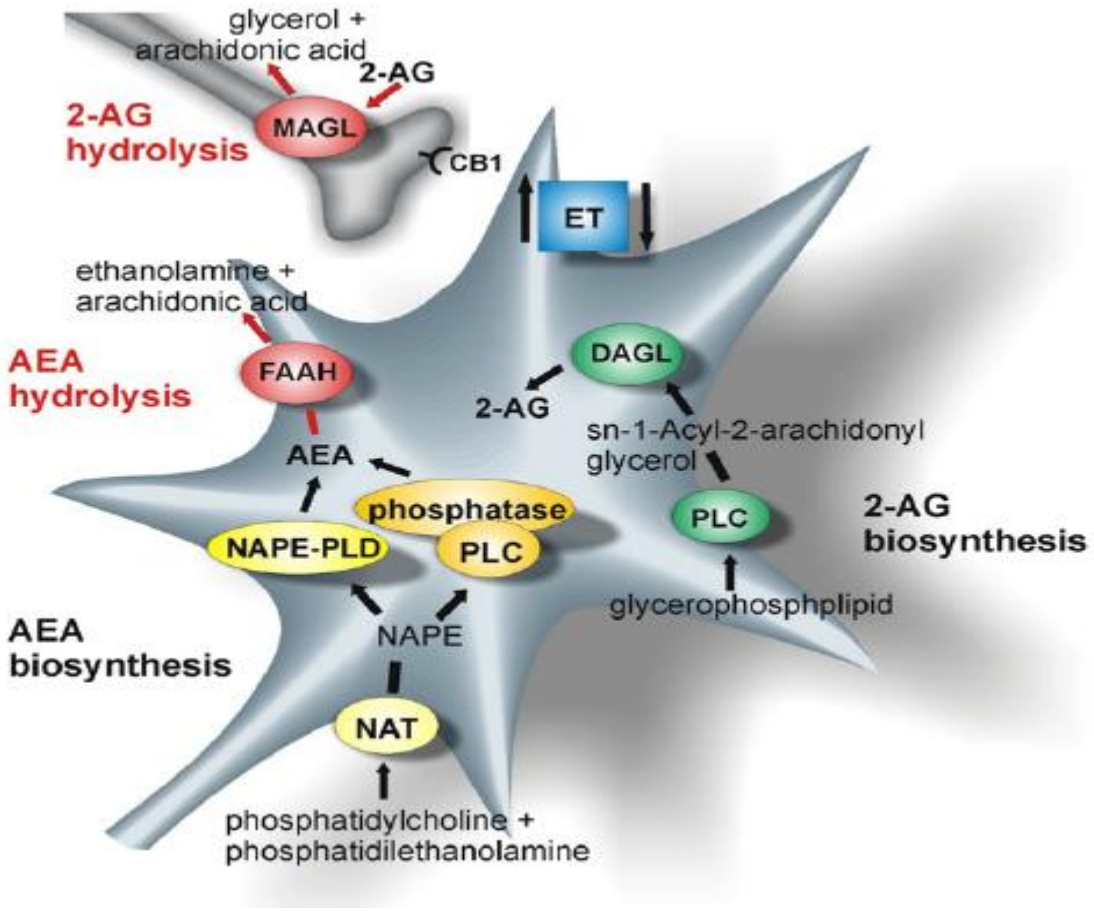


Fig. 1.4 Anandamide synthesis and degradation pathways.

The anandamide biosynthetic enzymes NAT catalyses the transfer of arachidonic acid from phosphatidylcholine to phosphatidylethanolamine, synthesizing the precursor NAPE of anandamide. A specific phospholipase D (NAPE-PLD) catalyses the release of anandamide. An alternative parallel pathway involves hydrolysis of NAPE by phospholipase C (PLC), followed by dephosphorylation through a phosphatase. The catabolic enzymes FAAH is responsible for the degradation of AEA. An endocannabinoid transporter (ET) on cellular membrane seems to facilitate both endocannabinoid release and re-uptake.

(Adapted from Rossi, S., G. Bernardi, et al. (2010). "The endocannabinoid system in the inflammatory and neurodegenerative processes of multiple sclerosis and of amyotrophic lateral sclerosis." Experimental Neurology **224**(1): 92-102.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents

Reagent	Supplier
Agar	Sigma
Agarose	Promega
Agarose, low melting point	Sigma
Alexa488	Invitrogen
Ampicillin	Sigma
AMV Reverse Transcriptase	Sigma
Antibodies (monoclonal) against human-	
β-Actin	Sigma
IRF3	Cell Signalling Tech.
IκBα	Santa Cruz
Phosho-IκBα/β	Cell Signaling Tech.
Antibodies (polyclonal) against human-	
Phospho-IRF3	Cell Signaling Tech.
IFN-β	Millipore
IRF3	Santa Cruz
APS	Sigma
Arachidonylethanolamide (Anandamide)	Sigma
Biotinylated Molecular Weight Standards	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
BX-795	Axon Medchem
Chamber Slides	NUNC
Chloroform	Sigma
Chloroquine	Sigma
Coelenterazine	Insight Biotech.

Cremophor El	Sigma
DAPI	Sigma
DEPC-treated water	Ambion
DMEM	Invitrogen
DMSO	Sigma
DNA ladder (1Kb) & Loading dye (6X)	Promega
dNTPs	Promega
DTT	Sigma
E.coli - TOP 10 competent cells	Invitrogen
EDTA	Sigma
Ethanol	Sigma
Ethidium bromide	Sigma
FBS	Invitrogen
Fenofibrate	Sigma
Glycerol	Sigma
Glycine	Sigma
Goat Serum	Vector Laboratories
Gradient gel (4% - 20%)	Pierce
GW6471	Sigma
GW9662	Sigma
Hydrogen Peroxide	Sigma
HEPES	Sigma
High speed plasmid midi kit	Qiagen
High speed plasmid mini kit	Qiagen
Human astrocytoma 1321N1	European Collection of Cell Cultures (ECACC)
Human HEK 293 TLR3/TLR4 cell line(s)	Invivogen
Hydrochloric acid	Merck
IRDye 800CW Goat Anti-Rabbit	Licor Biosciences
IRDye 680 Donkey Anti-Mouse	Licor Biosciences

IL-1β	R&D Systems
Isopropanol	Sigma
L-glutamine	Invitrogen
Lipofectamine 2000	Invitrogen
Lipopolysaccharide	Alexis
Luciferase substrate	Promega
Lymphoprep	Axis-Shield
Magnesium Chloride	Sigma
Nitrocellulose	Schleicher & Schuell
2-6-β-Mercaptoethanol	Sigma
Methanol	BDH
Microlon 96-well plates	Greiner
OptiMEM	Invitrogen
Paraformaldehyde	Sigma
PBS	Oxoid
pcDNA 3.1/Zeo	Invitrogen
pEGFP-N1	BD Biosciences
Penicillin / Streptomycin / Glutamine	Invitrogen
Pertussis Toxin	Sigma
PMSF	Sigma
Poly(I:C)	Sigma
Ponceau	Sigma
Prestained molecular weight marker (SeeBlue Plus)	Invitrogen
Protease inhibitor mixture	Roche
Protein A/G-agarose	Santa Cruz
Random primers	Invitrogen
Restriction enzymes	NewEnglandBiolabs
Reverse Transcription System	Promega
RNase Zap	Ambion
RPMI	Invitrogen
R(+)WIN55,212-2	Sigma

SDS	Sigma
Skim milk powder	Sigma
Sodium chloride (NaCl)	Sigma
Sodium hydroxide (NaOH)	Sigma
Sodium orthovanadate (Na_3VO_4)	Sigma
SR144528	National Institute of Mental Health (NIMH) Chemical Synthesis and Drug Supply Program
SR141716	NIMH Chemical Synthesis and Drug Supply Program
Sulphuric acid	Sigma
S(-)WIN55,212-2	Sigma
Synthetic oligonucleotides	MWG Biotech
Taq polymerase	Invitrogen
T4 DNA ligase	Promega
TEMED	Sigma
TMB	Sigma
Tissue culture ware	Greiner
TK Renilla	Promega
TNF-α (human) ELISA kit	R&D Systems
Tri reagent	Sigma
Tris-base	Sigma
Tris-HCl	Sigma
Triton-X	Sigma
Trypsin/EDTA	Invitrogen
Tryptone	DIFCO
Tween-20	Sigma
Whatmann paper	AGB
Yeast extract	DIFCO

2.1.2 Gifts

Cell lines:

- Bone marrow derived macrophages (BMDMs) from wildtype and TRIF deficient mice - Dr. Kate Fitzgerald (The University of Massachusetts Medical School, Worcestor, Massachusetts 01605, USA)
- U373 cell line – Dr. Sinead Miggin (Biology Department, NUI Maynooth, Ireland).

Constructs:

- TRIF, IRF-3/7 Gal4 reporter and IFN-β-luciferase reporter constructs – Dr. Kate Fitzgerald (The University of Massachusetts Medical School, Worcestor, Massachusetts 01605, USA).
- pFR-luciferase Gal4 reporter construct, green fluorescent protein (GFP)-tagged IRF3 and enhanced GFP (EGFP) construct – Dr. Andrew Bowie (Trinity College Dublin, Ireland).
- PRD II- and PRD I/III-luciferase reporter constructs - Dr. Sinead Miggin (Biology Department, NUI Maynooth, Ireland).
- NFκB-luciferase reporter construct – Prof. Luke O'Neill (Trinity College Dublin, Ireland).

Multiple Sclerosis Experiments:

- EAE mice – Prof. Padraic Fallon (Institute of Molecular Medicine, Trinity College Dublin and St. James's Hospital, Dublin 8, Ireland).
    - Multiple Sclerosis patients – Dr. Bruno Gran (Division of Clinical Neurology, University of Nottingham, Nottingham, United Kingdom).

2.1.3 Company Addresses

AGB Scientific:	AGB Scientific, Dublin Industrial Estate, Dublin 11.
Alexis:	Enzo Life Sciences (UK) Ltd. Palatine House, Matford Court, Exeter EX2 8NL, United Kingdom.
Ambion:	Ambion (Europe) Ltd. Ermine Business Park, Spitfire Close, Huntingdon, Cambridgeshire, PE29 6XY, United Kingdom.
Axis-Shield:	Axis-Shield PoC, P.O.Box 6863 Rodelokka, N-0504 Oslo, Norway.
Axon Medchem:	Axon Medchem BV. Biotech Center UMCG, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.

BD Biosciences:	BD Biosciences, 2350 Qume Drive, San Jose, CA 95131, USA.
Cell Signaling Tech:	Cell Signaling Technology, Inc., 3 Trask Lane, Danvers, MA 01923, USA.
DIFCO:	DIFCO Laboratories Detroit, Michigan, USA.
ECACC:	European Collection of Animal Cell Cultures, Salisbury, Wiltshire, SP4 0JG, United Kingdom.
Greiner:	Greiner Bio-One Ltd., Brunel Way, Stroudwater Business Park, Stonehouse, Gloucestershire, GL10 3SX, United Kingdom.

Insight Biotechnology:	Insight Biotechnology Ltd., Wembley, Middlesex, HA9 7YN, United Kingdom.
Invitrogen:	Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, United Kingdom.
Invivogen:	Cayla- InvivoGen Europe 5 rue Jean Rodier F-314000 Tououse, France.
Merck:	Merck Biosciences Ltd., Boulevard Industrial Park, Padge Road, Beeston, Nottingham NG9 2JR, United Kingdom.
Millipore:	Millipore, 290 Concord Road, Billerica, MA 01821, USA.

MWG Biotech: MWG Biotech AG,
Anzingerstr. 7a,
85560 Ebersberg,
Germany.

NEB: New England Biolabs (UK) Ltd.,
73 Knowl Piece,
Wilbury Way,
Hitchin,
Hertfordshire,
United Kingdom.

National Institute of Mental Health (NIMH): NIMH Chemical Synthesis and
Drug Supply Program,
6001 Executive Boulevard,
Rockville,
MD 20852,
USA.

NUNC: Nalge Nunc International,
75 Panorama Creek Drive,
Rochester,
NY 14625-2385,
USA.

Oxoid: Oxoid Ltd.
Wade Road,
Basingstoke,
Hampshire RG24 8PW,
United Kingdom.

Pierce:	Perbio Science UK Ltd., Unit 9, Atley Way, North Nelson Industrial Estate Cramlington, Northumberland NE231WA, United Kingdom.
Promega:	Promega US, 2800 Woods Hollow Road, Madison WI 53711, USA.
Qiagen:	Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, United Kingdom.
R & D:	R & D Systems Europe, Bartone Lane, Abington, Oxon, United Kingdom.
Roche:	Roche Diagnostics GmbH, Roche Applied Science, Nonnenwald 2, 82372 Penzberg, Germany.

Santa Cruz:	Santa Cruz Biotechnology Inc., 2161 Delaware Ave., Santa Cruz, CA 95060, USA.
Schleicher and Scheull:	Schleicher and Scheull Inc., 10 Optical Avenue, Keene, New Hampshire, USA.
Sigma:	Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland.
Vector Laboratories:	Vector Laboratories Inc., Burlingame, CA 94010, USA.

2.2 Methods

2.2.1 Mammalian cell culture

2.2.1.1 Adherent cell lines.

Human embryonic kidney (HEK) 293 cells stably transfected with the TLR3 or TLR4 receptors, human U373 astrocytoma cells stably transfected with CD14 (U373-CD14), human 1321N1 astrocytoma and murine BMDM cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a 37ºC humidified atmosphere with 5% CO_2. Cells were passaged every 2 to 3 days using 1% (w/v) Trypsin/ethylenediaminetetraacetic acid (EDTA) solution in phosphate-buffered saline (PBS). The neomycin analog G418 (500 µg/ml) was used to select for the stably transfected TLR cell lines and maintenance of CD14 expression while gentamicin (50 µg/ml) was used to maintain the transfected oncogene in BMDM.

2.2.1.2 Isolation of primary murine astrocytes.

Mixed glia were prepared from the cortices of 1-day old Wistar rats. For preparation of astrocytes, dissected cortices were chopped, added to Dulbecco's modified Eagle's medium (DMEM) triturated, passed through a sterile mesh filter (40µm) and centrifuged (2000 x g for 3 min at 20°C). The pellet was resuspended in DMEM and plated onto T25 flasks. Cells were maintained in a 37ºC humidified atmosphere with 5% CO_2. On day 14, non-adherent cells (microglia) were isolated by mechanical shaking and removed. The adherent astrocytes were isolated by harvesting in 1% (w/v) trypsin/ethylenediaminetetraacetic acid (EDTA) solution in phosphate-buffered saline (PBS). Cells were centrifuged (2000 x g for 5 min at 20°C) and the astrocyte-enriched pellet was resuspended in DMEM. Astrocytes were plated ($1x10^6$ cells/ml) in 12-well plates.

2.2.1.3 Isolation of human primary peripheral blood mononuclear cells (PBMCs)

Healthy donors and MS patients attending out-patient clinics at Queens Medical Centre University Hospital, University of Nottingham, UK, were recruited for this study. Written informed consent was obtained from each patient and the study received ethical approval from the Nottingham Research Ethics Committee. Patients with relapsing-remitting MS were clinically stable with an age ranging between 38-56 yrs (mean 48.4 ± 8.3; *n* =3). Patients were naïve to any disease modifying therapies including IFN-β, glatiramer acetate and natalizumab. Healthy individuals were recruited from the University of Nottingham (mean age 31 ± 2.6; n = 3).

Venous blood (30 ml) was obtained from each subject was collected into vacutainer tubes with 0.5% (w/v) EDTA. Blood was diluted 1:1 with Hank's Balanced Salt Solution (HBSS) medium containing 1% (v/v) FBS (which was heat inactivated for 30 min at 56^{o}C to avoid complement activation). Diluted blood (35 ml) was layered carefully onto Lymphoprep (15 ml) in 50 ml centrifuge tubes and centrifuged (400 x g for 25 min at 20°C), allowing deceleration to occur without braking. The cloudy layer sitting above the Lymphoprep contains the mononuclear cells and this was carefully collected with a Pasteur pipette and transferred to a new 50 ml centrifuge tube. HBSS medium was added to top up volume to 25 ml. Samples were centrifuged (400 x g for 10 min at 20°C). The supernatant was decanted off once and the pellet was resuspended in ~1 ml of remaining medium. Samples were vortexed and again topped up to 25 ml with HBSS medium and centrifuged at (400 x g for 10 min at 20°C). The supernatant was again decanted by tipping off once and the pellet was resuspended in ~10 ml RPMI 1640 containing 25 mM HEPES, 2mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin and 10% heat-inactivated FBS. Cells were counted and seeded at 1x10^{6} cells/ml in 24-well plates (1 ml per well).

2.2.2 Transient transfection of mammalian adherent cells

2.2.2.1 Transfection of cells for luciferase reporter assay and ELISA

HEK293 TLR3/TLR4 cells, human U373-CD14 and 1321N1 astrocytoma cells were seeded at $1.8x10^5$ cells/ml (200 µl DMEM/well) in 96-well plates and allowed to adhere for 24 h to approximately 80% confluency. Cells were transfected using Lipofectamine 2000. For each well to be transfected, 25 µl of OptiMEM was mixed with the DNA. DNA mixes were made up for the appropriate luciferase construct as outlined in section 2.2.4. Lipofectamine 2000 (0.4 µl) was diluited in OptiMEM (25 µl) per sample and the reaction was mixed gently and left at room temperature for 5 min. After the incubation, the Lipofectamine/OptiMEM solution was added to the DNA/OptiMEM mix (total volume 50 µl per well to be transfected) and the combined reaction was mixed gently and incubated at room temperature for 20 min. The transfection mixture was then added to each well and mixed gently by tapping the side of the plate. Each sample was transfected in triplicate. 24 h after transfection, cells were pre-treated with various agents for 1 hour followed by TLR ligand or IL-1β stimulation for 6 h. The supernatants were subsequently removed and stored at -20°C and then assayed for TNF-α production using sandwich enzyme-linked immunosorbent assay (ELISA), while cell lysates were generated and used to measure luciferase activity.

2.2.3 Propagation of DNA

2.2.3.1 Rapid transformation of competent *E.coli* cells

Commercially available TOP 10 competent *E.coli* bacterial cells were used for propagation of plasmids of interest. Plasmids (100 ng) were added to five times as much TOP10 cells, i.e. 1 µl of a 100 ng plasmid was added to 5 µl cells. DNA was mixed gently with cells by pipetting gently up and down and allowed to incubate on ice for 30 min. The

plasmids were allowed to enter the bacterial cells by heat shocking the mixture at 42 ^{o}C for 45 seconds. The cell membrane become permeable to allow easy entry of the plasmid and cooling on ice for 2 min makes the cell membrane once again impermeable. The transformed cells were then incubated in 1 ml Luria Bertoni (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 85 mM NaCl) at 37 ^{o}C on a shaker at 220 rpm for 1 h. Bacterial cells were centrifuged at 9000 rpm for 3 min and 850 µl supernatant was removed. The pellet was resuspended in the remaining LB broth and plated out onto LB agar plates (LB broth with 1.5% (w/v) agar) containing ampicillin (50 µg/ml). Plates were inverted and incubated overnight at 37 ^{o}C. Plates were then stored at 4 ^{o}C.

2.2.3.2 Small scale preparation of DNA from *E. coli* cells

LB broth (2 ml) containing the relevant antibiotic (50 µg/ml) was inoculated with a single transformed *E coli* colony from an agar plate. The culture was incubated overnight at 37 ^{o}C shaking at 220 rpm. Small plasmid preparations were made using the Qiaprep Spin Miniprep kit from Qiagen Inc. The bacterial cells were centrifuged at 9000 rpm for 3 min and the supernatant was discarded and the plasmid DNA was extracted as outlined in the manufacturer's handbook. DNA was quantified using a Cary spectrophotometer. After diluting the DNA appropriately in Tris-EDTA (TE) buffer, pH 8.0, (10 mM Tris-HCl, 1 mM EDTA) the absorbance of the solution was measured at 260 nm and 280 nm. All samples used had an optical density $(OD)_{260}/OD_{280}$ ratio in the range of 1.7 to 1.9. Ratios below 1.7 or above 1.9 indicated RNA or protein contamination, respectively. The concentration was calculated using the formula:

$$\mu g/ml\ DNA = 50\ \mu g/ml/OD_{260} \times (OD_{260}\ measured) \times (dilution\ factor).$$

2.2.3.3 Large scale preparation of DNA from *E. coli* cells

A starter culture of LB broth (2 ml) containing the relevant antibiotic (50 µg/ml) was inoculated with a single transformed *E.coli* colony and incubated at 37^{o}C with shaking

at 220 rpm for 6-8 h. This was then added to a larger volume of LB broth (100 ml) containing the relevant antibiotic and incubated at 37^{o}C overnight shaking at 220 rpm. Large plasmid preparations were made using the Qiagen high speed plasmid midi kit from Qiagen. The bacterial cells were centrifuged at 3000 rpm for 40 min and the supernatant was discarded and the plasmid DNA was extracted as outlined in the manufacturer's handbook. DNA was quantified as outlined in Section 2.2.3.2.

2.2.4 Luciferase assays

HEK293 TLR3, TLR4 cells and U373-CD14 and 1321N1 astrocytoma cells were all seeded at 1.8 x 10^5 cells/ml in 96-well plates (200 µl DMEM/well) and grown for 24 h. All transfections were performed using Lipofectamine 2000 transfection reagents (as described in sections 2.2.2.1). Details of the constructs transfected are given below. 24 h post-transfection, cells were pre-treated with various agents for 1 hour followed by TLR ligand or IL-1β stimulation for 6 h. The medium was then removed from the cells and reporter lysis buffer (100 µl, Promega) was added to each well using a multi-channel pipette. The plate was then wrapped in aluminium foil and placed on a rocker for 30 min at room temperature before being placed at -80^{o}C for a minimum of 1 h. After thawing at room temperature, aliquots (40 µl) of each were assayed for firefly luciferase activity using firefly luciferase substrate (40 µl, Promega), while *Renilla* luciferase activity was assayed using coelenterazine (0.1 µg/ml in PBS). Luminescence was monitored with a Glomax microplate luminometer (Promega).

2.2.4.1 NFκB assay

To measure activation of the NF-κB pathway, cells were transfected with NFκB -regulated firefly luciferase reporter plasmid (80 ng) and constitutively expressed *Renilla*-luciferase reporter construct phRL-TK (40 ng). The total amount of DNA was maintained at 240 ng using pcDNA3.1.

2.2.4.2 IFN-β assay

The activation of the IFN-β promoter was assessed by transfecting the cells with an IFN-β promoter-regulated luciferase construct (80 ng), or PRD-II-regulated-luciferase construct (80ng) or PRD-I/III-regulated-luciferase construct (80ng), phRL-TK (20 ng) and varying amounts of expression constructs (detailed in figure legends). The total amount of DNA was maintained at 240 ng using pcDNA3.1.

2.2.4.3 IRF3 assay

To measure the activation of IRF3, cells were transfected with pFR-Luc (60 ng), the trans-activator plasmid pFA-IRF3 (IRF3 fused downstream of the yeast Gal4 DNA binding domain, 30 ng), phRL-TK (20 ng) and varying amounts of expression constructs (detailed in figure legends). The total amount of DNA was maintained at 270 ng using pcDNA3.1.

2.2.4.4 IRF7 assay

To measure the activation of IRF7, cells were transfected with pFR-Luc (60 ng), the trans-activator plasmid pFA-IRF7 (IRF7 fused downstream of the yeast Gal4 DNA binding domain, 25 ng), phRL-TK (40 ng) and varying amounts of expression constructs (detailed in figure legends). The total amount of DNA was maintained at 270 ng using pcDNA3.1.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

U373-CD14 astrocytoma cells, BMDM were seeded in 12-well plates at a density of $1x10^5$ cells/ml. Primary astrocytes were seeded in 12-well plates at a density of $1x10^6$ cells/ml and human PBMCs were seeded in 24-well plates at a density of (1×10^6 cells/ml).. Cells were grown for 24 h to approximately 80% confluency. Next day, cells were stimulated with TLR ligands for 24 h (3 h for PBMCs) and pre-treatment with various agents for 1 hr, supernatants were collected from each well and stored at -20^{o}C until

analysis of TNF-α using Human TNF-α ELISA kits. 96-well NUNC "Maxisorb" plates were coated with mouse anti-human TNF-α capture antibody and were incubated overnight at room temperature. Plates were washed three times with wash buffer (PBS with 0.05% (v/v) Tween-20) and dried. Plates were blocked for 1 hour with PBS containing 1% (w/v) bovine serum albumin (BSA) and 5% sucrose (w/v). Plates were again washed three times and dried. Samples or standards (100 µl) diluted in reagent diluent (0.1% (w/v) BSA, 0.5% (v/v) Tween in 10 mM Tris–HCl, pH 7.5 containing 150 mM NaCl in Tris-buffer saline (TBS) were added to each well. TNF-α standard concentrations were between 0 and 2000 pg/ml. Plates were incubated with samples or standards for 2 h and then the series of washes was repeated. Detection antibody (100 µl) (biotinylated goat anti-human antibody) for human TNF-α diluted in reagent diluent was added to each well (20 ng/ml). Again plates were incubated for 2 h and washed. Streptavidin-horseradish peroxidase (HRP) conjugate (100 µl) was added to each well. The plates were incubated in the dark for 20 min and then the wash step was repeated. 3, 3', 5, 5'-TeTRAMethylbenzidine liquid substrate (TMB, 1.25 mM/L) (100 µl) solution was added to each well and again plates were incubated in the dark for no longer than 20 min. 1N sulphuric acid (H_2SO_4) (50 µl) was used to stop the reaction and the OD was measured for each well at 450 nm and 590 nm using a ELx800^{TM} microplate reader with Gen5 Data Analysis Software. The concentrations of TNF-α in each sample were extrapolated from a standard curve that related the OD of each standard amount to the known concentration. Standard samples were assayed in duplicate to generate the standard curve, while all samples were assayed in triplicate.

2.2.6 Western blot analysis

BMDM were seeded at a density of $1x10^5$ cells/ml in 6-well plates. Cells were grown for 24 h to approximately 80% confluency. Next day, cells were stimulated with TLR ligands for 1 h-3 h and pre-treatment with various agents for 1 hr. Cells were then washed in ice-cold PBS before being lysed on ice for 10 min in 150 µl lysis buffer (20mM HEPES, pH 7.4 containing 10mM KCl, 1.5mM MgCl2, 1mM EDTA, 1mM EGTA, 1 mM dithiothreitol, 0.1mM phenylmethylsulfonyl fluoride, pepstatin A (5 µg/ml), leupeptin (2

µg/ml), and aprotinin (2 µg/ml)). Cell lysates were centrifuged at 13,000*g* for 15 min at 4°C. The supernatant was mixed with SDS-PAGE sample buffer (0.125 Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 1.4 M β-mercaptoethanol and 0.0025% (w/v) bromophenol blue).

For *in vivo* experiments, samples of spinal cord were homogenised in lysis buffer and the resulting lysate was centrifuged (16000 x g for 15 min at 40°C). Supernatants were then further centrifuged (10000 x g for 1h at 40°C) and the supernatant (cytosolic fraction) added to sample buffer. All samples in sample buffer were boiled for 5-10 min and separated on SDS-PAGE gels.

2.2.6.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method of Laemmli (Laemmli, 1970), as modified by Studier (Studier, 1973). Samples and appropriate prestained (26.6-180 kDa) protein markers were loaded into separate wells. Electrophoresis was performed at 80 V through a 5% SDS polyacrylamide stacking gel and then through a 10% SDS polyacrylamide resolving gel at 100 V for 1.5-3 h, depending on the size of the proteins being electrophoresed.

2.2.6.2 Immunoblotting

Following separation by electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membranes in a Hoefer TE 70 Semiphor semi-dry transfer unit at 80 mA for 2 h using Whatmann and nitrocellulose pre-soaked in cold transfer buffer (25 mM Tris Base, 0.2 M glycine and 20% (v/v) methanol for 10 min. Following transfer, non-specific binding was blocked by incubating the nitrocellulose membranes at room temperature for 2 h (or overnight) in TBS (20 mM Tris-HCl pH 7.5, containing 0.05% (v/v) Tween 20 and 0.5 M NaCl) containing 5% (w/v) skimmed milk powder. The membranes were then washed 3 times for 10 min each in TBS prior to incubation at 4°C overnight with the primary antibodies diluted in TBS containing 2.5%

(w/v) skimmed milk powder. The membranes were subsequently subjected to 5 x 10 min washes in TBS prior to incubation with secondary antibody (1:5000 dilution) specific for the primary antibody in question (anti-rabbit or anti-mouse) in Odyssey Blocking Buffer (Licor, Bioscience) for 1 h in the dark at room temperature. The membranes were then washed a further 5 times for 10 min each in TBS in the dark. The immunoreactive bands were detected using Odyssey Infrared Imaging System from Licor Bioscience, according to the instructions of the manufacturer. Membranes were stripped and incubated with β-actin antibody, overnight at 4°C. Molecular weight markers were used to calculate molecular weights of proteins represented by immunoreactive bands. Densitometry was performed using ImageJ software, and values were normalized for protein loading relative to levels of total IRF3.

1° antibody	**Dilution**	**2° antibody***
β-actin	1:1000	mouse
IRF3	1:1000	rabbit
Phospho-IRF3	1:750	rabbit
IκBα	1:200	mouse
Phosho-IκBα/β	1:1000	mouse

***** All secondary antibodies were used at a dilution of 1:5000.

2.2.7 Isolation of RNA and cDNA synthesis

2.2.7.1 Isolation of total RNA from HEK293, U373-CD14 astrocytoma, BMDM cell, PBMCs and primary astrocytes

In order to minimise RNA degradation, a number of precautions were taken throughout the following procedures. All water and salt based solutions were treated with diethylpyrocarbonate (DEPC) (0.2% v/v) and then autoclaved. All plastic ware was

certified RNase-free. Before commencing, equipment was wiped with "RNase Zap" an RNase decontamination solution. Gloves were worn at all times and changed regularly.

To investigate the regulatory effects of various agents on TLR-induced activation of IFN-β, BMDM and primary astrocytes were seeded at $1x10^5$ cells/ml in 6-well plates (3 ml DMEM/well). PBMCs were seeded at $1x10^6$ cells/ml in 12-well plates. To investigate the expression of cannabinoid receptors, HEK293 TLR3/TLR4 cells and U373-CD14 astrocytoma cells and BMDM cells were seeded at $1x10^5$ cells/ml in 12-well plates (1 ml DMEM/well).

Total RNA was extracted from all cells as follows: after aspirating off the medium, Tri reagent was added (1 ml per well) and the plate was placed on a shaker at room temperature for 5 min. Following transfer to eppendorf tubes, insoluble material was removed by centrifugation (12000 x g for 10 min at 4°C). The supernatant was then moved to fresh eppendorf tubes and incubated at room temperature for 5 min. Chloroform was next added (0.2 ml). Samples were covered tightly and shaken vigorously for 15 s. This was followed by further incubation at room temperature for 15 min. Centrifugation at 12,000 x g for 15 min at 4^oC caused separation of the homogenate into an upper aqueous phase and lower organic phase. The aqueous phase containing RNA was transferred to fresh eppendorf tubes, taking care to avoid contamination with the lower organic layer. RNA was precipitated by adding isopropanol (0.5 ml per 1 ml of Tri reagent initially used) and maintaining samples at room temperature for 10 min. The precipitate was pelleted by centrifugation at 12,000 x g for 8 min at room temperature. The supernatant was discarded and the RNA pellet washed by vortexing with 70% ethanol (1 ml per 1 ml of Tri reagent initially used). The previous centrifugation step was repeated to collect the washed RNA pellet. Ethanol was then removed and the pellet was allowed to air-dry for up to 5 min. Care was taken not to allow the pellet to dry completely. RNA was dissolved in 30 µl RNAse-free water by passing the solution through a pipette several times. The amount of isolated RNA was quantified by measuring the absorbance at wavelengths of 260 nm and 280 nm on a spectrophotometer, where an absorbance of 1 unit at 260 nm is ~ 40 µg/ml. Pure RNA preparation have an OD_{260}/OD_{280} ration of 1.6-1.8. Extracted RNA was stored at -80^oC.

2.2.7.2 Synthesis of first strand cDNA from messenger RNA (mRNA)

1 µg of RNA was placed in nuclease-free microcentrifuge tubes and incubated for 10 min at 70^{o}C. The mixture was then chilled on ice and centrifuged briefly. The following components were then added:

Avian myeloblastosis virus reverse transcriptase (AMV RT) (15 U/µl)	0.75 µl
dNTPs (10 mM)	2 µl
10X AMV Buffer	2 µl
$MgCl_2$ (25 mM)	4 µl
RNasin (40 U/µl)	0.5 µl
Random Primers (0.5 µg/µl)	1 µl
Nuclease-Free Water	to 20 µl

The reaction mixture was incubated for 10 min at RT allowing the primers to anneal to the RNA. Next, The reaction was incubated at 42°C for 2 h. AMV RT was then deactivated by heating to 95^{o}C for 5 min followed by cooling to 0-5^{o}C for 5 min. Generated cDNA was stored at 4^{o}C for short-term storage or -20^{o}C for long-term storage.

2.2.7.3 PCR analysis of specific cDNA expression

Total RNA was isolated from HEK293 TLR3/TLR4 cells, U373-CD14 astrocytoma cells seeded at 1x10^{5} cells/ml in 12-well plates (1 ml DMEM/well) and BMDM seeded at 1 x 10^{5} cells/ml in 6-well plates (3 ml DMEM/well) using the Tri Reagent (Sigma) as per manufacturer's instructions. First strand cDNA synthesis was carried out using Superscript II reverse transcriptase and random oligonucleotide primers and PCR amplification was performed using TaqDNA polymerase and primers to selectively amplify regions of the human/mouse cannabinoid (CB) CB1 and CB2 receptors and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs.

The sequences of the forward and reverse oligonucleotide primers were as follows:

NAME	PRIMER SEQUENCE
human CB1 (a 500-bp fragment)	For: 5'- GAGACAACCCCCAGCTAGTCCCAGCAGACC-3' Rev: 5'-TGGGCCTGGTGACAATCCTCTTATAGGCC-3'
Human CB2 (a 400-bp fragment)	For: 5'-CTTCTGGCCCTGCTAAGTGCCCTGGAGAACG-3' Rev: 5'-CAGCAAGTCCATCCCATGAGGGGCAGCTAGG-3'
Mouse CB1 (a 400-bp fragment)	For: 5'-GTCACCAGTGTGCTGTTGCT-3' Rev: 5'-TGTCTCAGGTCCTTGCTCCT-3'
Mouse CB2 (a 374bp fragment)	For: 5'-TGCTGCTCATATGCTGGTTC-3' Rev: 5'-CTTCTGACTCGGGCTGTTTC-3'
Human GAPDH (a 450-bp fragment)	For: 5'-ACCACAGTCCATGCCATC-3' Rev: 5'-TCCACCACCCTGTTGCTG-3'
Mouse GAPDH (a 450-bp fragment)	For: 5'-GCACAGTCAAGGCCGAGAAT-3' Rev: 5'-GCCTTCTCCATGGTGGTGAA-3'

Amplification parameters were as follows: step 1: 35 cycles at 94 °C for 1 min, at 55 °C for 1 min and at 72 °C for 1 min; step 2: at 72 °C for 10 min. The PCR products

were subjected to electrophoresis on a 1% TAE agarose gel, containing 5 μg/ml ethidium bromide.

2.2.7.4. Agarose gel electrophoresis

Agarose gels were prepared by suspending agarose (0.8-1% (w/v)) in TAE (0.484% (w/v) Tris base, 0.1% (v/v) glacial acetic acid, 0.2 M EDTA). This was heated in a microwave until the agarose had completely dissolved, and was then cooled to less than 50^{o}C. Ethidium bromide (5 μg/ml) was added and the agarose poured into the gel tray. Following solidification, agarose gels were covered and electrophoresed in TAE (1X). Samples were mixed with loading dye (0.017% (w/v) xylene cyanol, 0.017% (w/v) bromophenol blue and 5% (w/v) glycerol). Samples were run simultaneously with molecular size markers, with the range of ladder chosen to suit the particular sample size. The voltage at which gels were run depended on the particular application. Nucleic acids were visualised under UV light (254 nm) and images acquired using the Syngene G box gel documentation system (Frederick, MD, USA).

2.2.7.5 Quantitative RT-PCR (QRT-PCR) of BMDM and primary mouse astrocytes

BMDM and primary astrocytes were seeded at a density of 1x10^{5} cells/ml in 12-well plates and 6-well plates respectively. PBMCs were seeded at a density of 1x106 cells/ml in 12-well plates. Cells were pre-treated with various agents for 1 h prior to TLR ligand exposure for 16 h (PBMCs for 3 h). RNA was extracted from cells using Tri Reagent™ (Invitrogen) and cDNA generated from normalized RNA using Superscript II reverse transcriptase. cDNA (1 μg) was amplified in the presence of SYBR® Green PCR mastermix.

Primers used were as follows:

NAME	PRIMER SEQUENCE
Mouse IFN-β	For: 5’-GGAGATGACGGAGAAGATGC-3’ Rev: 5’-CCCAGTGCTGGAGAAATTGT-3’
Human IFN-β	For: 5’-GACCAACAAGTGTCTCCTCCAAA-3’ Rev: 5’-CTCCTCAGGGATGTCAAAGTTCA-3’
Mouse PPARα	For: 5’-AAGTGACTTCGCTATCCAGG-3’ Rev: 5’-TAGGGGACCTTCCAAACATA-3’
Mouse PPARγ	For: 5’- GCGGAGATCTCCAGTGATATC -3’ Rev: 5’- TCAGCGACTGGGACTTTTCT -3’
Mouse GAPDH	For: 5’-AGGTCATCCCAGAGCTGAACG-3’ Rev: 5’-ACCCTGTTGCTGTAGCCGTA-3’
Human HPRT	For: 5’-TTGCTGACCTGCTGGATTAC-3’ Rev: 5’-TCTCCACCAATTACTTTTATGTCC-3’

Accumulation of gene-specific PCR products was measured continuously by means of fluorescence detection over 40 cycles. Samples were run in duplicate as follows: 10 min

at 95°C and for each cycle, 10 seconds at 95°C, 10 seconds at 55°C and 1 min at 72°C. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method.

2.2.8 Confocal Microscopy

For characterisation of endogenous IRF3, primary astrocytes were seeded at a density of $1x10^5$ cells/ml in 4-well chamber slides and grown for 24 h. Cells were pre-treated with various agents for 1 h prior to TLR ligand exposure for 1 h. Cells were fixed in 4% PFA, permeabilised with 0.2% Triton X-100 in PBS for 10 min at room temperature and blocked with 10% goat serum for 2 h. Cells were treated overnight at 4°C with primary antibody. Cells were washed and incubated with secondary antibody DAPI (1.5 μg/ml) in PBS, washed, and mounted (Vectashield; Vector Laboratories).

HEK293 TLR3 cells were seeded at a density of $1.5x10^5$ cells/ml in 4 well chamber slides for 24 h and transfected using Lipofectamine 2000 with an expression construct encoding GFP-tagged IRF3 (800 ng). Control slides were transfected with EGFP construct (800ng). Cells were pre-treated with various agents for 1 h prior to TLR ligand exposure for 1 h. Cells were fixed in 4% PFA, incubated with DAPI (1.5 μg/ml) in PBS for 30 min, washed, and mounted. All samples were viewed using an Olympus FluoView FV1000 confocal laser scanning microscope equipped with the appropriate filter sets. Acquired images were analysed using the Olympus FV-10 ASW imaging software. Negative control experiments were performed by replacing the primary antibody with isotype controls and using equal gain settings during acquisition and analysis.

2.2.9 Induction and assessment of EAE.

EAE was induced in mice as described (Smith et al., 2005). Female SJL/J mice (8 weeks old) were injected subcutaneously at 2 sites, with 2 injections (100 μl) of emulsified Freund's complete adjuvant containing 100 μg of Myelin proteolipid protein amino acid, 139-151 ($PLP_{139-151}$) and 200 μg *Mycobacterium tuberculosis* H37Ra followed 2 hours later

with 200ng Pertussis toxin injected intraperitoneally. The preparation and immunisation of the synthetic cannabinoid R(+)WIN55,212-2 was modified from previous studies (Croxford and Miller, 2003). R(+)WIN55,212-2 was prepared in Cremophor El and PBS (20:80) and administered (20 mg/Kg) intraperitoneally on days 0, 1, 2, 3, 4 and 5. Rabbit anti-mouse IFN-β polyclonal antibody was administered intraperitoneally (2 x 103 Neutralizing Units) on days 3 and 5 after PLP immunisation. Control mice received Cremophor:PBS (20:80) as vehicle. Data are from 4-8 mice per group. To ensure objective clinical scoring, all mice had electronic data chips placed subcutaneously prior to experiment and were subsequently tracked by barcode reader (AVID, UK). An investigator blinded to the treatment of the mice scored all animals by barcode number, to determine the mean clinical score as follows: 0, normal; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness, 3, partial hind limb paralysis; 4, complete hind limb paralysis, and 5, moribund.

2.2.10 Histology

Spinal cords were dissected and fixed in 10% formaldehyde saline. Spinal cords were sectioned and stained with haematoxylin and eosin for inflammatory scoring (Wraith et al., 2009). Inflammatory scores were as follows: 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, perivacular cuffing; 3, perivascular cuffing with extensions into adjacent parenchyma, or parenchymal infiltration without obvious cuffing. Demyelination was assessed on Luxol fast blue-stained spinal cord sections and scored as follows: 0, no evident demyelination; 1, decreased myelination with no foci; 2, obvious demyelination with evident foci; 3: severe demyelination. An investigator blinded to the treatment groups scored all stained sections, with slides labelled by mouse barcode number.

2.2.11 Statistical Analysis

Data is expressed as mean $\pm$ standard error of the mean (S.E.M.), and the results represent three independent experiments (nine independent experiments for primary cultures). Statistical comparisons of different treatments were done by a one-way analysis

of variance using a post hoc Student's Newman-Keuls test. Differences with a p value less than 0.05 were considered statistically significant.

Chapter 3

Defining the regulatory effects of WIN55,212-2 on TLR3 and TLR4 signalling

3.1 Introduction

TLRs and their adaptor molecules are essential regulators of the mammalian immune system and although have been studied for over a decade, their complexity continues to intrigue researchers. Since the discovery of the first mammalian TLR, TLR4, (Medzhitov et al., 1997) much work has been carried out in characterising these receptors and their central role in the innate and adaptive immune response. TLR recognition of diverse microorganisms and initiation of complex signalling pathways culminates in the induction of various transcription factors including NFκB, IRFs, AP-1 and CREB. However, dysregulation of the same pathways can result in the onset of various inflammatory and autoimmune diseases.

TLRs play a significant role in modulating M.S. (Hansen et al., 2006). Much of our insight has emerged from the use of the EAE model. Studies have confirmed that TLR expression is strongly increased within the CNS during EAE (Prinz et al., 2006). The absence of TLR4 exacerbates EAE and this clinical feature is associated with increased expression of IL-17, a potent inflammatory mediator of M.S. (Marta et al., 2008). Other studies have demonstrated how pertussis toxin (PTX) can facilitate leukocyte migration across the BBB by increasing its permeability, an effect that is mediated by activation of TLR4 (Kerfoot et al., 2004). Studies have also reported on the presence of the TLR2 ligand, PG, in the CNS of M.S. primates (Visser et al., 2006). PG has been found to be able to act as an adjuvant to induce EAE (Visser et al., 2005). Similarly, TLR9 agonists can also mediate the development of EAE (Waldner et al., 2004) as TLR9 deficient mice are partially resistant to the disease (Prinz et al., 2006)

MyD88, an essential adaptor in the activation of NFκB, also functions in the induction and progression of EAE as MyD88 knockout mice are completely resistant to EAE and show no brain inflammation (Prinz et al., 2006). This contrasts with the MyD88 independent pathway, the central pathway utilised by TLR3. Poly(I:C)-induced activation of TLR3 and subsequent induction of IFN-β suppresses relapsing EAE (Touil et al., 2006). In addition, IFN-β knockout (KO) mice are more susceptible to EAE and develop further extensive CNS inflammation and demyelination than wild-type mice (Teige et al., 2003).

One of the most recently introduced treatments for M.S. patients is the use of recombinant IFN-β. Transcriptional activation of the IFN-β gene is tightly regulated

and transcriptionally controlled by a limited number of transcription factors. The assembly of the transcription enhancer complex referred to as the enhanceosome is a key requirement for IFN-β transcription. Following TLR ligand stimulation, activated transcription factors e.g. NFκB and IRF3/7 translocate to the nucleus and bind to the PRD on the IFN-β promoter (Thanos and Maniatis, 1995). This results in high levels of IFN-β expression. While NFκB and IRF3 are expressed in multiple cell types, IRF7 expression is mainly confined to pDCs and dependent on activation of the ISGF3 transcriptional regulator (Taniguchi and Takaoka, 2002, Noppert et al., 2007).

The above studies demonstrate there is increasing evidence supporting a role for TLRs in M.S. Reports have also suggested that cannabinoids, known to exhibit general immunosuppressive properties, have potential therapeutic value in the treatment of neuroinflammatory conditions (Consroe et al., 1997). Furthermore, much interest has also been generated by reports of the therapeutic effects of cannabis in alleviating the symptoms of M.S. (Consroe et al., 1997) where patients with spasticity claim relief from using the drug (Iversen, 2003). In rats and guinea pigs, δ-9-THC suppressed both the histological and clinical manifestations of EAE (Lyman et al., 1989). The introduction of Sativex (a combination of δ-9-THC and cannabidiol) further demonstrates the therapeutic action of cannabinoids in M.S.

The advent of synthetic and endogenous cannabinoid compounds accompanied by the development of animal models of M.S. have enabled the pharmacology of cannabinoids to blossom and shed light on anecdotal reports of the medicinal value of cannabis.

EAE is one of the most intensively studied animal models of M.S. The origins of EAE date back to 1885 and arose from vaccination with rabies-infected rabbit spinal cord by Louis Pasteur (Baxter, 2007). Nowadays, EAE can be induced in a number of species, including mice, rats, guinea pigs, rabbits and primates. EAE may be induced via active immunisation with CNS tissue or myelin antigens in complete Freund's adjuvant or by adoptive transfer whereby $CD4^+$ T cells specifically reactive to myelin antigens that are transferred from affected animals to healthy recipients (Stromnes and Goverman, 2006). It has been previously been reported that treatment with high concentrations of R(+)WIN55,212-2 in EAE rats significantly improves the clinical performance of the animals during relapse (Hasseldam and Johansen, 2010).

R(+)WIN55,212-2 treatment reduced T cell, microglia production and axonal degeneration where high doses resulted in reduced demyelination.

Viral models of M.S. have also contributed in the understanding of the disease. Viral models are particularly relevant in the increasing evidence for viral mechanisms in the etiology of M.S. (Pohl, 2009). Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus natural pathogen in mice. Injection of TMEV in the CNS induces an immune-mediated demyelinating disease in susceptible mouse strains that resembles progressive M.S. (Dal Canto and Lipton, 1977). An immune response directed against viral and myelin epitopes takes place in the CNS of TMEV-infected mice and this event is considered to be the cause of TMEV-induced demyelinating disease (TMEV-IDD). In TMEV-IDD mice, treatment with R(+)WIN55,212-2 was shown to reduce microglial activation, diminish MHC class-II antigen expression and decreased the number of CD4+ infiltrating T cells in the spinal cord (Arevalo-Martin et al., 2003). R(+)WIN55,212-2 treatment has also been demonstrated to suppress ICAM-1 and VCAM-1 activation in this model (Mestre et al., 2009). Furthermore, intraperitoneal treatment of R(+)WIN55,212-2 at different time points in TMEV-IDD mice significantly inhibit the progression of clinical disease (Croxford and Miller, 2003). In chronic relapsing experimental allergic encephalomyelitis (CREAE), an additional mouse model of M.S., R(+)WIN55,212-2 treatment quantitatively ameliorated both tremor and spasticity in diseased mice (Baker et al., 2000).

R(+)WIN55,212-2 is anti-inflammatory in the brain by inhibiting leukocyte infiltration into the CNS (Arevalo-Martin et al., 2003). Our laboratory has previously elaborated on this study in 1321N1 astrocytoma, a cell model of human astrocytes (Curran et al., 2005). Astrocytes are resident CNS glial cells and play an important role in contributing to the pathogenesis of M.S. They secrete factors required to maintain the BBB, and provide physical support through foot processes found on the abluminal surface of the vascular endothelium. Astrocytes are also immunocompetent cells, responding to and producing cytokines, and expressing adhesion molecules and chemokines upon appropriate stimulation (Aloisi et al., 1992, Dong and Benveniste, 2001, Moynagh et al., 1994, Bourke and Moynagh, 1999). Their ability to express MHC class I and class II molecules, (Wong et al., 1984) gives them the machinery to activate a range of T cells in particular encephalitogenic CD4+ Th1 cells, the main cell type to mediate demyelination.

Curran and colleagues demonstrated the inhibitory effects of R(+)WIN55,212-2 on IL-1β-induced activation of NFκB (Curran et al., 2005). This study also encouraged an examination of the downstream signalling components involved in the transcriptional activation of NFκB. A number of molecules at various stages in the IL-1 signalling pathway were examined for their sensitivity to the inhibitory effects of R(+)WIN55,212-2. In each case, overexpression of MyD88, TAK-1 and IKKβ caused robust induction of the NFκB-regulated reporter gene. Treatment with R(+)WIN55,212-2 strongly inhibited the ability of each molecule to induce NFκB transcriptional activity. However the molecular basis of R(+)WIN55,212-2 on TLR signalling was not examined.

TLRs employ many of the same signalling components as the IL-1R (Martin and Wesche, 2002). As a result, it was deemed of interest to assess the regulatory effects of R(+)WIN55,212-2 on TLR activation of NFκB and other transcription factors.

3.2 Results

3.2.1 R(+)WIN55,212-2 inhibits NFκB activation by TLR ligands.

The initial focus of this study was to investigate if R(+)WIN55,212-2 regulated TLR-induced activation of NFκB. Various cell lines engineered to individually express TLR3 and TLR4 were utilised. TLR3 and TLR4 were selected as primary targets since they exert differential roles in the development of M.S. lesions (Racke and Drew, 2009, Bsibsi et al., 2006) and EAE inflammation (Hansen, Hussain et al. 2006). Poly(I:C)-induced activation of TLR3 and subsequent induction of IFN-β suppresses relapsing EAE (Touil et al., 2006) while PTX can facilitate leukocyte migration across the BBB by increasing its permeability, an effect that is mediated by activation of TLR4 (Kerfoot et al., 2004). Furthermore, studies in Lewis rats indicated that a combination of LPS and the TLR9 agonist CpG ODN are required for the development of EAE, while in contrast Poly(I:C) does not induce disease (Wolf et al., 2007).

R(+)WIN55,212-2 was first assessed for its regulatory effects on TLR-induced activation of NFκB. HEK293 cells stably tranfected with TLR3 or TLR4, were treated with Poly(I:C) and LPS respectively. The transcriptional activity of NFκB was measured by expression of a transfected NFκB-regulated reporter luciferase gene.

LPS significantly enhanced the transcriptional activation of the NFκB-regulated reporter gene (Fig. 3.1A). Pre-treatment with various concentrations of R(+)WIN55,212-2 caused a dose-dependent inhibition of LPS-induced NFκB-regulated luciferase expression. The WIN55,212-2 compound is available in two enantiomeric forms, R and S, with R being the active form (Herzberg et al., 1997). In contrast to the R form, the inactive chiral form S(-)WIN55,212-2 failed to affect the ability of LPS to induce NFκB activation (Fig. 3.1B), suggesting that a stereoselective mechanism underlies the inhibitory effects of R(+)WIN55,212-2.

A similar experiment was also preformed in HEK293 TLR3 cells where R(+)WIN55,212-2 treatment caused a dose-dependent inhibition of Poly(I:C)-induced activation of NFκB (Fig. 3.1C) whereas its chiral form S(-)WIN55,212-2 was ineffective (Fig. 3.1D).

Owing to the importance of astrocytes in neuroinflammation, it was next of interest to explore the regulatory effects of R(+)WIN55,212-2 on NFκB in this cell type.

1321N1 and U373-CD14 astrocytoma cells were used as cell line models of human astrocytes. Both astrocytoma cell lines failed to respond to LPS treatment but were strongly responsive to Poly(I:C) stimulation (Fig. 3.2). Pre-exposure of the cells to R(+)WIN55,212-2 dose-dependently attenuated the Poly(I:C)-induced activation of NFκB in U373-CD14 astrocytoma (Fig. 3.2A) and 1321N1 astrocytoma (Fig. 3.2B) cells.

3.2.2 R(+)WIN55,212-2 inhibits TNF-α expression by TLR ligands.

TNF-α production is predominantly regulated by NFκB. As a result, it was of interest to assess the regulatory effects of R(+)WIN55,212-2 in TLR-induced expression of TNF-α.

Stimulation of U373-CD14 astrocytoma cells with either LPS or Poly(I:C) for 24 hours induced a 10-fold increase in TNF-α expression and pre-treatment with R(+)WIN55,212-2 inhibited the induction of TNF-α in response to both TLR ligands (Fig. 3.3A and Fig. 3.3C). Specificity for the WIN55,212-2 compound was also established as S(-)WIN55,212-2 failed to inhibit the activation of TNF-α expression in LPS (Fig. 3.3B) or Poly (I:C) treated cells (Fig. 3.3D).

We next aimed to corroborate some of the above findings in primary cells. Primary mouse astrocytes were strongly responsive to LPS and Poly(I:C) stimulation (Fig. 3.4A and Fig. 3.4C) and showed significant TNF-α production after 24 hours of ligand treatment. In both cases R(+)WIN55,212-2 dose-dependently inhibited the response (Fig. 3.4A and Fig. 3.4C). Again, stereoselectivity for this effect was shown since S(-)WIN55,212-2 failed to affect TLR ligand induction of TNF-α expression (Fig. 3.4B and Fig. 3.4D).

R(+)WIN55,212-2 inhibits Poly(I:C) induction of TNF-α expression in a variety of human and animal cell models. It was next examined if the inhibitory effects of R(+)WIN55,212-2 on TNF-α production were extended to cells from M.S. patients. PBMCs which endogenously express TLR3 and TLR4 (XIN-YI et al., 2007) were isolated from healthy and M.S. patient subjects. PBMCs were responsive to Poly(I:C) and showed strong induction of TNF-α (Fig. 3.5A and Fig. 3.5B) expression. In both patient groups, pre-treatment with R(+)WIN55,212-2 robustly downregulated Poly(I:C)-induced activation of TNF-α expression (Fig. 3.5A and Fig. 3.5B).

3.2.3 R(+)WIN55,212-2 inhibits IRF7 activation by TLR ligands.

The above studies demonstrate the regulatory effects of R(+)WIN55,212-2 on NFκB and its responsive gene. Previous studies by our laboratory illustrated the inhibitory effects of R(+)WIN55,212-2 on MyD88 activation of NFκB (Curran et al., 2005). As MyD88 also activates the transcription factor IRF7, it was of significance to assess the effects of R(+)WIN55,212-2 on TLR-induced activation of IRF7.

In luciferase assays, the trans-activator plasmid pFA-IRF7 (where IRF7 is fused downstream of the yeast Gal4 DNA binding domain) was transfected into HEK293 TLR4 cells in conjunction with the Gal4-responsive pFR-luciferase construct. Upon activation of IRF7, the trans-activation capacity of the IRF7-Gal4 fusion protein is induced. This, in turn, induces the expression of luciferase from the Gal4-inducible promoter, pFR-Luc, which is used an index of IRF7 activity. Following transfection, the cells were treated with LPS which resulted in significant induction of IRF7-regulated reporter gene (Fig. 3.6A). Pre-treatment of cells with R(+)WIN55,212-2 dose-dependently and strongly attenuated LPS-induced IRF7 luciferase activity (Fig. 3.6A). Conversely, S(-)WIN55,212-2 exhibited no inhibition of LPS-induced activation of IRF7 (Fig. 3.6B).

R(+)WIN55,212-2 was next assessed for its regulatory effects on Poly(I:C)-induced activation of IRF7. Pre exposure of the cells to R(+)WIN55,212-2 inhibited Poly(I:C)-induced activation of IRF7 (Fig. 3.6C). Again, specificity was confirmed since the S(-)WIN55,212-2 compound had no effect on Poly(I:C) induced activation of IRF7 (Fig. 3.6D).

A similar profile was also observed in U373-CD14 astrocytoma cells where pre-exposure of the cells to R(+)WIN55,212-2 dose dependently attenuated LPS (Fig. 3.6E) and Poly(I:C)-induced (Fig. 3.6F) activation of IRF7.

3.2.4 R(+)WIN55,212-2 differentially regulates IRF3 activation by TLR ligands.

IRF3 is an additional transcription factor that is also activated by both TLR3 and TLR4 signalling events (Moynagh, 2005) which employs the signalling components TRIF and MyD88 respectively. As MyD88 deficiency is protective in EAE (Prinz et

al., 2006), while TRIF deficiency exacerbates the disease (Guo et al., 2008), the sensitivity of MyD88-dependent and -independent signalling to R(+)WIN55,212-2 exposure was further delineated. HEK293 TLR3 and TLR4 cells were transfected with the trans-activator plasmid pFA-IRF3 (where IRF3 is fused downstream of the yeast Gal4 DNA binding domain) in conjunction with the Gal4-responsive pFR-luciferase construct and stimulated with Poly(I:C) and LPS respectively. As illustrated in Fig. 3.7A, exposure of HEK293 TLR4 cells to LPS enhanced IRF3 luciferase activity and this was abrogated by R(+)WIN55,212-2 in a dose-dependent manner. Treatment of HEK293 TLR3 cells with Poly(I:C) enhanced IRF3 luciferase activity. However, in contrast to LPS treatment, pre-treatment with R(+)WIN55,212-2 potentiated Poly(I:C)-induced activation of IRF3 (Fig. 3.7B). Pre exposure of the cells to S(-)WIN55,212-2 failed to regulate Poly(I:C)-induced activation of IRF3 (Fig. 3.7C).

Similar results were also obtained in U373-CD14 astrocytoma (Fig. 3.7D) and 1321N1 astrocytoma cells (Fig. 3.7E), where R(+)WIN55,212-2 significantly augmented the Poly(I:C) induction of IRF3 in a dose-dependent manner.

The above data demonstrates that R(+)WIN55,212-2 differentially regulates IRF3 activation by TLR ligand stimulation and this observation is consistent across a number of different cell types.

3.2.5 R(+)WIN55,212-2 differentially regulates activation of the IFN-β promoter and induction of IFN-β mRNA by TLR ligands.

IRF3, IRF7 and NFκB are important regulators of type I IFN expression, including IFNβ (Taniguchi et al., 2001). As R(+)WIN55,212-2 exhibits diverse roles in the activation of these transcription factors, the functional consequences of R(+)WIN55,212-2 on TLR3/TLR4 activation of the IFN-β promoter, and the induction of IFN-β mRNA was next addressed.

HEK293 TRL3 cells were transfected with the IFN-β promoter luciferase construct and subsequently stimulated with Poly(I:C). This resulted in the Poly(I:C)-induced activation of the IFN-β promoter and pre-treatment with R(+)WIN55,212-2 significantly enhanced this response (Fig. 3.8A). Similar effects were also apparent in U373-CD14 astrocytoma cells (Fig. 3.8B), albeit at higher concentrations.

The regulatory effects of R(+)WIN55,212-2 on the activation of the IFN-β promoter were further characterised by dissecting its regulation of specific regions of the promoter. The PRDII region is recognised and regulated by NFκB while PRDI/III is recognised by IRF3/IRF7. Transfection with the PRD-I/III-luciferase construct and subsequent stimulation with Poly(I:C) resulted in significant activation of the PRD-I/III promoter (Fig. 3.9A). Pre-treatment with R(+)WIN55,212-2 inhibited this response (Fig. 3.9A). This possibly indicates a preference towards the activation of the IFN-β promoter by IRF7. Again, stereoselectivity for this effect was determined since S(-)WIN55,212-2 failed to affect Poly(I:C)-induced activation of the PRD-I/III domain (Fig. 3.9B).

A similar assay was also carried out in HEK293 TLR3 cells where the PRD-II-luciferase construct was transfected and followed by Poly(I:C) treatment. Pre-exposure of the cells to R(+)WIN55,212-2 inhibited the Poly(I:C) induction of the PRD-II (Fig. 3.9C) which further supports our previous data on the negative regulatory effects of R(+)WIN55,21-2 on Poly(I:C) induced activation of NFκB.

In order to further explore the mechanism underlying the regulatory effects of R(+)WIN55,212-2, transcriptional regulation of the IFN-β gene was probed as a potential target. Real-time PCR was used to quantitate levels of mRNA encoding IFN-β. Bone marrow derived macrophages (BMDM), endogenously expressing TLR3 and TLR4 were employed. Treatment of BMDM cells with LPS (Fig. 3.10A) or Poly(I:C) (Fig. 3.10C) enhanced the expression of IFNβ mRNA. Pre-exposure of the cells to R(+)WIN55,212-2 reduced LPS (Fig. 3.10A), but enhanced Poly(I:C)-induced (Fig. 3.10C) activation of IFNβ mRNA expression. Surprisingly, S(-)WIN55,212-2 also inhibited the activation of IFN-β mRNA by LPS (Fig. 3.10B) and Poly(I:C) (Fig. 3.10D) treatment.

Primary rat astrocytes were next used as a more physiologically relevant model system to further investigate the regulatory effects of R(+)WIN55,212-2 on IFN-β mRNA expression. Pre-exposure of the cells to R(+)WIN55,212-2 partially inhibited LPS induction of IFN-β mRNA, specifically at the 5μM and 50μM dose (Fig. 3.10E). In contrast, R(+)WIN55,212-2 treatment induced marginal augmentation of IFN-β mRNA expression following Poly(I:C) stimulation (Fig. 3.10F). This effect was again observed in higher concentrations of the drug.

Overall, these results further emphasise the differential effects of R(+)WIN55,212-2 on TLR3 and TLR4 signalling pathways indicating that R(+)WIN55,212-2 acts as a positive regulator of Poly(I:C)-induced activation IRF3 and its associated IFN-β expression.

Given that R(+)WIN55,212-2 can augment Poly(I:C)-induced activation of the IFN-β promoter and IFN-β mRNA in a variety of human and animal cell models, it was next examined if R(+)WIN55,212-2 could modulate IFN-β mRNA expression in cells from M.S. patients. PBMCs isolated from healthy subjects were responsive to Poly(I:C) treatment, with a significant increase in IFN-β mRNA expression, where pre-treatment with R(+)WIN55,212-2 ablated this response (Fig. 3.11A). In contrast to healthy subjects, PBMCs isolated from M.S. subjects were unresponsive to Poly(I:C) and no IFN-β mRNA expression was detected following Poly(I:C) treatment alone (Fig. 3.11B). Remarkably, PBMCs from M.S. subjects displayed exquisite sensitivity of exposure to R(+)WIN55,212-2, with treatment robustly inducing IFN-β mRNA expression in the absence of Poly(I:C). These findings are significant given that pDCs from M.S. patients produce lower levels of type I IFN (Stasiolek et al., 2006) and are weakly responsive to IFN-β-induced maturation (Kraus et al., 2008). This suggests that any therapeutic potential for R(+)WIN55,212-2 would depend on its ability to augment IFN-β in M.S. patients. The differential sensitivity of cells from healthy and M.S. subjects to R(+)WIN55,212-2 appear to be specific for IFN-β, since as previously shown, R(+)WIN55,212-2 blocks the Poly(I:C)-induced expression of TNF-α in PBMCs from both healthy (Fig. 3.5A) and M.S. (Fig. 3.5B) subjects.

3.2.6 R(+)WIN55,212-2 manifests protective effects in EAE in an IFN-β-dependent manner.

Given the therapeutic effects of IFN-β in the treatment of M.S. and the ability of R(+)WIN55,212-2 alone to augment IFN-β mRNA, it was intriguing to speculate that R(+)WIN55,212-2 may exert its therapeutic properties in M.S. animal models by inducing endogenous IFN-β. A relapsing mouse model of EAE involving immunisation with the myelin antigen, $PLP_{139-151}$ (PLP) was employed to address this hypothesis (Fig. 3.12A). PLP-immunised mice developed clinical symptoms of disease from day 5 post-immunisation, with disease severity peaking on day 16 followed by a relapse on day 26

(Fig. 3.12B). Mice treated with R(+)WIN55,212-2 (administered (20 mg/kg) showed delayed development of EAE and attenuated disease severity (Fig. 3.12B). However, PLP-immunised mice treated with R(+)WIN55,212-2 and anti-IFN-β antibody (αIFN-β) were not protected (Fig. 3.12B).

Scoring of histology sections confirmed R(+)WIN55,212-2 reduced lymphocytic infiltration (Fig. 3.13A and Fig. 3.13B) and demyelination of spinal cords (Fig. 3.13C). However, αIFN-β inhibited these protective effects (Fig. 3.13A, Fig. 3.13B and Fig. 3.13C). Animals that received αIFN-β antibody alone, displayed a similar degree of inflammation (Fig. 3.13B) and demyelination (Fig. 3.13C) compared to vehicle-treated mice. The effects of R(+)WIN55,212-2 on astrogliosis/microglial activation in PLP-immunised mice was also characterised. R(+)WIN55,212-2 attenuated both GFAP mRNA (Fig. 3.13D) and CD11b mRNA (Fig. 3.13E) in EAE spinal cord, and this was reversed by αIFN-β.

Finally, to characterise the anti-inflammatory effects of R(+)WIN55,212-2 at the molecular level, IκB proteins in spinal cords were analysed. As mentioned in Chapter 1, IκB proteins regulate NFκB by sequestering the transcription factor in the cytoplasm (Hacker and Karin, 2006) where NFκB activation is dependent on the phosphorylation and subsequent degradation of IκB. R(+)WIN55,212-2 reduced IκBα phosphorylation and degradation associated with EAE, and these effects were reversed by αIFN-β (Fig. 3.13F). This provides strong evidence that IFN-β plays a role in the protective effects of R(+)WIN55,212-2 in EAE.

3.3 Discussion

Although cannabis is illegal in many countries, it has been used for centuries as a therapeutic mediator for symptomatic relief in inflammatory and neuropathic disorders. Anecdotal evidence suggests that M.S. patients who consume whole extracts of cannabis claim relief from pain and spasticity (Consroe et al., 1997). Furthermore, similar results are also observed in M.S. patients administered with oral δ-9-THC (Pertwee, 2002). However, it is only in recent years that cannabinoid pharmacology has become better understood. The regulatory effects of the aminoalkylindole R(+)WIN55,212-2 was targeted in this study due to its impressive therapeutic effects in ameliorating disease progression in animal models of M.S. (Croxford and Miller, 2003, Arevalo-Martin et al., 2003). Furthermore, although R(+)WIN55,212-2 is structurally diverse from δ-9-THC, previous reports have identified the therapeutic value of R(+)WIN55,212-2 in attenuating EAE progression to a level that is comparable to the phytocannabinoid (Lyman et al., 1989).

The previously observed inhibitory effects of R(+)WIN55,212-2 in adhesion molecule expression in a human astrocyte cell line (Curran et al., 2005), may play a central role in mediating the therapeutic effects of the drug. Furthermore, adhesion molecules are expressed in elevated levels in astrocytes of acute M.S. lesions (Brosnan et al., 1995) and their expression is predominately mediated by IL-1β induced activation of NFκB. TLRs employ many of the same signalling components as the IL-1 pathway and also induce NFκB activation. As a result, the regulatory effects of R(+)WIN55,212-2 on TLR-induced activation of NFκB was deemed worthy of investigation .

NFκB and IRF7 are of fundamental importance in the induction of pro-inflammatory proteins, such as those encoding chemokines and cytokines, with MyD88 functioning as a potent activator of the transcription factors. Initial experiments using HEK293 cells and astrocytoma cells demonstrated that treatment with R(+)WIN55,212-2 inhibits TLR4-induced activation of NFκB and IRF7. Previous studies by our laboratory demonstrated that R(+)WIN55,212-2 strongly inhibits the ability of MyD88 to induce activation of NFκB (Curran et al., 2005). It is therefore suggested that R(+)WIN55,212-2 may target the TLR4 pathway downstream of MyD88, as both IRF7 and NFκB are regulated by the adaptor protein. Furthermore, R(+)WIN55,212-2 also

abolished TLR3 (MyD88-independent) activation of these transcription factors. Such targeting of the NFκB and IRF7 signalling pathways may provide major contributions to the inhibitory effects of R(+)WIN55,212-2 on pro-inflammatory gene expression. Indeed, this study also demonstrated that R(+)WIN55,212-2 blunts TLR3/4 induction of TNF-α in a variety of human and murine cells. Overall, the inhibitory effects attributed to R(+)WIN55,212-2 on inflammatory mediator production is comparable to other cannabinoid such as δ-9-THC (Puffenbarger et al., 2000), endocannabinoids (Facchinetti et al., 2003) and the synthetic cannabinoid ligand JWH-133 (Xu et al., 2007).

Interestingly, R(+)WIN55,212-2 exhibits contrasting effects on TLR3- and TLR4-induced activation of IRF3. While the synthetic cannabinoid strongly inhibits TLR4-induced activation of IRF3, in response to TLR3 stimulation, R(+)WIN55,212-2 profoundly augments IRF3 activation. Similar to TLR3-induced activation of IRF3, R(+)WIN55,212-2 also upregulated Poly(I:C)-induced activation of the IFN-β promoter and IFN-β mRNA expression. This effect was seen in astrocytoma cell lines and in both rodent and murine astrocytes. It appears that IFN-β may be selectively targeted by R(+)WIN55,212-2 following TLR3 stimulation and subsequent IRF3 activation. It is likely that R(+)WIN55,212-2 downregulation of LPS-induced activation of IFN-β mRNA expression is mediated via its inhibitory effects on NFκB and IRF3 in the same ligand response. The observed inhibitory effects of R(+)WIN55,212-2 on LPS-induced activation of IRF3 and IFN-β is consistent with a recent report describing δ-9-THC and cannabidiol ability to inhibit LPS-induced expression of IFN-β (Kozela et al., 2010). Despite this, the disparity between the regulatory effects of R(+)WIN55,212-2 on TLR3 and TLR4 activation of IRF3 and IFN-β is intriguing and highly novel. It is also extremely complex when studied in patient populations.

PBMCs from healthy donors responded to TLR3 stimulation by enhancing IFN-β production. Interestingly, this was absent in M.S. patient, suggesting that the TLR3 signalling pathway may be desensitised, at least in respect to IFN-β induction. Indeed, viral involvement in M.S. manifestation has been demonstrated (Pohl, 2009), and it is interesting to speculate that M.S. patients may be pre-sensitised to viral infection thus showing some form of TLR3 tolerance. Intriguingly, the non-responsiveness of M.S. patient PBMCs to Poly(I:C) is only relevant in the context of IFN-β induction since Poly(I:C) shows comparable efficacy in inducing TNF-α in cells from both healthy

subjects and MS patients. Therefore, any form of TLR3 tolerance that may exist, appears to be restricted to the pathway leading to IFN-β and this may explain why exogenous administration of IFN-β is effective in the treatment of M.S. Remarkably, R(+)WIN55,212-2 alone induced the expression of IFN-β in PBMCs from M.S. patients. Thus, whatever the basis underlying the refractory nature of M.S. cells to TLR3-induced IFN-β expression, R(+)WIN55,212-2 can bypass this blockage. This argues strongly in favour of the therapeutic potential of R(+)WIN55,212-2 in M.S. and presents an additional novel therapeutic strategy to the current exogenous administration of IFN-β. Furthermore, the induction of IFN-β by R(+)WIN55,212-2 in PBMCs from M.S. patients is strongly inhibited by Poly(I:C) treatment. This suggests that stimulation of TLR3 in cells from M.S. patients generates a negative input on IFN-β expression and is consistent with suggestions that viral infection can exacerbate disease.

The non polar nature of R(+)WIN55,212-2 raised the possibility that it mediates its effects via non-specific membrane disruption. However, the overall observed stereoselectivity of the response to the WIN55,212-2 enantiomers would seem to suggest receptor involvement. In the majority of experiments the inactive S(-)WIN55,212-2 enantiomer failed to affect TLR ligand activation of various transcription factors and their associated gene products. This is consistent with the lack of effect of S(-)WIN55,212-2 in animal models (Croxford and Miller, 2003). Furthermore, the concentrations of R(+)WIN55,212-2 used, are in line with those used *in vitro* (Facchinetti et al., 2003) and correlate well with the doses that are used to produce *in vivo* effects (Croxford and Miller, 2003).

In summation, the present study highlights the importance of IFN-β production as a mechanism underlying the protective effects of R(+)WIN55,212-2 in EAE and preliminary data suggests that a similar phenomenon may exist in humans. It is proposed that such effects are due to a combination of neuroprotection and dampening of inflammation. Whilst it is clear that the anti-inflammatory properties of R(+)WIN55,212-2 may be manifested directly by its inhibitory effects on NFκB, it is also apparent that *in vivo* anti-inflammatory effects of R(+)WIN55,212-2 are dependent on IFN-β and the immunomodulatory potential of the latter. Such directly and indirectly acting mechanisms of R(+)WIN55,212-2 may combine to explain its strong anti-inflammatory propensity.

Understanding the pharmacology of R(+)WIN55,212-2 is crucial in elucidating its anti-inflammatory effects. As a result, the involvement of the cannabinoid receptors in mediating the regulatory effects of R(+)WIN55,212-2 and the investigation of whether endocannabinoids mimic R(+)WIN55,212-2, forms the content of the next chapter.

Chapter 3

FIGURES

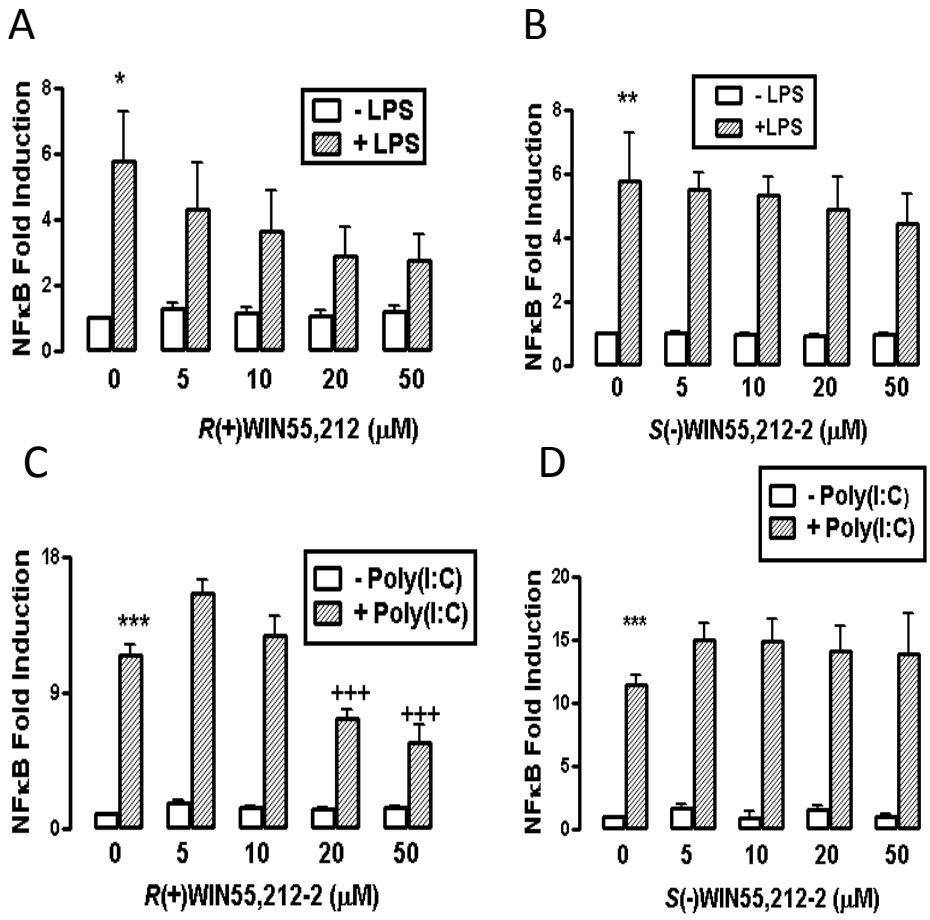


Figure 3.1 R(+)WIN55,212-2 inhibits LPS and Poly(I:C)-induced activation of NFκB in HEK293 TLR3/TLR4 cells.

HEK293 cells stably transfected with TLR4 or TLR3 were co-transfected with a NFκB-regulated firefly luciferase reporter (80 ng), phRL-TK (constitutively expressed Renilla luciferase) (40 ng), and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of R(+)WIN55,212-2 (A+C) or S(-)WIN55,212-2 (B+D) for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with vehicle-treated cells. +++P <0.001 compared with LPS or Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (9, 20) = 3.710. P < 0.01 (B) F value (9, 20) = 10.45. P < 0.001 (C) F value (9, 20) = 56.92. P < 0.001 (D) F value (9, 20) = 22.02. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

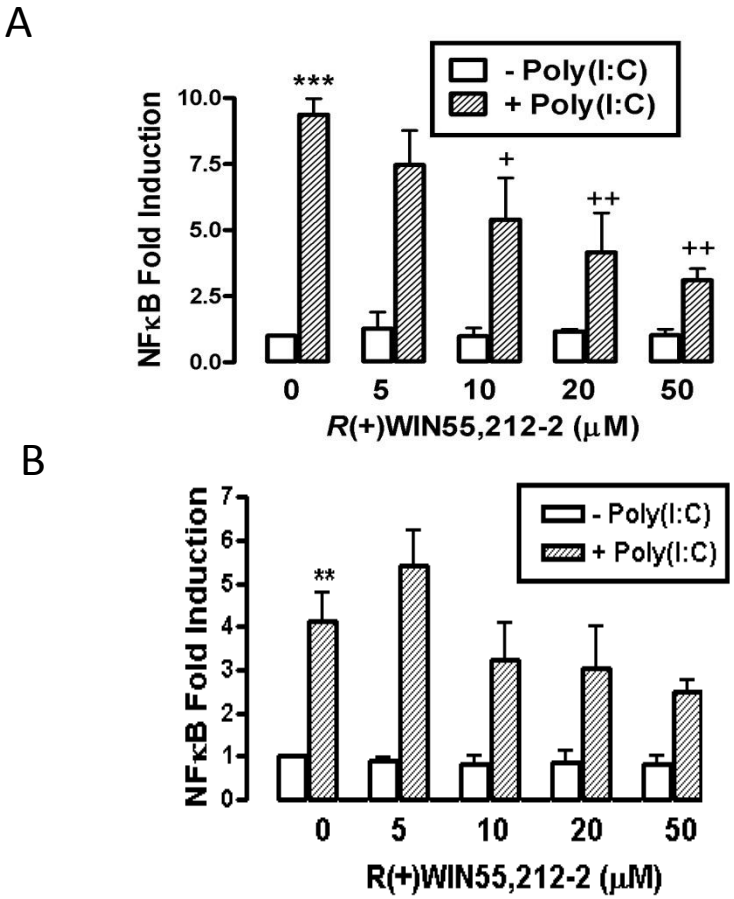


Figure 3.2 R(+)WIN55,212-2 inhibits Poly(I:C)-induced activation of NFκB in astrocytoma cells.

U373-CD14 astrocytoma (A) and 1321N1 astrocytoma cells (B) were co-transfected with a NFκB-regulated firefly luciferase reporter (80 ng), phRL-TK (constitutively expressed Renilla luciferase) (40 ng), and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of R(+)WIN55,212-2 for 1 h prior to stimulation in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. **$P < 0.01$ and ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$ and ++$P < 0.01$ compared with Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (9, 20) = 6.790. $P < 0.001$ (B) F value (13, 28) = 8.995. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

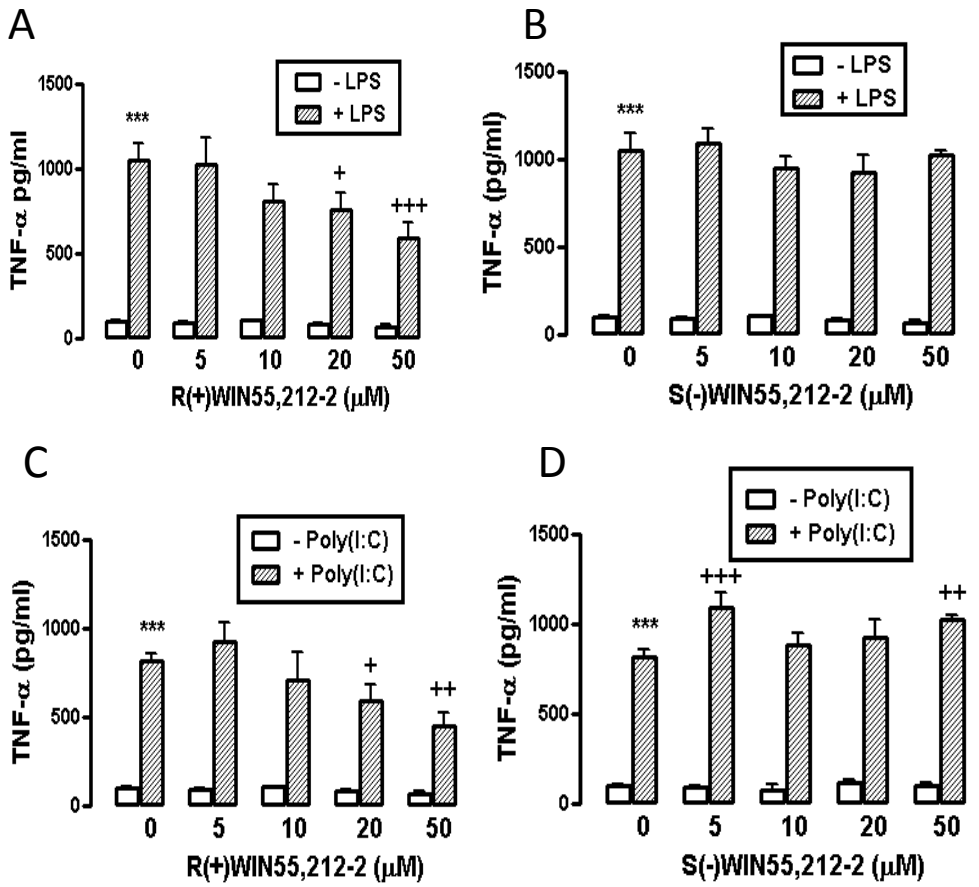


Figure 3.3 R(+)WIN55,212-2 inhibits LPS and Poly(I:C) induction of TNF-α expression in U373-CD14 astrocytoma cells.

U373-CD14 astrocytoma were pre-treated with or without various concentrations of R(+)WIN55,212-2 (A+C) or S(-)WIN55,212-2 (B+D) for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 24 h.

Supernatants were assayed for TNF-α levels by sandwich ELISA. ***P < 0.001 compared with vehicle-treated cells. +P < 0.05, ++P < 0.01 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (13, 28) = 34.13. P < 0.001 (B) F value (13, 28) = 94.90. P < 0.001 (C) F value (13, 28) = 30.22. P < 0.001 (D) F value (13, 28) = 99.20. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

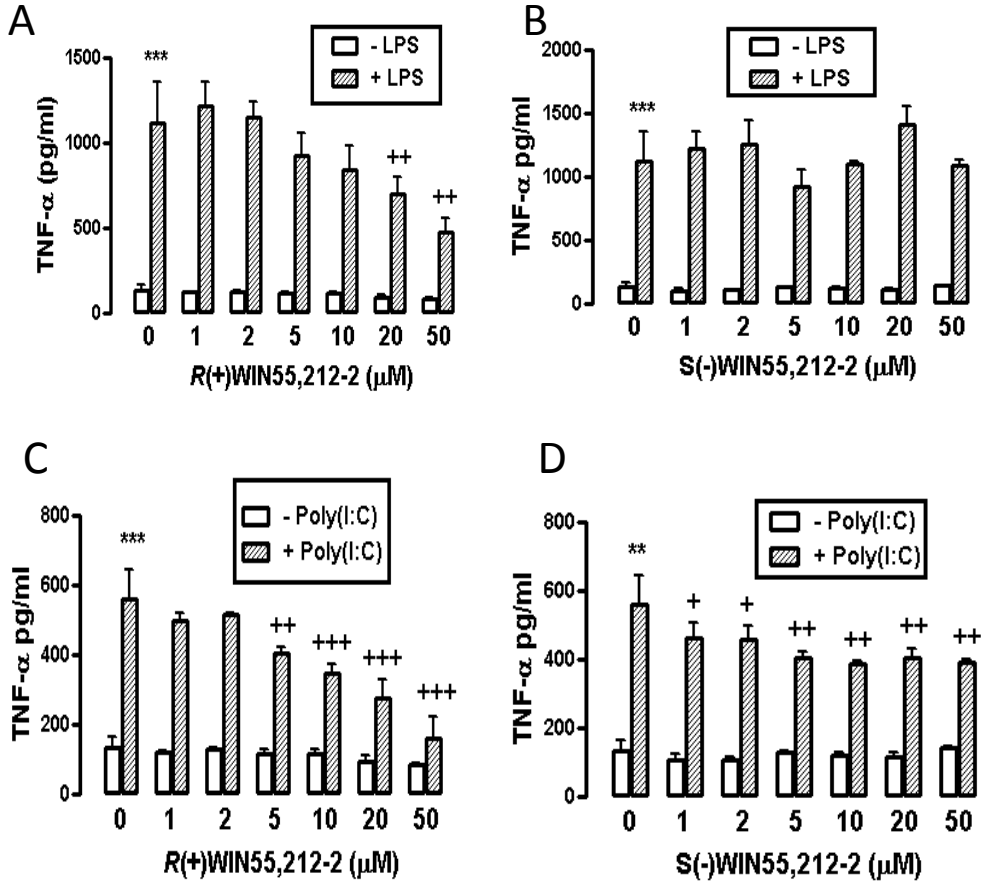
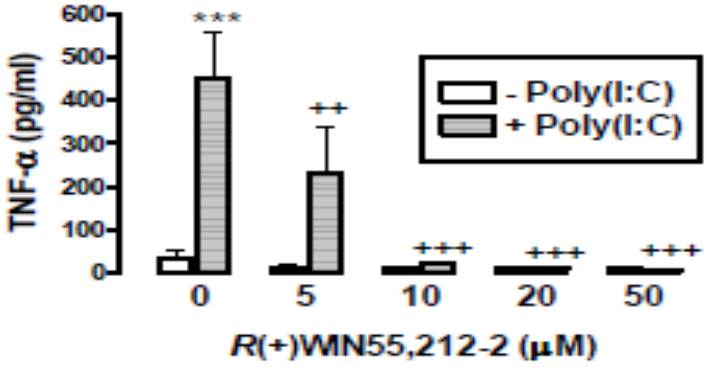


Figure 3.4 R(+)WIN55,212-2 inhibits LPS and Poly(I:C) induction of TNF-α expression in primary mouse astrocytes.

Primary astrocyte cultures prepared from neonatal murine brains were pre-treated with or without various concentrations of R(+)WIN55,212-2 (A+C) or S(-)WIN55,212-2 (B+D) for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 24 h. Supernatants were assayed for TNF-α levels by sandwich ELISA. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with vehicle-treated cells. $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (19, 40) = 57.76. $P < 0.001$ (B) F value (19, 40) = 8.564. $P < 0.001$ (C) F value (19, 40) = 15.62. $P < 0.001$ (D) F value (19, 40) = 24.05. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

A



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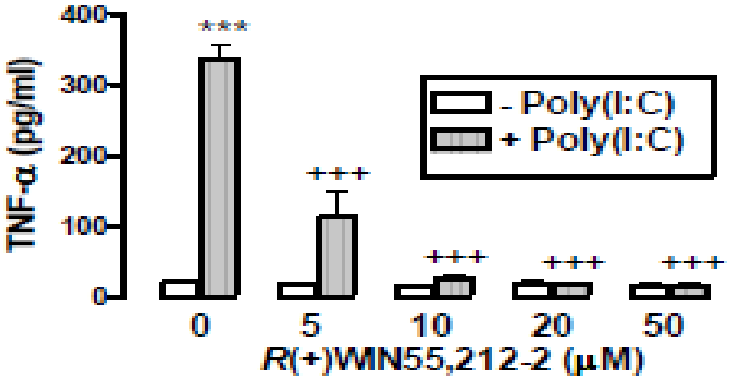


Figure 3.5 R(+)WIN55,212-2 inhibits Poly(I:C)-induced activation of TNF-α expression in PBMCs.

PBMCs isolated from healthy subjects (A) and M.S. patients (B) were pre-treated with or without various concentrations of R(+)WIN55,212-2 for 1 h prior to stimulation in the presence or absence of Poly(I:C) (25μg/ml) for a further 3 h.

Supernatants were assayed for TNF-α levels by sandwich ELISA. ***P < 0.001 compared with vehicle-treated cells. +++P < 0.001 compared with Poly(I:C)-treated cells. Results are mean +/- S.E.M. of three independent experiments.

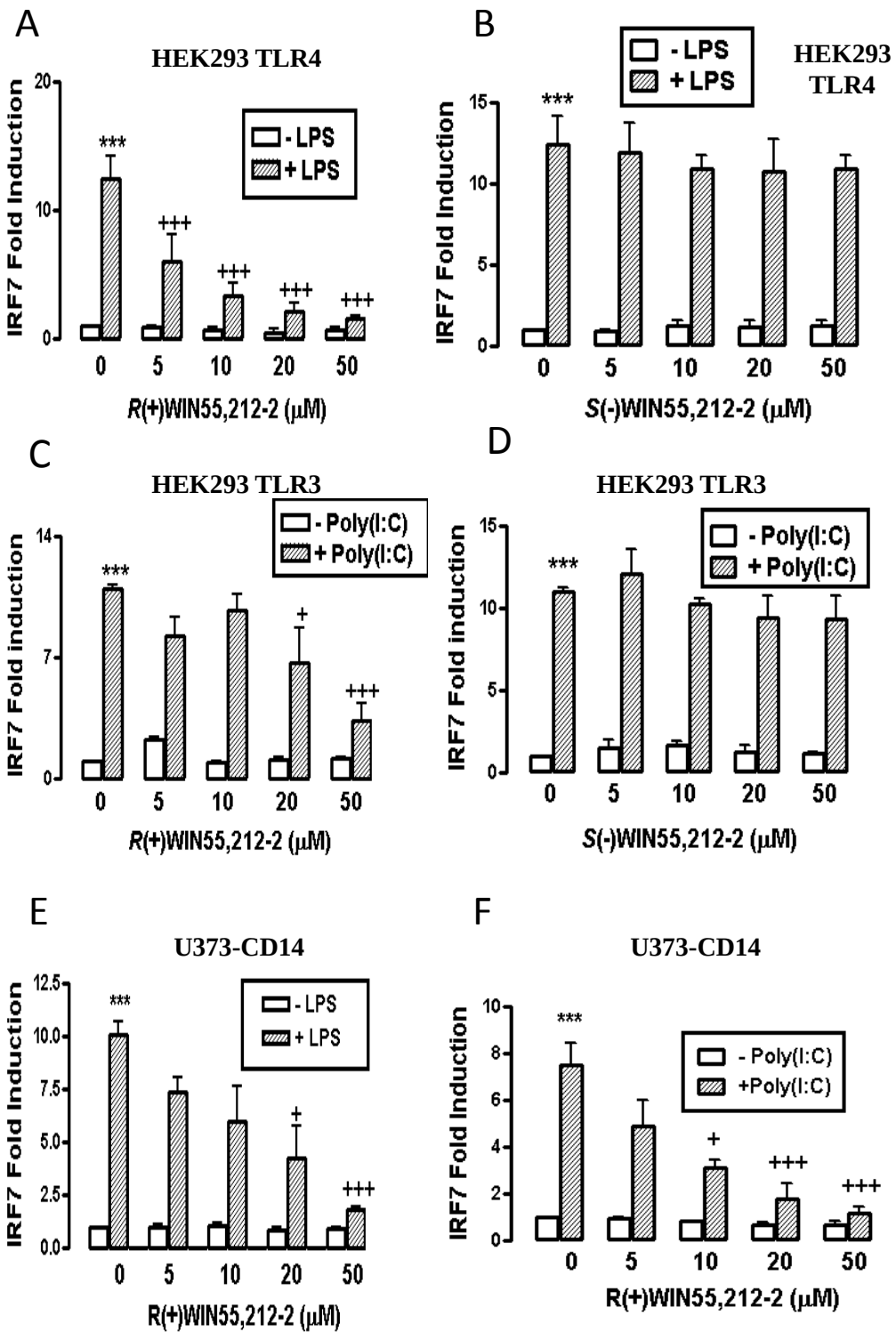

A
HEK293 TLR4
IRF7 Fold Induction
- LPS
+ LPS
***
+++
0
5
10
20
50
R(+)WIN55,212-2 (μM)
B
HEK293 TLR4
- LPS
+ LPS
IRF7 Fold Induction
***
S(-)WIN55,212-2 (μM)
C
HEK293 TLR3
IRF7 Fold induction
- Poly(I:C)
+ Poly(I:C)
***
+
+++
R(+)WIN55,212-2 (μM)
D
HEK293 TLR3
IRF7 Fold induction
- Poly(I:C)
+ Poly(I:C)
***
S(-)WIN55,212-2 (μM)
E
U373-CD14
IRF7 Fold Induction
- LPS
+ LPS
***
+
+++
R(+)WIN55,212-2 (μM)
F
U373-CD14
IRF7 Fold Induction
- Poly(I:C)
+Poly(I:C)
***
+
+++
R(+)WIN55,212-2 (μM)

Figure 3.6 R(+)WIN55,212-2 inhibits LPS and Poly(I:C)-induced activation of IRF7 in HEK293 TLR3/TLR4 and U373-CD14 astrocytoma cells .

HEK293 cells stably transfected with TLR4 (A+B) or TLR3 (C+D) and U373-CD14 astrocytoma cells (E+F) were co-transfected with the trans-activator plasmid pFA-IRF-7 (25ng), pFR-Luc (60ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (145ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of R(+)WIN55,212-2 or S(-)WIN55,212-2 for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***P < 0.001 compared with vehicle-treated cells. +P < 0.05 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (9, 20) = 24.10. P < 0.001 (B) F value (9, 20) = 29.04. P < 0.001 (C) F value (9, 20) = 21.55. P < 0.001 (D) F value (9, 20) = 34.68. P < 0.001 (E) F value (13, 28) = 23.10. P < 0.001 (F) F value (13, 28) = 23.12. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

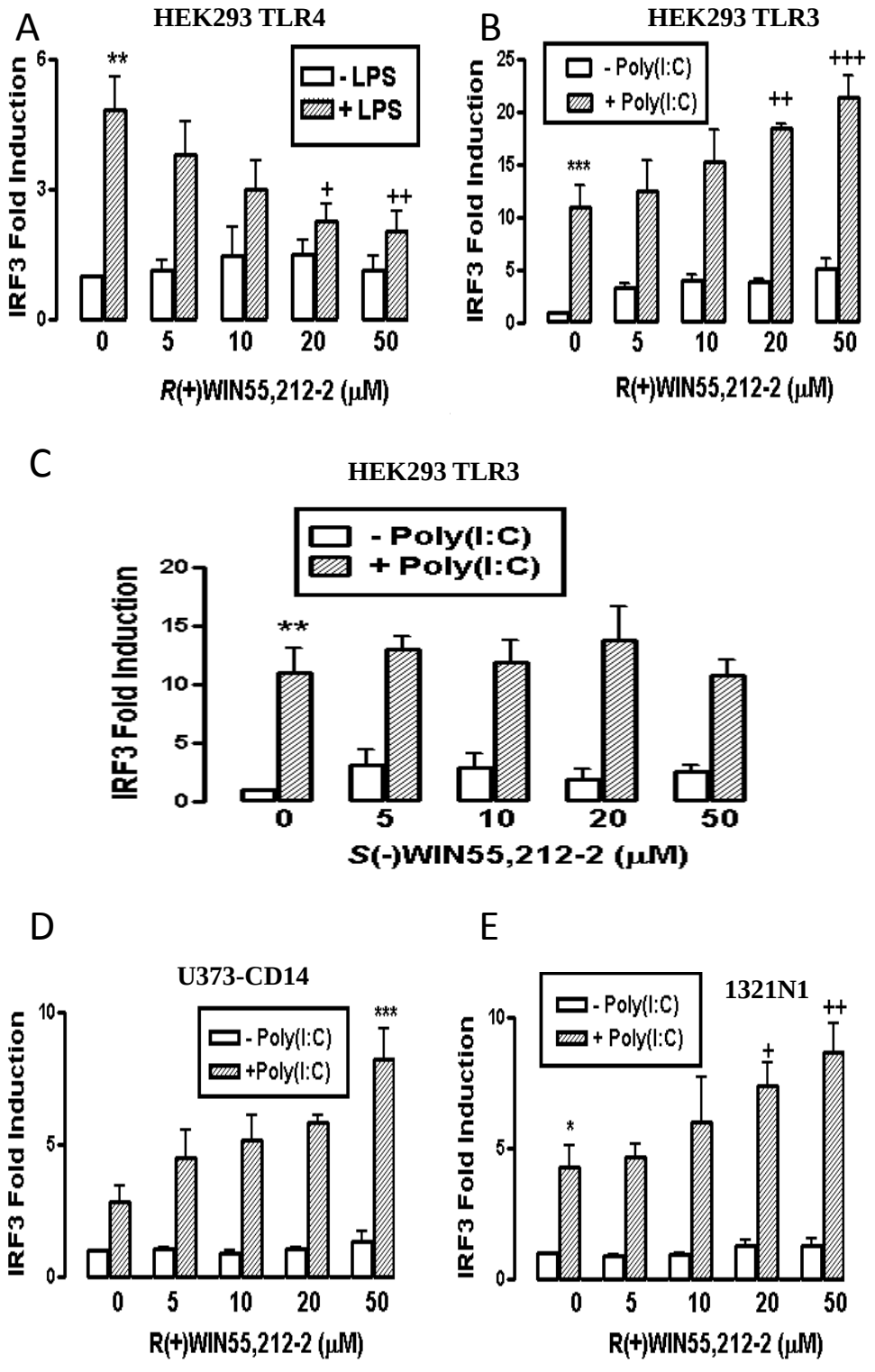

A
HEK293 TLR4
IRF3 Fold Induction
- LPS
+ LPS
**
+
++
0
5
10
20
50
R(+)WIN55,212-2 (μM)
B
HEK293 TLR3
IRF3 Fold Induction
- Poly(I:C)
+ Poly(I:C)
***
++
+++
0
5
10
20
50
R(+)WIN55,212-2 (μM)
C
HEK293 TLR3
IRF3 Fold Induction
- Poly(I:C)
+ Poly(I:C)
**
0
5
10
20
50
S(-)WIN55,212-2 (μM)
D
U373-CD14
IRF3 Fold Induction
- Poly(I:C)
+Poly(I:C)
***
0
5
10
20
50
R(+)WIN55,212-2 (μM)
E
1321N1
IRF3 Fold Induction
- Poly(I:C)
+ Poly(I:C)
*
+
++
0
5
10
20
50
R(+)WIN55,212-2 (μM)

Figure 3.7 R(+)WIN55,212-2 differentially regulates LPS and Poly(I:C)-induced activation of IRF3 in HEK293 TLR3/TLR4 and astrocytoma cells .

HEK293 cells stably transfected with TLR4 (A) or TLR3 (B+C), U373-CD14 astrocytoma cells (D) and 1321N1 astrocytoma cells (E) were were co-transfected with the trans-activator plasmid pFA-IRF-3 (30ng) pFR-Luc (60ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (140ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of R(+)WIN55,212-2 or S(-)WIN55,212-2 for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$ compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (9, 20) = 6.124. $P < 0.001$ (B) F value (13, 28) = 27.17. $P < 0.001$ (C) F value (9, 20) = 11.94. $P < 0.001$ (D) F value (13, 28) = 35.65. $P < 0.001$ (E) F value (13, 28) = 45.78. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

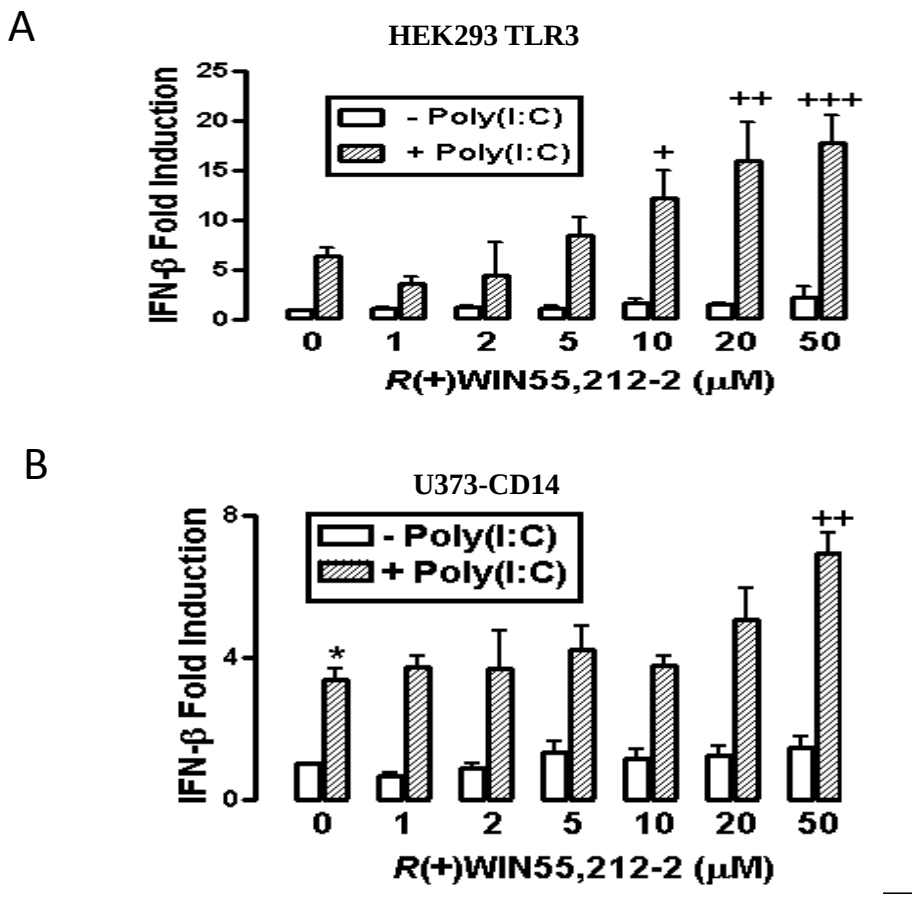


Figure 3.8 R(+)WIN55,212-2 augments Poly(I:C)-induced activation of the IFN-β promoter in HEK293 TLR3 and U373-CD14 astrocytoma cells .

HEK293 cells stably transfected with TLR3 (A) and U373-CD14 astrocytoma cells (B) were co-transfected with IFN-β luciferase reporter plasmid (80ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of R(+)WIN55,212-2 for 1 h prior to stimulation in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. *P < 0.05 compared with vehicle-treated cells. +P < 0.05, ++P < 0.01 and +++P < 0.001 compared with Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (19, 40) = 13.47. P < 0.001 (B) F value (13, 28) = 3.949. P < 0.01. Results are mean +/- S.E.M. of three independent experiments.

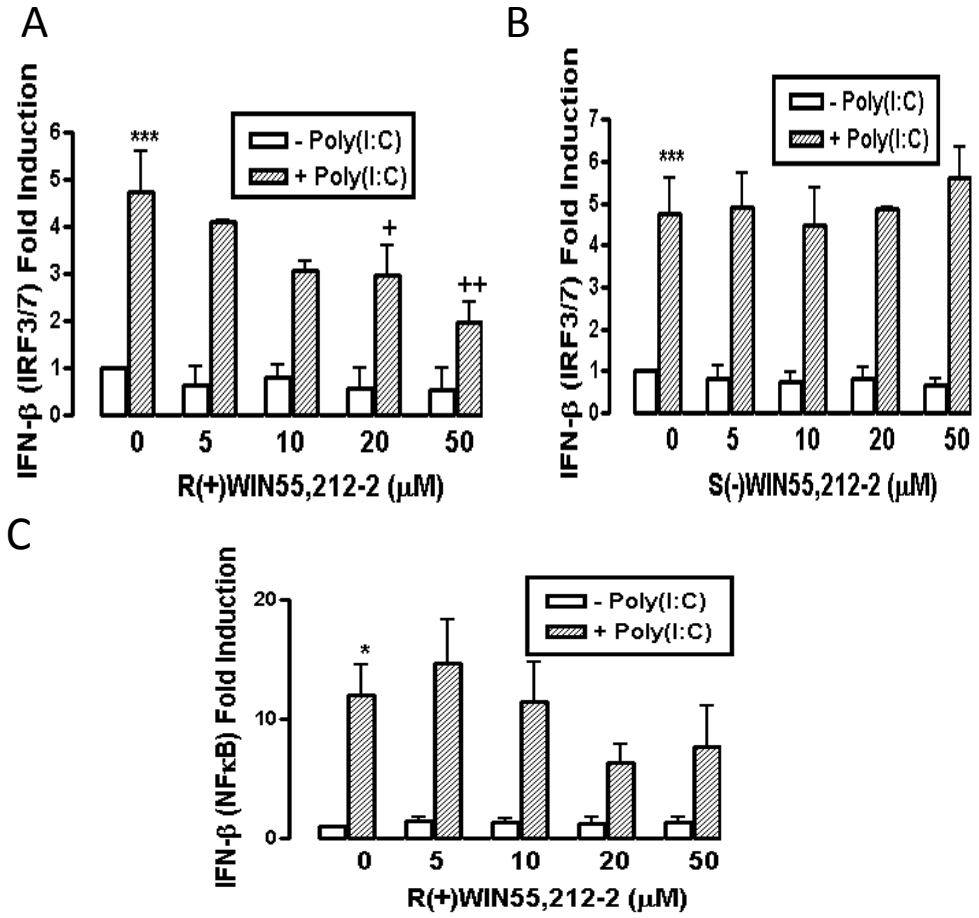


Figure 3.9 R(+)WIN55,212-2 inhibits Poly(I:C)-induced activation of PRDI/III and PRDII on the IFN-β promoter in HEK293 TLR3 cells .

HEK293 cells stably transfected with TLR3 were co-transfected with the luciferase reporter constructs, PRD-I/III-Luc (80ng) (A+B) or PRDII (80ng) (C), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of R(+)WIN55,212-2 or S(+)WIN55,212-2 for 1 h prior to stimulation in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. *$P < 0.05$ and ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$ and ++$P < 0.01$ compared with Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (13, 28) = 20.42. $P < 0.001$ (B) F value (13, 28) = 9.101. $P < 0.001$ (C) F value (13, 28) = 19.05. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

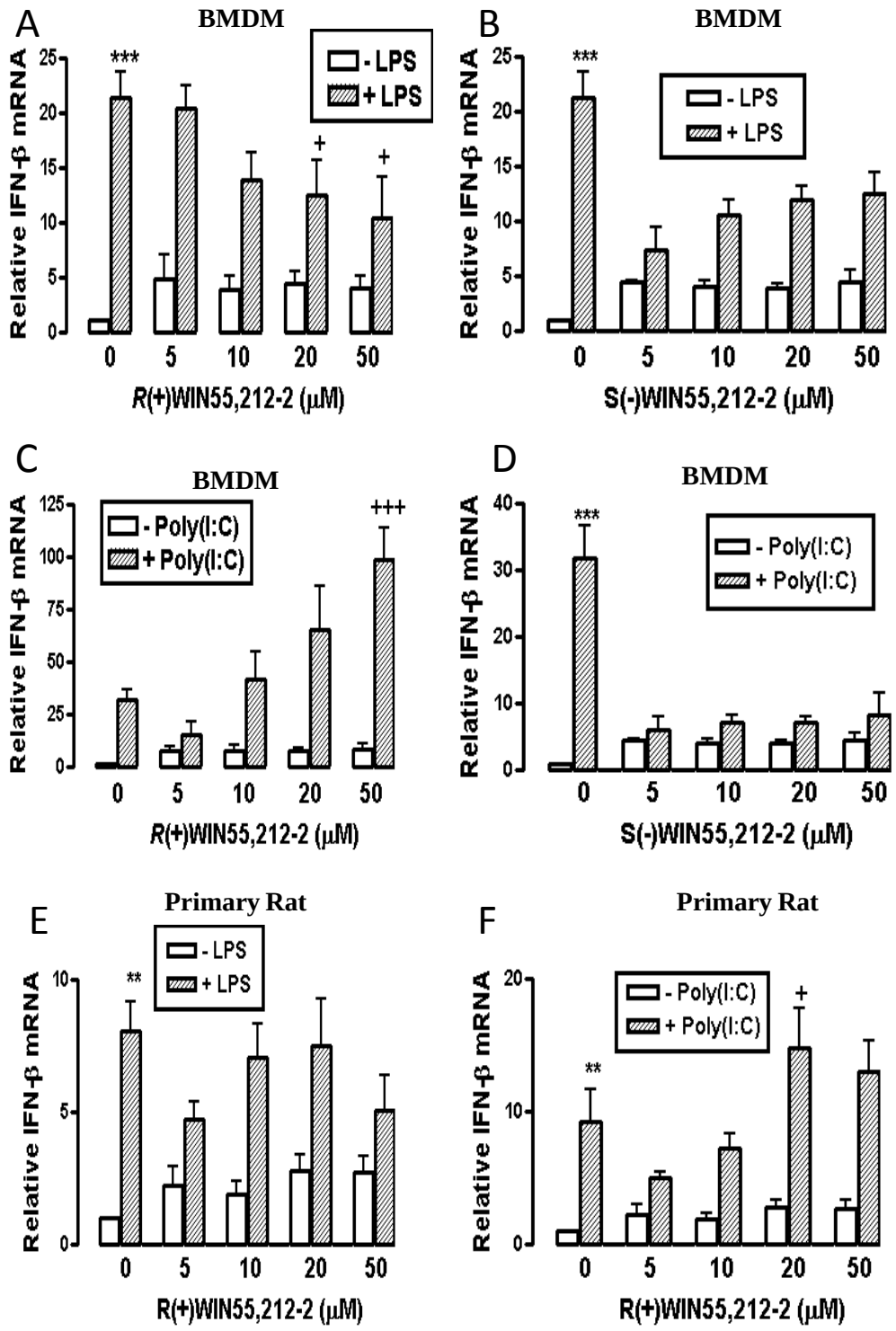

A
BMDM
- LPS
+ LPS
Relative IFN-β mRNA
***
+
+
0
5
10
20
50
R(+)WIN55,212-2 (μM)
B
BMDM
- LPS
+ LPS
Relative IFN-β mRNA
***
0
5
10
20
50
S(-)WIN55,212-2 (μM)
C
BMDM
- Poly(I:C)
+ Poly(I:C)
Relative IFN-β mRNA
+++
0
5
10
20
50
R(+)WIN55,212-2 (μM)
D
BMDM
- Poly(I:C)
+ Poly(I:C)
Relative IFN-β mRNA
***
0
5
10
20
50
S(-)WIN55,212-2 (μM)
E
Primary Rat
- LPS
+ LPS
Relative IFN-β mRNA
**
0
5
10
20
50
R(+)WIN55,212-2 (μM)
F
Primary Rat
- Poly(I:C)
+ Poly(I:C)
Relative IFN-β mRNA
**
+
0
5
10
20
50
R(+)WIN55,212-2 (μM)

Figure 3.10 R(+)WIN55,212-2 differentially regulates LPS and Poly(I:C)-induced activation of IFN-β mRNA in BMDM cells and primary rat astrocytes .

BMDM cells (A-D) and primary astrocyte cultures prepared from neonatal rat brains (E+F) were pre-treated with or without various concentrations of R(+)WIN55,212-2 or S(-)WIN55,212-2 for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 16 h.

RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. **P < 0.01 and ***P < 0.001 compared with vehicle-treated cells. +P < 0.05 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (9, 20) = 10.59. P < 0.001 (B) F value (13, 24) = 22.24. P < 0.001 (C) F value (9, 20) = 11.16. P < 0.001 (D) F value (13, 24) = 18.22. P < 0.001 (E) F value (13, 28) = 9.488. P < 0.001 (F) F value (13, 28) = 14.27. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

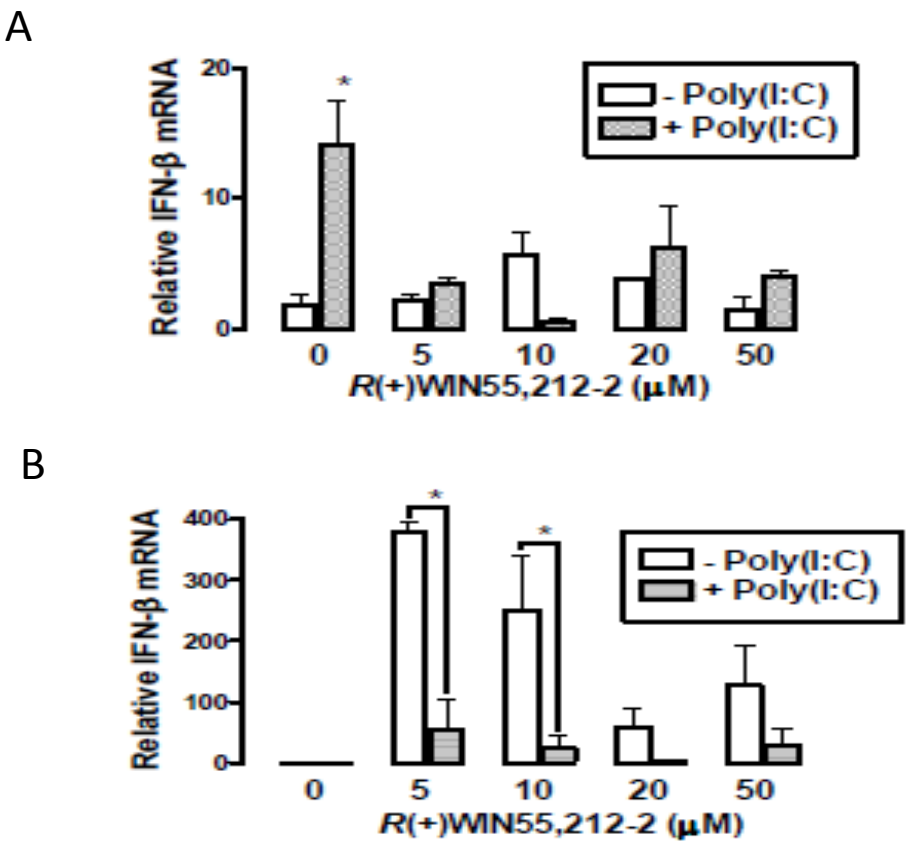


Figure 3.11 R(+)WIN55,212-2 inhibits Poly(I:C)-induced activation of IFN-β mRNA in PBMCs.

PBMCs isolated from healthy subjects (A) and M.S. patients (B) were pre-treated with or without various concentrations of R(+)WIN55,212-2 for 1 h prior to stimulation in the presence or absence of Poly(I:C) (25μg/ml) for a further 3 h.

RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. *P < 0.05 compared with vehicle-treated cells. Results are mean +/- S.E.M. of three independent experiments.

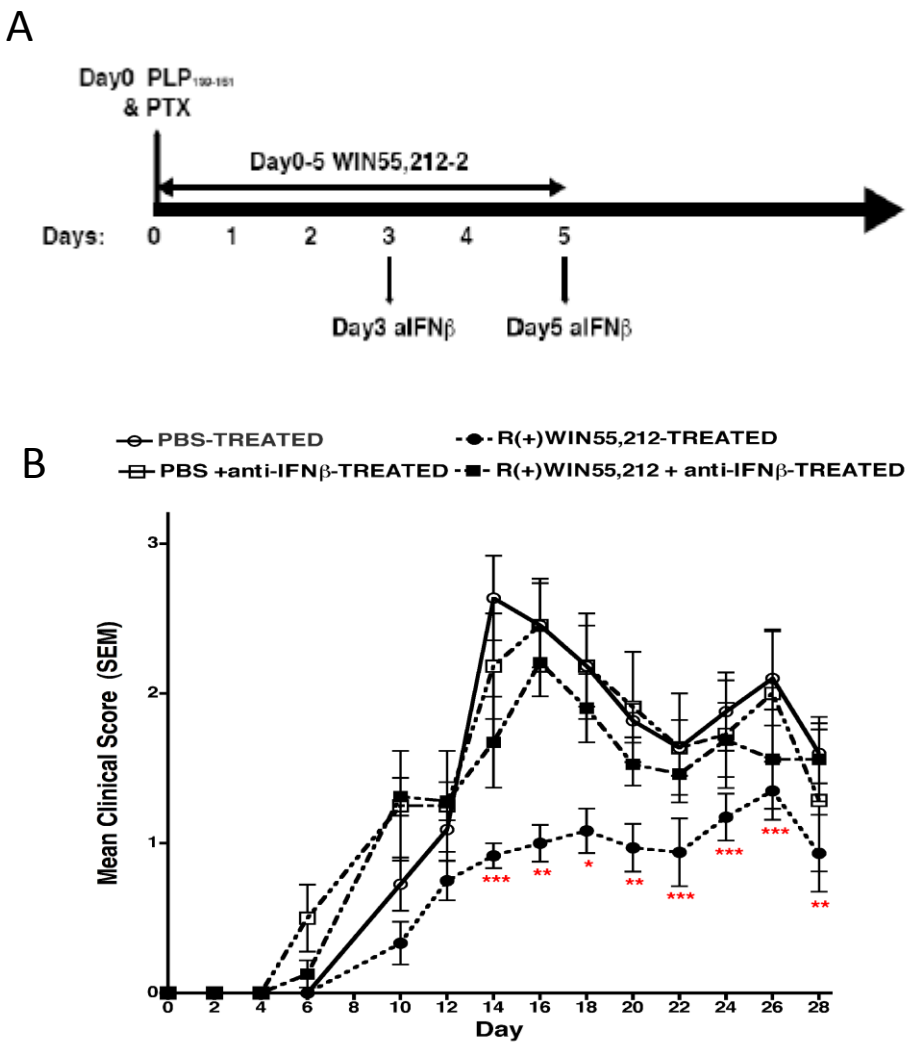
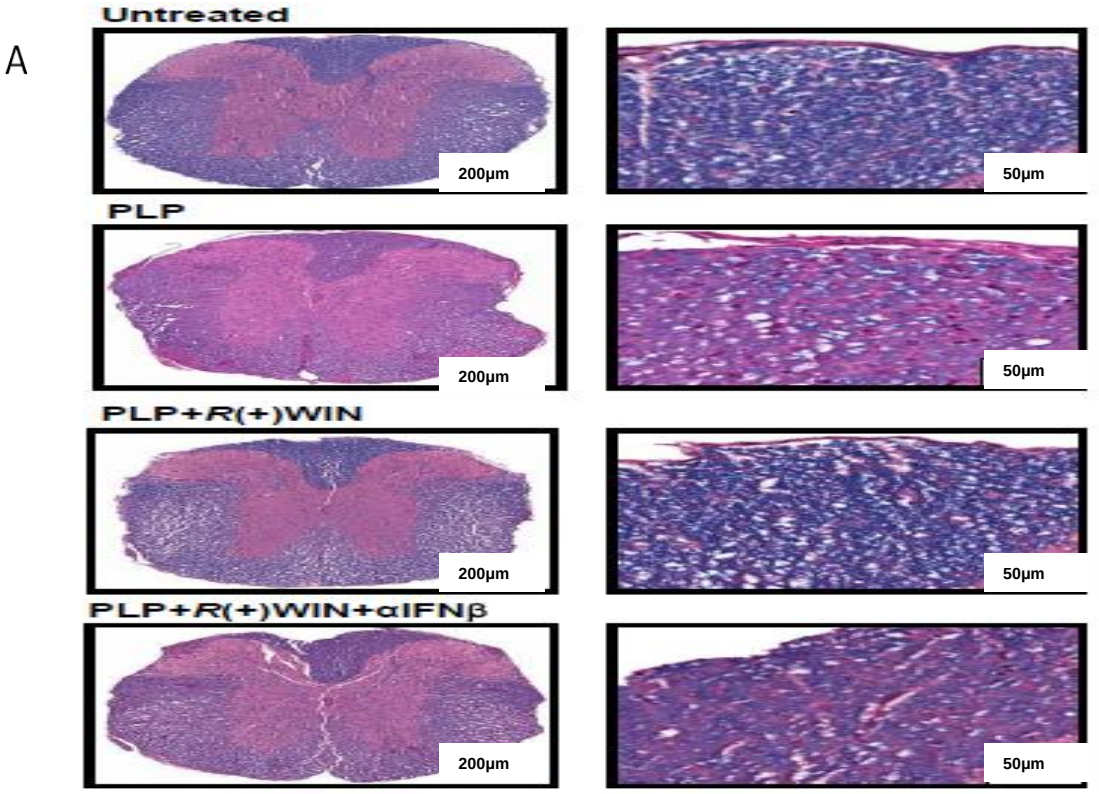
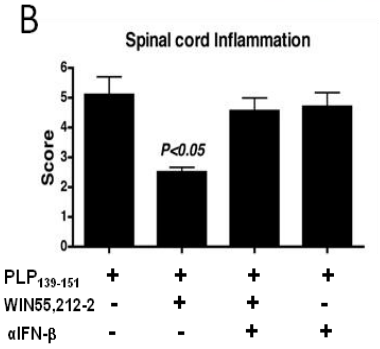
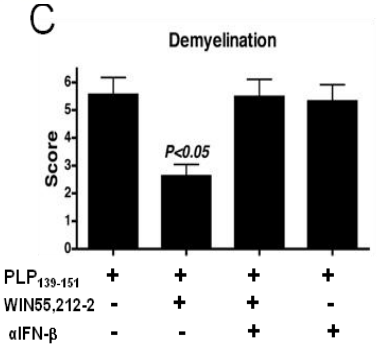


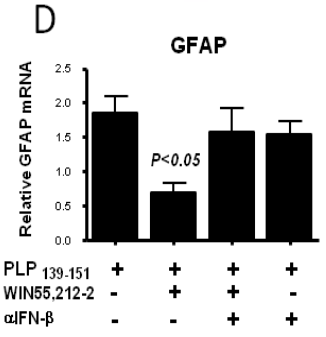
Figure 3.12 R(+)WIN55,212-2 ameliorates the clinical symptoms of EAE in an IFN-β-dependent manner.

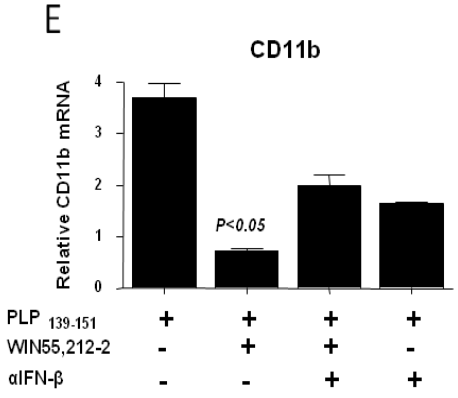
Protocol for induction of EAE by $PLP_{139-151}$ (PLP) immunisation (A). Mean clinical Score (B). EAE was induced in female SJL/J mice (8 weeks old). R(+)WIN55,212-2 was administered (20 mg/Kg) intraperitoneally on days 0, 1, 2, 3, 4 and 5. Rabbit anti-mouse IFN-β polyclonal antibody was administered intraperitoneally (2 x 103 Neutralizing Units) on days 3 and 5 after PLP immunisation. Control mice received Cremophor:PBS (20:80) as vehicle.


A
Untreated
200μm
50μm
PLP
200μm
50μm
PLP+R(+)WIN
200μm
50μm
PLP+R(+)WIN+αIFNβ
200μm
50μm


B
Spinal cord Inflammation
Score
P<0.05
PLP139-151 + + + +
WIN55,212-2 - + + -
αIFN-β - - + +


C
Demyelination
Score
P<0.05
PLP139-151 + + + +
WIN55,212-2 - + + -
αIFN-β - - + +


D
GFAP
Relative GFAP mRNA
P<0.05
PLP 139-151 + + + +
WIN55,212-2 - + + -
αIFN-β - - + +


E
CD11b
Relative CD11b mRNA
P<0.05
PLP 139-151 + + + +
WIN55,212-2 - + + -
αIFN-β - - + +

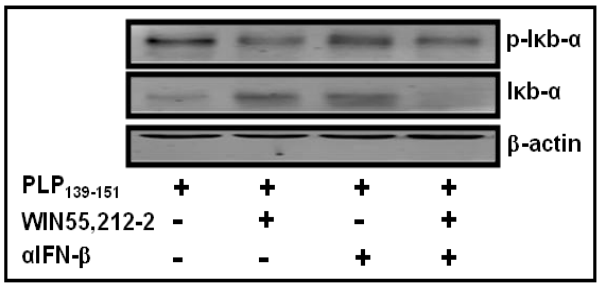

F
p-Ikb-α
Ikb-α
β-actin
PLP139-151 + + + +
WIN55,212-2 - + - +
αIFN-β - - + +

Figure 3.13 Characterising the effects of R(+)WIN55,212-2 treatment on the development of EAE.

Representative images of Luxol fast blue-stained spinal cord sections from untreated mice, PLP-treated, PLP+WIN-treated and PLP+WIN+antiIFN-β (αIFN-β)-treated mice illustrating the extent of demyelination and lymphocytic inflammation (A). The posterior funiculi of the spinal cord were observed under high power (right panels). Images are representative of data from 4 to 8 animals per treatment group. Scale bars are 200 and 50 m.

Quantification of (B) spinal cord inflammation and (C) extent of demyelination in treated groups. Relative (D) GFAP mRNA and (E) CD11b mRNA detection in spinal cord from vehicle treated, PLP-treated, PLP+WIN-treated and PLP+WIN+αIFN-β treated mice. Phosphorylated Iκb-α, total Iκb-α and β-actin protein expression in spinal cord cytosolic fractions from vehicle-treated, PLP-treated, PLP+WIN-treated and PLP+WIN+αIFN-β-treated mice (F). *P < 0.05, **P < 0.01 and ***P < 0.001 for differences between WIN-treated mice and other groups. All results are mean +/- S.E.M..

Summary of Results

Luciferase Assay (NFκB)	
HEK293 TLR3 and HEK293 TLR4	R(+)WIN55,212-2 **inhibits** LPS and Poly(I:C)-induced activation of NFκB
U373-CD14 and 1321N1	R(+)WIN55,212-2 **inhibits** Poly(I:C)-induced activation of NFκB
Luciferase Assay (IRF7)	
HEK293 TLR3 , HEK293 TLR4 and U373-CD14	R(+)WIN55,212-2 **inhibits** LPS and Poly(I:C)-induced activation of IRF7
Luciferase Assay (IRF3 & IFN-β promoter)	
HEK293 TLR3 , HEK293 TLR4 and U373-CD14	R(+)WIN55,212-2 **inhibits** LPS-induced activation of IRF3 R(+)WIN55,212-2 **augments** Poly(I:C)-induced activation of IRF3 & IFN-β promoter

Summary of Results

ELISA (TNF-α)	
Primary Mouse Astrocytes, U373-CD14 and PBMC's	R(+)WIN55,212-2 **inhibits** LPS and Poly(I:C) induction of TNF-α expression
QRT-PCR (IFN-β mRNA)	
Primary Mouse Astrocytes and BMDM	R(+)WIN55,212-2 **inhibits** LPS-induced activation of IFN-β mRNA R(+)WIN55,212-2 **augments** Poly(I:C)-induced activation of IFN-β mRNA
QRT-PCR (IFN-β mRNA)	
PBMC's	R(+)WIN55,212-2 **inhibits** Poly(I:C)-induced activation of IFN-β mRNA

Chapter 4

Pharmacological characterisation of the regulatory effects of WIN55,212-2 on TLR3 and TLR4 signalling

4.1 Introduction

Cannabinoids (CBs) are defined as compounds that are either structurally similar to δ-9-THC, possess cannabimimetic properties and/or bind to specific CB receptors. For a long time the regulatory effects of cannabinoids were attributed to their nonspecific intercalation into the lipid bilayer of the cell membrane. However, to date, five CB receptor subtypes have been identified; two cloned receptors (CB1 and CB2) (Munro et al., 1993, Matsuda et al., 1990), the orphan receptor (GPR55) (Baker et al., 2006) and two as yet molecularly uncharacterised receptors (Hajos et al., 2001, Jarai et al., 1999).

The CB1, CB2 and GPR55 receptors belong to the family of the 'seven trans-membrane spanning receptors', namely the G-protein coupled receptors (GPCR) (Maccarrone, 2009). The CB1 and CB2 receptors are single polypeptide receptors. Both possess an extracellular N-terminal domain, an intracellular C-terminal domain and seven transmembrane helices. Indeed, the first evidence of a specific CB receptor came from the CB inhibition of cyclic adenosine 3', 5'-monophosphate (cAMP) accumulation (Howlett et al., 1986). This strongly indicated a GPCR-mediated mechanism, as GPCR activation leads to the negative regulation of adenylyl cyclase (AC) and subsequent inhibition of cAMP.

The CB1 and CB2 receptors exhibit differential tissue expression patterns. The CB1 receptor is widely distributed in several brain regions (Matsuda et al., 1990) with highest density in the cortex, hippocampus, basal ganglia and cerebellum, and mediates most of the neurobehavioral effects associated with cannabinoids. CB1 receptor expression is sparse in areas of the brain stem, medulla, and thalamus (Pertwee, 1997) and may contribute to the general lack of life threatening effects associated with cannabis use. Over the last few years, the CB1 receptor has been localised in peripheral tissues including testis, guinea pig small intestine, the mouse urinary bladder and vas deferens (Pertwee, 1997). In EAE models, mice lacking the CB1 receptor develop more severe neuronal damage, thus supporting a role for the CB receptors in regulating inflammatory mediated neurodegeneration (Pryce et al., 2003). Furthermore, CB1 receptor expression by neurons is required for CB-mediated EAE suppression, while CB2 receptor knockdown exacerbates the inflammatory potential of autoreactive T cells in this model (Maresz et al., 2007). Microglia have also been reported to constitutively express low levels of the CB1 receptor, but to upregulate the CB2 receptor upon

activation (Cabral and Marciano-Cabral, 2005). The CB2 receptor is predominantly expressed in immune tissues such as the spleen and immunocompetent cells (Munro et al., 1993), with highest concentration in B cells, NK cells, mast cells and monocytes. This strategic location of the CB2 receptor in immunocompetent cells encourages the theory of cannabinoids exerting anti-inflammatory effects. However, CB1 receptor mRNA and protein expression have also been found in B cells and certain subsets of T cells (Galiègue et al., 1995). Therefore, the overall expression of CB receptors enables them to function as critical mediators in the regulation of the neuroimmune system.

CB receptors have been cloned from numerous species including rat, mouse and human (Howlett et al., 2002). The CB1 receptor exhibits approximately 98% amino acid sequence identity across species while the mouse CB2 receptor has an 82% sequence identity to the human CB2 receptor. The lack of genetic variance across species suggests that the CB receptors may be evolutionarily conserved. Distinction between the CB receptors is based on differences in their predicted amino acid sequence, signalling mechanisms and tissue distribution. The CB1 and CB2 receptors exhibit approximately 48% amino acid sequence identity (Shire et al., 1996). Both CB receptors primarily signal through the G proteins; G inhibitory (Gi) and G protein (Go). CB1 receptor activation of Gi leads to the negative regulation of AC and subsequent inhibition of cAMP, the activation of mitogen-activated protein kinase and the inhibition of certain voltage-gated calcium channels (Howlett, 2002). Stimulation of CB2 receptors has similar consequences (Bouaboula et al., 1996).

At submicromolar concentrations, the phytocannbinoid; δ-9-THC, binds to both the CB1 and CB2 receptors. At CB1 receptors, it behaves as a partial agonist and exhibits higher efficacy at the CB1 receptor relative to the CB2 receptor (Bayewitch et al., 1995). The synthetic aminoalkylindole; R(+)WIN55,212-2, displays high affinity and relative intrinsic activity at both the CB1 and CB2 receptors with a K_i range of 1.89-123 nM and 0.28-39.3 nM, respectively (Howlett et al., 2002). However, studies have demonstrated moderate selectivity of R(+)WIN55,212-2 in favour of the CB2 receptor (Bouaboula et al., 1997). R(+)WIN55,212-2 mimics δ-9-THC by producing the full spectrum of pharmacological effects of δ-9-THC *in vivo* (Martin et al., 1991). However, the inactive enantiomer S(-)WIN55,212-2, lacks activity both *in vivo* and *in vitro*.

The discovery of the CB receptors naturally led to the search for the endocannabinoid ligands e.g. AEA. AEA was the first endogenous ligand of the CB

receptors to be discovered in porcine brain (Devane et al., 1992). Subsequently, a further four arachidonic acid based natural ligands; 2-Arachidonoylglycerol (Mechoulam et al., 1995), 2-Arachidonylglyceryl ether (Hanuš et al., 2001), Virodhamine (Porter et al., 2002), and *N*-arachidonoyl-dopamine (NADA) (Huang et al., 2002) have been postulated to act as endocannabinoid ligands. Signal transduction studies and ligand binding studies have suggested that AEA can act at both the CB1 and CB2 receptors, although it may be more efficacious at the CB1 receptor (Felder et al., 1995).

Studies on the regulatory effects of AEA and R(+)WIN55,212-2 on the two molecularly uncharacterised receptors have been reported. R(+)WIN55,212-2 inhibits glutamate release in hippocampal pyramidal cells via a novel CB receptor (Hájos et al., 2001). Additionally, there have been indications that the vascular endothelium contains a novel CB receptor and AEA causes endothelium-dependent vasorelaxation in arteries from CB1/CB2 receptor knockout animals (Jarai et al., 1999).

Similar to R(+)WIN55,212-2, AEA may also have potential in regulating the progression of M.S. Increased levels of AEA have been reported in tissues from inflammatory lesions of M.S. patients (Eljaschewitsch et al., 2006). AEA ameliorates spasticity in CREAE models (Baker et. al. 2001) and inhibits TNF-α production in astrocytes in TMEV infected mice (Molina-Holgado et al., 1997). However, it has also been demonstrated that the expression of the enzyme involved in AEA degradation, namely FAAH, was upregulated in M.S. lesions, which indicates that the level of this endocannabinoid might be reduced in M.S. patients (Benito et al., 2007).

The discovery of the CB receptors also led to the development of selective CB1 and CB2 receptor antagonists, SR141716A and SR144528, respectively. These compounds, termed diarylpyrazoles, readily prevent or reverse the effects mediated by the CB1 and CB2 receptors (Rinaldi-Carmona et al., 1994, Rinaldi-Carmona et al., 1996). Although both antagonists are selective for their respective receptor, they are not CB-specific. As an example, SR141716A at concentrations of 10μM may block potassium channels and L-type voltage-operated calcium channels (White and Hiley, 1998), thereby acting as an inverse agonist. Interestingly, SR141716A is currently marketed in Europe under the name Acomplia^{TM} as a promising alternative therapy against cardiovascular and metabolic disease (André and Nicolas, 2009). However use of this treatment can potentially contribute to disease pathogenesis in M.S. (van Oosten

et al., 2004) and therefore highlights the neuroprotective and immunosuppressive effects of CB agonism.

Additional established CB1-selective antagonists include LY320135, AM251 and AM281. LY320135, developed by Eli Lilly, behaves as an inverse agonist where its CB1 affinity is less than that of SR141716A (Felder et al., 1998). AM251 and AM281 are both structural analogues of SR141716A and bind more readily to the CB1 receptor than the CB2 receptor (Lan et al., 1999a, Lan et al., 1999). Studies utilising the CB receptor antagonists have demonstrated that AEA inhibits LPS induced NFκB and IL-8 activation in human gingival fibroblasts, an effect that was antagonised by AM251 and SR144528 (Nakajima et al., 2006). However, others have reported on AEA inhibition of NFκB in adenocarcinoma cells in a CB receptor-independent manner (Sancho et al., 2003). Likewise, the inhibitory effects of R(+)WIN55,212-2 on NFκB activation (Curran et al., 2005) and adhesion molecule expression (Mestre et al., 2009, Curran et al., 2005) have also been demonstrated to be independent of the CB1 and CB2 receptors.

Various studies suggest that cannabinoids can also target the peroxisome proliferator activated receptors (PPAR) specifically the isoforms; PPARα and PPARγ (O'Sullivan and Kendall, 2009, Sun et al., 2007, Mestre et al., 2009). Indeed, the first evidence of CB interaction with PPARs was demonstrated in 2002, where lipoxygenase metabolism of 2-Arachidonoylglycerol produced a metabolite that increased the transcriptional activity of PPARα (Kozak et al., 2002).

PPARs belong to a family of nuclear receptors comprising three isoforms: α, β and γ. PPARα was the first isoform to be identified (Issemann and Green, 1990) and was named mostly due its ability to increase hepatic peroxisome volume and density, or induce peroxisome proliferation in rodent liver (Lee et al., 1995). PPARs contain an N-terminal domain with a ligand-independent transcription activation function 1 (AF-1) domain, a DNA binding domain with two zinc fingers, a hinge region, and a C-terminal ligand binding domain (LBD) followed by a ligand-dependent transcription AF-2 domain (Zoete et al., 2007). While the AF-2 domain interacts directly with several transcriptional co-activator complexes including steroid receptor co-activator 1 (SRC1) and CBP/p300, the AF-1 domain is responsible for enhancing the interaction between AF-2 and co-activator complexes (Kilroy et al., 2009, Bugge et al., 2009).

PPARs are ligand activated transcription factors. In resting cells, PPARs are mainly localised in the nucleus and bound to various transcription co-repressors

including nuclear receptor co-repressor/silencing mediator for retinoid and thyroid hormone receptors (NCoR/SMRT) (Dowell et al., 1999). NCoR/SMRT represses gene transcription through association with histone deacetylases. However, upon PPAR ligand activation via its LBD, a conformational change occurs where PPARs heterodimerise with the retinoid X receptor (RXR) (Daynes and Jones, 2002). Agonist induced activation of PPARs promotes co-repressor dissociation, co-activator association within the LBD and when complexed with RXR, PPARs bind to specific DNA response elements termed peroxisome proliferator response element (PPRE). PPRE have been found in the promoter regions of most PPAR regulated genes that are primarily involved in the regulation of metabolism and energy homeostasis, cell differentiation and inflammation (Sanderson and Kersten, 2010, Bishop-Bailey and Bystrom, 2009). Each PPAR isoform is encoded by separate genes and differentially expressed in various tissues including liver, heart, muscle and adipose tissue (Peters et al., 2005). All three PPAR isoforms are also expressed in the brain and peripheral nervous system (Moreno et al., 2004; Cimini et al., 2005).

Ligands of PPARα include members of the fibrate family such as fenofibrate that is used clinically in the treatment of cardiovascular disease while PPARγ ligands include the thiazolidinediones (TZDs) which is used to treat type-2 diabetes (Baranova, 2008).

In relation to the TLR4 signalling pathway, fenofibrate has been demonstrated to inhibit the expression of various members of the IL-12 family of cytokines including IL-12, IL-23, and IL-27 in LPS stimulated microglia (Xu et al., 2007). Xu and colleagues also confirmed the inhibitory effects of fenofibrate on mRNAs encoding TLR4, CD14 and MyD88, which all contribute to the activation of NFκB. A recent study also provides evidence for the ability of fenofibrate to downregulate the nuclear translocation of p65 and to significantly inhibit the DNA binding of NFκB in a dose-dependent manner (Zak et al., 2010), an effect that is distinct from R(+)WIN55,212-2 mechanism on NFκB (Curran et al., 2005). However, comparable to the synthetic cannabinoid (Arevalo-Martin et al., 2003), fenofibrate has also been reported to ameliorate the clinical signs of EAE by inhibiting $CD4^+$ T cells (Lovett-Racke et al., 2004).

The PPARγ ligand; 15d-PGJ2, a metabolite from the prostaglandin synthesis pathway (Forman et al., 1995), inhibits phorbol ester-induced nitric oxide, TNF-α, IL-1 and IL-6 production by antagonising the transcriptional activity of AP-1 and NFκB in

cells of the monocyte/macrophage lineage (Jiang et al., 1998). Similar to R(+)WIN55,212-2 and fenofibrate, 15d-PGJ2 treatment also significantly reduces the severity of clinical signs associated with EAE (Diab et al., 2002).

Selective PPAR antagonists are also available including MK886 and GW6471 (PPARα), and GW9662 (PPARγ). MK886 is an indole compound originally used to induce apoptosis and has now been shown to function as a non-competitive inhibitor of PPARα (Kehrer et al., 2001). GW6471 functions by completely inhibiting GW409544-induced activation of PPARα and induces a PPARα conformation that interacts efficiently with co-repressors (Xu et al., 2002). GW409544, an L-tyrosine analog, is a potent and selective full agonist for PPARα. The recruitment of co-repressors to PPARα is enhanced by GW6471. The chemical structure of the PPARα antagonist is similar to that of GW409544 except that the carboxylate group of GW409544 has been modified into an amide group in GW6741 (Xu et al., 2002). GW9662 is an irreversible PPARγ antagonist (Mestre et al., 2009).

Given the ability of cannabinoids to engage both CB- and non CB-receptors, this chapter aimed to characterise the pharmacology of the regulatory effects of R(+)WIN55,212-2 as described in the previous chapter. In addition to defining the receptors that mediate R(+)WIN55,212-2 actions, the endocannabinoid, AEA and PPARα agonist, fenofibrate, were also assessed for their ability to mimic the regulatory effects of R(+)WIN55,212-2..

4.2 Results

4.2.1 Regulatory effects of Anandamide on NFκB and IRF7 activation by TLR ligands.

In the previous chapter, it was observed that R(+)WIN55,212-2 inhibits TLR3 and TLR4 induced transcriptional activation of NFκB and IRF7. As a result, it was next of interest to investigate the regulatory effects of AEA and to determine whether this endocannabinoid behaved in a similar manner to R(+)WIN55,212-2.

The NFκB pathway was initially examined. Treatment with LPS significantly enhanced the transcriptional activation of the NFκB-regulated reporter gene where pre-treatment with various concentrations of AEA caused a dose-dependent inhibition of LPS-induced activation of NFκB (Fig. 4.1A) which is consistent with a previous study (Nakajima et al., 2006). However, pre-exposure of HEK293 TLR3 cells to AEA failed to influence the Poly(I:C)-induced activation of NFκB (Fig. 4.1B). This indicates that AEA differentially regulates NFκB activation by TLR ligand activation.

The previously reported inhibitory effects of R(+)WIN55,212-2 on IL-1β-induced activation of NFκB in 1321N1 astrocytoma cells (Curran et al., 2005) was next used as a cell model to investigate if AEA mimicked this effect. Stimulation of 1321N1 astrocytoma cells with IL-1β enhanced NFκB activation (Fig. 4.1C) and AEA treatment attenuated this response. The sensitivity of the TLR4 and IL-1 pathways to AEA suggests that the latter may target the MyD88 dependent pathway

The regulatory effect of AEA on TLR ligand activation of IRF7 was next assessed. LPS stimulation resulted in significant activation of IRF7 (Fig. 4.2A) where pre-treatment with AEA robustly and dose-dependently attenuated LPS-induced activation of IRF7. AEA also significantly inhibited Poly(I:C)-induced activation of IRF7 (Fig. 4.2B).

4.2.2 Anandamide inhibits TNF-α expression by TLR ligands.

R(+)WIN55,212-2 significantly inhibits TNF-α expression by TLR ligand activation. The regulatory effects of AEA on TNF-α production by TLR ligand activation was subsequently assessed in U373-CD14 astrocytoma cells. Cells showed robust induction of TNF-α expression in response to LPS (Fig. 4.3A) and Poly(I:C)

(Fig. 4.3B) stimulation and the higher concentrations of AEA blunted TNF-α expression in response to both ligands.

4.2.3 Anandamide differentially regulates IRF3 activation by TLR ligands.

R(+)WIN55,212-2 differentially regulates IRF3 activation by TLR3 and TLR4 ligand stimulation. As a result, AEA was assessed for its regulatory function on IRF3 activation by TLR ligands. Exposure of HEK293 TLR4 cells to LPS enhanced IRF3 luciferase activity, and this was abrogated by AEA in a dose-dependent manner (Fig. 4.4A). Treatment of HEK293 TLR3 cells with Poly(I:C) also enhanced IRF3 luciferase activity (Fig. 4.4B). Whilst AEA showed a trend towards augmentation of Poly(I:C)-induced activation of IRF3 (Fig 4.4B), unlike R(+)WIN55,212-2 treatment this failed to reach statistical significance.

4.2.4 Anandamide differentially regulates activation of the IFN-β promoter and IFN-β mRNA by TLR ligands.

Due to the therapeutic role of IFN-β in M.S. patients and the reported ability of AEA to act as a positive neuromodulator of the disease (Eljaschewitsch et al., 2006, Baker et al., 2003), it was of significance to assess the regulatory effects of AEA on the IFN-β promoter and IFN-β mRNA following TLR ligand activation. HEK293 TRL3 cells were transfected with the IFN-β luciferase reporter plasmid and subsequently stimulated with Poly(I:C). This resulted in significant activation of the IFN-β promoter (Fig. 4.5A) and pre-treatment with AEA significantly and dose dependently enhanced the response.

In an effort to delineate the mechanism underlying the regulatory actions of AEA, transcriptional regulation of the IFN-β gene by TLR ligand activation was probed as a potential target for AEA. Real-time PCR was used to quantitate levels of mRNA encoding IFN-β. LPS treatment enhanced the expression of IFN-β mRNA in BMDM cells, and this was blocked by pre-treatment with AEA (Fig. 4.6A). However, AEA treatment strongly augmented Poly(I:C) induction of IFN-β mRNA (Fig. 4.6B).

Primary rat astrocytes were next used as a more physiologically relevant model system to further investigate the regulatory effects of AEA on IFN-β mRNA. Similar to

results observed in BMDM cells, treatment with AEA reduced LPS (Fig. 4.6C), but enhanced Poly(I:C)-induced activation (Fig. 4.6D) of IFN-β mRNA.

In conclusion, AEA appears to largely mimic the regulatory effects of R(+)WIN55,212-2 on TLR signalling pathways.

4.2.5 R(+)WIN55,212-2 inhibits TLR3 ligand activation of NFκB and IRF7 in a cannabinoid receptor independent manner.

Recent studies of cannabinoid-mediated effects in various systems, including the immune system, have produced an increasing body of evidence suggesting R(+)WIN55,212-2 and other cannabinoid exerting their immunomodulatory effects in a CB receptor independent manner (Curran et al., 2005, Mestre et al., 2009, Kaplan et al., 2003, Sancho et al., 2003). As a result of the above studies, it was of interest now to assess the involvement of the CB receptors in mediating the inhibitory effects of R(+)WIN55,212-2 on NFκB and IRF7 activation. This was explored by assessing the ability of the CB1 and CB2 receptor antagonists, SR141716A and SR144528, respectively, to modulate the regulatory effects of R(+)WIN55,212-2 in HEK293 TLR3 cells. The doses used for the CB antagonists were 1μM and this was governed by their previously described affinities and inhibitory potential at the CB receptors (White and Hiley, 1998).

HEK293 TLR3 cells were pre-treated with the respective CB receptor antagonist (1μM) for 1 hour, prior to treatment with R(+)WIN55,212-2 (20μM) for 1 hour and Poly(I:C) treatment for a further 6 hours. Cells were subsequently assayed for the induction of a NFκB-regulated luciferase gene. SR141716A failed to influence either the Poly(I:C)-induced activation of NFκB, or the inhibitory effects of R(+)WIN55,212-2 on the induction process (Fig. 4.7A). Similar results were also obtained upon examination with the CB2 receptor antagonist, SR144528 (Fig. 4.7B).

SR141716A (Fig. 4.7C) or SR144528 (Fig. 4.7D) treatment also had no influence on the Poly(I:C)-induced activation of IRF7, or the inhibitory effects of R(+)WIN55,212-2 on the induction process. Overall, these results indicate the lack of involvement of the CB receptors in mediating the inhibitory effects of R(+)WIN55,212-2 on the IRF7 and NFκB pathways.

4.2.6 R(+)WIN55,212-2 and Anandamide inhibit TLR induction of TNF-α in a CB receptor independent manner.

To investigate whether the CB receptor antagonists, SR141716A and SR144528, were capable of blocking the inhibitory effect of R(+)WIN55,212-2 on TLR ligand activation of TNF-α expression, U373-CD14 astrocytoma cells were pre-treated for 1 hour with 1μM of SR141716A or SR144528 prior to treatment with 20μM of R(+)WIN55,212-2 for 1 hour followed by TLR ligand activation for 24 hours. U373-CD14 astrocytoma supernatants were subsequently assayed for TNF-α production. Both antagonists failed to block the inhibitory effect of R(+)WIN55,212-2 on TNF-α expression by LPS (Fig. 4.8A and Fig. 4.8B) and Poly(I:C) (Fig. 4.8C and Fig. 4.8D).

AEA can also signal through the CB receptors (Felder et al., 1995), however research has also demonstrated AEA exerting its regulatory effects independent of both CB receptors (Sancho et al., 2003). Similar to R(+)WIN55,212-2, SR141716A failed to block the inhibitory effect of AEA on TNF-α expression induced by LPS (Fig. 4.9A) and Poly(I:C) (Fig. 4.9C) treatment. A comparable result was also observed with SR144528 treatment in LPS (Fig. 4.9B) and Poly(I:C) (Fig. 4.9D) stimulated cells and is consistent with a recent report (Cencioni et al., 2010). The above results again suggest a CB receptor independent mechanism for the endocannabinoid.

4.2.7 R(+)WIN55,212-2 augments TLR3 ligand activation of IRF3 in a cannabinoid receptor independent manner.

We have previously shown that R(+)WIN55,212-2 augments Poly(I:C)-induced activation of IRF3. As a result, the role of the CB receptors in this process was next examined. HEK293 TLR3 cells were pre-treated with the specific antagonist (1μM) for 1 hour, prior to treatment with R(+)WIN55,212-2 (20μM) for 1 hour and Poly(I:C) treatment for a further 6 hours. The cells were subsequently assayed for IRF3 activation. Both SR141716A and SR144528 failed to affect R(+)WIN55,212-2 induced potentiation of IRF3 activation by Poly(I:C) (Fig. 4.10A and Fig. 4.10B) respectively. These results additionally indicate the clear lack of involvement of the CB receptors in mediating the regulatory effects of R(+)WIN55,212-2 on various transcription factors employed by the TLR3 signalling pathway.

4.2.8 R(+)WIN55,212-2 augments TLR3 ligand activation of IFN-β mRNA in a cannabinoid receptor independent manner.

As previously observed, R(+)WIN55,212-2 augments Poly(I:C) activation of IFN-β mRNA following TLR3 ligand activation. The CBs receptors were now probed as potential targets in mediating this increased activation. BMDM cells were treated with the specific CB receptor antagonists (1μM) for 1 hour, prior to treatment with R(+)WIN55,212-2 (20μM) for 1 hour and Poly(I:C) treatment for a further 16 hours. The CB1 and CB2 selective receptor antagonists, SR141716A and SR144528 respectively, both failed to regulate the effects of R(+)WIN55,212-2 on Poly(I:C)-induced expression of IFN-β mRNA (Fig. 411A and Fig. 4.11B). Overall, these findings indicate that the regulatory effects of R(+)WIN55,212-2 on the TLR3-IRF3-IFN-β axis are mediated independently of the CB1 and CB2 receptors.

4.2.9 The regulatory effects of R(+)WIN55,212-2 on TLR3 ligand activation of IRF3, IRF7 and IFN-β mRNA are independent of Gi protein signalling.

Both CB receptors primarily signal through Gi proteins. To further confirm the lack of involvement of the CB receptors in the observed effects of R(+)WIN55,212-2, the dependence of the latter on Gi-protein signalling was explored. Cannabinoid receptors when coupled to a heterotrimeric Gi-protein can be selectively inactivated by pre-treatment with pertussis toxin (PTX). As a result, it was of interest to negate any possible contribution of Gi-proteins to the observed regulatory effects of R(+)WIN55,212-2 on TLR3-induced activation of IRF7, IRF3 and IFN-β mRNA.

HEK293-TLR3 cells were challenged with PTX (50 ng/ml) for 1 hour prior to treatment with R(+)WIN55,212-2 (20μM) for 1 hour and further stimulated with Poly(I:C) for 6 hours. PTX failed to modulate the regulatory effects of R(+)WIN55,212-2 on Poly(I:C)-mediated induction of IRF3 (Fig 4.12A), and IRF7 (Fig 4.12B), thus further arguing against a role for the CB receptors in mediating the regulatory effects of R(+)WIN55,212-2.

The sensitivity of PTX treatment on R(+)WIN55,212-2 regulatory effects of IFN-β mRNA was also assessed in BMDM cells treated with LPS (Fig. 4.13A) and Poly(I:C) (Fig. 4.13B) The inability of PTX to modulate the regulatory effects of R(+)WIN55,212-2 on LPS/Poly(I:C)-mediated induction of IFN-β mRNA expression

indicates no involvement of the classical CB receptors and also their coupling to Gi proteins.

4.2.10 The regulatory effects of R(+)WIN55,212-2 on TLR ligand activation of TNF-α expression are independent of Gi protein signalling.

The role of the CB receptors in mediating the inhibitory effects of R(+)WIN55,212-2 on pro-inflammatory TNF expression was next assessed. U373-CD14 astrocytoma cells were challenged with PTX (50 ng/ml) for 1 hour, prior to treatment with R(+)WIN55,212-2 (20μM) for 1 hour and further stimulated with LPS or Poly(I:C) for 24 hours. The cells were assayed for TNF-α expression in response to LPS (Fig. 4.14A) and Poly(I:C) (Fig. 4.14B) treatment. In both assays, R(+)WIN55,212-2 inhibitory effects on TNF-α expression were insensitive to PTX treatment which is consistent with a previous report (Facchinetti et al., 2003).

The above findings prompted an analysis of the expression levels of the two CB receptors in various cell models. It has previously been demonstrated that 1321N1 astrocytoma cells do not express the CB1 or CB2 receptors (Curran et al., 2005). As a result, it was of particular interest to assess CB receptor expression in U373-CD14 astrocytoma cells owning to the TLR expression similarity between the cell models. The expression of the CB receptors was also examined in BMDM cells. CB receptor expression was approached using RT-PCR to specifically amplify regions of the CB1 and CB2 receptor cDNAs. As it had previously been shown that B cells express high levels of CB receptors (Galiegue, 1995), the Burkitt lymphoma B (BJAB) cell line was utilised as a positive control both for the primers and the RT-PCR procedure. RT-PCR analysis resulted in the generation of products with the predicted sizes of 500 bp (CB1) and 400 bp (CB2) from U373-CD14 astrocytoma cDNA (Fig. 4.15A) demonstrating the presence of the CB receptors in this cell line. Interestingly, subjecting cDNA from BMDM cells to RT-PCR analysis also resulted in the expression of such products (Fig. 4.15B). To confirm the viability of the cell's RNA as a source for RT-PCR analysis, primers specific for the housekeeping human and mouse GAPDH gene were used to amplify an approx 450 bp product (not shown). Overall, this illustrates the presence of the CB1 and CB2 receptors in the U373-CD14 astrocytoma and BMDM cells and is consistent with previous data demonstrating the expression of the CB receptors in U373-CD14 astrocytoma cells (Hart et al., 2004). Despite the presence of both CB

receptors in both tested cell models, it is clearly evident that the current studied CBs regulatory effects are not mediated via the CB receptors. Subjecting cDNA from HEK293 cells to RT-PCR analysis resulted in the failure to generate any CB receptor products, indicating the absence of known CB receptors in this cell line (data not shown). This consistent with CB receptor studies having to transfect each CB receptor into HEK293 cells (Vásquez et al., 2003, Lauckner et al., 2005). In addition, the absence of the CB receptors in HEK293 cells further argues against a role for the CB receptors in mediating the regulatory effects of R(+)WIN55,212-2 and AEA.

4.2.11 Fenofibrate inhibits NFκB and IRF7 activation by TLR ligands.

Due to the ability of R(+)WIN55,212-2 and AEA to interact with PPARα (O'Sullivan and Kendall, 2009, Sun et al., 2007, Mestre et al., 2009), its respective agonist, fenofibrate, was probed for its ability to mimic the regulatory effects of the cannabinoids.

Pre-treatment of HEK293 TLR4 or TLR3 cells with various concentrations of fenofibrate caused a dose-dependent inhibition of LPS- (Fig. 4.16A) and Poly(I:C)-induced (Fig. 4.16B) activation of NFκB. A comparable, yet more potent profile was also observed in U373-CD14 astrocytoma cells (Fig 4.16C and Fig. 4.16D), indicating that this effect is not cell type-specific.

In IRF7 luciferase assays, fenofibrate strongly and dose dependently inhibited both LPS- (Fig. 4.17A) and Poly (I:C)-induced (Fig. 4.17B) activation of the transcription factor. Similar results were also observed in U373-CD14 astrocytoma cells (Fig. 4.17C and Fig. 4.17D), where higher concentrations were more effective.

4.2.12 Fenofibrate inhibits TNF-α expression by TLR ligands.

The regulatory effect of fenofibrate on TLR-induced expression of the proinflammatory protein TNF-α was next assessed. Stimulation of BMDM cells with LPS and Poly(I:C) for 24 hours induced significant TNF-α expression and fenofibrate treatment inhibited expression in both cases in a dose dependent manner (Fig. 4.18A and Fig. 4.18B). The inhibitory effects of fenofibrate on TNF-α expression were also consistent in U373-CD14 astrocytoma cells following LPS (Fig. 4.18C) and Poly(I:C) (Fig. 4.18D) treatment.

4.2.13 Fenofibrate differentially regulates IRF3 activation by TLR ligands.

The regulatory effects of fenofibrate on the activation of the IRF3 pathway were next assessed. As illustrated in Fig. 4.19A, exposure of HEK293 TLR4 cells to LPS enhanced IRF3 luciferase activity, and this was abrogated by fenofibrate treatment which is consistent with a recent study (Koch et al., 2010). Treatment of HEK293 TLR3 cells with Poly(I:C) enhanced IRF3 luciferase activity and similar to R(+)WIN55,212-2, pre-treatment with fenofibrate also potentiated the response (Fig. 4.19B). Similar results were also evident in U373-CD14 astrocytoma cells (Fig. 4.19C) following Poly(I:C) treatment.

4.2.14 Fenofibrate differentially regulates activation of the IFN-β promoter and IFN-β mRNA by TLR ligands.

The regulatory effects of fenofibrate on activation of the IFN-β promoter were next examined. Pre-treatment with fenofibrate enhanced Poly(I:C)-induced activation of the IFN-β promoter in HEK293 TLR3 cells (Fig. 4.20A) and this was apparent at low concentrations of fenofibrate. Pre-exposure of U373-CD14 astrocytoma cells to fenofibrate also notably augmented Poly(I:C)-induced activation of the IFN-β promoter (Fig.. 4.20B). However this was evident at higher concentrations of the drug.

The regulatory actions of fenofibrate on IFN-β mRNA expression were then explored. Treatment of BMDM cells with Poly(I:C) caused robust activation of IFN-β mRNA and this activation was further amplified by pre-treatment with fenofibrate (Fig. 4.21B). However, in contrast, pre-treatment with fenofibrate reduced LPS induction of IFN-β mRNA (Fig. 4.21A). These results are also reproduced in primary rat astrocytes where pre-treatment with fenofibrate repressed LPS-induced (Fig. 4.21C) but augmented Poly(I:C)-induced expression of IFN-β mRNA (Fig. 4.21D).

These results further emphasise the differential role of fenofibrate on TLR3 and TLR4 signalling pathways. Comparable to R(+)WIN55,212-2, fenofibrate also acts as a positive regulator of Poly(I:C)-induced activation of IRF3 and its associated IFN-β mRNA expression.

4.2.15 The PPARα antagonist, GW6471 induces selective effects on the regulatory actions of R(+)WIN55,212-2 and Anandamide.

The regulatory effects of the PPARα agonist fenofibrate are very similar to the regulatory effects of cannabinoids on TLR signalling. This hinted at a potential role for PPARα in mediating the effects of cannabinoids and this was directly addressed by use of a PPARα antagonist.

The ability of the PPARα antagonist; GW6471 to alter the inhibitory effects of R(+)WIN55,212-2 and AEA on NFκB and IRF7 was initially examined. HEK293 TLR3 cells were pre-treated with GW6471 (1μM) for 1 hour, prior to treatment with R(+)WIN55,212-2 or AEA (20μM) for 1 hour and Poly(I:C) treatment for a further 6 hours. In both cannabinoid treatment groups, GW6471 failed to influence either the Poly(I:C)-induced activation of NFκB, or the strong inhibitory effects of each drug on the transcriptional activity of NFκB (Fig. 4.22A and Fig. 4.22B). In parallel, GW6471 treatment also failed to regulate the strong inhibitory effects of R(+)WIN55,212-2 on Poly(I:C)-induced activation of IRF7 (Fig. 4.23A). The results presented here clearly indicate that the inhibitory effects of R(+)WIN55,212-2 and AEA on NFκB or IRF7 activity are insensitive to antagonism by GW6471. Despite this, the upregulation of IRF3 induced by R(+)WIN55,212-2 in response to Poly(I:C) treatment was slightly reversed by the addition of GW6471 (Fig. 4.23B).

The involvement of PPARα in regulating the positive effects of R(+)WIN55,212-2 and AEA on Poly(I:C)-induced activation of the IFN-β promoter was next examined. The PPARα antagonist reversed the regulatory effects of R(+)WIN55,212-2 on Poly(I:C)-induced activation of the IFN-β promoter (Fig. 4.24A). However it failed to affect the regulatory actions of AEA on the same response (Fig. 4.24B). This was further corroborated in BMDM cells where GW6471 blocked the regulatory effects of R(+)WIN55,212-2 on Poly(I:C) mediated induction of IFN-β mRNA (Fig. 4.25B). However, the PPARα antagonist failed to affect the inhibitory effects of R(+)WIN55,212-2 on LPS mediated induction of IFN-β mRNA (Fig. 4.25A).

To investigate whether GW6471 was capable of blocking the inhibitory effect of R(+)WIN55,212-2 on TLR-induced activation of TNF-α, U373-CD14 astrocytoma cells and primary mouse astrocytes were employed. Both cell types were pre-treated for 1 hour with 1μM of GW6471 prior to treatment with 20μM of R(+)WIN55,212-2 for 1 hour followed by LPS or Poly(I:C) treatment for 24 hours. In U373-CD14 astrocytoma

cells, GW6471 failed to block the inhibitory effect of R(+)WIN55,212-2 on TNF-α production induced by LPS (Fig. 4.26A) and Poly(I:C) (Fig. 4.26B) treatment. This observation was also apparent in primary mouse astrocytes following LPS (Fig. 4.26C) and Poly(I:C) (Fig. 4.26D) treatment.

4.2.16 The PPARγ antagonist, GW9662 induces selective effects on the regulatory actions of R(+)WIN55,212-2.

Although much evidence is attributed to R(+)WIN55,212-2 regulatory effects via PPARα, a current study (Mestre et al., 2009) has elaborated on findings by our laboratory (Curran et al., 2005). Mestre and colleagues described the ability of R(+)WIN55,212-2 to suppress ICAM-1 and VCAM-1 in the brain endothelium of TMEV-IDD mice. This effect was again independent of the CB receptors but dependent on PPARγ, as its selective antagonist; GW9662, blocked R(+)WIN55,212-2 inhibitory effects on adhesion molecule expression.

Due to the selective role of PPARα in mediating the regulatory effects of the studied cannabinoid compounds and taking into consideration the above observations, it was now of interest to assess PPARγ as a potential target in the pharmacological profile of R(+)WIN55,212-2.

The ability of GW9662 to alter the regulatory effects of R(+)WIN55,212-2 on TLR3 induction of IRF7 and IRF3 activity was initially examined. Following transfection of HEK293 TLR3 cells with the relevant constructs, the cells were pre-treated with GW9662 (1μM) for 1 hour, prior to treatment with R(+)WIN55,212-2 (20μM) for 1 hour and Poly(I:C) treatment for a further 6 hours. GW9662 partially blocked R(+)WIN55,212-2 inhibitory effects on Poly(I:C)-induced activation of IRF7 (Fig. 4.27A). However the PPARγ antagonist robustly reversed R(+)WIN55,212-2 augmentation of Poly(I:C)-induced activation of IRF3 (Fig. 4.27B). Similar results were also observed upon examination of IFN-β mRNA expression in BMDM cells where the PPARγ antagonist blocked the regulatory effects of R(+)WIN55,212-2 on Poly(I:C)-induced activation of IFN-β mRNA (Fig. 4.28B). However, GW9662 failed to affect R(+)WIN55,212-2 inhibitory effects on LPS-induced activation of IFN-β mRNA expression (Fig 4.28A).

The role of PPARγ in mediating the inhibitory effects of R(+)WIN55,212-2 on TLR-induced expression of TNF-α was next examined in U373-CD14 astrocytoma cells

and primary mouse astrocytes. Both cell types were pre-treated for 1 hour with 1μM of GW9662 prior to treatment with 20μM of R(+)WIN55,212-2 for 1 hour followed by LPS or Poly(I:C) treatment for 24 hours. GW9662 slightly reversed the inhibitory effects of R(+)WIN55,212-2 on Poly(I:C) induction of TNF-α expression in U373-CD14 astrocytoma cells (Fig. 4.29B) where complete reversal was observed in primary astrocytes (Fig. 4.29D). However, the inhibitory effects of R(+)WIN55,212-2 on LPS-induced activation (Fig. 4.29A and Fig. 4.29C) of TNF-α expression was insensitive to GW9662 treatment.

Given that some of the effects of the cannabinoids appear to be PPAR-mediated, the ability of cannabinoids to regulate PPAR expression was next investigated. BMDM cells were treated with R(+)WIN55,212-2 or AEA (20μM) at various time points. It was found that R(+)WIN55,212-2 treatment resulted in an increase of approximately 7-fold in PPARα mRNA expression after 1 hour, with highest upregulation of the receptor after 16 hours (Fig. 4.30A). PPARγ mRNA expression was also significantly induced by R(+)WIN55,212-2 (Fig. 4.30C). Furthermore AEA treatment also resulted in an increased expression of both PPARα and PPARγ mRNAs after 1 hour (Fig. 4.30B and Fig. 4.30D), however, in comparison to R(+)WIN55,212-2, AEA failed to retain this significant activation as increased time points resulted in downregulation of the PPARs, more notably in PPARγ mRNA expression. This suggests that cannabinoid may exert some of their responses by controlling the expression levels of PPARs.

4.3 Discussion

The initial part of this chapter focused on the regulatory effects of the endocannabinoid, AEA, and it was of interest to assess if its regulatory mechanism was similar to R(+)WIN55,212-2. AEA was found to be a strong suppressor of TLR4 induced activation of IRF3, NFκB and IRF7. AEA was also a potent regulator of TLR3 ligand activation of IRF7, but not of NFκB or IRF3. Similar to R(+)WIN55,212-2, AEA also augmented TLR3 activation of IRF3 albeit significantly less than the synthetic cannabinoid. In respect to transcription factor activation, the TLR4 signalling pathway appears to be a more potent target for the regulatory effects of AEA. This translated into effects on downstream gene expression with AEA inhibiting TLR-induction of TNF-α protein expression whilst enhancing TLR-induced activation of the IFN-β promoter and IFN-β mRNA expression

In an effort to characterise the pharmacological characteristics of R(+)WIN55,212-2 and AEA, two independent approaches indicate that the regulatory effects of the cannabinoid compounds on various transcription factors and their related cytokines, are largely mediated by a mechanism independent of the CB1 and CB2 receptors. Firstly, selective antagonists for these receptors mostly failed to influence the regulatory action of the cannabinoids. Secondly, the failure of PTX to interfere with R(+)WIN55,212-2 and AEA activity, effectively excludes any contribution of Gi-proteins to the observed effects. Indeed, Sagan and colleagues report on AEA and R(+)WIN55,212-2 inhibiting cAMP formation through GPCRs distinct from the CB receptors (Sagan et al., 1999), which further supports our theory on the pharmacological effects of the studied cannabinoids.

Recent reports on the ability of R(+)WIN55,212-2 and AEA to target PPARs lead us to explore the importance of this receptor in mediating the regulatory effects of the cannabinoids. The PPARα agonist; fenofibrate, was initially studied to examine if its regulatory role was similar to cannabinoids. Comparable to R(+)WIN55,212-2 and AEA, fenofibrate also inhibited several of the studied transcription factors and TNF-α expression. More importantly, fenofibrate augmented TLR3-induced activation of IRF3 and IFN-β, thus further supporting an anti-inflammatory role for this fibrate.

Owing to the regulatory similarities between cannabinoids and fenofibrate, the involvement of PPARα in mediating the regulatory effects of R(+)WIN55,212-2 and AEA was probed. The large LBD of PPARα allows for the interaction with a wide

variety of fatty acid-derived molecules (Wang et al., 2004). Despite this, cannabinoids are highly lipophilic agents and their ability to overcome the hydrophilic diffusional barrier of the cell membrane to access intracellular and nuclear receptors remains largely unknown.

The PPARα antagonist, GW6471, failed to induce any significant effect on the inhibitory actions of cannabinoids in the TLR4 signalling pathway. While agonists of PPARα e.g. fenofibrate can strongly suppress the activation of the TLR4 signalling pathway (Xu et al., 2007), it appears that PPARα is not the target in mediating the regulatory effects of R(+)WIN55,212-2 on TLR4 activation of various transcription factors and responsive genes. This observation is partially consistent with recent preliminary work (S Grassin Delyle, 2010). In addition, similar to our data, AEA has also been reported to be insensitive to antagonism by GW6471 (Alhamoruni et al., 2010). Furthermore, AEA failed to retain significant activation of PPARα mRNA over a longer time period.

R(+)WIN55,212-2 has also been reported to bind to and increase the transcriptional activity of PPARα (Sun et al., 2007). Likewise, the data presented here provides evidence for the ability of R(+)WIN55,212-2 to activate PPARα mRNA. This may contribute to PPARα ligand activation with endogenous targets e.g. hydroxydecanoic acid and certain long chain fatty acids (Ziouzenkova and Plutzky, 2004). However, while PPARα may not directly facilitate the inhibition of R(+)WIN55,212-2 on pro-inflammatory pathways, our preliminary data suggest that PPARα may be a potential target site for the regulatory effects of R(+)WIN55,212-2 on the TLR3-IRF3-IFN-β signalling axis. Further studies are necessary to unequivocally demonstrate this. It should be emphasised that not all of the effects of R(+)WIN55,212-2 on the IFN-β promoter are mediated by PPARα. Indeed the former can augment TLR3-induced activation of IRF3 in a PPAR-independent manner whilst in contrast fenofibrate inhibits activation of IRF3 in response to polyI:C. Thus whilst R(+)WIN55,212-2 employs PPARα to promote activation of AP-1 and the PRDIV domain of the IFN-β promoter, it can also utilise a PPARα-independent mechanism that overrides any negative regulatory effects of PPARα on IRF3 and the PRDI-III regions of the promoter.

Much research has provided evidence on AEA targeting PPARγ, including its ability to increase transcriptional activation of and bind directly to PPARγ (Bouaboula et al., 2005, Gasperi et al., 2007) and inhibit IL-2 in a partial PPARγ dependent mechanism (Rockwell and Kaminski, 2004). The data presented here additionally describes AEA increasing PPARγ mRNA; however, similar to its effects on PPARα mRNA, AEA decreased activation of PPARγ mRNA over a longer time period. Despite ample literature on AEA interacting with PPARγ, it is still not clear whether PPARγ activation is due to direct interaction with AEA or secondary metabolism generating a PPARγ active ligand.

R(+)WIN55,212-2 has also been reported to increase the transcriptional activity of PPARγ (O'Sullivan et al., 2009). Our data suggests that R(+)WIN55,212-2 is a potent inducer of PPARγ mRNA. Furthermore the combination of GW9662 and Poly(I:C) treatment slightly blocked R(+)WIN55,212-2 inhibitory effects on IRF7 activation but completely reversed R(+)WIN55,212-2 augmentation of IRF3 activity and IFN-β mRNA expression.

Much data indicate that cannabinoids, both synthetic and plant-derived, manifest at least some effects via PPARs. Such effects include palmitoylethanolamide (PEA)-induced blunting of β-amyloid (Aβ)-induced inflammation (Scuderi et al., 2011), R(+)methanandamide-induced apoptosis (Eichele et al., 2009), cannabidiol- and tetrahydrocannabinol-induced vasorelaxation (O'Sullivan et al., 2006; O'Sullivan et al., 2009), N-Oleoylethanolamine-induced protection following middle cerebral artery occlusion (Sun et al., 2007) and ajulemic acid-induced fibroblast differentiation and IL-8 promoter activity inhibition (Liu et al., 2003). In addition, data presented herein demonstrate such PPAR-dependency for the synthetic cannabinoid R(+)WIN55,212-2. R(+)WIN55,212-2 belongs to the family of aminoalkylindoles that possess cannabimimetic properties despite being structurally dissimilar to plant-derived cannabinoids (Pacheco et al., 1991). Although R(+)WIN55,212-2 displays high affinity for both CB1 and CB2 cannabinoid receptors, with moderate selectivity for CB2 (Howlett, 2002), our laboratory (Curran et al., 2005; Downer et al., 2011) and others (Marchalant et al., 2007, Facchinetti et al., 2003, Germain et al., 2002, Nilsson et al., 2006, Sánchez et al., 2006) have demonstrated cannabinoid receptor-independent effects of this aminoalkylindole. Indeed our findings are consistent with PPAR-dependent effects of R(+)WIN55,212-2 on cell viability (Giuliano et al., 2009) and adhesion molecule expression (Mestre et al.,2009). It is noteworthy that Mestre and colleagues

(2009) demonstrated that R(+)WIN55,212-2 regulates endothelial expression of VCAM-1 adhesion molecule independent of CB1 and CB2 receptors, but involvement of PPAR receptors was identified, further demonstrating a non cannabinoid-dependent effect for this aminoalkylindole. The selectivity of R(+)WIN55,212-2 in targeting PPARα independently of CB1 or CB2 is further supported by data indicating that ligands (pyrazol fatty acid amides) incorporating cannabinoid and PPARα features, may lack cannabinoid activity (hypothermia, locomotor activity) but behave as potent activators of PPARs (Alvarado et al., 2008).

Since their initial characterisation, PPARs have attracted an increasing amount of experimental attention particularly in their role in inflammation. The mechanistic involvement of the PPARs in the innate and adaptive immune systems is a relatively new research area. Despite this, recent observations of the potent anti-inflammatory activities of PPARα- and PPARγ-specific ligands in various disease models have been reported (Lovett-Racke et al., 2004, Xu et al., 2007).

In conclusion, it appears that PPARγ, rather than PPARα, is a more potent endogenous target in mediating the anti-inflammatory effects of R(+)WIN55,212-2. This is particularly evident in relation to the TLR3 signalling pathway. The overall ability of R(+)WIN55,212-2 to augment the Poly(I:C)-induced transcriptional activity of IRF3, activation of IFN-β mRNA and the potential of PPARγ to mediate this mechanism, is a novel finding. Furthermore, it appears that the molecular target governing R(+)WIN55,212-2 anti-inflammatory effects is distinct from TLR4 signalling and is certainly independent of PPARγ.

This observation, not only broadens the potential use of cannabinoids as therapeutic agents, but also supports PPARγ as the likely target. Whilst PPARγ may act as a mediator in manifesting neuroprotective effects of some cannabinoids, it would also be of great value to identify the components of the TLR3 signalling pathway that are principally targeted in mediating these effects. This constitutes the major objective of the following chapter.

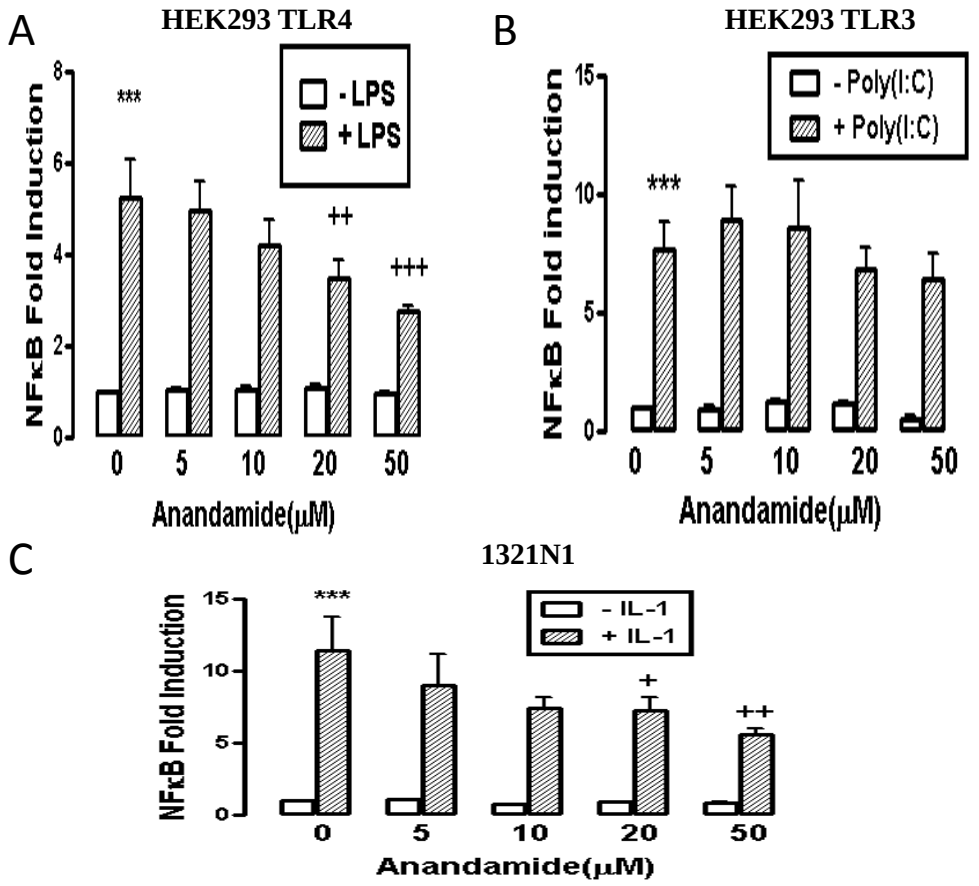


Figure 4.1 Anandamide inhibits LPS, Poly(I:C) and IL-1β-induced activation of NFκB in HEK293 TLR3/TLR4 and 1321N1 astrocytoma cells.

HEK293 cells stably transfected with TLR4 (A) or TLR3 (B) and 1321N1 astrocytoma cells (C) were co-transfected with a NFκB-regulated firefly luciferase reporter (80 ng), phRL-TK (constitutively expressed Renilla luciferase) (40 ng), and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of Anandamide for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml), Poly(I:C) (25μg/ml) or IL-1β (10ng/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$ compared with ligand treated cells. One way ANOVA effect of treatment (A) F value (14, 30) = 33.23. $P < 0.001$ (B) F value (15, 32) = 21.43. $P < 0.001$ (C) F value (13, 28) = 13.36. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

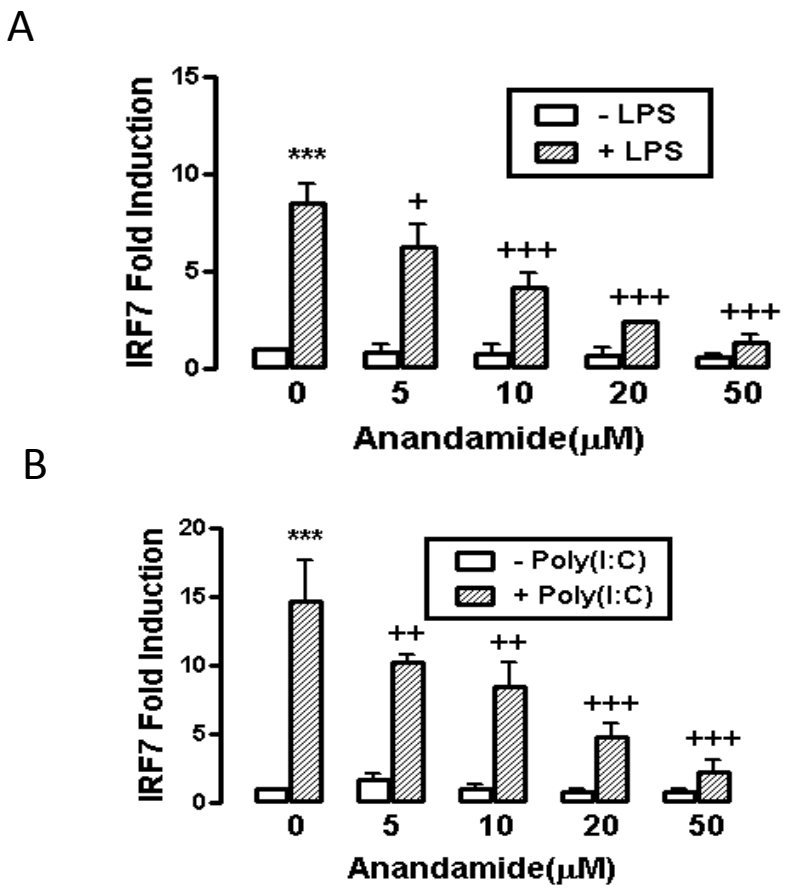


Figure 4.2 Anandamide inhibits LPS and Poly(I:C)-induced activation of IRF7 in HEK293 TLR3/TLR4 cells.

HEK293 cells stably transfected with TLR4 (A) or TLR3 (B) were co-transfected with the trans-activator plasmid pFA-IRF-7 (25ng), pFR-Luc (60ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (145ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of Anandamide for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$ compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (13, 28) = 37.73. $P < 0.001$ (B) F value (13, 28) = 20.79. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

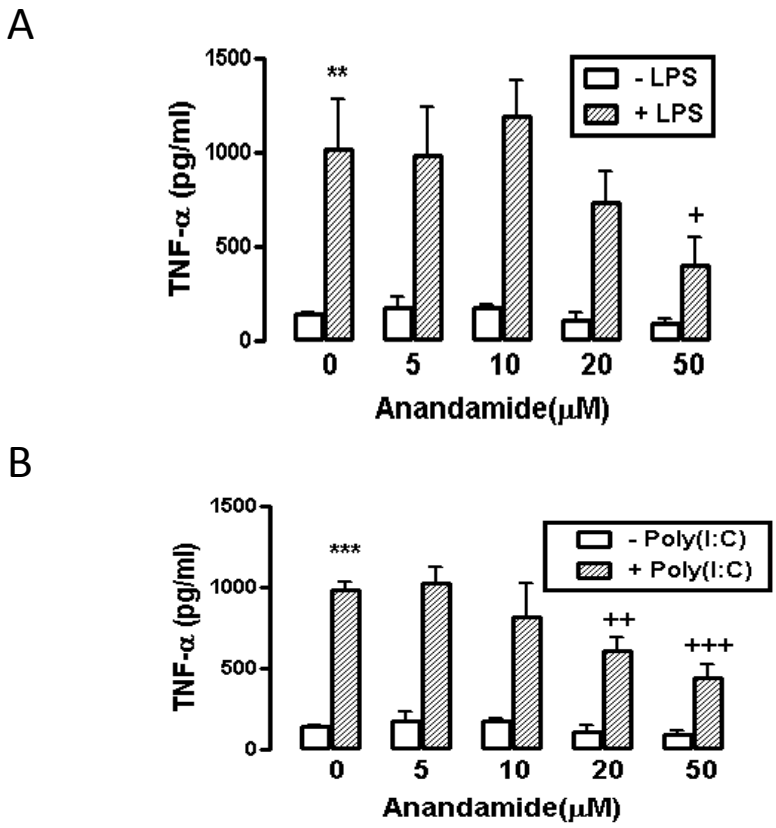


Figure 4.3 Anandamide inhibits LPS and Poly(I:C) induction of TNF-α expression in U373-CD14 astrocytoma cells.

U373-CD14 astrocytoma cells were pre-treated with or without various concentrations of Anandamide for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) (A) or Poly(I:C) (25μg/ml) (B) for a further 24 h.

Supernatants were assayed for TNF-α levels by sandwich ELISA. **P< 0.01 and ***P < 0.001 compared with vehicle-treated cells. +P < 0.05, ++P < 0.01 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (13, 24) = 8.605. P < 0.001 (B) F value (13, 24) = 21.08. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

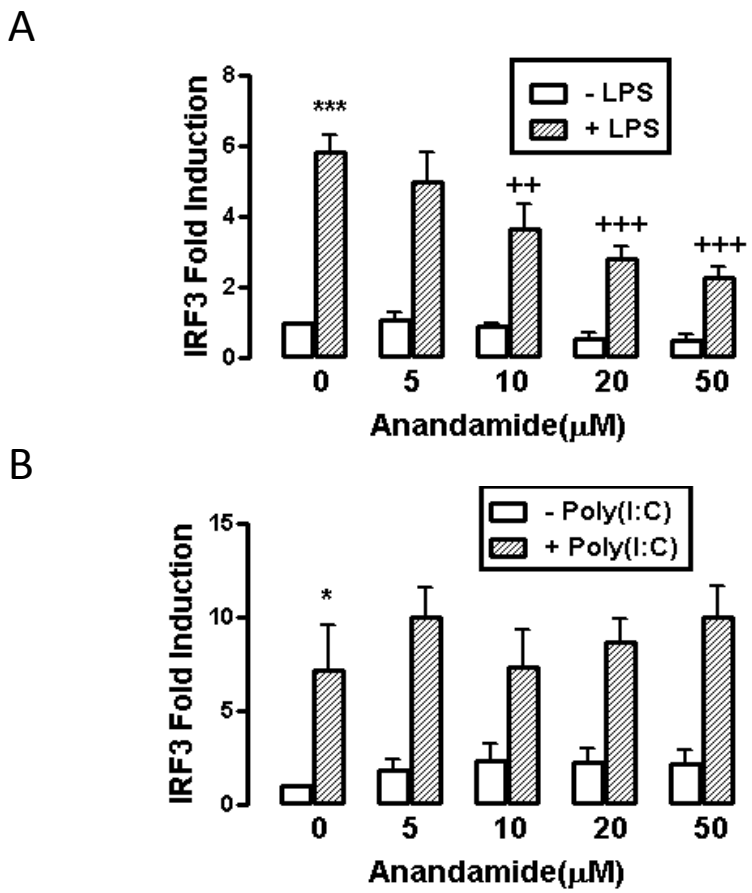


Figure 4.4 Anandamide differentially regulates LPS and Poly(I:C)-induced activation of IRF3 in HEK293 TLR3/TLR4 cells.

HEK293 cells stably transfected with TLR4 (A) or TLR3 (B) were were co-transfected with the trans-activator plasmid pFA-IRF-3 (30ng) pFR-Luc (60ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (140ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of Anandamide for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. *P < 0.05 and ***P < 0.001 compared with vehicle-treated cells. ++P < 0.01 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (13, 24) = 22.74. P < 0.001 (B) F value (13, 28) = 11.26. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

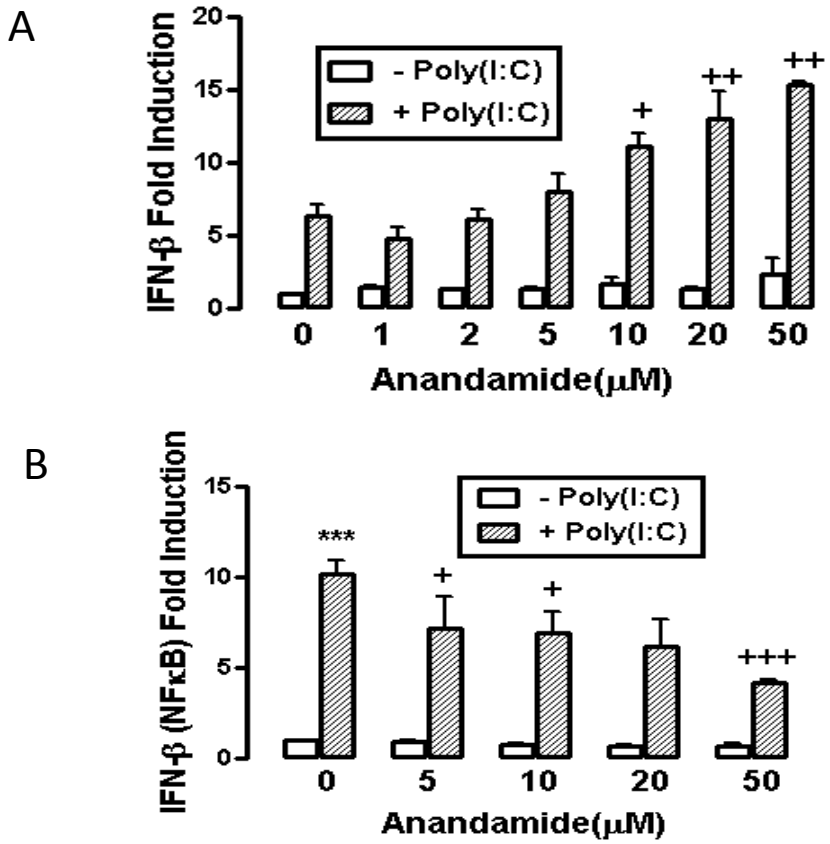


Figure 4.5 Anandamide regulatory effects on Poly(I:C)-induced activation of the IFN-β promoter and activation of PRDII on the IFN-β promoter in HEK 293 TLR3 cells.

HEK293 cells stably transfected with TLR3 were co-transfected with IFN-β luciferase reporter plasmid (80ng) (A) or the luciferase reporter construct; PRD-II-Luc (80ng) (B), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of Anandamide for 1 h prior to stimulation in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h.

Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$ compared with Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (19, 40) = 117.1. $P < 0.001$ (B) F value (13, 28) = 20.94. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

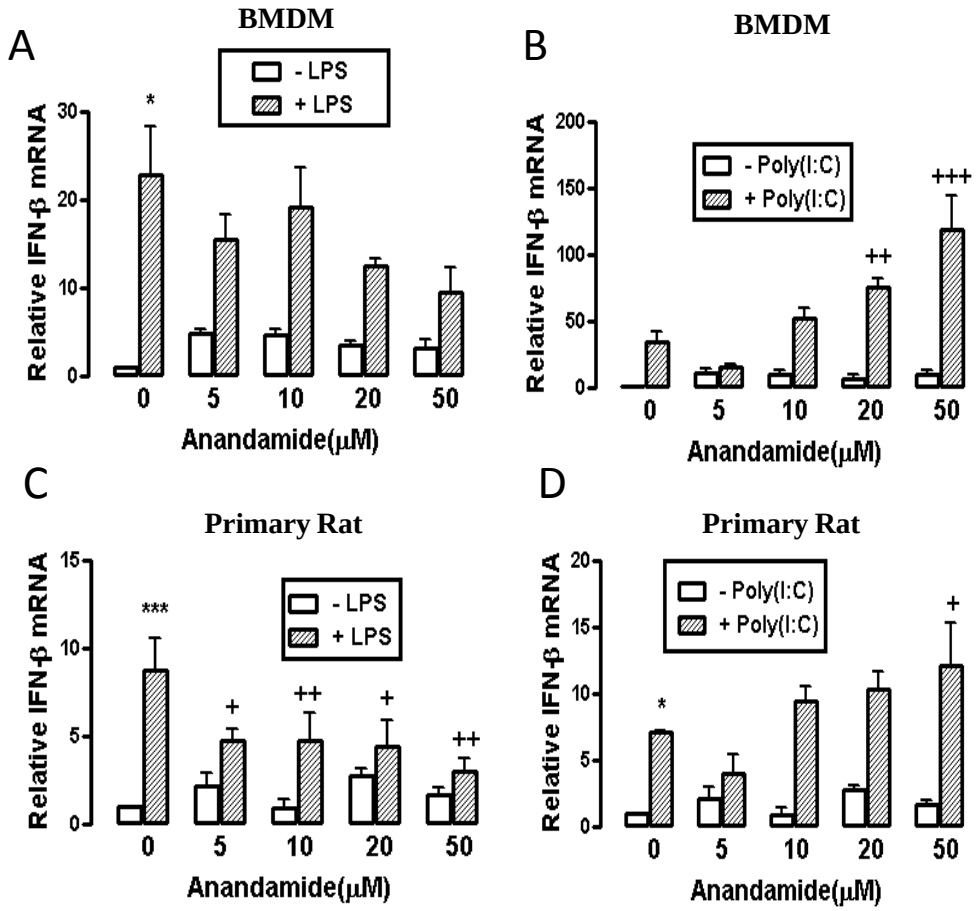


Figure 4.6 Anandamide differentially regulates LPS and Poly(I:C)-induced activation of IFN-β mRNA in BMDM cells and primary rat astrocytes.

BMDM cells (A+B) and primary astrocyte cultures prepared from neonatal rat brains (C+D) were pre-treated with or without various concentrations of Anandamide for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 16 h. RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. *P < 0.5 and ***P < 0.001 compared with vehicle-treated cells. +P < 0.05, ++P < 0.01 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (13, 25) = 10.29. P < 0.001 (B) F value (13, 28) = 15.37. P < 0.001 (C) F value (13, 28) = 10.68. P < 0.001 (D) F value (13, 28) = 24.99. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

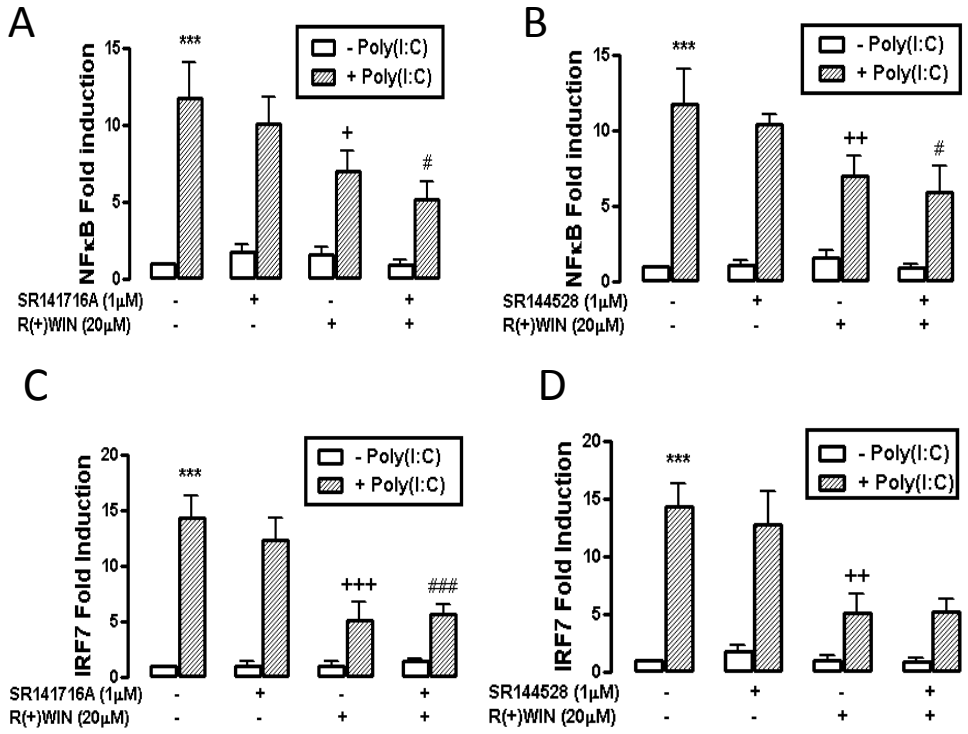


Figure 4.7 R(+)WIN55,212-2 inhibits Poly(I:C)-induced activation of NFκB and IRF7 in HEK293 TLR3 cells in a cannabinoid receptor independent manner.

HEK293 cells stably transfected with TLR3 were co-transfected with a NFκB-regulated firefly luciferase reporter (80 ng), phRL-TK (constitutively expressed Renilla luciferase) (40 ng), and pcDNA3.1 (120 ng) (A+B). In an separate assay, HEK293 cells stably transfected with TLR3 were co-transfected with the trans-activator plasmid pFA-IRF-7 (25ng), pFR-Luc (60ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (145ng) (C+D). Cells were allowed to recover overnight then challenged for 1 h with SR141716A (1μM) or SR144528 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$ compared with Poly(I:C)-treated cells. #$P < 0.05$ and ###$P < 0.001$ compared with cells treated with R(+)WIN55,212-2 in the presence of Poly(I:C). One way ANOVA effect of treatment (A) F value (10, 19) = 7.946. $P < 0.001$ (B) F value (10, 22) = 11.39. $P < 0.001$ (C) F value (10, 22) = 17.75. $P < 0.001$ (D) F value (10, 22) = 18.05. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

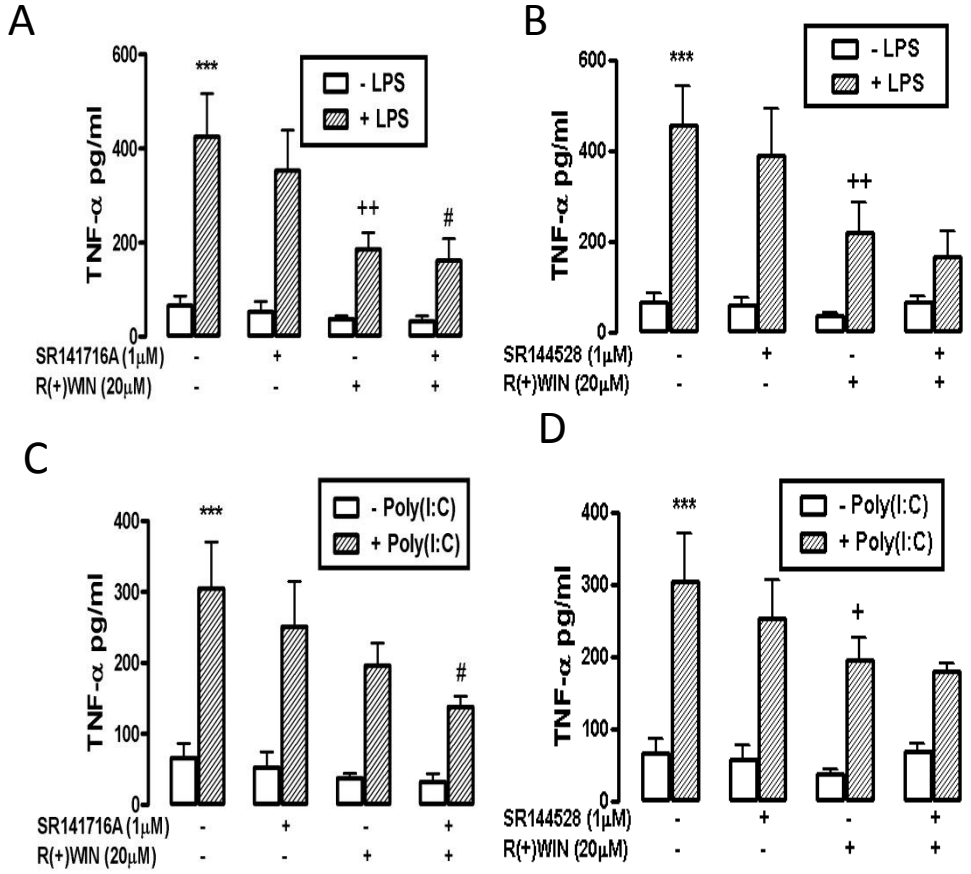


Figure 4.8 R(+)WIN55,212-2 inhibits LPS and Poly(I:C) induction of TNF-α expression in U373-CD14 astrocytoma cells in a cannabinoid receptor independent manner.

U373-CD14 astrocytoma cells were challenged for 1 h with SR141716A (1μM) (A+C) or SR144528 (1μM) (B+D) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 24 h. Supernatants were assayed for TNF-α levels by sandwich ELISA. ***P < 0.001 compared with vehicle-treated cells. +P < 0.05 and ++P < 0.01 compared with LPS or Poly(I:C)-treated cells. #P < 0.05 compared with cells treated with R(+)WIN55,212-2 in the presence of LPS. One way ANOVA effect of treatment (A) F value (10, 22) = 11.74. P < 0.001 (B) F value (10, 22) = 9.392. P < 0.001 (C) F value (10, 22) = 12.01. P < 0.001 (D) F value (10, 22) = 14.21. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

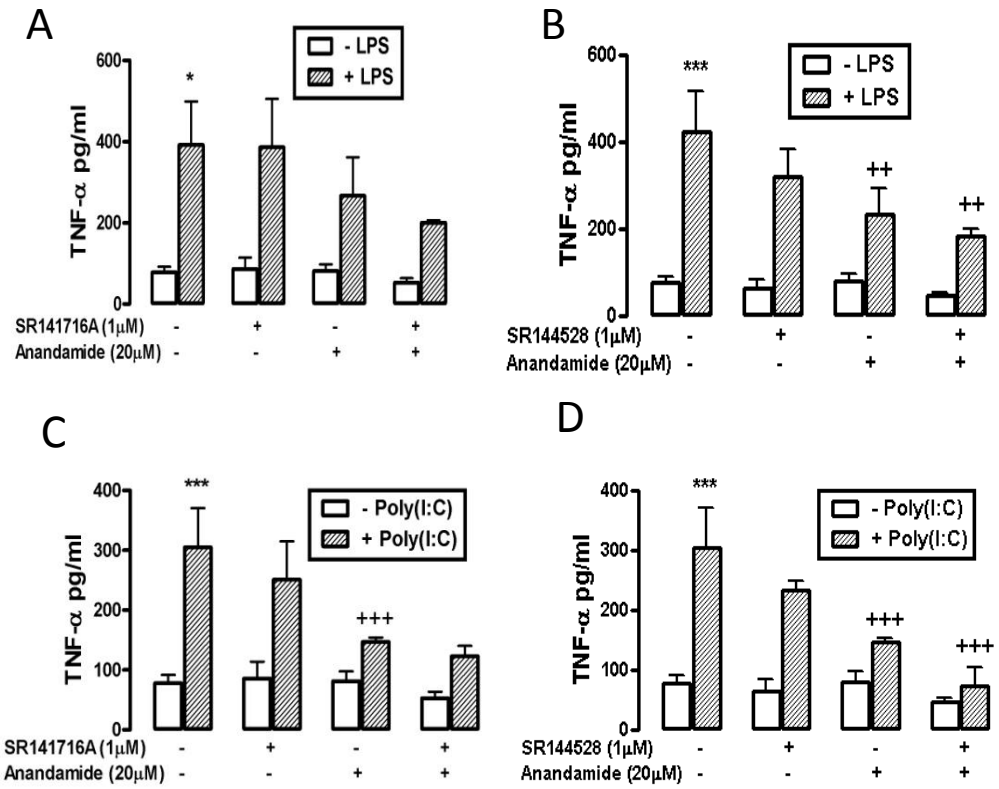


Figure 4.9 Anandamide inhibits LPS and Poly(I:C) induction of TNF-α expression in U373-CD14 astrocytoma cells in a cannabinoid receptor independent manner.

U373-CD14 astrocytoma cells were challenged for 1 h with SR141716A (1μM) (A+C) or SR144528 (1μM) (B+D) prior to treatment with or without Anandamide (20μM) for 1 h and further stimulated in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 24 h. Supernatants were assayed for TNF-α levels by sandwich ELISA. *P < 0.05 and ***P < 0.001 compared with vehicle-treated cells. ++P < 0.01 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (10, 22) = 7.866. P < 0.001 (B) F value (10, 22) = 12.77. P < 0.001 (C) F value (10, 22) = 10.93. P < 0.001 (D) F value (10, 22) = 16.60. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

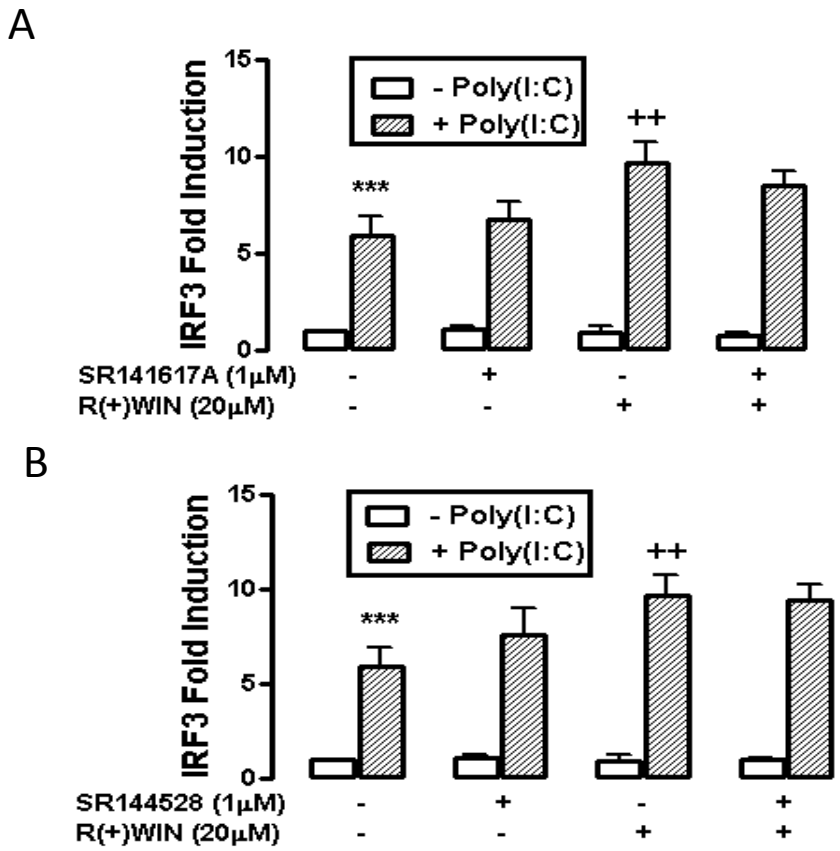


Figure 4.10 R(+)WIN55,212-2 augments Poly(I:C)-induced activation of IRF3 in HEK293 TLR3 cells in a cannabinoid receptor independent manner.

HEK293 cells stably transfected with TLR3 were were co-transfected with the trans-activator plasmid pFA-IRF-3 (30ng) pFR-Luc (60ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (140ng). Cells were allowed to recover overnight then challenged for 1 h with SR141716A (1μM) (A) or SR144528 (1μM) (B) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. ++$P < 0.01$ compared with Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (10, 22) = 40.10. $P < 0.001$ (B) F value (10, 22) = 33.29. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

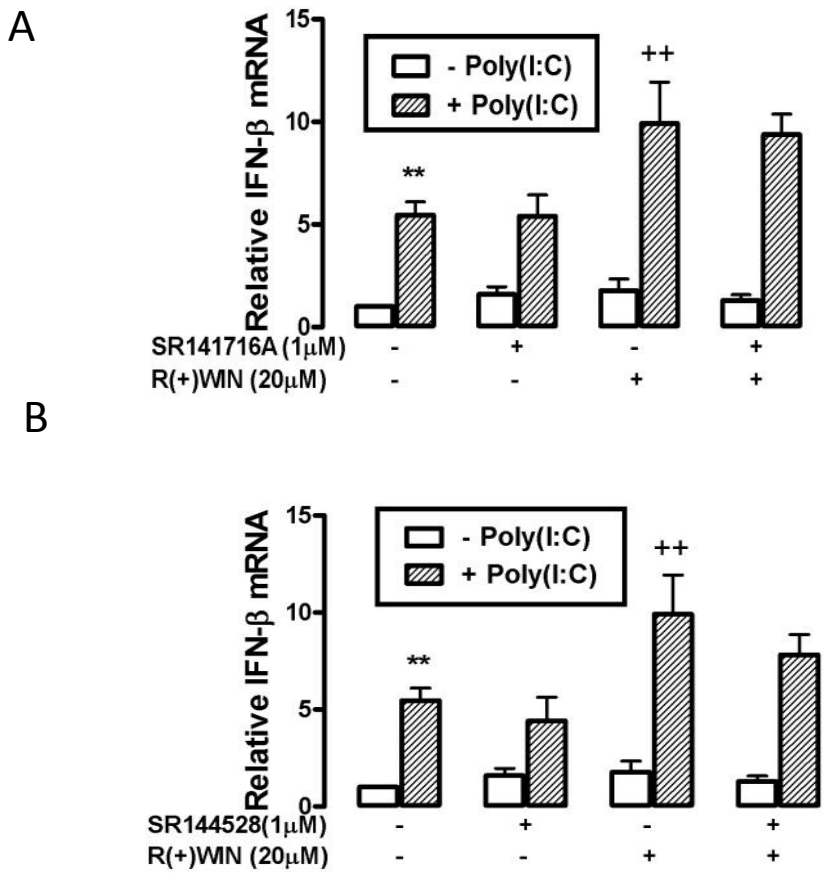


Figure 4.11 R(+)WIN55,212-2 augments Poly(I:C)-induced activation of IFN-β mRNA in BMDM cells in a cannabinoid receptor independent manner.

BMDM cells were challenged for 1 h with SR141716A (1μM) (A) or SR144528 (1μM) (B) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 16 h. RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. **P < 0.01 compared with vehicle-treated cells. ++P < 0.01 compared with Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (10, 22) = 20.60. P < 0.001 (B) F value (10, 22) = 16.87. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

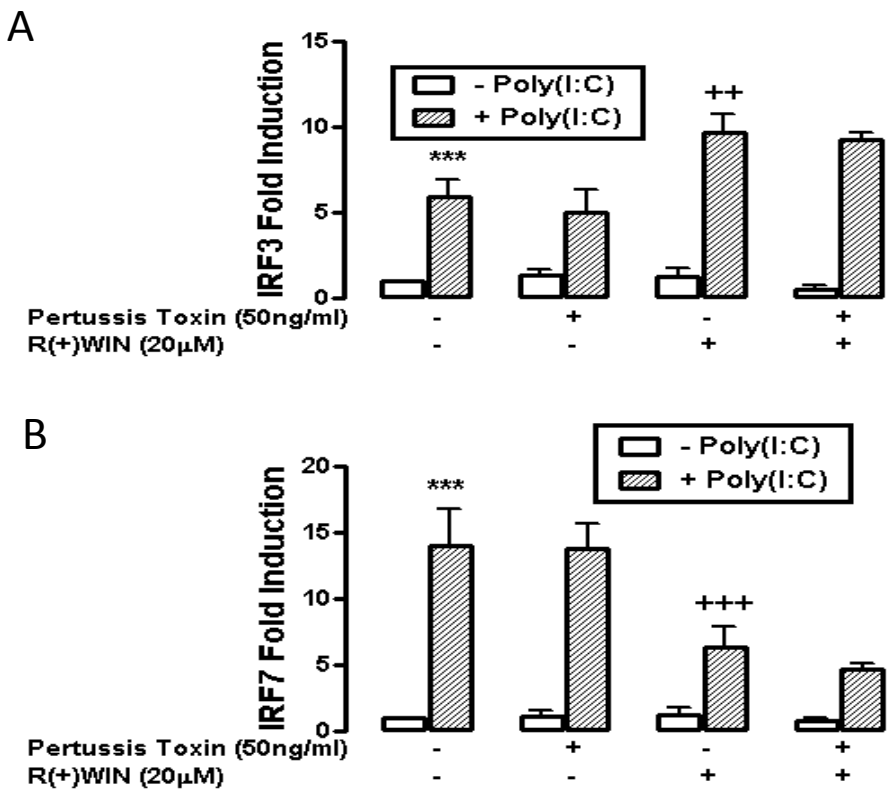


Figure 4.12 Effects of R(+)WIN55,212-2 on Poly(I:C)-induced activation of IRF3 and IRF7 are independent of Gi protein signalling.

HEK293 cells stably transfected with TLR3 were were co-transfected with the trans-activator plasmid pFA-IRF-3 (30ng) pFR-Luc (60ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (140ng) (A). In an separate assay, HEK293 cells stably transfected with TLR3 were co-transfected with the trans-activator plasmid pFA-IRF-7 (25ng), pFR-Luc (60ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (145ng) (B). Cells were allowed to recover overnight then challenged for 1 h with pertussis toxin (50ng/ml) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. ++$P < 0.01$ and +++$P < 0.001$ compared with Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (10, 22) = 34.42. $P < 0.001$ (B) F value (10, 22) = 20.97. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

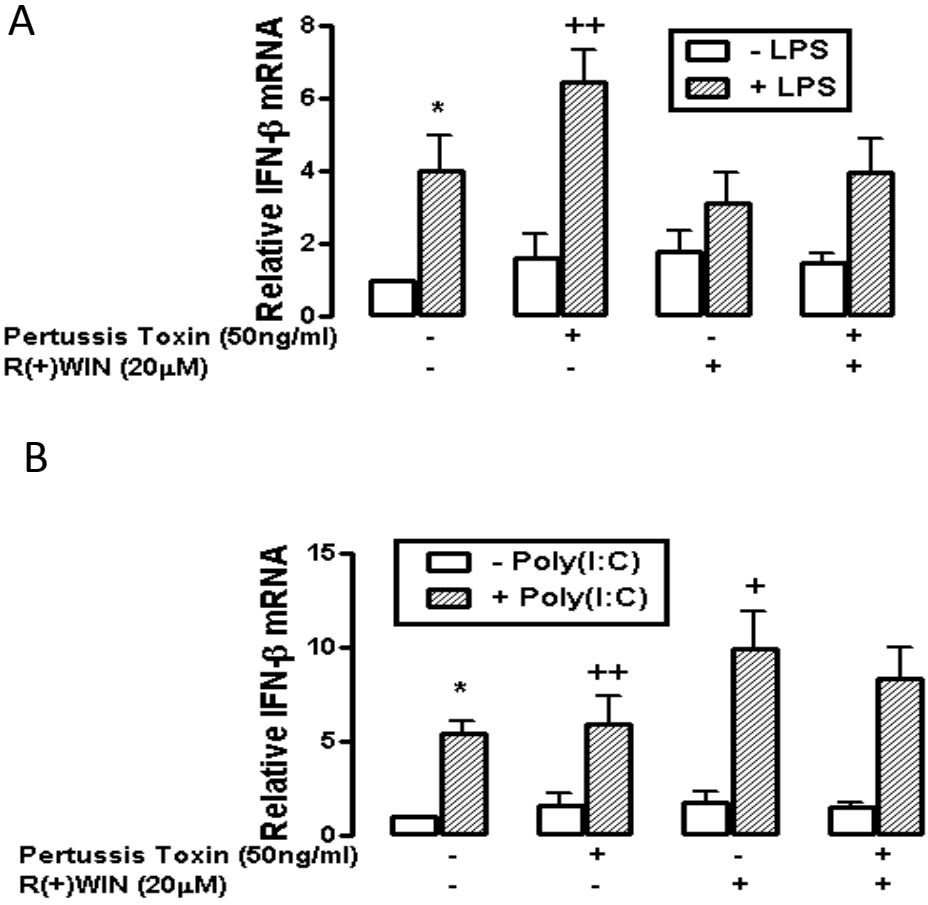


Figure 4.13 Effects of R(+)WIN55,212-2 on LPS and Poly(I:C)-induced activation of IFN-β mRNA in BMDM cells are independent of Gi protein signalling.

BMDM cells were pre-treated for 1 h with pertussis toxin (50ng/ml) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of LPS(100ng/ml) (A) or Poly(I:C) (25μg/ml) (B) for a further 16 h. RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. *$P < 0.05$ compared with vehicle-treated cells. +$P < 0.05$ and ++$P < 0.01$ compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (10, 22) = 9.194. $P < 0.001$ (B) F value (10, 22) = 11.82. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

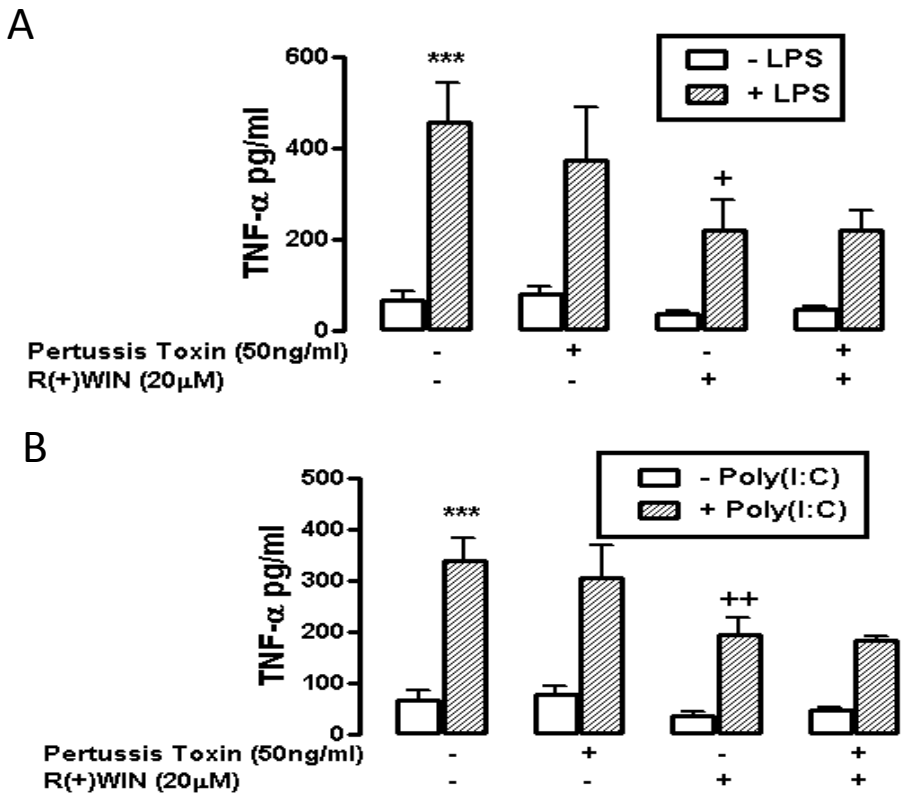


Figure 4.14 Effects of R(+)WIN55,212-2 on LPS and Poly(I:C)-induced activation of TNF-α expression in U373-CD14 astrocytoma cells are independent of Gi protein signalling.

U373-CD14 astrocytoma cells were pre-treated for 1 h with pertussis toxin (50ng/ml) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of LPS (100ng/ml) (A) or Poly(I:C) (25μg/ml) (B) for a further 24 h. Supernatants were assayed for TNF-α levels by sandwich ELISA. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$ and ++$P < 0.01$ compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (10, 22) = 9.916. $P < 0.001$ (B) F value (10, 22) = 20.21. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

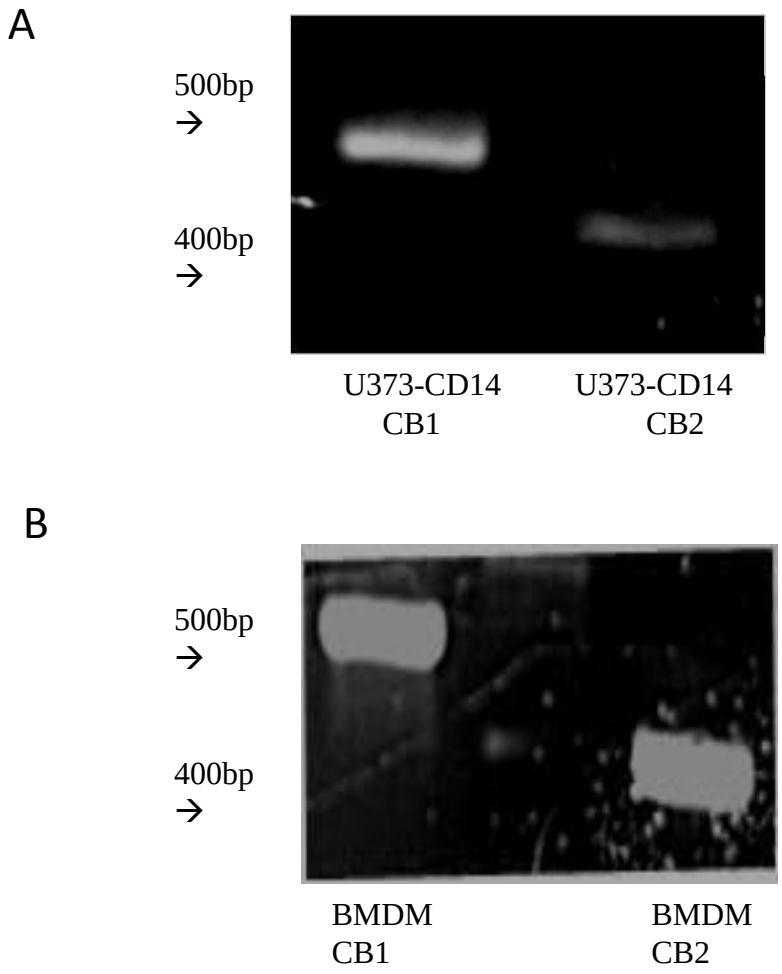


Figure 4.15 Expression of CB1 and CB2 receptors in U373-CD14 astrocytoma and BMDM cells.

Total cellular RNA was prepared from U373-CD14 astrocytoma (A) and BMDM (B) cells and subjected to first strand cDNA synthesis using Superscript II reverse transcriptase and random oligonucleotide primers. PCR amplification was preformed to selectively amplify regions of CB1 and CB2 cDNA. This resulted in the generation of products with the predicted size of 500bp (CB1) and 400bp (CB2).

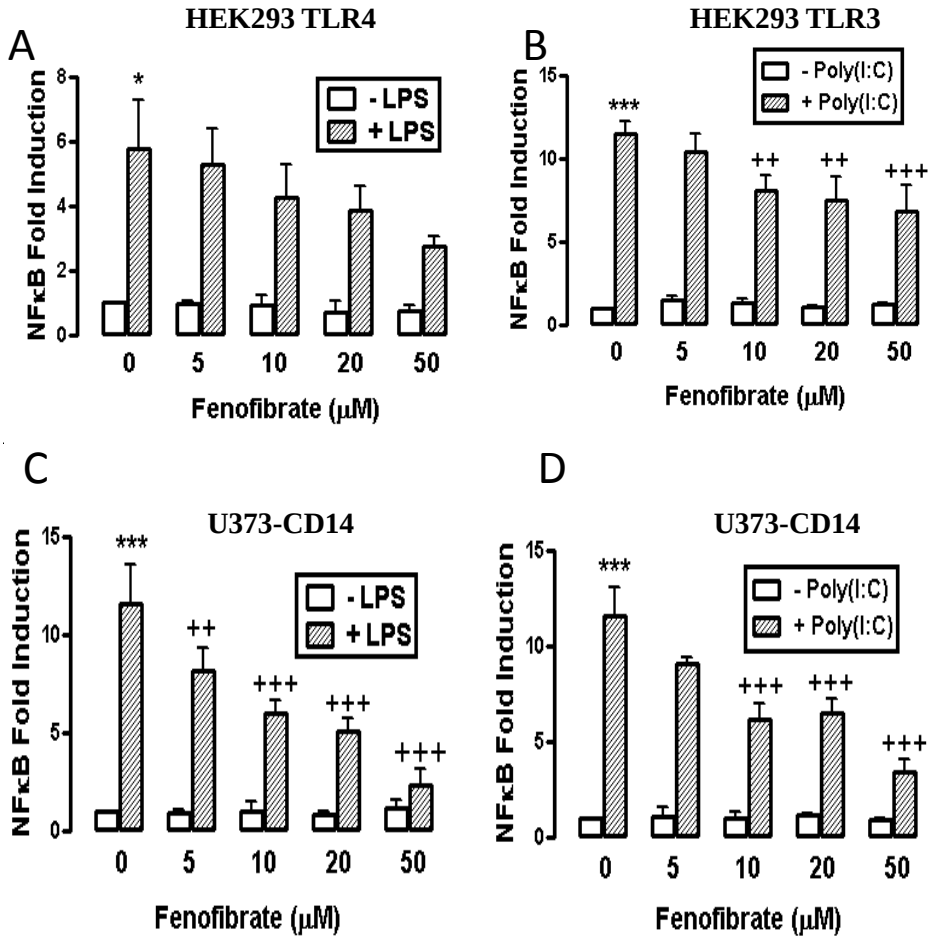


Figure 4.16 Fenofibrate inhibits LPS and Poly(I:C)-induced activation of NFκB in HEK293 TLR3/TLR4 and U373-CD14 astrocytoma cells.

HEK293 cells stably transfected with TLR4 (A) or TLR3 (B) and U373-CD14 astrocytoma cells (C+D) were co-transfected with a NFκB-regulated firefly luciferase reporter (80 ng), phRL-TK (constitutively expressed Renilla luciferase) (40 ng), and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of Fenofibrate for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with vehicle-treated cells. ++P < 0.01 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (13, 28) = 10.97. P < 0.001 (B) F value (13, 28) = 33.96. P < 0.001 (C) F value (13, 28) = 39.61. P < 0.001 (D) F value (13, 28) = 77.08. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

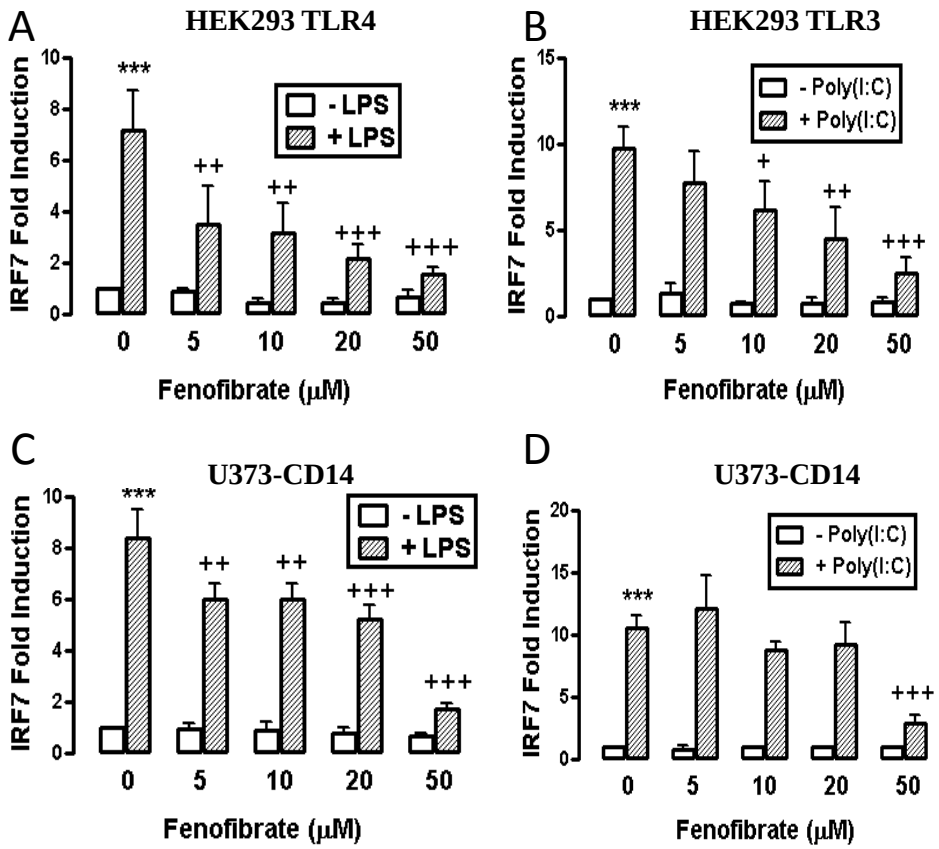


Figure 4.17 Fenofibrate inhibits LPS and Poly(I:C)-induced activation of IRF7 in HEK293 TLR3/TLR4 and U373-CD14 astrocytoma cells.

HEK293 cells stably transfected with TLR4 (A) or TLR3 (B) and U373-CD14 astrocytoma cells (C+D) were co-transfected with the trans-activator plasmid pFA-IRF-7 (25ng), pFR-Luc (60ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (145ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of Fenofibrate for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$ compared with LPS or Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (13, 28) = 12.00. $P < 0.001$ (B) F value (13, 28) = 33.96. $P < 0.001$ (C) F value (13, 28) = 11.36. $P < 0.001$ (D) F value (13, 28) = 40.35. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

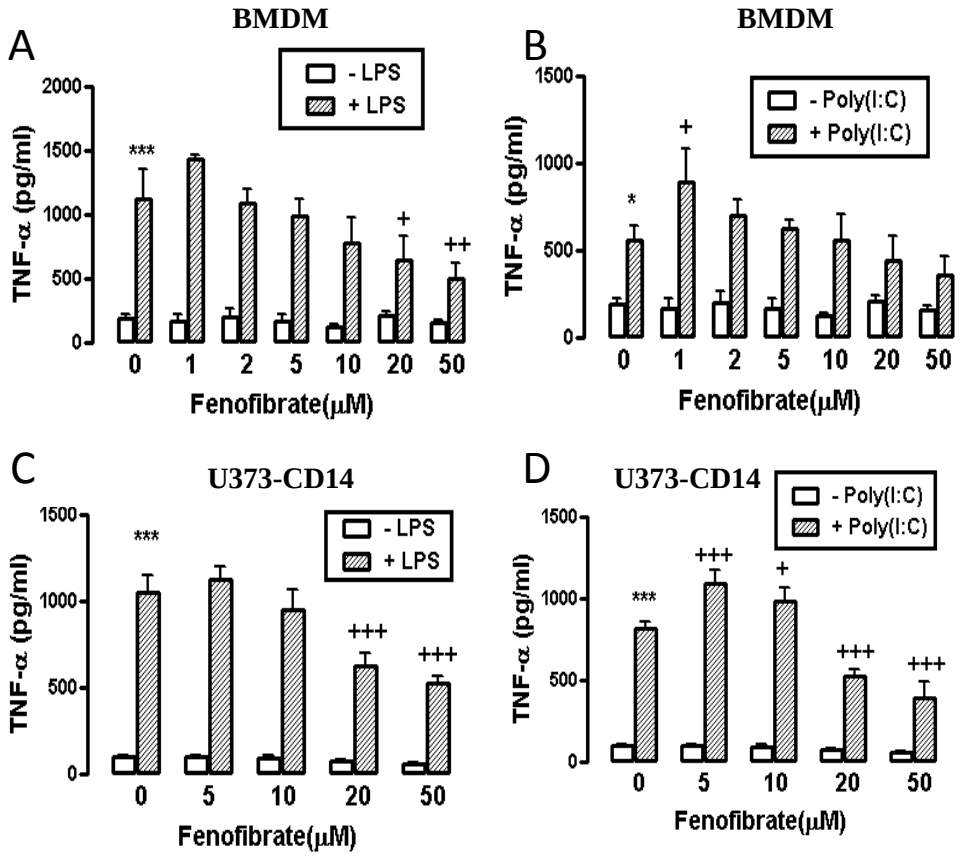


Figure 4.18 Fenofibrate inhibits LPS and Poly(I:C) induction of TNF-α expression in BMDM and U373-CD14 astrocytoma cells.

BMDM (A+B) and U373-CD14 astrocytoma (C+D) cells were pre-treated with or without various concentrations of Fenofibrate for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 24 h. Supernatants were assayed for TNF-α levels by sandwich ELISA. *P< 0.05 and ***P < 0.001 compared with vehicle-treated cells. +P < 0.05, ++P < 0.01 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (19, 34) = 20.09. P < 0.001 (B) F value (19, 34) = 18.30. P < 0.001 (C) F value (13, 28) = 67.40. P < 0.001 (D) F value (13, 28) = 77.39. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

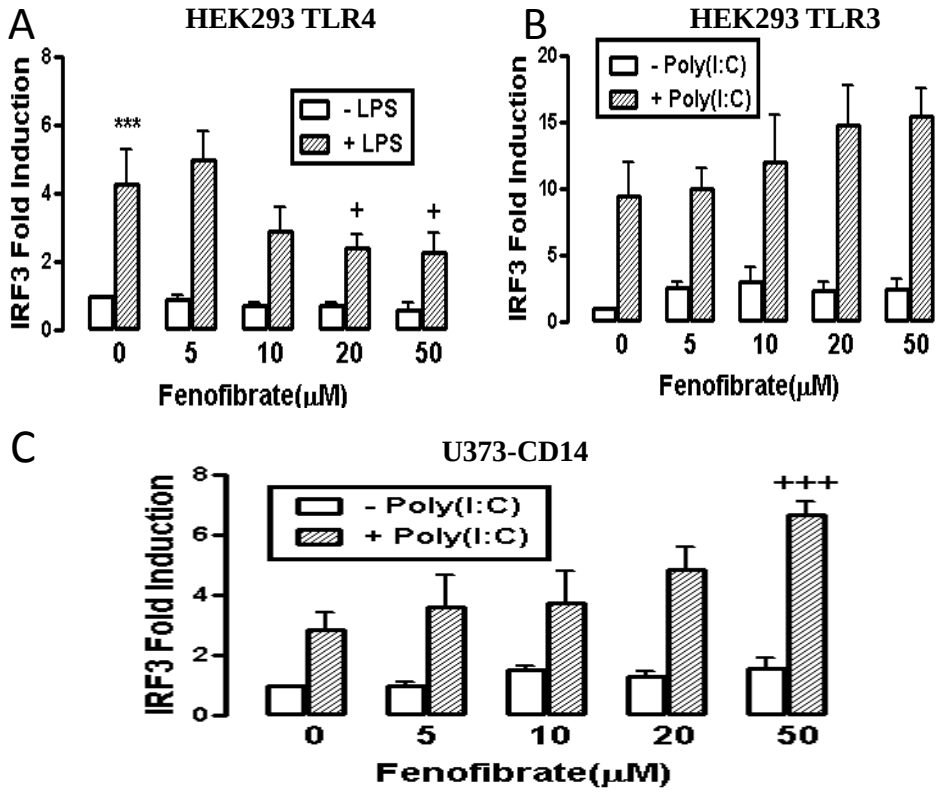


Figure 4.19 Fenofibrate regulatory effects on LPS and Poly(I:C)-induced activation of IRF3 in HEK293 TLR3/TLR4 and U373-CD14 astrocytoma cells.

HEK293 cells stably transfected with TLR4 (A) or TLR3 (B) and U373-CD14 astrocytoma cells (C) were co-transfected with the trans-activator plasmid pFA-IRF-3 (30ng), pFR-Luc (60ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (140ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of Fenofibrate for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$ and +++$P < 0.001$ compared with LPS or Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (13, 24) = 10.36. $P < 0.001$ (B) F value (13, 24) = 9.527. $P < 0.001$ (C) F value (13, 28) = 8.635. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

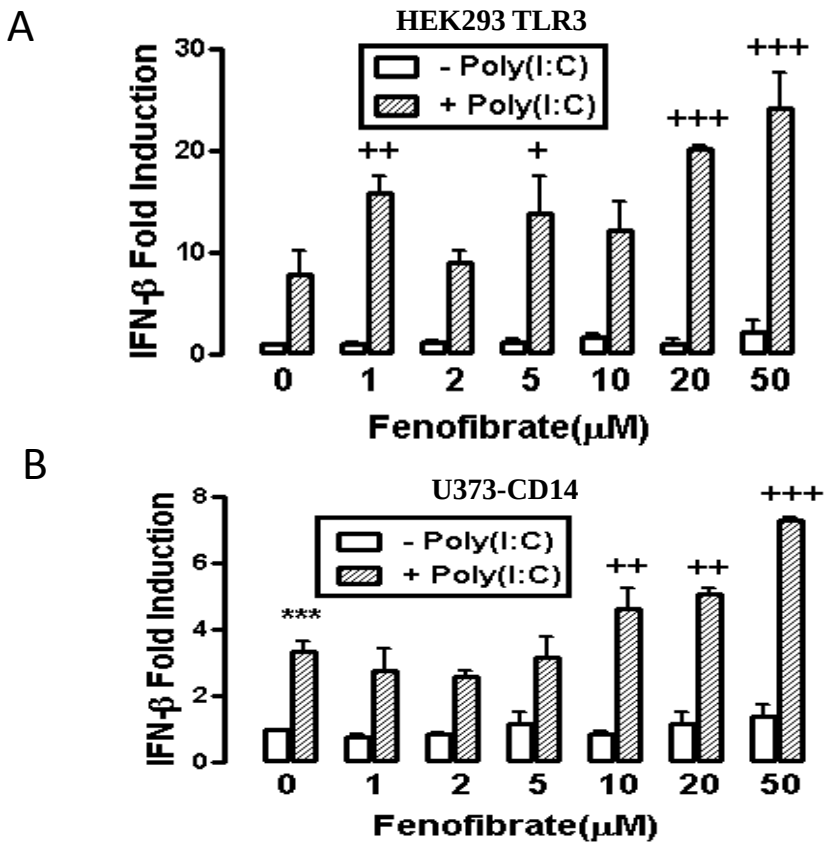


Figure 4.20 Fenofibrate augments Poly(I:C)-induced activation of the IFN-β promoter in HEK293 TLR3 and U373-CD14 astrocytoma cells.

HEK293 cells stably transfected with TLR3 (A) and U373-CD14 astrocytoma (B) cells were co-transfected with IFN-β luciferase reporter plasmid (80ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then pre-treated with or without various concentrations of Fenofibrate for 1 h prior to stimulation in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h.

Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$ compared with Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (19, 40) = 13.36. $P < 0.001$ (B) F value (19, 40) = 70.52. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

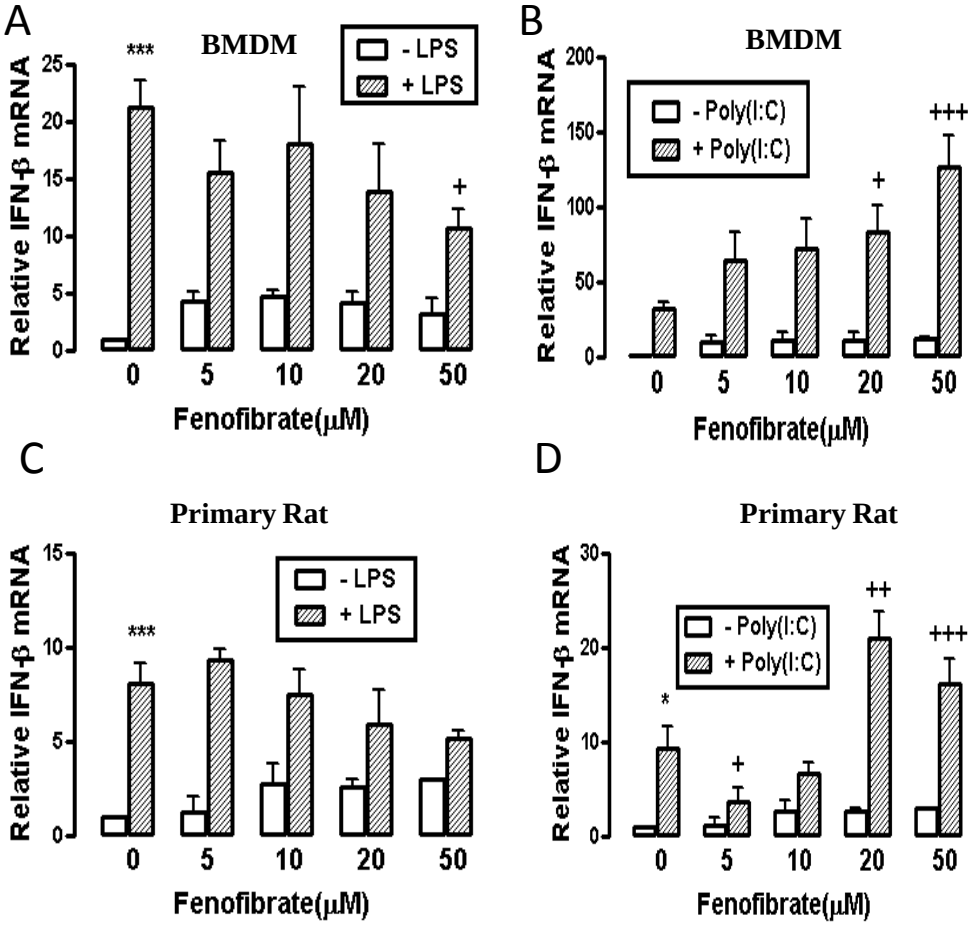


Figure 4.21 Fenofibrate differentially regulates LPS and Poly(I:C)-induced activation of IFN-β mRNA in BMDM cells and primary rat astrocytes.

BMDM cells (A+B) and primary astrocyte cultures prepared from neonatal rat brains (C+D) were pre-treated with or without various concentrations of Fenofibrate for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 16 h. RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. *P < 0.5 and ***P < 0.001 compared with vehicle-treated cells. +P < 0.05, ++P < 0.01 and +++P < 0.001 compared with LPS or Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (13, 24) = 9.864. P < 0.001 (B) F value (13, 27) = 13.30. P < 0.001 (C) F value (13, 28) = 35.95. P < 0.001 (D) F value (13, 28) = 22.37. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

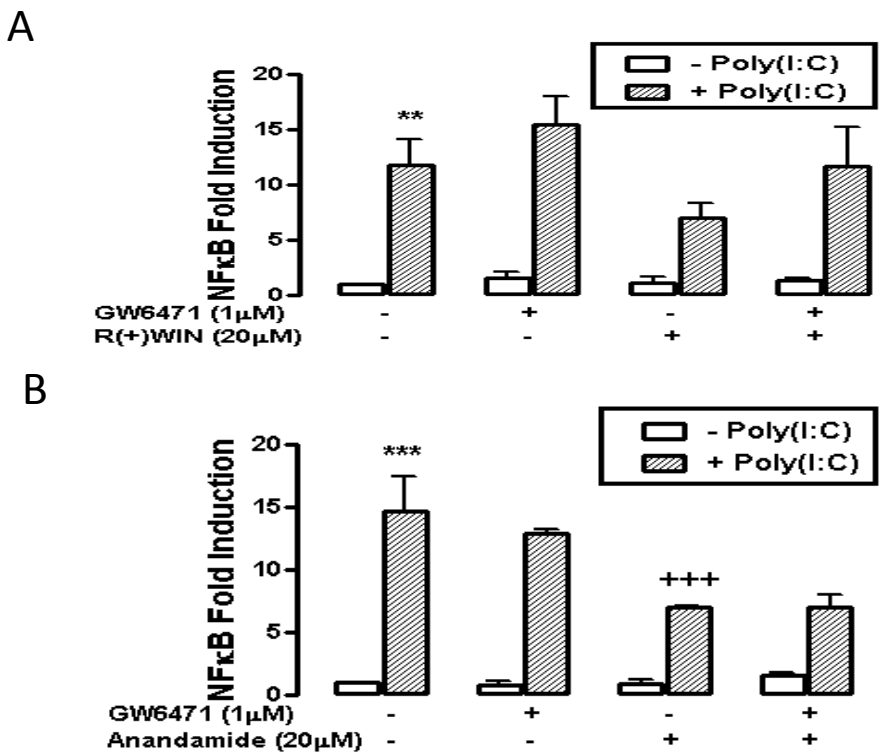


Figure 4.22 Effects of PPARα antagonism on the regulatory effects of cannabinoids on the Poly(I:C)-induced activation of NFκB in HEK293 TLR3 cells.

HEK293 cells stably transfected with TLR3 were co-transfected with a NFκB-regulated firefly luciferase reporter (80 ng), phRL-TK (constitutively expressed Renilla luciferase) (40 ng), and pcDNA3.1 (120 ng). Cells were allowed to recover overnight then challenged for 1 h with GW6471 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) (A) or Anandamide (20μM) (B) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. **P < 0.05 and ***P < 0.001 compared with vehicle-treated cells. +++P < 0.001 compared with Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (10, 22) = 12.64. P < 0.001 (B) F value (10, 22) = 36.18 P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

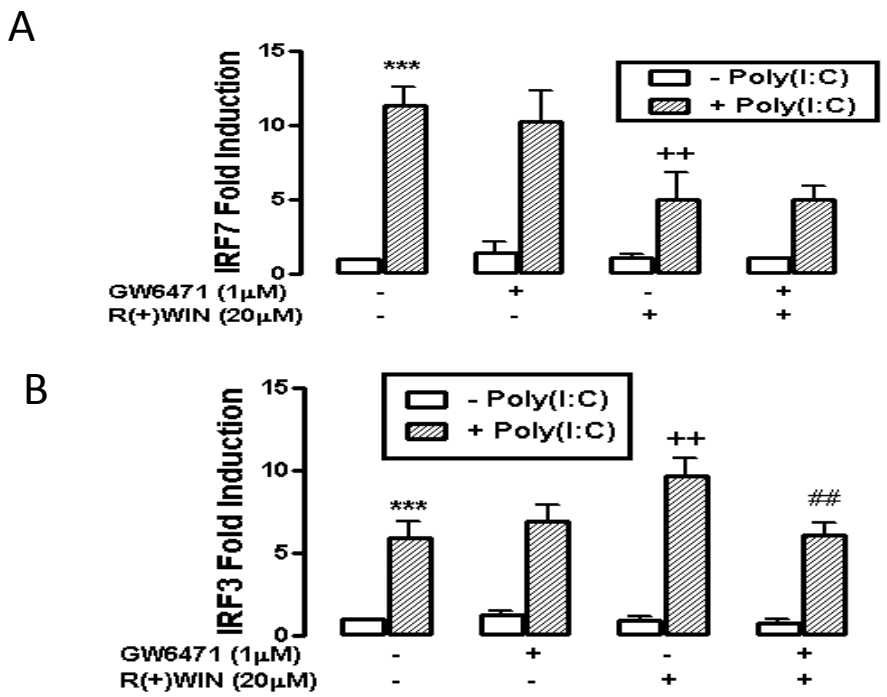


Figure 4.23 Effects of PPARα antagonism on the regulatory effects of R(+)WIN55,212-2 on the Poly(I:C)-induced activation of IRF7 and IRF3 in HEK293 TLR3 cells.

HEK293 cells stably transfected with TLR3 were co-transfected with the trans-activator plasmid pFA-IRF-7 (25ng), pFR-Luc (60ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (145ng) (A). In a separate assay HEK293 cells stably transfected with TLR3 were co-transfected with the trans-activator plasmid pFA-IRF-3 (30ng), pFR-Luc (60ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (140ng) (B). Cells were allowed to recover overnight then challenged for 1 h with GW6471 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. ++$P < 0.01$ compared with Poly(I:C)-treated cells. ##$P < 0.01$ compared with cells treated with R(+)WIN55,212-2 in the presence of Poly(I:C). One way ANOVA effect of treatment (A) F value (10, 19) = 20.84. $P < 0.001$ (B) F value (10, 19) = 26.77 $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

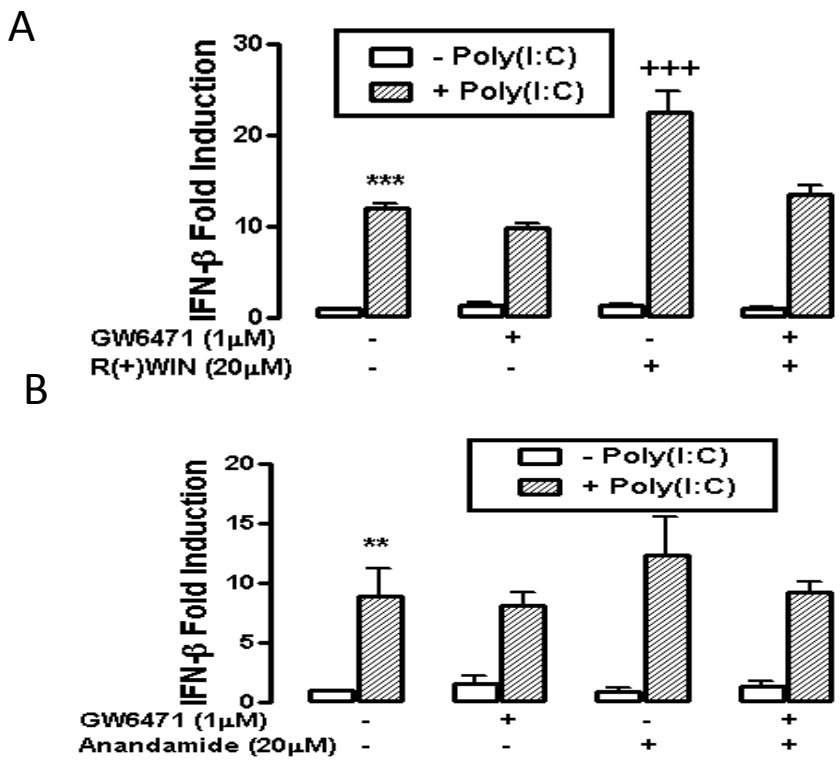


Figure 4.24 Effects of PPARα antagonism on the regulatory effects of cannabinoids on the Poly(I:C)-induced activation of the IFN-β promoter in HEK293 TLR3 cells.

HEK293 cells stably transfected with TLR3 cells were co-transfected with IFN-β luciferase reporter plasmid (80ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (120 ng). Cells were allowed to recover overnight then challenged for 1 h with GW6471 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) (A) or Anandamide (20μM) (B) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. **P < 0.01 and ***P < 0.001 compared with vehicle-treated cells. +++P < 0.001 compared with Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (10, 19) = 73.17. P < 0.001 (B) F value (10, 19) = 10.45 P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

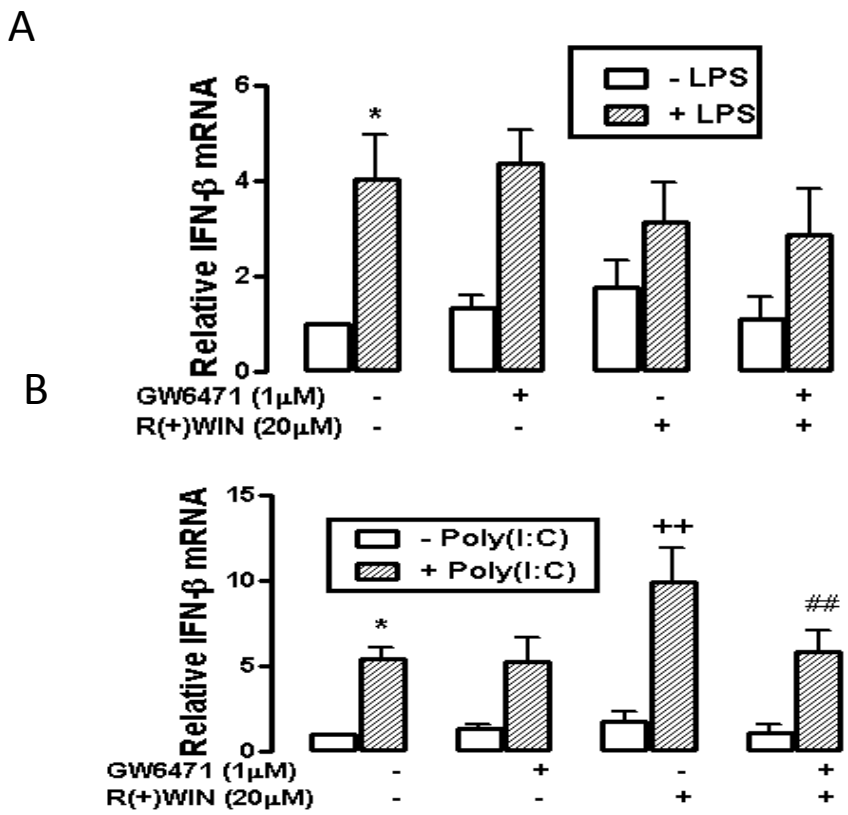


Figure 4.25 Effects of PPARα antagonism on the regulatory effects of R(+)WIN55,212-2 on IFN-β mRNA expression in BMDM cells.

BMDM cells were pre-treated for 1 h with GW6471 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of LPS (100ng/ml) (A) or Poly(I:C) (25μg/ml) (B) for a further 16 h. RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. *P < 0.05 compared with vehicle-treated cells. ++P < 0.01 compared with Poly(I:C)-treated cells. ##P < 0.01 compared with cells treated with R(+)WIN55,212-2 in the presence of Poly(I:C).

One way ANOVA effect of treatment (A) F value (10, 19) = 5.465. P < 0.001 (B) F value (10, 19) = 13.41 P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

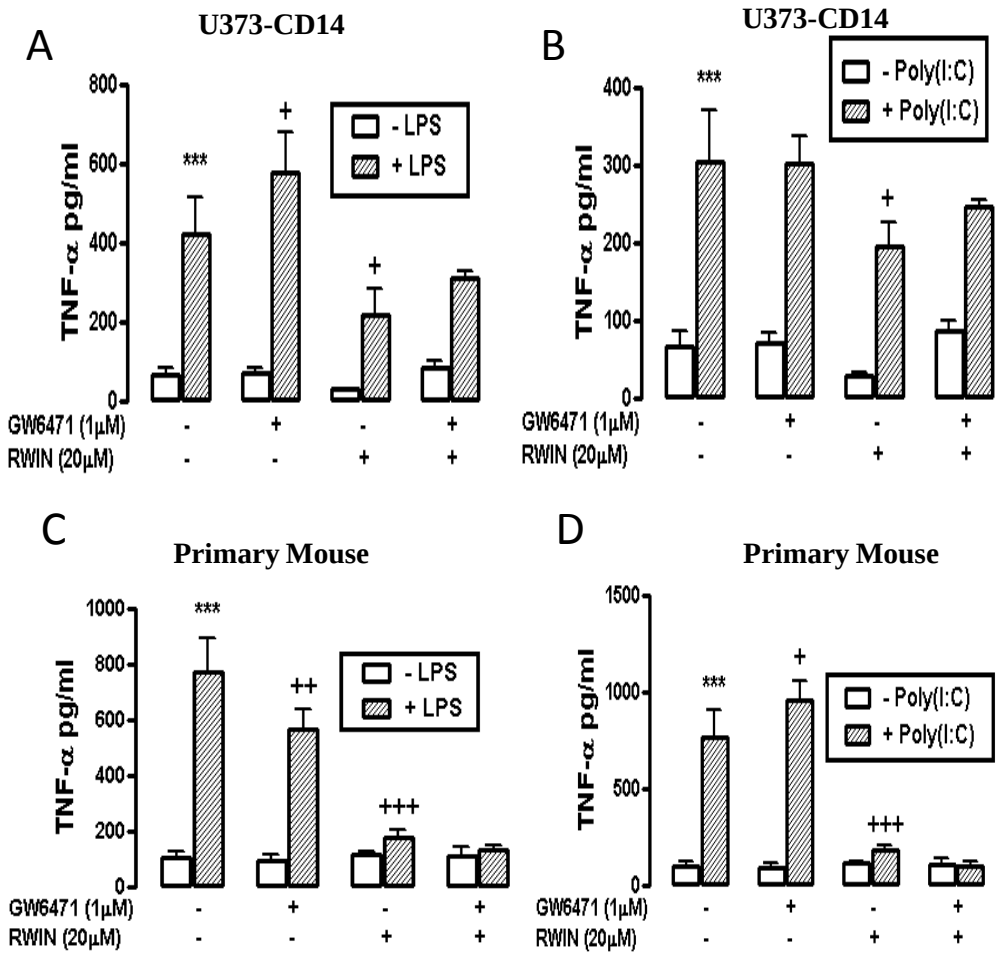


Figure 4.26 Effects of PPARα antagonism on the regulatory effects of R(+)WIN55,212-2 on TNF-α expression in U373-CD14 astrocytoma cells and primary mouse astrocytes.

U373-CD14 astrocytoma cells (A+B) and primary astrocyte cultures prepared from neonatal murine brains (C+D) were pre-treated for 1 h with GW6471 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 24 h. Supernatants were assayed for TNF-α levels by sandwich ELISA. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$, ++$P < 0.01$ and +++$P < 0.001$ compared with LPS or Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (10, 22) = 16.99. $P < 0.001$ (B) F value (10, 22) = 21.74. $P < 0.001$ (C) F value (10, 44) = 28.70. $P < 0.001$ (D) F value (10, 44) = 34.09. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments (five independent experiments for primary mouse).

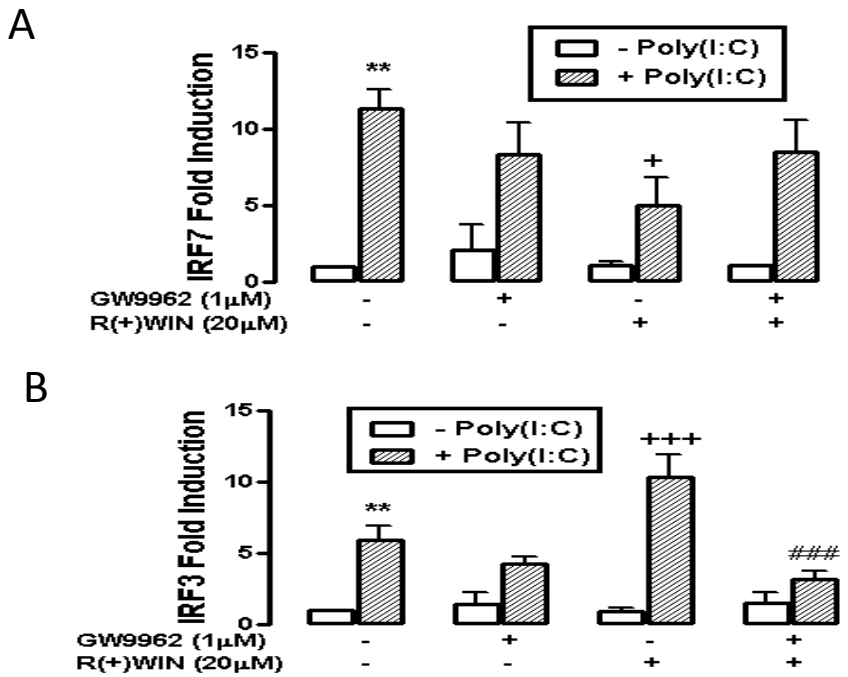


Figure 4.27 Effects of PPARγ antagonism on the regulatory effects of R(+)WIN55,212-2 on the Poly(I:C)-induced activation of IRF7 and IRF3 in HEK293 TLR3 cells.

HEK293 cells stably transfected with TLR3 were co-transfected with the trans-activator plasmid pFA-IRF-7 (25ng), pFR-Luc (60ng), phRL-TK (constitutively expressed Renilla luciferase) (40ng) and pcDNA3.1 (145ng) (A). In a separate assay HEK293 cells stably transfected with TLR3 were co-transfected with the trans-activator plasmid pFA-IRF-3 (30ng), pFR-Luc (60ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (140ng) (B). Cells were allowed to recover overnight then challenged for 1 h with GW9662 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. **P < 0.01 compared with vehicle-treated cells. +P < 0.05 and +++P < 0.001 compared with Poly(I:C)-treated cells. ###P < 0.01 compared with cells treated with R(+)WIN55,212-2 in the presence of Poly(I:C). One way ANOVA effect of treatment (A) F value (10, 22) = 13.38. P < 0.001 (B) F value (10, 22) = 20.96 P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

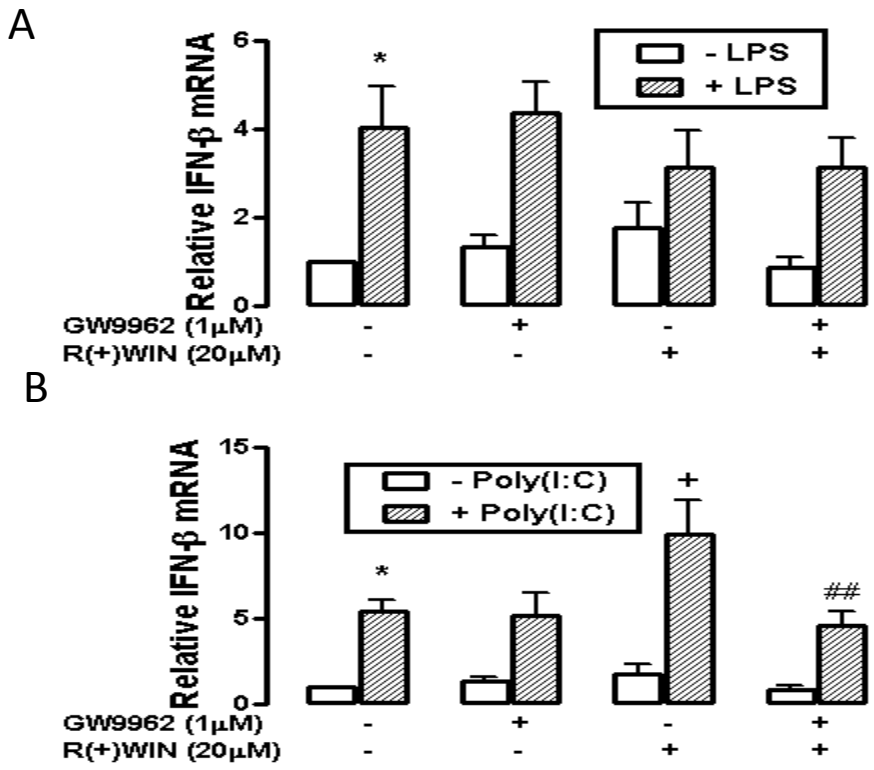


Figure 4.28 Effects of PPARγ antagonism on the regulatory effects of R(+)WIN55,212-2 on IFN-β mRNA expression in BMDM cells.

BMDM cells were pre-treated for 1 h with GW9662 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of LPS (100ng/ml) (A) or Poly(I:C) (25μg/ml) (B) for a further 16 h. RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. *P < 0.05 compared with vehicle-treated cells. +P < 0.05 compared with Poly(I:C)-treated cells. ##P < 0.01 compared with cells treated with R(+)WIN55,212-2 in the presence of Poly(I:C).

One way ANOVA effect of treatment (A) F value (10, 19) = 5.465. P < 0.001 (B) F value (10, 22) = 17.04 P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

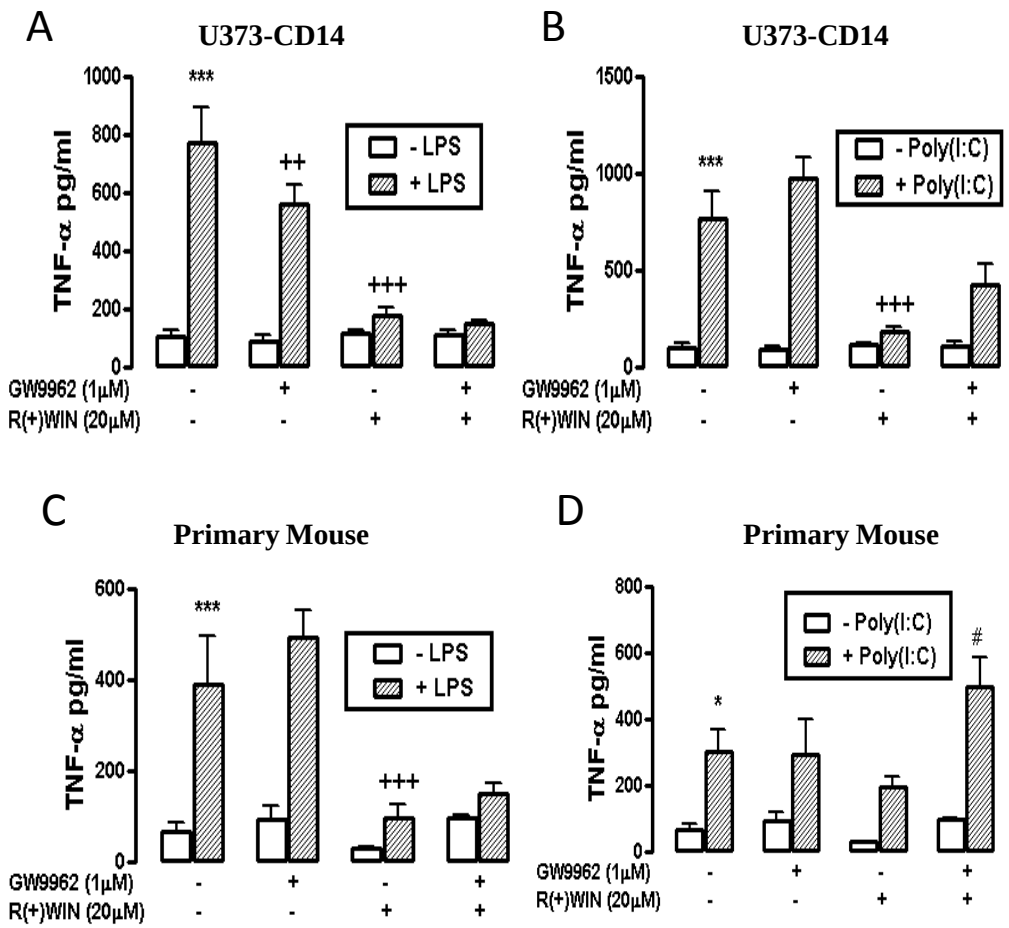


Figure 4.29 Effects of PPARγ antagonism on the regulatory effects of R(+)WIN55,212-2 on TNF-α expression in U373-CD14 astrocytoma cells and primary mouse astrocytes.

U373-CD14 astrocytoma cells (A+B) and primary astrocyte cultures prepared from neonatal murine brains (C+D) were pre-treated for 1 h with GW9662 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 24 h. Supernatants were assayed for TNF-α levels by sandwich ELISA. *$P < 0.05$ and ***$P < 0.001$ compared with vehicle-treated cells. ++$P < 0.01$ and +++$P < 0.001$ compared with LPS or Poly(I:C)-treated cells. #$P < 0.05$ compared with cells treated with R(+)WIN55,212-2 in the presence of Poly(I:C). One way ANOVA effect of treatment (A) F value (10, 22) = 16.73. $P < 0.001$ (B) F value (10, 19) = 8.876. $P < 0.001$ (C) F value (10, 44) = 25.65. $P < 0.001$ (D) F value (10, 44) = 31.32. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments (five independent experiments for primary mouse).

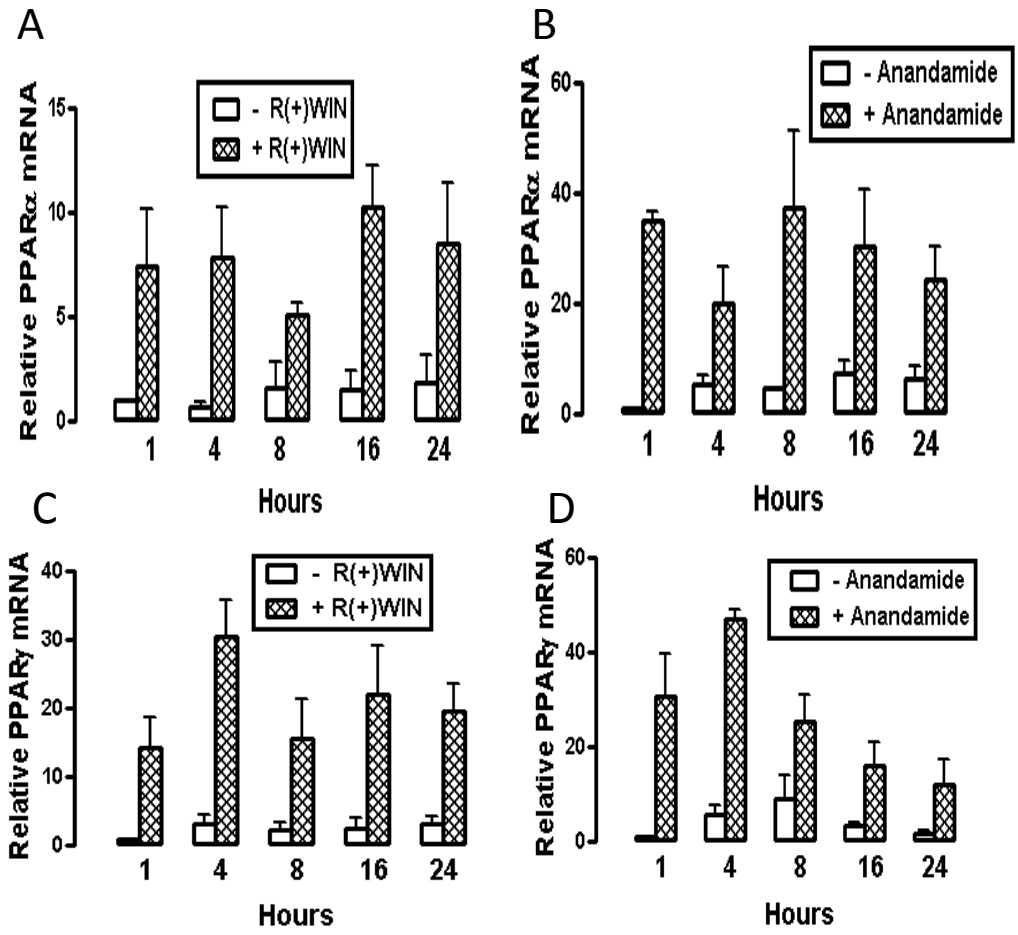


Figure 4.30 R(+)WIN55,212-2 and Anandamide regulatory effects on PPARα mRNA and PPARγ mRNA in BMDM cells.

BMDM cells were treated at various time points with or without R(+)WIN55,212-2 (20μM) or Anandamide (20μM). RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of PPARα (A+B) and PPARγ (C+D) mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Results are mean +/- S.E.M. of three independent experiments .

Summary of Results

Luciferase Assay (NFκB)	
HEK293 TLR3 , HEK293 TLR4 and U373-CD14	Anandamide and Fenofibrate **inhibit** LPS and Poly(I:C)-induced activation of NFκB
Luciferase Assay (IRF7)	
HEK293 TLR3 and HEK293 TLR4	Anandamide and Fenofibrate **inhibits** LPS and Poly(I:C)-induced activation of IRF7
U373-CD14	Fenofibrate, **inhibits** LPS and Poly(I:C)-induced activation of IRF7
Luciferase Assay (IRF3 & IFN-β promoter)	
HEK293 TLR3 and HEK293 TLR4	Anandamide and Fenofibrate **inhibit** LPS-induced activation of IRF3 Anandamide and Fenofibrate **augment** Poly(I:C)-induced activation of IRF3 & IFN-β promoter
U373-CD14	Fenofibrate **augments** Poly(I:C)-induced activation of IRF3 & IFN-β promoter

Summary of Results	
ELISA (TNF-α)	
U373-CD14	Anandamide and Fenofibrate **inhibit** LPS and Poly(I:C) induction of TNF-α expression
BMDM	Fenofibrate **inhibits** LPS and Poly(I:C) induction of TNF-α expression
QRT-PCR (IFN-β mRNA)	
Primary Rat Astrocytes and BMDM	Anandamide and Fenofibrate **inhibit** LPS-induced activation of IFN-β mRNA Anandamide and Fenofibrate **augment** Poly(I:C)-induced activation of IFN-β mRNA

Chapter 5

Defining the molecular target for the action of WIN55,212-2 on TLR3 signalling

5.1 Introduction

TLR4 is widely recognised for its ability to induce the transcription of many pro-inflammatory targets, while TLR3 activation is a strong inducer of anti-viral genes. Upon ligand activation of TLR3, a TRIF-dependent/MyD88-independent signalling response occurs to regulate IRF3 and late phase NFκB activity. TRIF interacts with TLR3 through its TIR domain while its N terminal domain forms a complex with TBK1/IKKε (Fitzgerald et al., 2003).

When first characterised, it was thought that the major role of TBK1 and IKKε was to activate NFκB (Tojima et al., 2000). Later studies demonstrated that when murine double TBK1/IKKε knockout cells were treated with various inflammatory stimuli, there was no effect on NFκB-dependent gene transcription (Hemmi et al., 2004). Instead, it was found that TBK1/IKKε play an essential role in regulating the production of type I interferons by phosphorylating IRF3 on its C-terminal regulatory domain (Fitzgerald et al., 2003). Despite these results, numerous reports continue to publish and propose roles for TBK1/IKKε in phosphorylating and regulating nuclear accumulation of the NFκB subunits; p65 and c-Rel (Harris et al., 2006, Buss et al., 2004a, Mattioli et al., 2006).

To date, TBK1 and IKKε appear to be the main kinases involved in the catalysing phosphorylation of IRF3. Gene targeting and knockout studies have revealed that each kinase may behave in an independent manner with respect to IRF3 activation (Hemmi et al., 2004, McWhirter et al., 2004). For example, cells deficient in TBK1 exhibit severe impairment of IRF3 activation, nuclear translocation and subsequent induction of IFN-β and regulated on activation normal T cell expressed and secreted (RANTES) in response to Poly(I:C) or LPS stimulation. Despite this, some residual gene expression was still apparent, presumably due to the action of IKKε. On the other hand, IKKε deficient cells displayed normal expression of IRF3 target genes in response to dsRNA, suggesting that TBK1 can fully compensate for the lack of IKKε. Therefore, it is proposed that both TBK1 and, albeit to a lesser extent, IKKε, are necessary for the activation of IRF3 and the induction of IFN-inducible genes.

IRF3 contains two phosphorylation sites within its C terminal domain which plays crucially important roles in regulating the activation of IRF3. Site 1 includes Ser385 and Ser386 residues while site 2 includes the residues Ser396, Ser398, Ser402, Thr404 and Ser405 (Tamura et al., 2008). The activation of IRF3 appears to be a

sequential phosphorylation process where various groups have reported on the phosphoacceptor site(s) targeted by TBK1/IKKε. Ser396 appears to be a key residue in IRF3 activation that is initially phosphorylated (Lin et al., 1998) and is required for IRF3 dimerisation (Servant et al., 2003). However as phosphoacceptor sites in site 1 are the most accessible amino acids, others have demonstrated that Ser386 could be the initial and critical target for TBK1/IKKε mediated phosphorylation (Mori, Yoneyama et al. 2004) and IRF3 dimerisation (Takahasi et al., 2010). A mutation of Ser386 to an alanine residue abolishes the ability of IRF3 to dimerise, a function that is critical in the nuclear translocation of IRF3 (Mori et al., 2004). Furthermore, *in vitro* assays using either recombinant or immunoprecipitated kinases from overexpressing cells and truncated recombinant IRF3, suggest that Ser402 is also targeted by TBK1/IKKε (Sharma et al., 2003, tenOever et al., 2004). As a result, the precise sites targeted by TBK1/IKKε remain to be characterised.

A recent study has reported on the ability of c-Jun-NH_2-terminal kinase (JNK) to phosphorylate IRF3 in its N terminal domain (Zhang et al., 2009). Consequently, TAK1 (a common activator of NFκB, p38 and JNK pathways) can stimulate phosphorylated IRF3 via JNK without interfering with the C terminus of IRF3. IRF3 can also be phosphorylated within its N terminus in response to stress inducers including anisomycin, sorbitol, and DNA-damaging agents such as doxorubicin (Servant et al., 2001). LPS has also been shown to induce N-terminal phosphorylation of IRF3 (Solis et al., 2007).

Under basal conditions IRF3 remains in the cytoplasm but following TBK1/IKKε-mediated phosphorylation, IRF3 dimerises and translocates to the nucleus. Upon nuclear localisation and preceding DNA binding, IRF3 associates with the co-activator CBP/p300 on its hydrophobic surface (Kumar et al., 2000, Lin et al., 1998). The direct interaction of CBP/p300 with DNA is indispensable for the DNA binding activity of phosphorylated IRF3 (Suhara et al., 2002). Upon association with CBP/p300, IRF3 binds to the PRDI/III elements of the IFN-β promoter (Thanos and Maniatis, 1995) or the PRD-like element of the IFNα4 promoter. IFNβ and IFNα4 subsequently signal via IFNAR and the JAK/STAT pathway to activate the ISGF3 transcriptional regulator which induces IRF7 expression (Fig 1.3). In a similar manner to IRF3, phosphorylated IRF7 translocates to the nucleus and binds to PRD on the IFN-α promoter.

The activation of IRF3 is not exclusive to TLR signalling events. RIG-I and MDA5 (Fig 1.2) are two cytoplasmic PRRs that recognise dsRNA independently of TLR3 and function in the activation of IRF3. RIG-I and MDA5 both contain a C terminal DExD/H box RNA helicase domain, a central ATPase domain and two N-terminal caspase-recruitment domain (CARD)-like domains (Yoneyama et al., 2004). Following ligand binding, both proteins undergo a conformational shift facilitated by their ATPase domain. This allows for recruitment of the downstream adaptor protein, IFN-β promoter stimulator (IPS1) via their CARD domains (Potter et al., 2008). IPS1 is localised to mitochondria where it initiates diverse signalling pathways that culminate in the activation of IRF3 and NFκB. IPS1 interacts with TRAF3 via its C-terminal domain (Saha et al., 2006). TRAF3 subsequently forms a multiprotein signalling complex with TBK1/IKKε to induce IRF3 phosphorylation and nuclear translocation.

The compound, BX-795, was originally developed as a small molecular inhibitor of 3-phosphoinositidedependent protein kinase 1 (PDK1) (Feldman et al., 2005). However it has recently been demonstrated that BX-795 can inhibit TBK1 and IKKε at low nanomolar concentrations (Bain et al., 2007) and is now been recognised as a potent and relatively competitive inhibitor of TBK1/IKKε (Clark et al., 2009). Indeed, BX-795 has previously been reported to be unsuccessful in blocking the transcriptional activity of NFκB by TLR ligands and TNF-α stimulation (Clark et al., 2009). Furthermore, BX-795 does not inhibit IKKβ (Bain et al., 2007) or IKKα (Clark et al., 2009), critical kinases involved in the nuclear translocation of NFκB. In relation to IRF3 regulation by TBK1/IKKε, treatment of macrophages with BX-795 was shown to block the phosphorylation, nuclear translocation and transcriptional activity of IRF3 and also IFN-β production induced by TLR3 and TLR4 stimulation (Clark et al., 2009).

In the previous chapter it was intriguing to note that cannabinoids and fenofibrate augmented TLR3 signalling. Given that the TLR3 pathway employs the TRIF/TBK1/IKKε/IRF3 axis, we next aimed to identify the molecular targets for the pharmacological agents in this pathway. This also offered the potential to further understand the molecular basis to the therapeutic effects of cannabinoids and fenofibrate with relevance to M.S.

5.2 Results

5.2.1 R(+)WIN55,212-2 targets TRIF-meditated signalling.

In the preceding chapters, it was observed that R(+)WIN55,212-2 augments Poly(I:C)-induced activation of IRF3. In order to pinpoint the molecular targets that mediate this effect, the sensitivity of TRIF-mediated signalling to the pharmacological agent was initially explored.

HEK293 TLR3 cells were transfected with the trans-activator plasmid pFA-IRF3 (where IRF3 is fused downstream of the yeast Gal4 DNA binding domain) and with or without a construct encoding TRIF. Following transfection, in separate assays, cells were treated with or without various concentrations of R(+)WIN55,212-2 or S(-)WIN55,212-2 for 7 hours. Overexpression of TRIF promoted strong induction of IRF3-regulated luciferase expression (Fig. 5.1A) and this was dose-dependently augmented by R(+)WIN55,212-2 (Fig. 5.1A). This effect was again stereoselective since S(-)WIN55,212-2 failed to affect the ability of TRIF to activate IRF3 (Fig.5.1B). These findings suggested that R(+)WIN55,212-2 can directly target TRIF-mediated signalling.

5.2.2 Regulatory effects of R(+)WIN55,212-2 on TLR induction of IFN-β mRNA in TRIF-deficient cells

TRIF-deficient BMDM (TRIF KO) cells were next employed to evaluate the importance of TRIF in manifesting the positive effects of R(+)WIN55,212-2 on IFN-β mRNA expression. The responsiveness to Poly(I:C) and LPS was greatly reduced in TRIF KO cells with only modest induction of IFN-β mRNA following ligand treatment (Fig. 5.2A and Fig. 5.2C). Interestingly, R(+)WIN55,212-2 failed to modulate this effect in response to Poly(I:C) treatment (Fig. 5.2C), thus further suggesting that R(+)WIN55,212-2 targets a TRIF-mediated pathway.

In LPS treated cells, pre-exposure of the cells to R(+)WIN55,212-2 (Fig 5.2A) inhibited LPS-induced activation of IFN-β mRNA expression which is consistent with its effect in wild type BMDM cells. S(-)WIN55,212-2 failed to influence IFN-β mRNA expression by LPS (Fig. 5.2B) or Poly(I:C) (Fig. 5.2D) which again verifies specificity for the WIN55,212-2 compound.

5.2.3 Use of the TBK1/IKKε inhibitor, BX-795, to explore TBK1/IKKε as possible targets for R(+)WIN55,212-2.

As TRIF is an upstream regulator of TBK1 and IKKε (Fitzgerald et al., 2003), the importance of these kinases in manifesting the regulatory effects of R(+)WIN55,212-2 were next examined by using the potent and selective TBK1/IKKε inhibitor; BX-795. Given the previous controversy with respect to regulation of the NFκB pathway by BX-795 (Clark et al., 2009), it was necessary to investigate the sensitivity of NFκB activation to BX-795 in our experimental systems. This was evaluated by treating HEK293 TLR3 cells with BX-795 (1μM) for 1 hour followed by treatment with R(+)WIN55,212-2 or S(-)WIN55,212-2 (20μM) for 1 hour and subsequent Poly(I:C) treatment for 6 hours. In both assays, treatment with BX-795 failed to affect the ability of Poly(I:C) to regulate NFκB-regulated luciferase expression. As previously observed, R(+)WIN55,212-2 inhibited Poly(I:C)-induced activation of NFκB whereas S(-)WIN55,212-2 was ineffective. The inhibitory effects of R(+)WIN55,212-2 on NFκB were partially reversed but not totally removed by BX-795 treatment (Fig. 5.3A). As expected, S(-)WIN55,212-2 was insensitive to BX-795 treatment (Fig. 5.3B).

In order to further explore the effects of BX-795 on R(+)WIN55,212-2 and the NFκB pathway, their regulatory potential on the expression of the NFκB-responsive gene, TNF-α, was explored. This was examined in two model systems; U373-CD14 astrocytoma cells and primary mouse astrocytes. Both cells types were treated with for BX-795 (1μM) for 1 hour, prior to treatment with R(+)WIN55,212-2 (20μM) for 1 hour and Poly(I:C) treatment for 24 hours. R(+)WIN55,212-2 inhibited Poly(I:C) induced expression of TNF-α in both cell types (Fig. 5.4A and Fig 5.4B) with almost complete abolition of TNF-α expression in primary mouse astrocytes (Fig 5.4B). Pre-treatment of both cells types with BX-795 failed to affect the regulatory effects of R(+)WIN55,212-2 on TNF-α expression suggesting that the inhibitory activity of R(+)WIN55,212-2 on pro-inflammatory gene expression is independent of TBK1/IKKε.

5.2.4 Regulatory effects of R(+)WIN55,212-2 on TLR3 induced activation of IRF3, the IFN-β promoter and IFN-β mRNA is dependent on the TLR3-TRIF-TBK1/IKKε signalling pathway.

Given that TBK1/IKKε are upstream kinases of IRF3, the role of these kinases as potential targets for R(+)WIN55,212-2 was next examined. BX-795 was employed for this purpose. Cells were treated with BX-795 (1μM) for 1 hour followed by treatment with R(+)WIN55,212-2 (20μM) for 1 hour and Poly(I:C) stimulation for 6 hours. As expected, BX-795 showed strong inhibitory effects on Poly(I:C)-induced activation of IRF3 (Fig. 5.5A) and the IFN-β promoter (Fig. 5.5B). Some residual activity was apparent in both cases but in the presence of BX-795 neither the activation of IRF3 (Fig. 5.5A) or the IFN-β promoter (Fig. 5.5B) was augmented by. R(+)WIN55,212-2. This suggests that the latter requires functional TBK1/IKKε in order to enhance the IRF3 pathway.

The transcriptional regulation of IFN-β mRNA was also assessed in BMDM cells. Similar to the above data, BX-795 strongly inhibited Poly(I:C)-induced expression of IFN-β mRNA and the residual expression in the presence of BX-795 was not potentiated by R(+)WIN55,212-2 (Fig. 5.6A). This again confirms the need for the catalytic activity of TBK1 and IKKε in order for R(+)WIN55,212-2 to manifest its anti-inflammatory effects. .

5.2.5 R(+)WIN55,212-2 enhances phosphorylation of IRF3 by TLR3 ligand activation.

Due to the ability of R(+)WIN55,212-2 to augment Poly(I:C)-induced activation of IRF3 in a TBK1/IKKε dependent manner, it was next examined whether R(+)WIN55,212-2 could regulate the phosphorylation status of IRF3. BMDM cells were treated with R(+)WIN55,212-2 (20μM) for 1 hour followed by Poly(I:C) treatment at various time points (30, 60, 120 and 180 minutes). To establish if R(+)WIN55,212-2 could increase the C-terminal phosphorylation of IRF3, the lysates were immunoblotted for Ser396, a critical residue in IRF3 phosphorylation. Increased phosphorylation of Ser396, albeit to modest levels, was detected after Poly(I:C) treatment time of 60 minutes and this response was augmented by R(+)WIN55,212-2 pre-treatment (Fig. 5.7A). BMDM cells were also treated with S(-)WIN55,212-2 and Poly(I:C) in a similar

manner. As shown in (Fig. 5.7B), pre-treatment with S(+)WIN55,212-2 had no effect on the Poly(I:C)-induced phosphorylation of Ser396 which again confirms the specificity of the regulatory effects of R(+)WIN55,212-2.

5.2.6 R(+)WIN55,212-2 enhances nuclear localisation of IRF3.

Due the ability of R(+)WIN55,212-2 to enhance the Poly(I:C)-induced phosphorylation of IRF3 and as phosphorylation of IRF3 is a pre-requisite to nuclear translocation of the transcription factor, the regulatory effects of R(+)WIN55,212-2 on subcellular localisation of IRF3 was assessed in primary mouse astrocytes and HEK293 TLR3 cells, by use of confocal microscopy.

Primary murine astrocytes were pre-treated with R(+)WIN55,212-2 or S(-)WIN55,212-2 (20 μM) for 1 hour prior to Poly(I:C) exposure for an additional hour. As shown in (Fig. 5.8A), IRF3 localises predominantly to the cytoplasm. However, stimulation of cells with Poly(I:C) leads to the nuclear translocation of IRF3. Intriguingly, R(+)WIN55,212-2 promotes the nuclear localisation of IRF3 both in the presence and absence of Poly(I:C) (Fig. 5.8A) whilst S(-)WIN55,212-2 is without effect (Fig. 5.8B).

To further confirm these findings, HEK293 TLR3 cells were transfected with an expression construct encoding GFP-tagged IRF3 (800 ng). Control slides were transfected with EGFP construct (800 ng). Cells were allowed to recover overnight and pre-treated with R(+)WIN55,212-2 (20 μM) for 1 hour prior to Poly(I:C) exposure for an additional hour. The ability of R(+)WIN55,212-2 to promote the nuclear translocation of IRF3 was also confirmed in HEK293 cells by monitoring the translocation of IRF3-GFP fusion protein (Fig. 5.9A). The positive effects of R(+)WIN55,212-2 on enhancing the nuclear localisation of IRF3 provides a novel mechanistic basis to the enhancement of the TLR3-TRIF-TBK1/IKKε-IRF3-IFN-β pathway.

5.3 Discussion

The aim of this chapter was to identify molecular targets that may underpin the regulatory effects of R(+)WIN55,212-2 on the TLR3 pathway. As previously explained, TRIF is a critical adaptor molecule employed in the TLR3 signalling pathway. In overexpression studies, it was found that R(+)WIN55,212-2 augmented TRIF-induced activation of IRF3. TRIF knockout studies also revealed the importance of this adaptor molecule in mediating the positive regulatory effects of the synthetic cannabinoid. Interestingly Poly(I:C) showed some residual activity in TRIF knockout cells. It is not immediately obvious since one would expect total abolition of TLR3 signalling in the absence of TRIF. A plausible explanation may lie in the fact that Poly(I:C) can also trigger cytosolic receptors such as RIG-I or MDA5 and this may account for the residual activity. However in order to trigger the RIG-I pathway, Poly(I:C) would have to gain access to the cytosol. Generally in experimental model systems this is achieved by use of liposomal-based transfection reagents e.g. lipofectamine. In the present study, lipofectamine was not used at the time of addition of Poly(I:C). However, some residual lipopfectamine may have remained in the cell culture from the transfection protocol on the preceding day and this may facilitate entry of Poly(I:C) into the cytosol and its subsequent triggering of the RIG-I pathway. Irrespective of the exact mechanism underlying the ability of Poly(I:C) to signal in TRIF-deficient cells, R(+)WIN55,212-2 fails to augment activation of IRF3 or the induction of IFN-β in the absence of TRIF. These results strongly suggest that R(+)WIN55,212-2 targets a TRIF-mediated signalling pathway.

The use of the TBK1/IKKε inhibitor; BX-795 strongly suppressed the activation of IRF3 and the IFN-β promoter in response to TLR3 ligand stimulation whilst having no effect on the activation of NFκB. It was interesting to note this lack of effect of BX-795 on NFκB, since previous studies have implicated a role for TBK1/IKKε in promoting the phosphorylation and nuclear accumulation of p65 (Buss et al., 2004b) and in facilitating the nuclear accumulation of c-Rel (Harris et al., 2006). However unlike the present study, the latter studies did not employ Poly(I:C) as a stimulus. Furthermore we also show that the Poly(I:C)-induced expression of the NFκB-responsive gene, TNF-α is insensitive to BX-795. This again confirms the absence of a role for TBK1/IKKε in regulation of the NFκB pathway, at least with respect to TLR3 signalling. It is also worth noting that the selectivity of BX-795 for the IRF3 pathway is

consistent with a previous report using TBK1/IKKε murine knockout models that highlighted important roles for these kinases in the IRF3-IFN-β pathway and not the NFκB pathway (Hemmi et al., 2004).

Interestingly, BX-795 failed to totally abolish the ability of Poly(I:C) to activate IRF3 and induce IFN-β suggesting that some of its signalling may be mediated independently of TBK1/IKKε. This may be due to the recently described role for JNK in phosphorylating IRF3 in its N terminal domain (Zhang et al., 2009) and lead to a situation where IRF3 is activated without requiring the C terminal phosphorylation of IRF3. Such N terminal phosphorylation of IRF3 is also apparent in response to stress inducers (Servant et al., 2001). Thus the residual activity of Poly(I:C) in the presence of BX-795 may be attributable to JNK signalling but whatever the molecular basis, it is clear that R(+)WIN55,212-2 fails to regulate activation of IRF3 and induction of IFN-β in the absence of a functional TBK1/IKKε complex.

Given the positive effects of R(+)WIN55,212-2 on the IRF3 pathway appear to be dependent on TBK1/IKKε and the latter are the immediate upstream kinases of IRF3, the regulatory effects of the synthetic cannabinoid on the phosphorylation status of IRF3 was explored. Intriguingly R(+)WIN55,212-2 enhances Poly(I:C)-induced phosphorylation of IRF3 and this is consistent with its augmentation of Poly(I:C)-induced activation of IRF3 and induction of IFN-β. However, a difference arises when the regulatory effects of R(+)WIN55,212-2 on the subcellular localisation of IRF3 was explored. Intriguingly, even in the absence of Poly(I:C), R(+)WIN55,212-2 appears to facilitate the nuclear accumulation of IRF3. This was surprising given that R(+)WIN55,212-2 failed to affect the basal phosphorylation of IRF3 in the absence of Poly(I:C) and such phosphorylation is required for nuclear translocation. However it should be noted that in detecting phosphorylation of IRF3, a phosphospecific antibody that specifically detects phosphorylation of Ser396 was employed. This does not exclude the possibility that R(+)WIN55,212-2 may regulate the phosphorylation of other sites that may also facilitate nuclear accumulation of IRF3. Indeed, as already described above, JNK is known to phosphorylate IRF3 on its N terminus thus leading to its activation.

Whilst the increased nuclear localisation of IRF3 in response to R(+)WIN55,212-2 may be due to increasing its nuclear translocation another possibility exists that IRF3 may undergo a basal cycle of cytoplasmic-nuclear-cytoplasmic shuttling. This is particularly common for other transcription factors such as NFκB. In

such a scenario, R(+)WIN55,212-2 may sequester IRF3 in the nucleus as part of this shuttling process. It is interesting to speculate from the previous chapter that given the possible role of PPARs in mediating the regulatory effects of R(+)WIN55,212-2, the latter may induce expression of PPARs that can then interact with IRF3 and retain it in the nucleus. This hypothesis is deserving of future exploration.

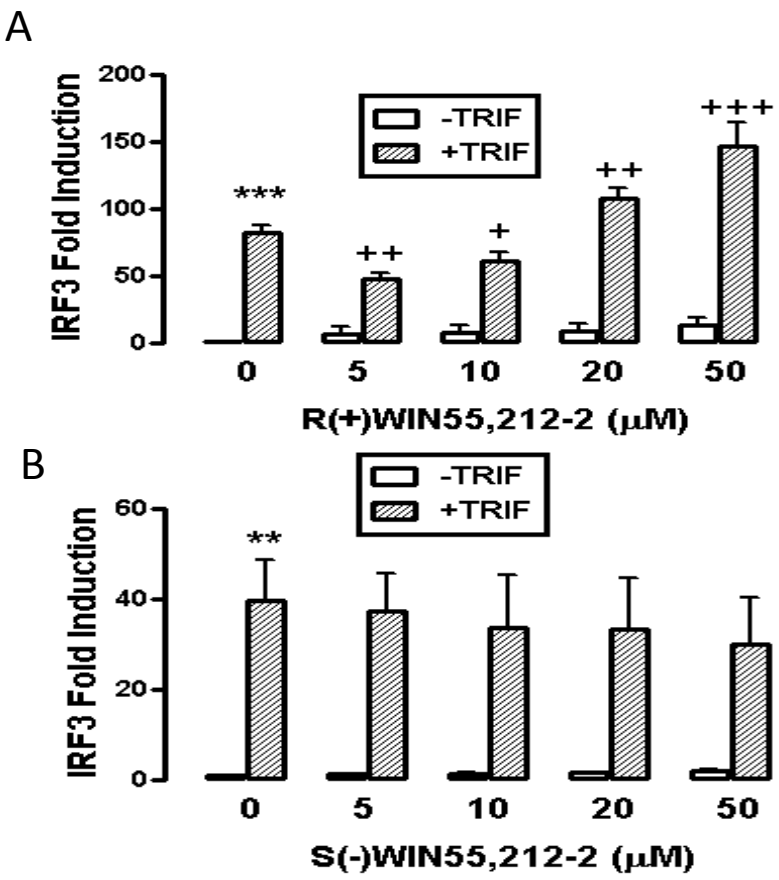


Figure 5.1 R(+)WIN55,212-2 targets TRIF mediated signalling in HEK293 TLR3 cells.

HEK293 cells stably transfected with TLR3 were co-transfected with the trans-activator plasmid pFA-IRF3 (30ng), pFR-Luc (60ng), phRL-TK (constitutively expressed renilla luciferase) (40ng), a construct encoding TRIF (40ng) and pcDNA3.1 (100ng). Cells were allowed to recover overnight and then treated with or without various concentrations of R(+)WIN55,212-2 (A) or S(-)WIN55,212-2 (B) for 7 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. **P < 0.01 and ***P < 0.001 compared with vehicle-treated cells. +P < 0.05, ++P < 0.01 and +++P < 0.001 compared with TRIF-transfected treated cells.

One way ANOVA effect of treatment (A) F value (13, 28) = 31.32. P < 0.001 (B) F value (13, 28) = 9.120. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

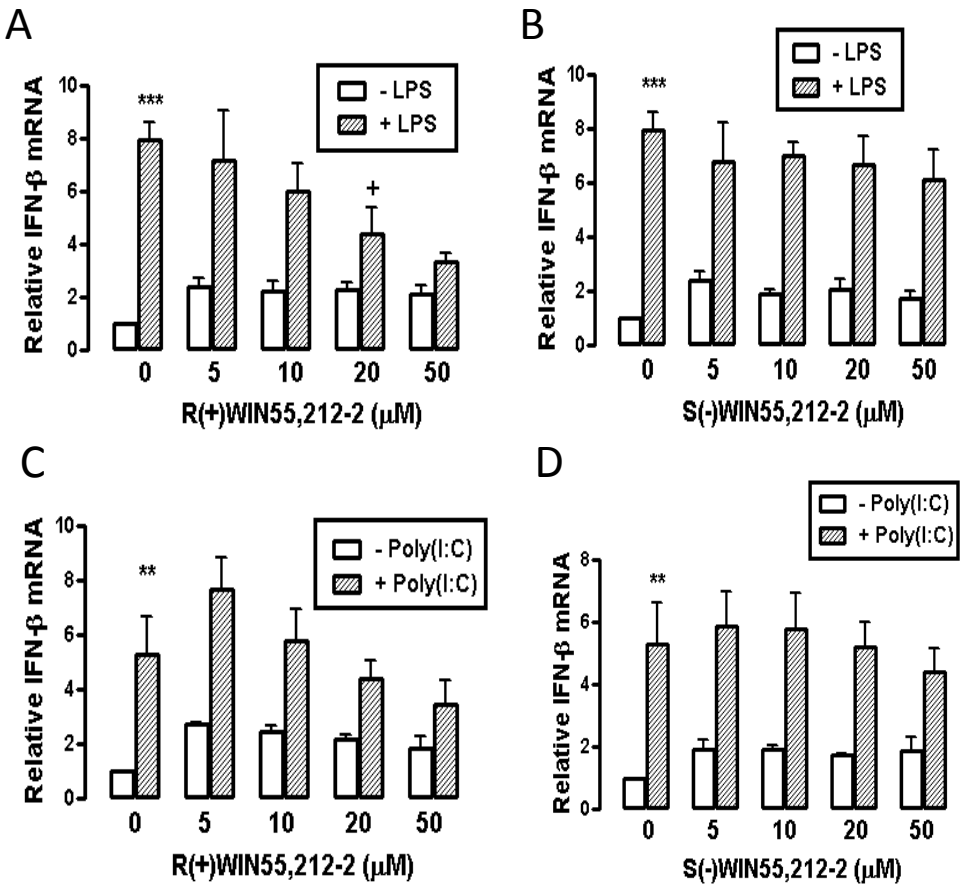


Figure 5.2 R(+)WIN55,212-2 regulatory effects on LPS and Poly(I:C)-induced activation of IFN-β mRNA in TRIF deficient BMDM cells.

TRIF deficient BMDM cells were pre-treated with or without various concentrations of R(+)WIN55,212-2 (A+C) or S(-)WIN55,212-2 (B+D) for 1 h prior to stimulation in the presence or absence of LPS (100ng/ml) or Poly(I:C) (25μg/ml) for a further 16 h.

RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. **P < 0.01 and ***P < 0.001 compared with vehicle-treated cells. +P < 0.05 compared with LPS-treated cells. One way ANOVA effect of treatment (A) F value (13, 24) = 11.58. P < 0.001 (B) F value (13, 24) = 18.20. P < 0.001 (C) F value (13, 24) = 10.25. P < 0.001 (D) F value (13, 28) = 12.76. P < 0.001. Results are mean +/- S.E.M. of three independent experiments.

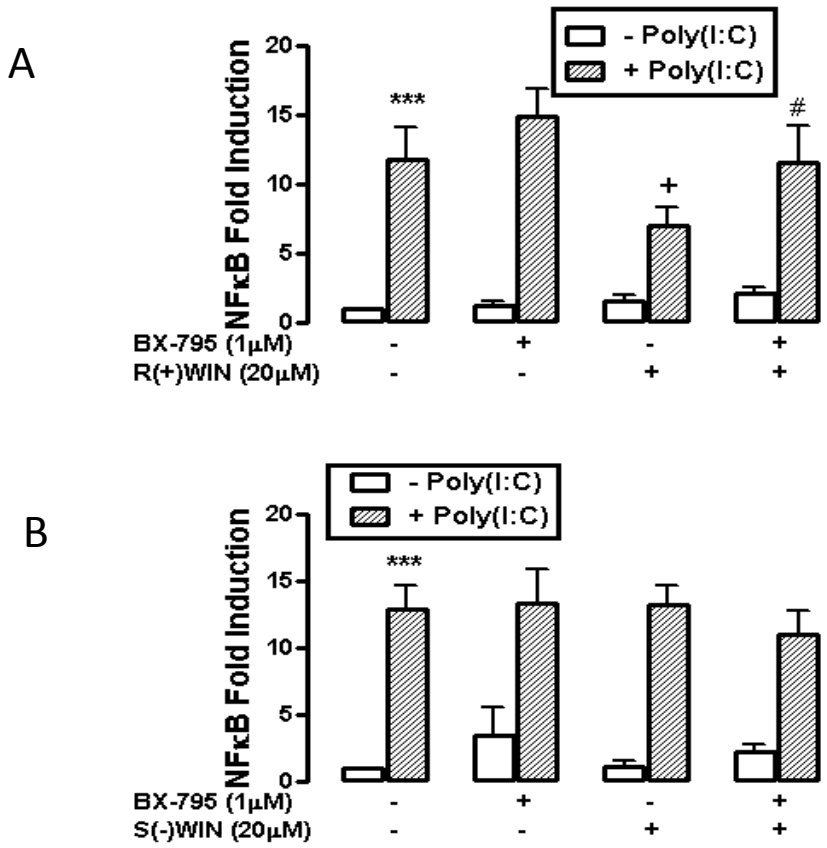


Figure 5.3 R(+)WIN55,212-2 inhibitory effect on Poly(I:C)-induced activation of NFκB in HEK293 TLR3 cells in the TBK1/IKKε signalling pathway.

HEK293 cells stably transfected with TLR3 were co-transfected with a NFκB-regulated firefly luciferase reporter (80 ng), phRL-TK (constitutively expressed Renilla luciferase) (40 ng), and pcDNA3.1 (120 ng). Cells were allowed to recover overnight then challenged for 1 h with BX-795 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) (A) or S(-)WIN55,212-2 (20μM) (B) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$ compared with Poly(I:C)-treated cells. #$P < 0.05$ compared with cells treated with R(+)WIN55,212-2 in the presence of Poly(I:C).

One way ANOVA effect of treatment (A) F value (10, 22) = 12.09. $P < 0.001$ (B) F value (10, 22) = 19.06. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

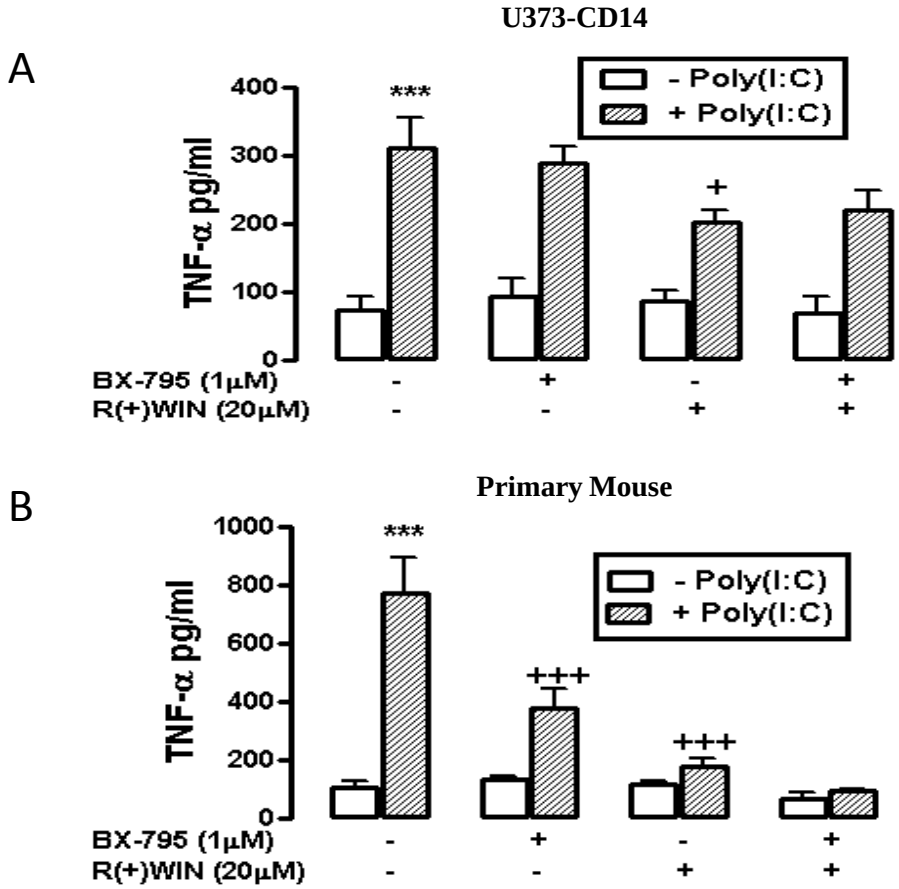


Figure 5.4 R+)WIN55,212-2 inhibitory effect on Poly(I:C)-induced activation of TNF-α expression in U373-CD14 astrocytoma cells and primary mouse astrocytes is independent of the TBK1/IKKε signalling pathway.

U373-CD14 astrocytoma cells (A) and primary astrocyte cultures prepared from neonatal murine brains (B) were challenged for 1 h with BX-795 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 24 h. Supernatants were assayed for TNF-α levels by sandwich ELISA. ***$P < 0.001$ compared with vehicle-treated cells. +$P < 0.05$ and +++$P < 0.001$ compared with Poly(I:C)-treated cells. One way ANOVA effect of treatment (A) F value (10, 22) = 27.78. $P < 0.001$ (B) F value (10, 41) = 27.79. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments (five independent experiments for primary mouse astrocytes).

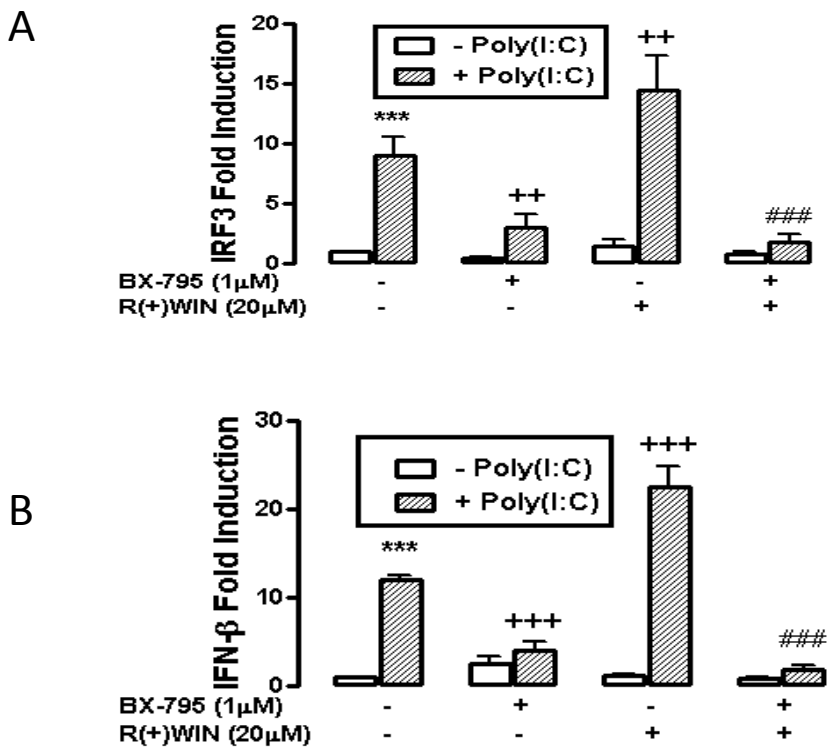


Figure 5.5 R+)WIN55,212-2 regulatory effects on Poly(I:C)-induced activation of IRF3 and the IFN-β promoter in HEK293 TLR3 cells is dependent on the TBK1/IKKε signalling pathway.

HEK293 cells stably transfected with TLR3 were were co-transfected with the trans-activator plasmid pFA-IRF3 (30ng) pFR-Luc (60ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (140ng). In a separate assay, HEK293 cells stably transfected with TLR3 were co-transfected with IFN-β luciferase reporter plasmid (80ng), phRL-TK (constitutively expressed renilla luciferase) (40ng) and pcDNA3.1 (120 ng). Cells were allowed to recover overnight and then challenged for 1 h with BX-795 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) (A+B) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 6 h. Cell extracts were generated and assayed for firefly and Renilla (for normalizing transfection efficiency) luciferase. ***$P < 0.001$ compared with vehicle-treated cells. ++$P < 0.01$ and +++$P < 0.001$ compared with Poly(I:C)-treated cells. ###$P < 0.001$ compared with cells treated with R(+)WIN55,212-2 in the presence of Poly(I:C). One way ANOVA effect of treatment (A) F value (10, 22) = 18.60. $P < 0.001$ (B) F value (10, 22) = 71.14. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.

A

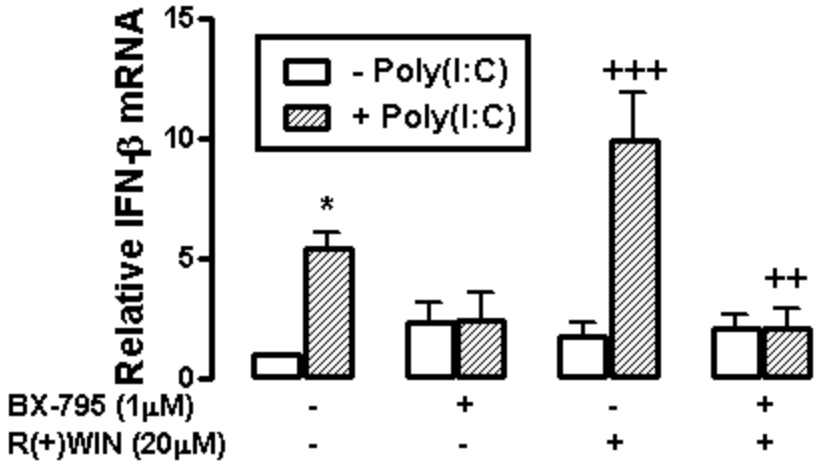
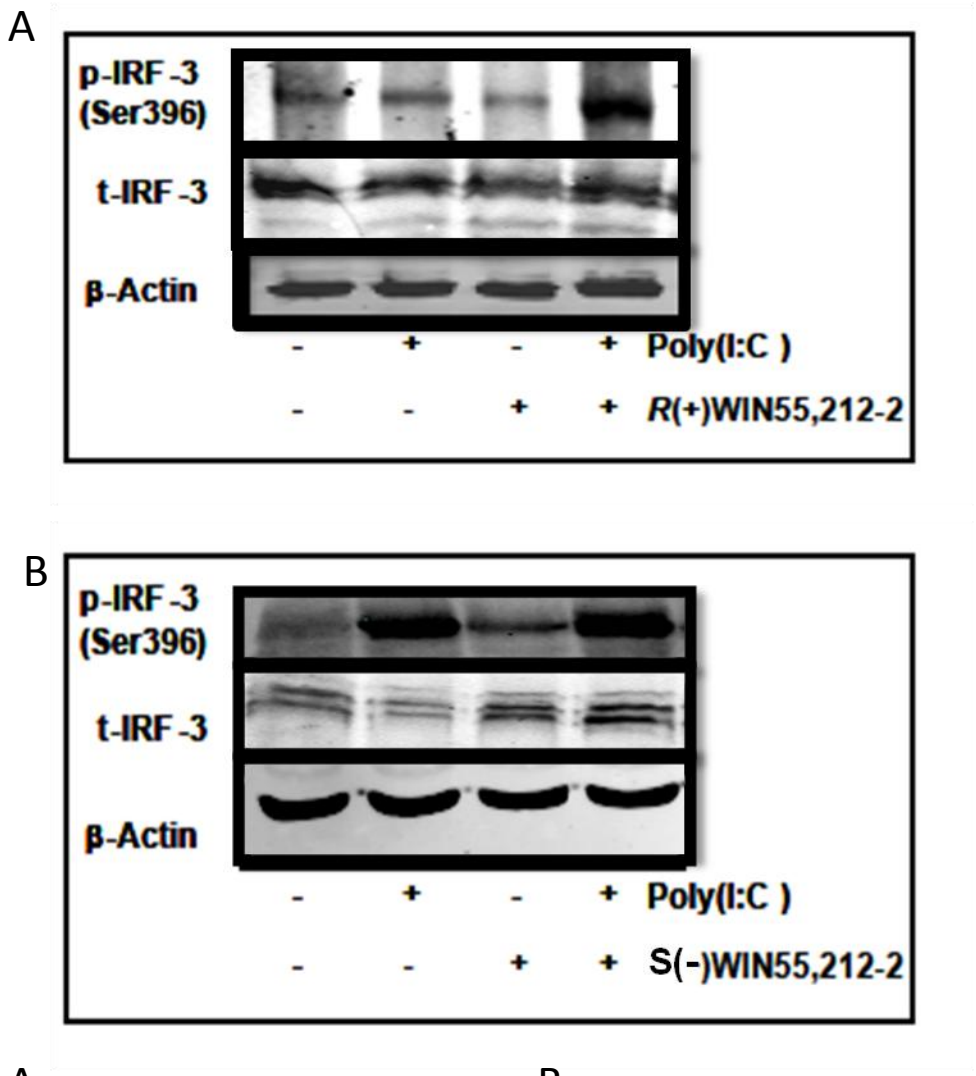


Figure 5.6 R+)WIN55,212-2 regulatory effects on Poly(I:C)-induced activation of IFN-β mRNA in BMDM cells is dependent on the TBK1/IKKε signalling pathway.

BMDM cells were pre-treated for 1 h with BX-795 (1μM) prior to treatment with or without R(+)WIN55,212-2 (20μM) (A) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 16 h. RNA extracts were generated and converted into cDNA. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN-β mRNA. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. *$P < 0.05$ compared with vehicle-treated cells. +++$P < 0.001$ compared with Poly(I:C)-treated cells.

One way ANOVA effect of treatment (A) F value (10, 22) = 12.90. $P < 0.001$. Results are mean +/- S.E.M. of three independent experiments.


A
p-IRF-3
(Ser396)
t-IRF-3
β-Actin
- + - + Poly(I:C)
- - + + R(+)WIN55,212-2
B
p-IRF-3
(Ser396)
t-IRF-3
β-Actin
- + - + Poly(I:C)
- - + + S(-)WIN55,212-2

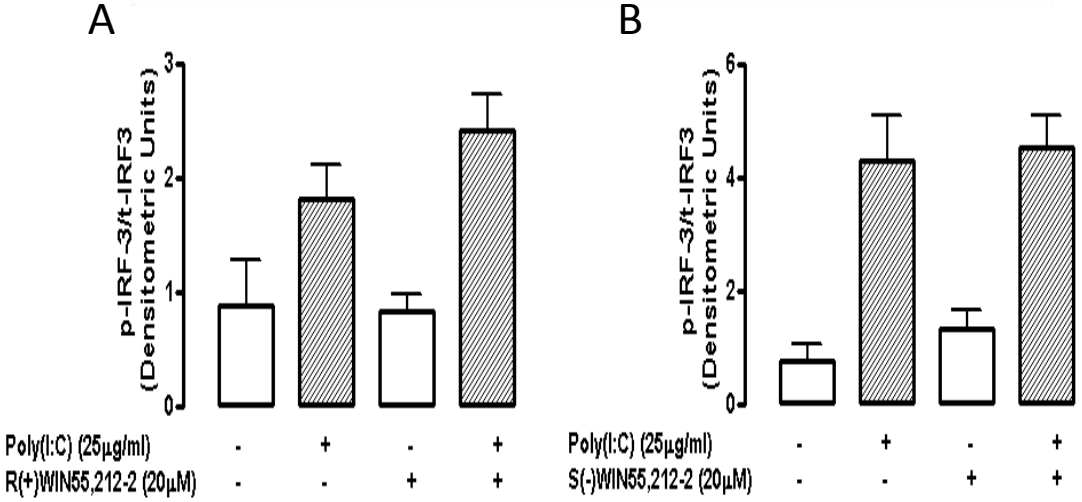

A
p-IRF-3/t-IRF3
(Densitometric Units)
0
1
2
3
Poly(I:C) (25μg/ml) - + - +
R(+)WIN55,212-2 (20μM) - - + +
B
p-IRF-3/t-IRF3
(Densitometric Units)
0
2
4
6
Poly(I:C) (25μg/ml) - + - +
S(-)WIN55,212-2 (20μM) - - + +

Figure 5.7 R(+)WIN55,212-2 enhances Poly(I:C) phosphorylation of IRF3 in BMDM cells.

BMDM cells were pre-treated with or without R(+)WIN55,212-2 (20μM) (A) or S(-)WIN55,212-2 (B) for 1 h and further stimulated in the presence or absence of Poly(I:C) (25μg/ml) for a further 1 h. Cells were harvested in SDS-PAGE sample buffer and subjected to western blotting using antibodies recognizing IRF3, IRF3 phosphorylated at Ser396 and β-Actin. All immunoblots were subjected to densitometric analysis with levels of phospho-IRF3 normalized to total levels of IRF3 (A+B lower panels). Results are mean +/- S.E.M. of three independent experiments.

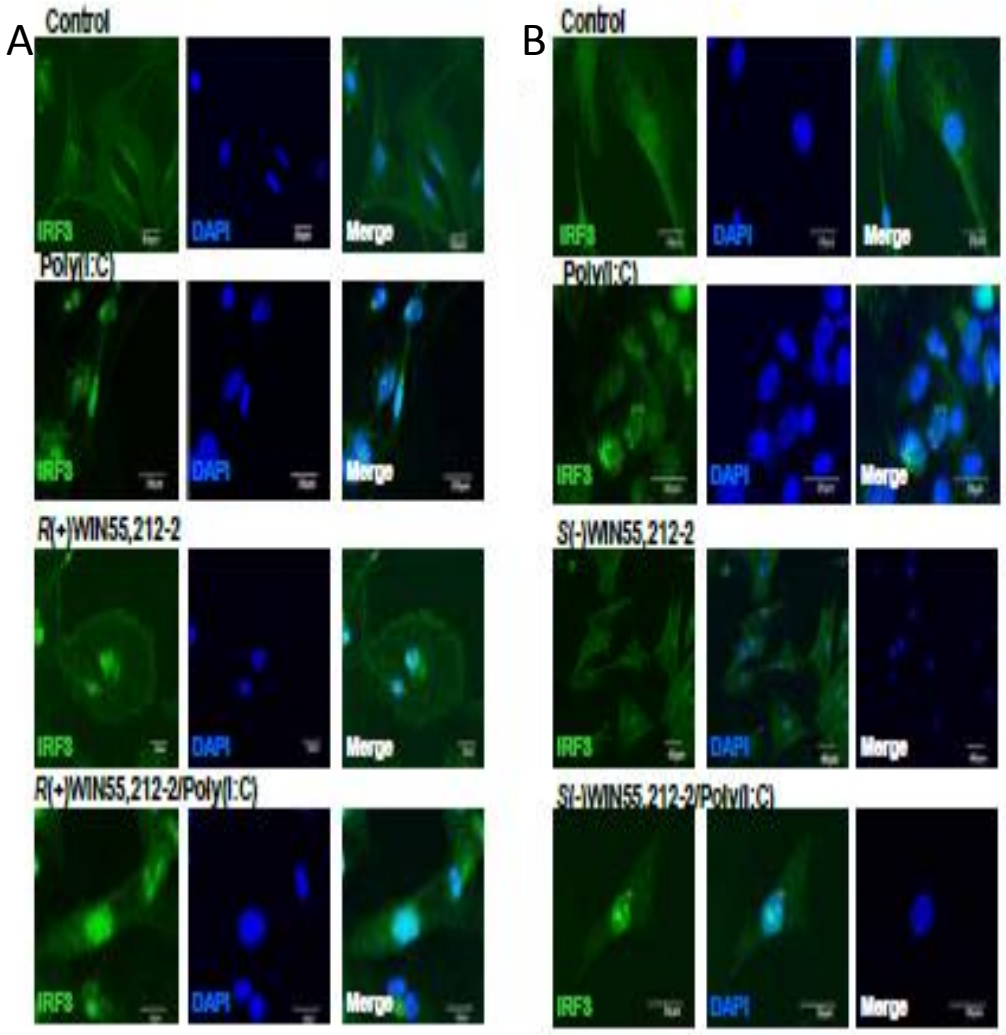


Figure 5.8 R(+)WIN55,212-2 promotes IRF3 translocation to the nucleus in primary mouse astrocytes.

Primary astrocyte cultures prepared from neonatal murine brains were pre-treated with R(+)WIN55,212-2 (20 µM) (A) or S(-)WIN55,212-2 (20 µM) (B) for 1 h prior to Poly(I:C) (25 µg/ml) exposure for 1 h. Cells were fixed, mounted in anti fade medium with DAPI and visualised using confocal microscopy. Confocal images were captured using a UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets. Data analysis was performed using the LSM 5 browser imaging software. Images are representative of three independent experiments. Scale bars are 20 µm.

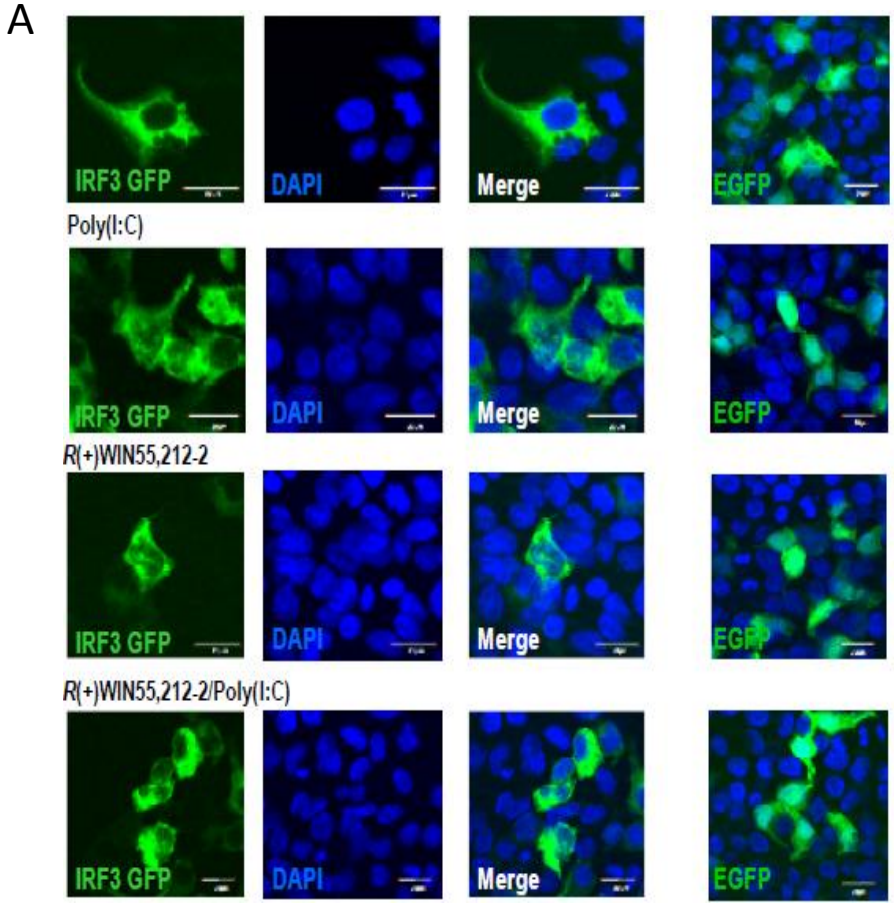


Figure 5.9 R(+)WIN55,212-2 promotes IRF3 translocation to the nucleus in HEK293 TLR3 cells

HEK293 TLR3 cells were transfected with an expression construct encoding GFP-tagged IRF3 (800 ng). Control slides were transfected with EGFP construct (800 ng). Cells were allowed to recover overnight and pre-treated with R(+)WIN55,212-2 (20 µM) for 1 h prior to Poly(I:C) (25 µg/ml) exposure for 1 h.

Cells were fixed in 4% PFA, incubated with DAPI (1.5 µg/ml) in PBS for 30 min, washed, and mounted. All samples were viewed using an Olympus FluoView FV1000 confocal laser scanning microscope equipped with the appropriate filter sets. Acquired images were analysed using the Olympus FV-10 ASW imaging software. Negative control experiments were performed by replacing the primary antibody with isotype controls and using equal gain settings during acquisition and analysis.

Summary of Results

Luciferase Assay (IRF3)	
HEK293 TLR3	R(+)WIN55,212-2 **augments** TRIF-induced activation of IRF3
QRT-PCR (IFN-β mRNA)	
TRIF deficient BMDM	R(+)WIN55,212-2 **inhibits** LPS-induced activation of IFN-β mRNA R(+)WIN55,212-2 **inhibits** Poly(I:C)-induced activation of IFN-β mRNA

Chapter 6

Concluding Remarks

6.1 Concluding Remarks

Over the past decade, considerable progress has been made in improving our understanding of the mammalian immune system and in particular the role of TLR signalling pathways. The function of various transcription factors in the regulation of TLR signalling and in the development of T and B cell subsets, has demonstrated the complex link between the innate and adaptive immune response. However, dysregulation of a variety of transcription factors can bestow autoimmunity in certain individuals and can be a triggering factor in the pathogenesis of autoimmune diseases such M.S.

It is widely accepted that M.S. in an autoimmune disease involving autoreactive $CD4^+$ T lymphocytes, B lymphocytes, plasma lymphocytes, extensive macrophage/glial activation which act cooperatively in the destructive process of demyleination.

Currently there are few effective treatments for the symptoms of M.S. Most of the current drugs only benefit a minority of people and frequently have adverse side effects. In the absence of such effective treatments, M.S. patients are willing to seek alternative therapies such as those which come in the form of cannabis. Anecdotal and scientific evidence both report on M.S. patients to self-medicate with and perceive benefit from the consumption of cannabis (Consroe et al., 1997).

Cannabis has been used medicinally for thousands of years to alleviate a wide variety of conditions including pain, dysentery, sleep disturbance, nausea and vomiting (Di Marzo et al., 1998, Russo, 1998). Cannabinoids affect almost every system in the human body including the cardiovascular, respiratory, immune, reproductive and nervous systems due to the presence of the high expression of cannabinoid receptors. Up until 1971, doctors in the U.K. were able to prescribe oral tinctures of cannabis to patients. However, this was outlawed by the issue of the Misuse of Drugs Act. This act completely prohibited cannabis use unless under exceptional circumstances, such as strictly monitored scientific research. The advent of synthetic and endogenous cannabinoid compounds, coupled with the development of animal models of disease, enabled the biology of cannabinoids to blossom and shed light on the anecdotal reports on the medicinal value of cannabis.

The largest randomised controlled trial studying the effects of cannabis on M.S. was carried out in 2003 (Zajicek et al., 2003). The “Cannabinoids for Treatment of Spasticity

and other Symptoms Related to Multiple Sclerosis" (CAMS) study involved 667 patients with stable M.S. and muscle spasticity, and compared the oral synthetic δ-9-THC molecules, Marinol and Cannador, to placebo. The primary outcome measure, using the Ashworth scale, displayed no statistically significant improvements in spasticity and no effect on tremor. Despite this, patients self-reported improvements in pain, muscle spasms, spasticity and sleep disturbance. The follow-up CAMS study in 2005 was more encouraging. This involved 630 patients where two thirds of patients involved in the original study opted to continue treatment over a 12 month period (Zajicek et al., 2005). Objective improvements in spasticity, as measured by the Ashworth Scale and general disability indices were reported, as well as amelioration in pain and tremor. Following on from the CAMS study, a new study, "Cannabinoid Use in Progressive Inflammatory brain Disease" CUPID began in 2006 and is expected to be completed in 2012. The main differences in this trial are the randomised recruitment of M.S. patients with either primary or secondary progressive M.S. who will receive the cannabinoid treatments over a longer time period.

The oromucosal spray, Sativex (a combination of δ-9-THC and cannabidiol), developed by GW Pharmaceuticals under licence of the British Home Office is approved for the treatment of spasticity associated with M.S. In 2005, Canadian authorities approved the marketing of Sativex, while in 2010, Sativex was licensed as a prescription only medicine in the U.K. (Kmietowicz, 2010). Several studies (Collin et al., 2007, Wade et al., 2004) have tested the therapeutic effects of Sativex on spasticity in M.S. patients but no major improvement in spasticity was observed as measured by the Ashworth scale. This may be due to cannabidiol having no demonstrable activity in experimental spasticity (Baker et al., 2000) and its ability to act as a cannabinoid receptor antagonist (Thomas et al., 2007). Although Sativex may have better pharmacokinetics than ingested agents, it is not as favourable as smoked cannabis. This is because it is administered oromucosally and therefore enters the bloodstream directly. Furthermore, much greater dose-titrations are possible. However, the side effects associated with smoked cannabis are more significant than those obtained from the spray form. Common side effects include anxiety, panic, paranoia, acute psychosis and hallucinations where users also experience a brief euphoria state (JOHNS, 2001).

Despite the use of the Ashworth Scale in the above clinical trials, several studies (Shakespeare et al., 2003, Fleuren et al., 2010) have reported on its lack of reliability and sensitivity to measure the significant functional change in spasticity. As a result, there is a clear need to find alternatives.

Since the initial discovery of various endocannabinoids and development of synthetic cannabinoids, numerous studies have reported on their protective neuroinflammatory role in both autoimmune (EAE) and viral (TMEV) models of M.S. (Croxford and Miller, 2003, Jean-Gilles et al., 2009, Mestre et al., 2009). Furthermore, various groups have also determined the significant involvement of both the CB1 and CB2 receptors (Pryce et al., 2003, Sanchez et al., 2006, Palazuelos et al., 2008) and the role of TLRs (Prinz et al., 2006, Visser et al., 2005) in EAE and M.S. pathology. However, despite the vast amount of published information, limited research has described the regulatory effects of cannabinoids on TLR signalling events with respect to M.S. As a result, this forms the basis for the work described in this thesis.

Both the canonical and non-canonical NFκB pathways are involved in the pathogenesis of autoimmune diseases and play crucial roles in the development, maturation, and homeostasis of T and B lymphocytes (Hayden et al., 2006). NFκB subunits including p50, p65 and c-Rel have all been found in the nuclei of infiltrating macrophages in active M.S. plaques (Bonetti et al., 1999) where p65 expression is also increased in the nuclei of certain oligodendrocytes. In addition, studies investigating the role of NFκB in EAE demonstrate that p65 and p50, but not c-Rel, RelB or p52, are the prototypic inducible subunits in the CNS during EAE (Hilliard et al., 1999, Pahan and Schmid, 2000). However, these findings require some caution, as EAE is normally induced via active immunization with CNS tissue or myelin antigens in complete Freund's adjuvant which is a potent activator of the canonical NFκB pathway.

Much controversy also surrounds the use of EAE as an animal model of M.S. particularly due to the inability of the EAE model to adequately reflect the pathology of M.S. (t Hart et al., 2011, O'Brien et al., 2010) i.e. immunopathogenic mechanisms inducing CNS inflammation in EAE models favours CD4+/MHC class II restricted T cells driven autoimmune mechanisms, whereas CD8+/MHC class I T cells are prevalent in M.S. lesions. Furthermore, there are obvious dissimilarities between innate and adaptive

immune functions in humans and rodents (Mestas and Hughes, 2004, Gibbons and Spencer, 2011). It is worth nothing that while some therapies have proven positive in EAE but failed to succeed in clinical trials e.g. TNF-α neutralizing agents (Sriram and Steiner, 2005, Fromont et al., 2009), others have successfully translated into clinical practice in humans including many of the currently known M.S. treatments for example, glatiramer acetate (Teitelbaum et al., 1971) mitoxantrone (Lublin et al., 1987) and natalizumab (Yednock et al., 1992). In addition, EAE studies have demonstrated various disease modifying drugs which can lead to protection against EAE by inhibiting NFκB activation. For example, Beta-interferon has been proposed to function by shifting the immune response in M.S. from a pro-inflammatory Th1 to anti-inflammatory Th2 type, an effect that is mediated by NFκB inhibition (Martín-Saavedra et al., 2007, Hamamcioglu and Reder, 2007). As a result, agents that inhibit NFκB are attractive therapeutic options for M.S. patients and further clarifies why NFκB was the initial target reported in this thesis in assessing the possible therapeutic role of R(+)WIN55,212-2 in M.S.

Although it was initially assumed that the anti-inflammatory effects exhibited by the cannabinoid compounds were mediated by their ability to inhibit NFκB, regardless of the triggering stimulus, further work revealed that IRF3 is also a target. The knowledge that MyD88 deficiency is protective in EAE (Prinz et al., 2006), while TRIF deficiency exacerbates the disease (Guo et al., 2008) and since IRF3 is regulated by the latter, provided a suitable platform for investigating the regulatory effects of the cannabinoid compounds on IRF3 activation. R(+)WIN55,212-2 treatment was found to enhance IRF3 phosphorylation and nuclear translocation, and positively impact on it and IFN-β expression in response to TLR3 signalling. In addition, the TLR3 signalling events leading to NFκB and IRF3 activation is differentially sensitive to R(+)WIN55,212-2 and suggests that the latter target a component of the IRF3 pathway that is not in common to the NFκB pathway. This target may well be TRIF but it is more likely that the target is TBK1/IKKε due to the loss of the observed effects of each drug on IRF3 and IFN-β, when these kinases are blocked. As a result, it is now proposed, that R(+)WIN55,212-2, by directly regulating the TLR3-TRIF-TBK1/IKKε signalling axis, controls the expression pattern of IRF3 and IFN-β. Furthermore, despite the opposing effects of R(+)WIN55,212-2 on the NFκB and

IRF3 signalling pathways, it is interesting to note that R(+)WIN55,212-2 targets both transcription factors at the nuclear level.

The present study also highlighted the importance of IFN-β production as a key mechanism underlying the protective effects of R(+)WIN55,212-2 in EAE and M.S. It is proposed that such protective effects are due to combined neuroprotection and a dampening of inflammation that is specific to disease models

The regulatory effects, particularly of R(+)WIN55,212-2 cannot be explained by mere virtue of its lipophilic characteristics since its enantiomeric form is ineffective. Furthermore, the often observed dose-response nature of regulatory effects of R(+)WIN55,212-2 induced downregulation of various transcription factors by TLR ligand stimulation additionally argues for a receptor mediated effect. However the primary receptor target that mediates the effects of both cannabinoids on the IFN-β pathway remains undefined. AEA, like R(+)WIN55,212-2 binds to both the CB1 and CB2 receptors. Despite this, the use of selective CB1/2 receptor antagonists, in addition the Gi protein inhibitor, PTX, failed to influence the regulatory effects of both cannabinoids on various transcription factors and their responsive genes. While, the inhibitory effects of AEA may be due to the presence of FAAH, a previous study has demonstrated that FAAH is not involved in AEA inhibition of TNF-α induced activation of NFκB (Sancho et al., 2003). Indeed, both CB1- (Curran et al., 2005b, Facchinetti et al., 2003, Germain et al., 2002, Nilsson et al., 2006, Marchalant et al., 2007, Sanchez et al., 2006) and CB2- (Curran et al., 2005b, Facchinetti et al., 2003, Smith et al., 2000, Nilsson et al., 2006, Sancho et al., 2003) independent effects of R(+)WIN55,212-2 and AEA have been reported. The ability of the PPARα agonist, fenofibrate, to mimic certain regulatory effects of the cannabinoids along with the above information, resulted in PPARα been subsequently assessed as potential target in the pharmacological profile of R(+)WIN55,212-2 and AEA. However, further studies implicated that PPARγ may be a more relevant endogenous target in mediating the anti-inflammatory effects of R(+)WIN55,212-2, particularly in response to the TLR3 signalling pathway. While it now appears that TBK1/IKKε may be pivotal kinases in mediating R(+)WIN55,212-2 anti-inflammatory effects, further studies would need to assess if PPARγ also targets TBK1/IKKε or alternatively if PPARs may have

exclusively a nuclear role and act to physically interact with IRF3 and promote its nuclear retention and so facilitate its transactivation potential.

Approximately 30% of treatments currently on the market are reported to interact with GPCR (Wise et al., 2002). This statistic alone emphasises the importance of cannabinoid receptors and their associated ligands in the treatment of various conditions. Furthermore, as GPCRs constitute the most widely targeted proteins to modify physiological functions and pathological processes, the development of pharmacological agents that selectively target such receptors opens the prospect for entirely new therapeutic venues. In terms of targeting drugs for clinical studies, the psychotropic effects exerted by activation of CB1 in particular, coupled with law enforcement, have somewhat diminished the enthusiasm for promoting the medicinal value of cannabis. Conversely, selectively targeting the CB2 receptor system appears to be neuroprotective in M.S., where disease symptoms and underling pathology are controlled (Baker et al., 2000). However, as described in this work, the cannabinoid regulatory effects are not mediated by their interaction with GPCRs and as previously reported; δ-9-THC has also been demonstrated to interact with novel receptors distinct from the cannabinoid receptors (O'Sullivan and Kendall, 2009, Sun et al., 2007). The existence of novel cannabinoid receptors and the discovery of cannabinoids interacting with PPARs have generated a new wave of interest in the area of neuroimmunology. This discovery may well lead to pharmacological interventions that are devoid of the psychotropic and euphoric effects attributed to the activation of cannabinoid receptors.

As presented here, both R(+)WIN55,212-2 and AEA display numerous anti-inflammatory effects and offer a novel therapeutic approach in the treatment of M.S. However, before proceeding to clinical trials, additional research on the psychotropic and euphoric effects of these cannabinoid compounds will need to be examined. Anecdotal evidence has reported on both the psychotropic and euphoric effects obtained from R(+)WIN55,212-2 consumption. This is probably due to R(+)WIN55,212-2 activating the CB receptors. Furthermore, in terms of legal substitutes for cannabis, it has recently been reported that analyses of Spice (brand name of, and generic slang, for a herbal mixture sold in head shops) show that it contains synthetic cannabinoids such as JWH-018 and HU-210 (Schifano et al., 2009) which produce the psychotropic and euphoric effects associated with

consuming this legal drug. Another factor in terms of clinical trial drug suitability is the toxicity of the substance. Our laboratory previously demonstrated that R(+)WIN55,212-2 did not cause any detrimental effects on cellular viability (Curran et al., 2005a).

In order to extend the findings described in this work, it would be of particular interest to assess R(+)WIN55,212-2 and AEA ability to promote PPARα and PPARγ transactivation and expression. As each cannabinoids regulatory effects are independent of the cannabinoid receptors, to further extend this work, one should assess that the CB1 and CB2 antagonists and pertussis toxin are active in the experimental systems. Transcriptional activation of the IFN-β gene is tightly regulated and transcriptionally controlled by a limited number of transcription factors. We have already investigated R(+)WIN55,212-2 and AEA regulatory effects on the positive regulated domains recognised and regulated by NFκB and by IRF3/IRF7. Transcriptional activation of the IFN-β gene is also regulated by the transcription factor AP-1. Therefore, to extend the above findings, cannabinoid regulatory effects on the AP-1-binding enhancer element of the IFN-β promoter should be considered. In relation to M.S. work, the numbers of patient subjects should be increased. Furthermore, it would be of interest to perform this experiment using S(-)WIN55,212-2.

In conclusion, this work describes the anti-inflammatory effects of the synthetic cannabinoid; R(+)WIN55,212-2. This work suggests that the innate arm of the immune response is a novel target for the anti-inflammatory actions where a novel dual molecular mechanism of action is described. R(+)WIN55,212-2 can exert direct anti-inflammatory properties by downregulating the TLR induced activation of NFκB and induction of its pro-inflammatory targets. In parallel, by enhancing the activation of IRF3 and subsequent induction of its anti-inflammatory target; IFN-β, R(+)WIN55,212-2 can boost an endogenous protective system. Such effects of R(+)WIN55,212-2, in particular its capacity to induce endogenous expression of IFN-β, offers a potentially attractive alternative to the currently marketed Beta-interferon therapies. Overall, these findings encourage clinical trials to assess if the therapeutic application of R(+)WIN55,212-2 in M.S. can extend beyond symptomatic relief.

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Communications

Communications

Title of Conference: Immunology PhD Masterclass

Dates: 16th July 2007

Location: NUIM

Form of Participation: Poster Presentation

Title of Presentation: The anti-inflammatory and therapeutic effects of cannabinoids: relevance to multiple sclerosis.

Eileen Clifford and Paul Moynagh

Title of Conference: Irish Society for Immunology (ISI)

Dates: 9th and 10th August 2007

Location: DCU

Form of Participation: Poster Presentation

Title of Presentation: The anti-inflammatory and therapeutic effects of cannabinoids: relevance to multiple sclerosis

Eileen Clifford and Paul Moynagh

Title of Conference: Northern Neuro-Immunology Interest Group Meeting

Dates: 2nd November 2007

Location: Nottingham, UK

Form of Participation: Meeting Participant

Title of Conference: Immunology PhD Masterclass

Dates: 18th July 2008

Location: NUIM

Form of Participation: Poster Presentation

Title of Presentation: Identification of the synthetic cannabinoid R(+)WIN55,212-2 as a novel regulator of TLR signalling pathways

Eileen Clifford and Paul Moynagh

Title of Conference: Cannabinoid Ireland Workshop 2008

Dates: 25th July 2008

Location: NUIG

Form of Participation: Oral Presentation

Title of Presentation: The anti-inflammatory and therapeutic effects of cannabinoids: relevance to multiple sclerosis

Eileen Clifford and Paul Moynagh

Title of Conference: Irish Society for Immunology (ISI)

Dates: 15th and 16th September 2008

Location: RDS

Form of Participation: Poster Presentation

Title of Presentation: Identification of the synthetic cannabinoid R(+)WIN55,212-2 as a novel regulator of TLR signalling pathways

Eileen Clifford and Paul Moynagh

Title of Conference: Keystone Symposia 'Multiple Sclerosis'

Dates: 21st - 25th January 2009

Location: New Mexico, USA

Form of Participation: Poster Presentation

Title of Presentation: Probing the molecular basis to the anti-inflammatory effects of R(+)WIN55,212-2 in multiple sclerosis

Eileen Clifford and Paul Moynagh

Title of Conference: Cannabinoid Ireland Workshop 2009

Dates: 9th September 2009

Location: NUIM

Form of Participation: Oral Presentation

Title of Presentation: Are the anti-inflammatory effects of R(+)WIN55,212-2 PPAR dependent?

Eileen Clifford and Paul Moynagh

Publication: Downer, E. J., E. Clifford, et al. (2011). "Identification of the Synthetic Cannabinoid R(+)WIN55,212-2 as a Novel Regulator of IFN Regulatory Factor 3 Activation and IFN-β Expression." Journal of Biological Chemistry **286**(12): 10316-10328.