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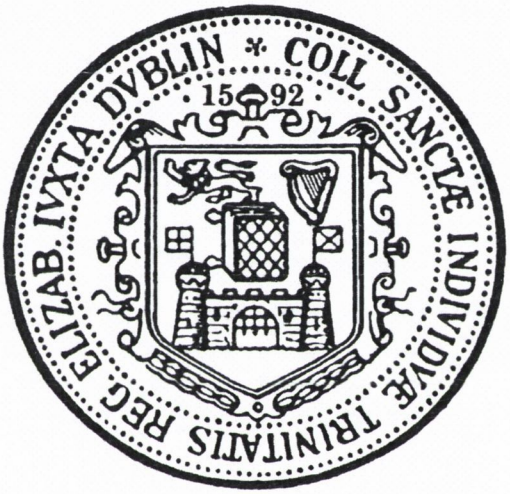
Analysis of the impact of SIGIRR on glial function

Dónal Carney



A thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy

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2013

I. Declaration

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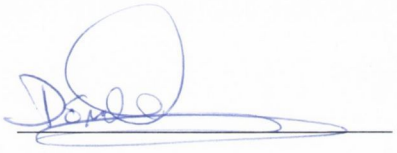

Dónal Carney

II. Abstract

Many neurodegenerative diseases are associated with a shift towards a pro-inflammatory environment within the CNS, and this is believed to contribute to the progressive decline in cognitive function associated with these diseases. Activated glial cells are thought to be the driving force behind the development of a pro-inflammatory environment, and endogenous modulators of glial activity have emerged as targets for the treatment of chronic neuroinflammation. The objective of this thesis was to analyse the ability of one such molecule, SIGIRR, to act as a modulator of glial activation.

The effects of LPS, IL-1β, Pam_3CSK_4 and Aβ on markers of microglial activation and production of cytokines was assessed in mixed glia and isolated astrocytes and microglia prepared from wildtype and SIGIRR-deficient mice. Next, the effect of an acute intrahippocampal injection of Aβ in young and middle-aged, wildtype and SIGIRR-deficient mice was assessed. Finally, IL-1F5, SIGIRR/Fc and TREM-1/Fc were assessed for their ability to promote SIGIRR expression and/or activate the receptor.

Mixed glia and astrocytes from SIGIRR-deficient mice exhibited increased basal expression of TLR2 and TLR4 compared to cells from wildtype mice. It is proposed that this is responsible for the observed exaggerated production of IL-6 and TNF-α in mixed glia from SIGIRR-deficient mice compared with wildtype mice in response to LPS, IL-1β, Pam_3CSK_4 and Aβ. Isolated microglia, but not astrocytes, from SIGIRR-deficient mice, exhibited and an exaggerated production of IL-6 compared with wildtype mice in response to LPS, however this was seen in both astrocytes and microglia in response to Pam_3CSK_4. Pre-treatment of mixed glia with an anti-TLR2 antibody attenuated the Aβ-induced increase in IL-6 and TNF-α suggesting that TLR2 acts as a receptor for Aβ.

Pre-treatment of mixed glia with SIGIRR/Fc attenuated the LPS-induced increase in CD11b mRNA expression, IL-6 and TNF-α, and the IL-1β-induced increase in IL-6, in a

dose-dependent manner. Pre-treatment with TREM-1/Fc attenuated the LPS-induced increase in TNF-α from mixed glia and isolated microglia in a dose-dependent manner. TREM-1/Fc reversed the LPS-induced decrease in SIGIRR mRNA expression.

IL-1β induced the mRNA expression of markers of microglial activation, CD11b and CD68, in mixed glia, which was exaggerated in cells prepared from SIGIRR-deficient mice. Similarly the IL-1β-induced increases in IL-6, TNF-α and MPC-1 in mixed glia were enhanced in cells prepared from SIGIRR-deficient mice. Pre-treatment with IL-1F5 was found to attenuate the IL-1β-induced increase in IL-6 in mixed glia prepared from wildtype mice but this effect was absent in cells prepared from SIGIRR-deficient mice.

Analysis of changes *in vivo* revealed that there was an increase in mRNA expression of TLR2 and RAGE in the cortex and hippocampus, respectively, of SIGIRR-deficient mice compared with wildtype mice, and there was an increase in CD68 and phosphorylated IκBα in the hippocampus of SIGIRR-deficient mice. The data presented in this study was designed to characterise the response of glial cells from SIGIRR-deficient mice to IL-1R/TLR ligands. The evidence presented supports the hypothesis that SIGIRR acts as a modulator of TLR2, TLR4 and IL-1R1 signaling in glial cells. A significant finding is that SIGIRR also modulates Aβ-induced changes in cytokine release from glial cells. However, IL-1F5, SIGIRR/Fc and TREM-1/Fc do not appear to be viable tools for the activation of SIGIRR, and that future studies should focus on overexpression of the receptor as a means of modulating glial activation.

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Finally, I'd like to thank Ran for being a pie.

IV. Abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
Aβ	Amyloid-beta peptide
ANOVA	Analysis of variance
APC	Antigen presenting cells
ApoE	Apolipoprotein E
BACE	β-APP Cleaving Enzyme
BBB	Blood brain barrier
BSA	Bovine serum albumin
CNS	Central nervous system
CSF	Cerebrospinal fluid
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
dH_20	Deionised water
Da	Dalton
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DMEM	Dulbecco's modified eagle medium
ELISA	Enzyme linked immunosorbent assay
EAE	Experimental autoimmune encephalomyelitis
Epo	Erythropoietin
FBS	Foetal bovine serum
Fc	Fragment, crystalisable
GFAP	Glial fibrillary acidic protein
g	Gram

GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor (GM-CSF)
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HMGB1	High motility group box 1
HRP	Horse radish peroxidase
ICAM	Intracellular adhesion molecule
IκK	IκB kinase
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL-1RAcP	IL-1R accessory protein
IRAK	IL-1R associated kinases
Ig	Immunoglobulin
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1F5	Interleukin-1 family member 5
ICAM-1	Intracellular adhension molecule-1
IRAK	Interleukin-1 receptor associated kinase
i.h.	Intrahippocampal
i.p.	Intraperitoneal
i.v.	Intravenously
JNK	Jun N-terminal kinase
JAK	Janus Kinase
k	Kilo
-/-	Knock out
L	Ligand
LPS	Lipopolysaccharide
LTP	Long-term potentiation
M	Molar
MAL	MyD88-adaptor like

MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MHC class II	Major histocompatibility complex class II
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
μ	Micro
ml	Milliliter
mm	Millimetre
MAPK	Mitogen-activated protein kinases
mM	Molar
MS	Multiple sclerosis
MyD88	Myeloid differentiation factor 88
n	Number
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nm	Nanometre
NK	Natural killer
NO	Nitric oxide
NGS	Normal goat serum
NHS	Normal horse serum
NF-κB	Nuclear factor-κB
Pam_3Csk_4	$Pam_3CysSerLys_4$
PAMPs	Pathogen associated moleculare patterns
PRRs	Pathogen recognition receptors
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline containing tween
PCR	Polymerase chain reaction
PD	Parkinson's Disease
RNase	Ribonuclease
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature

SIGIRR	Single-immunoglobulin interleukin-1 receptor related
SEM	Standard error of mean
Strep-HRP	Streptavidin-horseradish peroxidase linked
H_2S0_4	Sulphuric acid
TAB1	TGF-beta activated kinase 1
TIR Domain	Toll/interleukin-1 receptor domain
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TNFR	TNF receptor
TRAF	TNF receptor associated factor
TREM-1	Triggering receptor expressed on myeloid cells 1
TRIF	TIR-domain-containing adapter-inducing interferon-β
TLR	Toll like receptor
Tg	Transgenic
TNF-α	Tumour necrosis factor-alpha

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Chapter 1: Introduction

1.1. Immunity in the Central Nervous System (CNS)

The increasing prevalence of neurodegenerative disorders because of the increase in the aging population has accelerated the efforts of researchers to develop new therapeutics aimed at combating the factors which contribute to the pathogenesis of these disorders. Many neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS), are associated with inflammatory changes in the central nervous system (CNS). This is thought to contribute to neuronal damage and therefore may contribute to the progressive decline in cognitive function. Therefore the inflammatory response which is associated with glial activation and the secretion of pro-inflammatory cytokines and chemokines by activated astrocytes and microglia has been targeted in the development of novel drugs (Storer et al., 2005).

1.1.2. Astrocytes

Astrocytes are the major glial cell type found within the CNS. They are characterised by their star shape, a cell body surrounded by a multitude of branched processes – and by the expression of glial fibrillary acidic protein (GFAP). Astrocytes perform a wide array of functions within the CNS. These include structural support within the brain, including forming part of the blood brain barrier (BBB), neurotransmitter uptake and release via specialised cell-surface transporters, maintenance of ionic homeostasis within the CNS through uptake of excess potassium in the extracellular space and communication with other cell types within the CNS through gap junctions (Bush et al., 1999, Bennett et al., 2003). In addition to these and many other roles played by astrocytes, it is increasingly recognised that astrocytes have a significant immune function within the CNS (Dong and Benveniste, 2001). Although their role in the maintenance of the structural and functional characteristics of the BBB has been described in detail, evidence now points to an additional role for astrocytes as antigen-presenting cells. Astrocytes have been shown to express the cell surface protein major histocompatibility complex II (MHCII) which presents extracellular antigens to T-cells (Wong and Battisto, 1984). Although expression is low in comparison to professional antigen presenting cells (APC) such as microglia, it does indicate that astrocytes are capable of communicating with T-cells and contributing to the inflammatory response within the CNS. Perhaps the greatest contribution made by astrocytes to the

inflammatory response in the brain is through the production of cytokines and chemokines. This cytokine-secreting capability is particularly pronounced when the CNS is in a diseased state. Astrocytic production of pro-inflammatory cytokines and chemokines is considerably more pronounced in AD, PD and MS (Benveniste, 1998, Hesselgesser and Horuk, 1999, Akiyama et al., 2000). Astrocytes are known to be the primary source of interleukin (IL)-6 and Chemokine (C-C motif) ligand 2 (CCL2). Both molecules play a significant role in the chemotaxis of peripheral immune cells to the CNS among other functions (see sections 1.3.2 and 1.3.4).

1.1.3. Microglia

Microglia are immune cells localised entirely within the CNS, and they are responsible for removing toxic substances that emerge from within the CNS, or that enter it by permeating the BBB (Perry, 2007, Soriano and Piva, 2008). They are monocyte-lineage cells and share many characteristics with macrophages, which perform a similar role in the periphery. Microglia can exist in several functional states, which correspond at least loosely with a particular morphological phenotype. Ramified microglia extend processes into their immediate surroundings allowing them to survey the extracellular space for any changes in the normally highly-stable CNS microenvironment. Microglia displaying such a phenotype are said to be in a "resting" state and lack the ability to present antigens to peripheral T-cells. Their cell bodies are immobile while the cells continuously release a variety of neurotrophic and anti-inflammatory cytokines including IL-4 and IL-10 (Block et al., 2007). These cytokines help to maintain a healthy environment to support neuronal growth and survival. The detection of an insult i.e. neurotoxin, pathogen-associated molecular pattern (PAMP), cell debris or cytokine, can induce an alteration in the phenotypic and functional state of microglia. Following stimulation, microglia adopt a more amoeboid morphology, and this is often accompanied by the upregulation of cell surface membrane proteins which facilitate functions ranging from antigen presentation to phagocytosis (Block et al., 2007, Lynch, 2009). In addition to these phenotypic changes, activated microglia typically exhibit upregulated transcription of genes coding for pro-inflammatory cytokines and a corresponding increase in the secretion of such molecules. This feature of activated microglia allows them to propagate the pro-inflammatory signal by

activating nearby microglia and other immune cells and establishing a inflammatory environment in the CNS (Perry, 2007, Soriano and Piva, 2008).

1.1.4. Inflammation

The inflammatory response is generally beneficial, neutralising the effects of stimuli, infection or trauma. However dysregulation of inflammatory signaling is thought to cause or contribute to a wide range of diseases. Crohn's Disease and Guillain-Barre Syndrome are known to arise from acute inflammatory conditions. Persistence of the inflammatory response is associated with the development of chronic diseases such as rheumatoid arthritis. In addition, inflammatory mediators released from cancerous cells may contribute to malignant progression of tumours (Kulbe et al., 2005). More recently, it has been recognised that inflammatory conditions that arise secondary to many neurodegenerative diseases have been shown to contribute to the decline in cognitive function associated with neurodegenerative diseases (Griffin et al., 1995). A common link between these chronic conditions is the efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) in providing symptomatic relief to the patient, further indicating the role of inflammation in their pathology (Rich et al., 1995, Harris et al., 1996, Deeks et al., 2002). The role of chronic inflammation in neurodegenerative diseases is discussed in more detail in section 1.5. The immune cells responsible for driving this inflammation e.g. macrophages, dendritic cells and microglia, are activated through activation of cell surface receptors which sense damage. A cascade of intracellular signaling often accompanies this process, which results in the upregulation of inflammatory mediators e.g. cytokines, complement proteins, and cluster of differentiation (CD) molecules.

1.1.5. LPS

PAMPs are molecules associated with pathogens that are recognised by pattern recognition receptors (PRR) on the surface membranes of immune cells. Experimental tools used in the activation of PRRs include the synthetic molecule Pam_3CSK_4 and the endogenous bacterial-associated molecule lipopolysaccharide (LPS), which activate toll-like receptor (TLR) 2 and TLR4 respectively. LPS is a large molecule which contributes to the structure of the outer

membranes of gram-negative bacterial cell walls. It consists of covalently-bound molecules of lipid and polysaccharide. TLR4 recognises LPS and interaction between the two initiates an intracellular signalling cascade which results in the upregulation pro-inflammatory gene transcription. This process is described in more detail below.

1.2. Markers of microglial activation

A number of proteins are recognised as markers of microglial activation i.e. proteins that show a consistent pattern of upregulation in response to stimuli known to cause a change in the activation state of microglia. Although the upregulation of these proteins is indicative of functional changes within the microglial cell, it is unclear whether they play a direct role in these functional changes. **Table 1.1** outlines a number of these markers. The markers examined in this thesis – CD11b, CD40 and CD68 – are discussed in more detail below.

Table 1.1. Markers of microglial activation

Marker	Function	Cell Types
CD11b	Chemotaxis; Leukocyte adhesion and migration.	Microglia, monocytes
CD40	T-cell restimulation; Phagocytosis	Microglia, macrophages, astrocytes, endothelial cells
CD68	Antigen presentation; Phagocytosis	Microglia, macrophages
CD86	Co-stimulation; antigen presentation	Microglia, astrocytes
CD80	Co-stimulation; antigen presentation	Microglia, macrophages
MHCII	Antigen presentation; Phagocytosis	Microglia, macrophages, astrocytes
ICAM	Cell adhesion	Microglia, macrophages, leukocytes, endothelial cells
CD200R	Microglial deactivation	Microglia, macrophages, monocytes
CX3CR1	Microglial deactivation; T-cell migration	Microglia, macrophages, leukocytes, NK cells, astrocytes.

Adapted from Lynch, 2009.

1.2.1. CD11b

Integrins are a family of receptors which mediate cell-cell interaction, cell adhesion to extracellular matrix proteins and facilitate signalling pathways through their intracellular domains. They play key roles in development, homeostasis, immune response and leukocyte trafficking. The latter characteristics have implicated integrins in the development of auto-immune diseases. CD11b is a member of the integrin family which is constitutively expressed on monocytes, granulocytes, natural killer cells, macrophages and microglia (Hynes, 2002, Solovjov et al., 2005). CD11b can bind to CD18 to form the two subunits making up integrin $\alpha_M\beta_2$, which is involved in the adhesion and migration of immune cells. CD11b expression is markedly increased in microglia when the cells are in an active state and upregulated in response to LPS and amyloid-β (Aβ) (Perera et al., 2001, Seabrook et al., 2006). In addition, CD11b is upregulated in brain tissue in many disease models including APP/PS1 transgenic mouse models of AD and experimental autoimmune encephalomyelitis (EAE), a model for MS (Agnello et al., 2002, Yan et al., 2003).

1.2.2. CD40

CD40 is a co-stimulation molecule constitutively expressed at low levels on endothelial cells, astrocytes, macrophages, microglia and other antigen-presenting cells. It is a member of the tumour necrosis factor (TNF) receptor superfamily of proteins. Interaction of CD40 with its ligand CD40L induces signalling cascades, often by associating with TNF-receptor associated factor (TRAF) adaptor proteins, resulting in activation of immune cells. Signaling events downstream of CD40-CD40L interaction can result in pro-apoptotic gene transcription, cell proliferation and differentiation. In addition, the upregulation of other proteins involved in antigen presentation, such as MHCII, and of genes encoding pro-inflammatory cytokines IL-1β, tumour necrosis factor-α (TNF-α) and IL-6 has been associated with CD40-CD40L interaction. CD40 expression and signaling has been show to be upregulated in response to a variety of stimuli, including LPS and TNF-α (Arrighi et al., 2001, Qin et al., 2005).

1.2.3. CD68

CD68 is a 110kD, heavily glycosylated, mucin-like membrane protein found on endosomal and cell-surface membranes of macrophages and microglia. It shares significant sequence homology of the membrane proximal and cytoplasmic domains with a family of lysosomal proteins including LAMP-1 (Holness and Simmons, 1993). CD68 is thought to play a role similar to MHCII in antigen presentation and may also maintain the integrity of the lysosomal membrane (Kurushima et al., 2000). Digestion of materials phagocytosed by cells occurs in the lysosome, and the upregulation of CD68 on the surface of the lysosome is indicative of lysosomal, and hence phagocytic, activity.

1.3. Cytokines

The pro-inflammatory cytokines upregulated upon activation of microglia and astrocytes exist in low concentrations in the healthy brain, where they play important roles in cell-cell signalling. However under conditions of neurological stress their expression is increased and may lead to cytotoxicity. The main pro-inflammatory cytokines implicated in neurodegeneration are IL-1β, IL-6 and TNF-α. Glial cells may also release chemokines which create a chemotactic gradient to promote the migration of immune cells such as macrophages and T-cells. Chemokines play an important role in the progression of chronic neurodegeneration, especially when altered BBB permeability allows peripheral immune cells to enter the CNS. Among the most studied chemokines are chemokine (C-C motif) ligand 2 (CCL2), interferon C-X-C motif chemokine 10 (CXCL10) and (C-C motif) ligand 3 (CCL3). The major families of cytokines and chemokines are outlined in **Table 1.2.** while the cytokines examined in this study are described in more detail below.

Table 1.2. Cytokine classification and function

Classification	Cytokine	Function
Pro-inflammatory	IL-1α, IL-1β, IL-6, IL-7, IL-16, IL-17, IL-18	Promotion of systemic inflammation
Anti-inflammatory	IL-1RA, IL-4, IL-10, TGF-β1, TGF-β2, IL-1F5, IL-1F6	Suppression of systemic inflammation
CXC Chemokines	CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10	Chemotaxis of neutrophils, lymphocytes
CC Chemokines	CCL2, CCL3, CCL4, CCL17, CCL18, CCL19, CCL20, CCL22	Chemotaxis of monocytes, NK cells, dendritic cells and T-cells
C Chemokines	XCL1, XCL2	Chemotaxis of T-cells
CX_3C Chemokines	CX_3CL1	Chemotaxis of T-cells, monocytes; leukocyte adhesion
Immunoregulatory	IFNα, IFNγ, IL-12, IL-23	Immune cell activation; T-cell differentiation
Colony-stimulating factors	G-CSF, M-CSF, GM-CSF, IL3, SCF	Immune cell proliferation and differentiation
Angiogenic/fibrogenic	HB-EGF, HGF, FGF-2, TGFα, VEGF	Angiogenesis; Fibrogenesis Cell growth, proliferation, differentiation
TNF Superfamily	TNFα, BAFF, CD30L, CD95L, LTβ, RANKL	Initiation of apoptotic cell death
Other	BDNF, NGF, Amphiregulin, oncostatin, PBEF	

1.3.1. IL-1β

IL-1β is believed to be secreted primarily by microglial cell. Two stimuli combine to initiate the assembly of inflammasomes. These multi-protein complexes contain a proteolytic enzyme, caspase-1, which cleaves pro-IL-1β into its soluble active product IL-1β (Basu et al., 2004). In the presence of neurotoxic stimuli, such as would occur following brain trauma or in neurodegenerative diseases, microglia upregulate the synthesis of IL-1β where it contributes to an exaggerated inflammatory response. The IL-1β receptor has been localised on microglial cell membranes and this suggests that IL-1β is involved in a feedback pathway, amplifying the expression of other pro-inflammatory mediators such as IL-6, reactive oxygen species (ROS), nitric oxide and prostaglandins (Vitkovic et al., 2000).

1.3.2. IL-6

IL-6 operates primarily as a pro-inflammatory cytokine in the CNS, with some reported anti-inflammatory effects (Pais et al., 2008). Like IL-1β, IL-6 is secreted from both activated microglia and astrocytes, with the latter seeming to bear responsibility for the majority of IL-6 production (Van Wagoner et al., 1999). LPS has been identified as a potent stimulator of IL-6 from glial cells, and similar effects were seen in glia cultured from brains of aged rats (Xie et al., 2003). The upregulation of IL-6 is accompanied by an amplification of the inflammatory response. Although IL-6 itself contributes to this directly through its role as a pro-inflammatory cytokine, it also exacerbates neuroinflammation further through its actions on other cells i.e. astrocytes (Xie et al., 2003). Binding of IL-6 to it's receptor leads to phosphorylation of two kinases that associate with the receptor, Janus kinase (JAK)1 and JAK2. These kinases subsequently activate STAT-1 and STAT3 which can initiate transcription of pro-inflammatory genes by translocating to the nucleus (Van Wagoner and Benveniste, 1999). This pathway is known as the JAK-STAT signaling pathway and it is the primary route by which IL-6 signaling is transduced. Increased concentrations of IL-6 in the CNS have been reported in several neurodegenerative diseases including AD and PD (Mogi et al., 1996, Baranowska-Bik et al., 2008).

1.3.3. TNF-α

TNF-α is believed to be absent, or present at extremely low concentrations, in the healthy CNS. TNF-α mRNA is expressed in much greater abundance than protein, and it was therefore suggested that upon appropriate stimulation, TNF-α mRNA is rapidly translated, leading to a spike in concentration of the cytokine immediately following the insult or injury (Vitkovic et al., 2000). Secreted TNF-α then acts in a similar way to IL-1β and IL-6, initiating a series of inflammatory responses in the brain. TNF-α acts on its receptors TNFR1 and TNFR2 which differ in their cellular distribution and in their ability to bind membrane-bound and/or soluble TNF-α. Binding of TNF-α results in trimerisation of TNF receptors and the dissociation of an inhibitory associated protein silencer of death domains (SODD). This facilitates the recruitment

of TNFR1-associated death domain protein (TRADD) to the death domain of the TNFR trimer. The binding of TRADD to TNFR may facilitate several signaling pathways with varying end results (Wajant et al., 2003). These include stimulation of the mitogen activated protein kinase (MAPK) pathway which may lead to cell proliferation, pro-apoptotic pathways that result in cell death and the NF-κB pathway which, as described earlier, results in the upregulation of pro-inflammatory genes. Microglial-derived TNF-α has been implicated in cell death associated with neurodegenerative diseases including AD and PD (Tweedie et al., 2007).

1.3.4. CCL2

CCL2 is classed as a chemokine due to its small size, four conserved cysteine residues and ability to facilitate chemotaxis of nearby cells. Although CCL2 can be secreted by neurons and astrocytes, the primary source of the chemokine in the CNS are microglial cells (Ransohoff et al., 1993, Ishizuka et al., 1997) although both astrocytes and microglia release CCL2 when exposed to LPS (Ling et al., 2010). Increased concentrations of CCL2 have also been reported to be associated with certain neurodegenerative disorders and animal disease models, including AD and EAE (Ransohoff et al., 1993, Godiska et al., 1995, Ishizuka et al., 1997). In these cases, CCL2 was found to recruit macrophages and T-cells into the CNS. Pro-inflammatory cytokines i.e. IL-1β, IL-6 and TNF-α are thought to induce the release of CCL2, which in turn may act on nearby cells to facilitate the release of more pro-inflammatory cytokines in a positive feedback cycle (Cota et al., 2000, Rankine et al., 2006).

1.3.5. CXCL10

Like CCL2, CXCL10 is classed as a chemokine and acts primarily to recruit macrophages, T-cells, NK cells and dendritic cells. It is released by several cell types including fibroblasts and endothelial cells however the primary source of the chemokine are cells of the monocyte lineage i.e. macrophages and microglia (Ransohoff et al., 1993, Luster et al., 1995). CXCL10 can also facilitate the binding of T-cells to endothelial cells, and thus plays an important role in the trafficking of peripheral immune cells across the BBB (Dufour et al., 2002). Interferon-γ (IFN-γ) and LPS are potent stimulators of CXCL10 release from microglial cells (Luster et al., 1995, Ren

et al., 1998). Increased concentrations of CXCL10 have also been reported in the CNS in EAE and in brains from patients with multiple sclerosis (Ransohoff et al., 1993, Gerard and Rollins, 2001).

1.3.6. CCL3

CCL3 is a chemokine known for its ability to selectively promote the migration of neutrophils and other granulocytes. It has also been shown to promote the upregulation of pro-inflammatory cytokine genes (Karpus and Kennedy, 1997). Constitutive expression of this chemokine is low in resting microglia, however following exposure to LPS, TNF-α or IL-1β the CCL3 gene is upregulated and there is increased secretion of the protein (McManus et al., 1998). As with CCL2 and CXCL10, increased expression and secretion of CCL3 has been associated with several disease models, including EAE and focal cerebral ischemia (Karpus and Kennedy, 1997, Takami et al., 1997).

1.4. Pro-inflammatory signal transduction

Glial cells become activated through a variety of external stimuli. These include such diverse elements as cytokines, bacterial endotoxins, reactive oxygen species and toxic peptides e.g. Aβ. Glia recognise these elements through a diverse array of cell surface receptors which vary in their location on the cell membrane, the molecules they recognise and in the signals that they transduce. Extracellular stimuli are transduced into the cell through the initiation of signalling cascades following receptor activation. These cascades ultimately result in the modulation of gene transcription and changes in protein production which affect the functional state of the cell. The IL1/TLR superfamily is one such group cell surface receptors which include the receptor for the pro-inflammatory cytokine IL-1β, IL-1 receptor 1 (IL-1R1) and the TLRs and some of the structural features of these receptors are illustrated in **Figure 1.1.** The signaling pathways initiated by this family of proteins have emerged as a possible target for modulation as a means of achieving a balance in the immune system. A member of the IL-1/TLR family, single immunoglobulin IL-1β receptor related molecule (SIGIRR) appears to have a negative

regulatory effect on interleukin-1/toll-like receptor signaling. It has emerged as a possible target for novel anti-inflammatory therapies.

1.4.1. Interleukin-1/toll-like receptor signaling

Members of the IL-1/TLR superfamily of receptors are characterized by the presence of a conserved sequence called the Toll/IL-1R (TIR) domain. This domain, located in the cytosolic region, is thought to participate in homotypic interactions with the TIR domains of other receptors and accessory proteins. It is through such interactions that signaling cascades initiated by members of the IL-1/TLR superfamily are facilitated (Fitzgerald and O'Neill, 2000). The superfamily can be subdivided into two additional protein families based on their extracellular domains – the IL-1R1 and TLR subgroups. Member of these sub-families are represented in **Figure 1.1.** The extracellular domains of members of the IL-1R1 subgroup are distinguishable by the presence of immunoglobulin (Ig)-like domains, whereas TLRs typically consist of leucine-rich repeats. The IL-1R1 subgroup takes its name from the first protein in the IL-1/TLR superfamily to be described: the type 1 receptor, IL-1R1. In its role as receptor for the ubiquitous pro-inflammatory cytokine IL-1β, IL-1R1 initiates signaling cascades that lead to the phosphorylation of the transcription factor NF-κB and the MAPKs, p38 and JNK (O'Neill and Dinarello, 2000). The toll subgroup comprises 13 members (TLR1-13) which are expressed in humans. TLR4, a receptor found on the surface of immune cells and is the most studied member of the family. The signaling cascades that follow LPS-induced activation of TLR4 are similar to those associated with interaction between IL-1β-IL-1R1 and result in phosphorylation of NF-κB and the MAPKs (Chow et al., 1999). This signaling cascade is illustrated in **Figure 1.2.** Certain accessory proteins thus have the ability to interact with either activated IL-1R1 or TLR4, so both receptors employ common signaling pathways in response to different stimuli (O'Neill and Dinarello, 2000). The end biological effect of this signaling pathway is the upregulation of genes that encode for pro-inflammatory molecules. The IL-1R/TLR superfamily is outlined in **Table 1.3**.

Table 1.3. Interleukin-1 Receptor/Toll-like Receptor Superfamily

Receptor	Function	Ligand(s)
IL-1R1	Cytokine receptor; pro-inflammatory signal transduction	IL-1
IL-1RAcP	Pro-inflammatory signal transduction	IL-1
IL-1Rrp2	Pro-inflammatory signal transduction	IL-1F6
IL-18R	Pro-inflammatory signal transduction	IL-18
IL-RAPL	Pro-inflammatory signal transduction	N/A
SIGIRR	Decoy receptor	N/A
TIGIRR	Decoy receptor	N/A
TLR1	Forms dimer with TLR2; pro-inflammatory signal transduction	Peptidoglycan, triacyl lipoproteins, zymosan
TLR2	Forms dimer with TLR1; pro-inflammatory signal transduction	Peptidoglycan, lipoteichoic acid, lipoprotein, lipoarabinomannan, zymosan, amyloid-β
TLR3	Pro-inflammatory signal transduction	ds RNA
TLR4	Pro-inflammatory signal transduction	LPS, F protein, hsp60, ethanol, amyloid-β
TLR5	Pro-inflammatory signal transduction	Flagellin
TLR6	Interacts with TLR2; pro-inflammatory signal transduction	Lipoprotein
TLR7	Pro-inflammatory signal transduction	ss RNA, imiquimod
TLR8	Pro-inflammatory signal transduction	ss RNA, imiquimod
TLR9	Pro-inflammatory signal transduction	Unmethylated CpG DNA
TLR10	Pro-inflammatory signal transduction	N/A
MYD88	Universal TLR adaptor	N/A
Mal	Adaptor for TLR2, TLR4	N/A
Trif	Adaptor for TLR3	N/A
Tram	Adaptor for TLR4	N/A

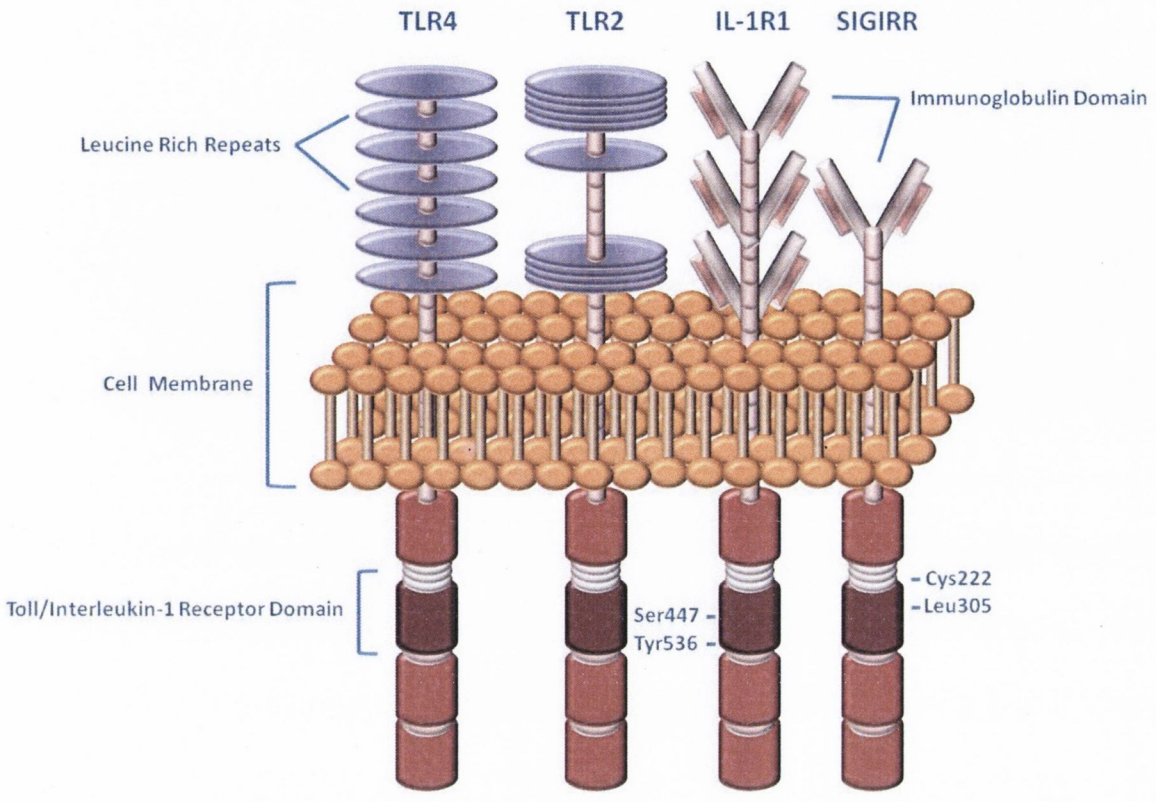


Figure 1.1. The Interleukin-1 Receptor/Toll-Like Receptor Superfamily

Figure 1.1. illustrates the structural features of a subset of the IL-1R/TLR superfamily of proteins. Members of the IL-1R/TLR superfamily share a highly conserved intracellular domain known as a TIR domain. For almost all members of the family, the TIR domain enables the propagation of downstream signaling cascades by binding to intracellular TIR domain-containing proteins such as MYD88. An exception to this rule is SIGIRR, which has two point mutation in its TIR domain which renders it unable to initiate signaling cascades. SIGIRR is also exceptional in its extracellular region, whose single Ig domain is not known to bind any ligand. The three Ig domains of IL-1R1 act as a receptor for the pro-inflammatory cytokine IL-1β, while the leucine rich extracellular regions of TLR4 and TLR2 enable recognition of bacterial molecular patterns, LPS and flagellin respectively.

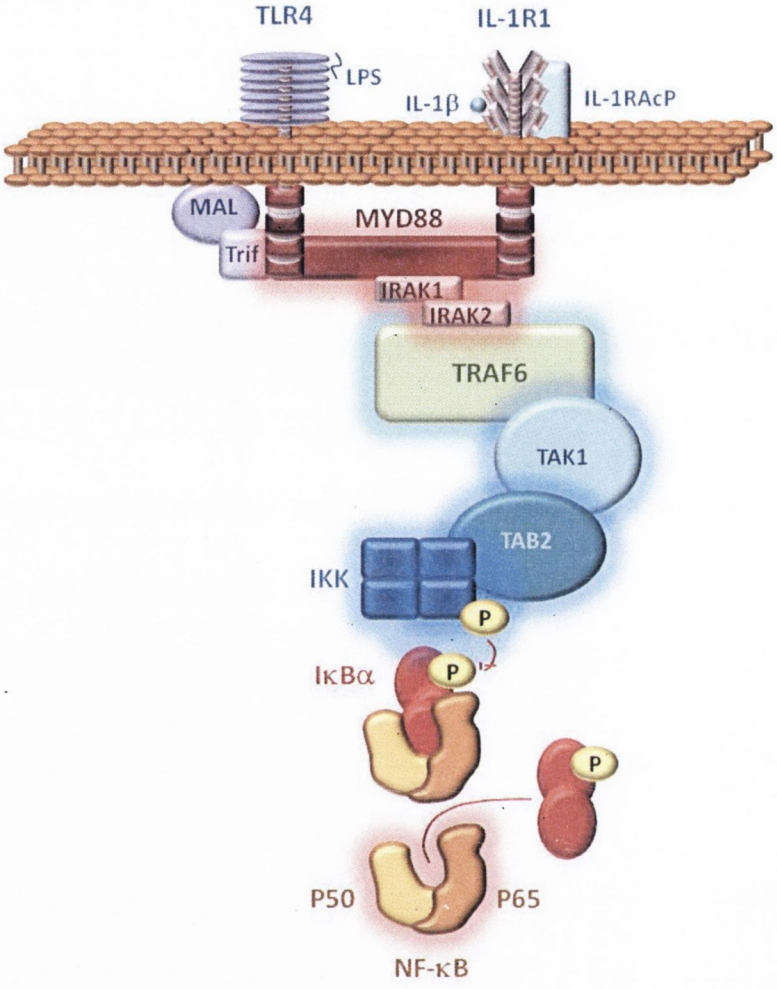


Figure 1.2. IL-1R1/TLR4 signaling pathway

Figure 1.2. illustrates a signaling pathway common to IL-1R1, TLR4 and TLR2 when stimulated. These receptors recruit the TIR domain-containing accessory protein MyD88, which allows them to form heterodimers with IL-1R-associated kinase 1 and 4 (IRAK1 and 4), (Auron, 1998). IRAK1 and IRAK4 are serine/threonine protein kinases which recruit the adapter protein TRAF-6. The receptor-IRAK-TRAF6 complex activates TAB-1 and TAK-1 which form a signalosome with IkB kinase (IKK) The signalosome removes the inhibitory protein nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-α (IkBα) from NF-kB, rendering it active. MyD88 and IRAK-1 may activate the MAPKs p38 and JNK independently of the signalosome, although any additional proteins required in this pathway remain unknown. Although the pathway described is common to both receptors, a number of other accessory proteins unique to each receptor determine the specificity of IL-1RI and TLR4 signaling (Auron, 1998, Fitzgerald and O'Neill, 2000, O'Neill and Dinarello, 2000).

The IL-1R1 and TLR signaling pathways play an integral role in the host response to infection. However, inappropriate or excessive inflammatory signaling may cause damage to the host tissue and can often give rise to secondary diseases or exacerbate an existing condition. Abnormally high concentrations of IL-1 and over-expression of gene products of IL-1R1 signaling have been reported in patients with inflammatory diseases such as rheumatoid arthritis and Guillain-Barre syndrome and have been implicated in their pathogenesis (Eastgate et al., 1988, Sharief et al., 1999). Chronic over-expression of IL-1β by activated microglia in the CNS has been associated with accumulation of Aβ into plaques in neurons, and elevated concentrations of the cytokine have been shown to exert a negative impact on neuronal and synaptic function e.g., inhibiting spatial learning and long-term potentiation (LTP) in the rat dentate gyrus (Vereker et al., 2000, Mrak and Griffin, 2001). This suggests that a persistent increase in IL-1β and Aβ signaling may be associated with the decline in cognitive function associated with AD.

Given the number and severity of diseases that may result from an excessive inflammatory response, anti-inflammatory drugs have long been prescribed for auto-immune and auto-toxic diseases. Non-steroidal anti-inflammatory drugs (NSAIDs) have proved effective in reducing the symptoms of diseases such as rheumatoid arthritis. NSAIDs have a broad spectrum anti-inflammatory effect, primarily achieved through cyclo-oxygenase inhibition. However, long-term treatment of chronic inflammation with NSAIDs is inadvisable due to reports of side-effects in the kidney and gastrointestinal tract (Whelton and Hamilton, 1991, Laine et al., 2003). It is now thought that modulation, rather than suppression of the inflammatory response is a more appropriate method of treating inflammatory diseases. This has created a demand for small molecules which interact with and modulate specific inflammatory signaling pathways.

1.5. Chronic neuroinflammation

While it is acknowledged that the inflammatory response is a necessary feature of host defence against infection and tissue damage, dysregulation leads to dysfunction. Disorders resulting from an abnormal acute inflammatory response vary in severity from painful swelling, partial

paralysis associated with Guillain-Barré syndrome, to potentially fatal allergic reactions. In the context of the CNS, however, chronic inflammation, that is, an inability of CNS immune cells to remove a stimulus, results in a persistently activated phenotype and a shift in the ratio of pro-inflammatory cytokines to anti-inflammatory cytokines in the microenvironment. Chronic inflammation can be induced by a number of factors which may be related to changes in physiological processes with aging, through the presence of invading pathogens, chemical or physical insults or as a response to toxic peptide accumulation as occurs in some neurodegenerative disorders.

1.5.1. Aging and chronic neuroinflammation

Aging is associated with significant neuronal loss and cognitive decline, and it is believed to be the strongest risk factor for the development of neurodegenerative diseases (Lucin and Wyss-Coray, 2009). There is growing evidence to suggest that the negative impact of aging on cognitive processes is due to chronic inflammatory changes that lead to the loss of neuronal fibers (Licastro et al., 2005, Griffin et al., 2006). The major cause of age-related chronic inflammation is thought to be increased oxidative stress resulting from impaired anti-oxidative mechanisms in cells which results in a build-up of ROS over time (Chung et al., 2009). It has been suggested that over time, mitochondrial DNA may become more susceptible to mutations, which alter respiratory function and anti-oxidant capacity of mitochondria leading to an imbalance between the production and clearance of ROS (Lin and Beal, 2006). Thus age-related mitochondrial dysfunction can result in an accumulation of ROS and ATP, both of which are capable of activating intracellular signaling proteins such as stress-activated protein kinases and NF-κB, although ATP is also recognised as a DAMP, inducing neuronal damage by interacting with P2X7R (Kim et al., 2002, Murphy et al., 2012). ROS accumulation in the CNS can enhance constitutive pro-inflammatory signaling cascades in microglia and other immune cells leading to the production of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 and further production of ROS. These in turn are released from glial cells to act on neurons and other glial cells to amplify pro-inflammatory signaling cascades and contribute to neuronal damage and cell death, and helping to maintain an inflammatory microenvironment within the CNS. While age-related chronic inflammation alone can contribute toward neurodegeneration and cognitive

decline, it also has the effect of maintaining microglia in a hypersensitive, or primed, state. There is evidence to suggest that the primed microglia of the aged brain exhibit increased expression of MHCII and CD11b, indicative of activation, and exaggerated responses to pro-inflammatory stimuli that may exacerbate inflammation and consequent tissue damage (Block et al., 2007, Lyons et al., 2007a, Cox et al., 2012). This is one of the reasons why age is the major risk factor for neurodegenerative disorders driven by inflammation.

1.5.2. Neurodegenerative disease and chronic neuroinflammation

Neurodegenerative diseases are characterised by the progressive loss, or deterioration in function, of neurons and neurite outgrowths in the peripheral nervous system (PNS) and CNS. These diseases may be induced or exacerbated by environmental factors but many are also associated with an underlying genetic mutation. A common factor in many neurodegenerative disorders is the accumulation of toxic peptides, often misfolded variants of endogenously-occurring proteins. One of the most well characterised proteopathic neurodegenerative disorders is Creutzfeld-Jakob Disease (CJD), a condition in which native proteins are induced to misfold and accumulate in toxic concentrations within cells (Niimi et al., 2008). The result is progressive cell loss and deterioration in cognitive function. Misfolded and aggregated toxic peptides have been associated with other neurodegenerative diseases, including PD and AD. In these diseases, protein aggregations may, in addition to directly inducing cell death, act as PAMPs and stimulate a robust inflammatory response which can exacerbate the effects of the disease.

1.5.3. Parkinson's Disease

PD is characterised by the profound loss of dopaminergic neurons in the substantia nigra which progressively impairs motor function and other forms of cognition. One of the factors associated with the neuronal loss typical of PD is the accumulation of α-synuclein peptides into inclusions known as Lewy bodies, which can disrupt neuronal functioning. Dopaminergic neurons in the PD brain are known to release a variety of factors, such as α-synuclein and matrix metalloproteinase 3 (MMP3), which can induce the activation of microglia and the release of ROS and pro-inflammatory cytokines, which exacerbate the neuronal damage and death typical

of the disease (Kim et al., 2005, Zhang et al., 2005). Although the activated microglia accumulate in large numbers in the substantia nigra in the PD brain, they are also present in a number of other brain regions, including the hippocampus (Imamura et al., 2003). Mouse models in which a dopaminergic neurotoxin, 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), is used to mimic the neurodegeneration seen in PD, exhibit chronic microglial activation, and a similar effect is seen in transgenic models of α-synuclein overexpression (Harvey et al., 2008, Theodore et al., 2008). It has also been reported that TNF-α and IL-1β as well as ROS, can contribute to neurodgeneration in PD (Hirsch and Hunot, 2009). It is widely believed that the widespread microglial activation and chronic inflammation typical of PD contributes to the progression of the disease and exacerbates many of its symptoms (Long-Smith et al., 2009).

1.5.4. Alzheimer's disease

AD is the most common form of dementia. The causes of the disease are not understood, however the greatest risk factor is age, while there is a strong genetic element associated with the early onset form of AD (Brookmeyer et al., 1998, Waring and Rosenberg, 2008). The presence of plaques composed of amyloid peptides and accumulated hyperphosphorylated tau proteins in neurofibrillary tangles are indicative of the disease (Tiraboschi et al., 2004). There is evidence that sporadic AD is associated with persistently-activated microglia and a chronic inflammatory response which may contribute to progression of the disease and to the cognitive decline associated with AD (Khandelwal et al., 2011). This is also supported by early studies which indicated that long-term treatment with anti-inflammatories appears to delay the onset of AD (McGeer and Rogers, 1992). One hypothesis is that the stimulus for the chronic inflammation associated with AD are Aβ peptides. Aβ is formed when the proteolytic enzymes, β-secretase and γ-secretase, cleave the amyloid precursor protein (APP) releasing the toxic Aβ peptides. This process is illustrated in **Figure 1.3**. It is believed that mutations in the genes coding for APP and presenlin-1, a component of γ-secretase, are responsible for early onset AD and transgenic mouse models overexpressing these genes have been developed to help elucidate the mechanisms underlying amyloid toxicity (Kurt et al., 2001). The Aβ peptides interact with cell surface receptors on glial cells and neurons e.g. RAGE, TLR2 and TLR4, to trigger signaling cascades and initiate inflammatory responses. Receptors thought to interact with Aβ are listed in **Table**

1.4. Aβ peptides also aggregate in and around neurons to form senile plaques which disrupt synaptic function and attract activated microglia, ultimately leading to neurodegeneration (Yankner et al., 1990, Qin et al., 2002). *In vitro* studies have demonstrated the ability of Aβ to induce the activation of microglia and to promote the release of pro-inflammatory cytokines (Tan et al., 2002, Lyons et al., 2007b, Jiao et al., 2008). It was also demonstrated that ICV injection of Aβ induced an increase in the hippocampal expression of MHCII and IL-1β (Lyons et al., 2007b). Microglia that accumulate around senile plaques have been shown to be immunopositive for MHCII and CD68 (Akiyama et al., 2000, Gallagher et al., 2012). These studies demonstrate the close relationship between microglial activation and AD, and highlight the importance of microglial activation as a target for treatments of AD.

Table 1.4. Receptors for Amyloid-β

Receptor	Other ligands
TLR2	Peptidoglycan, lipoteichoic acid, lipoprotein, lipoarabinomannan, zymosan
TLR4	LPS, F protein, hsp60, ethanol
RAGE	AGE, HMGB1, S100b, Mac-1, phosphatidyl serine
CD36/CD47/α6β1-integrin	Collagen, thrombospondin, LDL, lipoproteins, laminin
SR-A1	Modified LDL
SR-B1	LDL, HDL
α7-nicotinic receptor	Acetylcholine, nicotine, anabasine
Formyl peptide receptor	N-formylmethionine

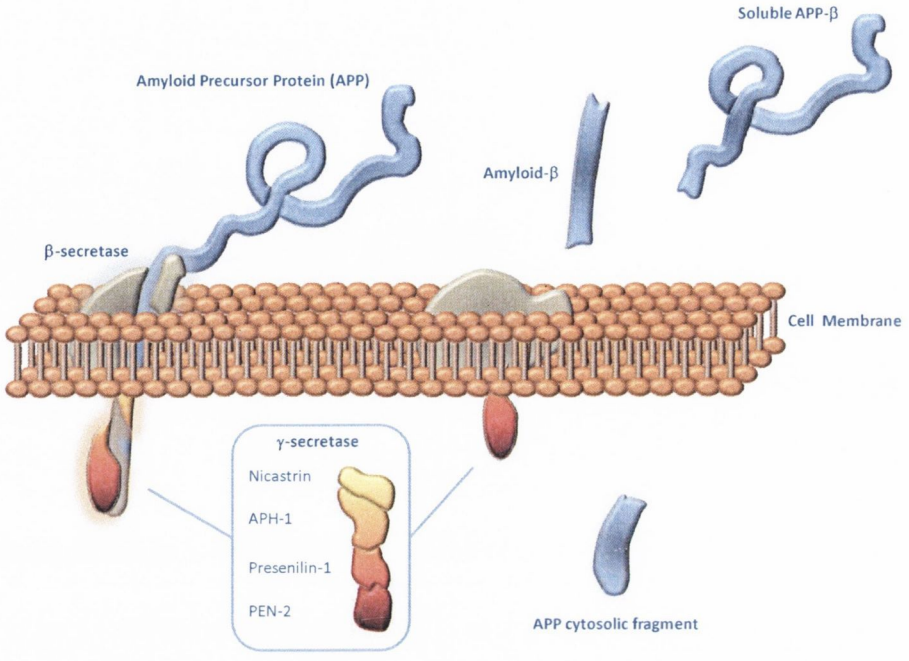


Figure 1.3. Formation of amyloid-β peptides

Aβ peptides are formed when integral membrane APP is sequentially cleaved by transmembrane proteolytic enzymes. γ-secretase cleaves APP in the transmembrane region of APP while β-secretase, whose active site is located extracellularly, cleaves the extracellular portion of APP (Bennett et al., 2003, Portelius et al., 2011). The process causes the release of soluble APP fragments and toxic Aβ peptides of varying lengths. The most common species of Aβ produced by this process are composed of 40 and 42 amino acid residues. Although both species are toxic to neurons and stimulate glial activation, $A\beta_{1\text{-}42}$ is more fibrillogenic and is therefore more associated with the formation of plaques seen in AD. A third enzyme, α-secretase, is capable of cleaving APP closer to the membrane than γ-secretase and represents a non-amyloidogenic form of APP processing (Yin et al., 2007, Valle-Delgado et al., 2010). Early-onset AD has been associated with mutations in the genes coding for APP and the presenilin-1 component of γ-secretase, illustrating the central role of amyloidogenesis in AD pathology (Murrell et al., 1991, Scheuner et al., 1996).

1.6. Modulation of inflammatory signalling

It is evident that inflammatory signalling plays an important role in the response to insults to the brain. It has also become clear that the dysregulation of inflammatory signalling associated with age and neurodegenerative disorders can have a devastating impact on neuronal viability and consequently on neuronal and synaptic function and therefore cognition. There are a number of endogenous factors which exert direct and indirect effects on inflammatory cascades to either promote or inhibit inflammatory signalling. Changes in the expression or function of these factors are a characteristic of many neurodegenerative disorders. The following section will focus on a number of proteins which exert a negative regulatory effect on inflammatory signalling, acting as a break on the inflammatory response.

1.6.1. Fractalkine

Fractalkine is a chemokine which is expressed in a variety of tissues and can exist in a membrane-bound or soluble form (Bazan et al., 1997, Lyons et al., 2009). The membrane-bound form of fractalkine is found in abundance on neurons while its receptor is highly expressed on microglia (Harrison et al., 1998). This complementary expression of the chemokine and its receptor supports a growing body of evidence that describes a modulatory role for fractalkine on microglial activation. Exogenous application of soluble fractalkine has been shown to attenuate the LPS-induced increase in IL-1β from rat mixed glia and microglia. This effect was also seen when glial cells were co-cultured with neurons, but the effect was lost following treatment with a fractalkine blocking antibody, suggesting that the neuronal membrane-bound form of fractalkine plays a similar role (Lyons et al., 2009). There is evidence which links this ability to modulate cytokine release from glia to cognition, as fractalkine-deficient mice exhibit reduced learning ability and LTP, which was reversed following application of IL-1Ra (Rogers et al., 2011). Fractalkine deficiency has been shown to negatively impact the regeneration of the olfactory epithelium due to an inhibition in the proliferation of progenitor cells and the increased recruitment and activation of macrophages (Blomster et al., 2011). However, there is evidence of an alternative role for soluble fractalkine as a chemoattractant to peripheral immune cells. Increased fractalkine concentrations have been found in CSF of MS patients and fractalkine gradients have been shown to promote the migration of T-cells into the MS brain (Broux et al.,

2012). A number of studies have investigated the role of fractalkine in other neurodegenerative diseases. There is conflicting evidence about its role in PD. Fractalkine concentrations in the CSF of PD patients appears to increase with the severity of the disease but it is unclear whether this is an indication of a protective response or due to the role of fractalkine as a chemoattractant for immune cells (Shi et al., 2011). Exogenously-applied fractalkine has shown differential effects in different models of PD, with microglial activation and dopaminergic neuron depletion observed in an MPP^+ model whereas, in a 6-hydroxydopamine (6-OHDA) infusion model, fractalkine suppressed microglial activation and reduced lesion size (Pabon et al., 2011, Shan et al., 2011). There have also been conflicting reports of the role of fractalkine in AD. Reduced fractalkine concentrations have been observed in the plasma of patients with severe AD compared with mild AD or controls (Kim et al., 2008). Interestingly, fractalkine was also shown to be reduced in the brains of aged APP_{swe} transgenic mice (Duan et al., 2008). Fractalkine-deficient APP-PS1 mice have a profoundly altered CNS cytokine environment with reduced mRNA expression of TNF-α and CCL2 but increased IL-1β mRNA (Lee et al., 2010). It remains unclear whether fractalkine has a beneficial or detrimental role in PD and AD. It is possible that the multiple functions of the chemokine cause it to have differential effects as the disease progresses, contributing to deleterious effects early on by promoting the migration of peripheral immune cells which exacerbate the negative effects of inflammation, but acting as an essential modulator of microglia as the cells develop and become chronically over-active in later stages.

1.6.2. Cluster of Differentiation 200 (CD200)

CD200 is a transmembrane glycoprotein and member of the Ig superfamily of proteins (Wright et al., 2000). It is widely distributed on a variety of cell types, including neurons, and interacts with its structurally similar receptor CD200R which is located primarily on cells of the myeloid lineage (Wright et al., 2003, Lyons et al., 2007a). CD200 is thought to mediate in cell-cell interactions and is an important modulator of myeloid cells, including microglia (Lyons et al., 2007a). Evidence to date suggests that it is one of the most important mechanisms of neuronal modulation of microglial function. CD200 exerts its effects through inhibition of the ras/MAPK signalling pathway. CD200R binds to CD200 and is tyrosine phosphorylated leading to the recruitment of adaptor protein downstream of tyrosine kinase 1 (DOK1) and DOK2. DOK1 and

DOK2 are capable of interacting with and activating ras GTPase activating protein (RasGAP), which acts as an inhibitor of ras by enhancing its GTPase activity, inducing the non-signalling GDP-bound form of ras. This ultimately reduces the activity of the transcription factors ERK, JNK and p38 and dampens down the pro-inflammatory gene expression they induce (Zhang et al., 2004). Studies have revealed that ligand-bound CD200R interacts more strongly with DOK2 than DOK1 and that siRNA knock-down of DOK2, but not DOK1, disrupted CD200 signalling (Mihrshahi et al., 2009). It has been suggested that DOK1 may be a negative regulator of DOK2 signalling (Mihrshahi and Brown, 2010). Hoek and colleagues (2000) first revealed that microglia from CD200-deficient mice exhibited increased proliferation and activation compared with controls and that lack of CD200 was associated with a more rapid onset of EAE (Hoek et al., 2000). Glia prepared from CD200-deficient mice were found to have increased expression of markers of microglial activation in response to LPS and Pam_3CSK_4 (Costello et al., 2011a). The modulatory effect of CD200 on glia may be important in maintaining the integrity of synaptic transmission, as impaired LTP was noted in hippocampal slices from CD200-deficient mice (Costello et al., 2011a). The effect of CD200 was investigated further in vivo and it was reported that a CD200 fusion protein (CD200Fc) was capable of attenuating age and LPS-induced deficits in LTP in the rat hippocampus. CD200Fc treatment attenuated both age and LPS-induced microglial activation, suggesting that modulation of microglial activation by CD200 may have therapeutic potential in neurodegenerative disorders (Cox et al., 2012). This hypothesis is supported with evidence suggesting an impaired ability to induce CD200R on macrophages from patients with PD (Luo et al., 2010). In addition, there was an increase in microglial activation and neuronal loss, and an exacerbated impairment of motor skills in a 6-OHDA mouse model of PD treated with a CD200R blocking antibody compared with vehicle treated mice (Zhang et al., 2011). Reduced expression of CD200 and CD200R mRNA was also observed in hippocampus from AD patients with specific reductions in neuronally-localised CD200 in regions affected by the disease (Walker et al., 2009). In addition, treatment with CD200Fc was capable of attenuating Aβ-induced activation of glia and it was also capable of reversing the Aβ-induced impairment of LTP in hippocampal slices (Lyons et al., 2012). These studies indicate that modulation of microglial activation by CD200 may be impaired in neurodegenerative diseases and that the CD200-CD200R complex may be a useful target for potential therapeutics.

1.6.3. SIGIRR

SIGIRR was first discovered through expressed sequence tag database searching for proteins containing TIR domains. It was classified as a member of the IL-1/TLR superfamily because of the TIR domain contained within its intracellular domain. Although it is structurally similar to IL-1RI, several differences distinguish them. The extracellular domain of IL-1RI consists of three immunoglobulin-like domains, whereas SIGIRR possesses only one such domain and cannot bind IL-1 (Thomassen et al., 1999). The amino acid residues Ser447 and Tyr536 found in the conserved intracellular region of IL-1RI have been replaced in SIGIRR with Cys222 and Leu305 respectively, rendering SIGIRR incapable of normal IL-1 signaling (Thomassen et al., 1999, Wald et al., 2003). In addition, the intracellular domain of SIGIRR is 56 amino acids longer than that of IL-1RI. Although SIGIRR is more closely related to the IL-1R subfamily than the TLR subfamily, its elongated intracellular domain is similar in length to the Drosophila toll protein and it is capable of modulating TLR signaling (Thomassen et al., 1999). RNA blot analyses have shown that SIGIRR is ubiquitously but differentially expressed in mouse tissue and is found in greatest abundance in the kidney. It is also highly expressed in the colon, small intestine, and moderately expressed in the lung and in the liver on hepatic cells and in the spleen on splenocytes. Epithelial cell lines were found to have the highest cell-specific expression. SIGIRR is moderately expressed in dendritic cells with lower expression on macrophages (Wald et al., 2003). Isolated and cultured human NK cells express high levels of SIGIRR (Polentarutti et al., 2003). Although initial findings suggested that SIGIRR is weakly expressed in the brain, subsequent studies using RT-PCR and immunohistochemistry showed that SIGIRR is present on primary neuronal cultures and secondary astrocyte and microglia cultures (Wald et al., 2003, Andre et al., 2005, Costelloe et al., 2008). In 2003, Wald and colleagues identified several features of SIGIRR expression which provided initial evidence that it acted as a negative regulator of TLR/IL-1 signaling. Exposure to LPS typically results in the upregulation of members of the IL-1/TLR superfamily, particularly IL-1R1 and TLR4. In contrast, SIGIRR is down-regulated in mouse tissue by low concentrations of LPS. Jurkat and HepG2 cells which over-express SIGIRR exhibit markedly reduced NF-κB activation when exposed to IL-1 but not IFN-γ.

In 2004, Garlanda and colleagues generated SIGIRR-deficient mice by homologous recombination, and this represented a major leap forward in the understanding of the function of SIGIRR. It was reported that dendritic cells from SIGIRR-deficient mice showed increase production of cytokines in response to a number of TLR agonists, including LPS, and SIGIRR-deficient mice exhibited increased susceptibility to dextran sulphate sodium (DSS)-induced intestinal inflammation, underlining the importance of SIGIRR in the gut (Garlanda et al., 2004). SIGIRR-deficient mice, injected intraperitoneally with LPS displayed a mortality rate three times greater than wildtype mice. In addition, intraperitoneal injection of IL-1 in SIGIRR-deficient and wildtype mice resulted in the induction of greater concentrations of inflammatory chemokines for a longer duration in the SIGIRR-deficient mice. Enhanced NF-κB and JNK activation was observed in primary kidney epithelial and splenocyte cell lines prepared from SIGIRR-deficient mice exposed to IL-1 and LPS, but not TNF-α. Polentarutti and colleagues (2003), injected wild-type mice with LPS and observed a partial down-regulation of SIGIRR in all tissue types that express the receptor under normal circumstances (Polentarutti et al., 2003). These results gave a strong indication that SIGIRR has a negative regulatory role in inflammatory processes. More recently, the effect of SIGIRR over-expression in 293 cells transfected with IL-1RI and TLR4 was examined. It was reported that following exposure to IL-1 or LPS, SIGIRR over-expression significantly attenuated NF-κB activation. No change in NF-κB activation was observed in cells treated with TNF-α (Wald et al., 2003). Since the anti-inflammatory effects of SIGIRR were observed in IL-1 and LPS-induced signaling but not in TNF or IFN-γ-induced signaling, it appears that SIGIRR act specifically through the IL-1/TLR superfamily.

In order to identify the mechanisms by which SIGIRR modulates TLR/IL-1R signaling, 293 cells were transfected with an NF-κB responsive construct. They were then co-transfected with a full-length SIGIRR construct, or a truncated version of SIGIRR lacking either the 56 amino acid intracellular sequence absent in IL-1R1 or the entire intracellular portion of the receptor. Following exposure to IL-1, IL-1R1-mediated NF-κB activation was inhibited by full length SIGIRR, but not by the truncated versions (Polentarutti et al., 2003). NF-κB activation was inhibited by a SIGIRR chimera lacking an extracellular domain but was unaffected by the chimera consisting of the extracellular domain only. From these results the authors concluded that the intracellular domain, but not the extracellular domain, of SIGIRR was necessary for

inhibition of IL-1R1 signaling (Polentarutti et al., 2003). Subsequent research by Wald and colleagues in 2005 investigated the interaction between SIGIRR and several components of the signaling cascades induced by TLR4/IL-1R1 activation. A cell line was transfected with IL-1R1, TLR4 and MD2, exposed to IL-1 or LPS for 5, 10 and 30 minutes and then immunoprecipitated with SIGIRR. It was shown that SIGIRR interacts with the IL-1R1 signaling complex in a ligand-dependant manner as exposure to IL-1 resulted in endogenous SIGIRR interacting with all analysed components of the TLR4/IL-1RI signaling pathways transiently. This experiment was repeated using LPS as the stimulant and a similar interaction was observed between SIGIRR and components of the TLR4 signaling complex. Further study investigated the domains involved in SIGIRR-associated inhibition. Wildtype SIGIRR or SIGIRR deletion mutants lacking the extracellular immunoglobulin domain, the C-terminal domain or the TIR domain were transfected into 293IL-1R1/TLR4/MD2 cells and subjected to analysis for interactions. The results showed that following exposure to IL-1, wild-type SIGIRR interacted with IL-1R1, MYD88, IRAK and TRAF6. However SIGIRR lacking an extracellular domain was unable to interact with any component of the IL-1R1 signaling complex. This ability was also reduced in the mutant lacking a TIR domain, however deletion of the C-terminal domain had a negligible effect. The role of each domain in the inhibition of IL-1 signaling was determined using SIGIRR deletion mutants and observing IL-1 induced NF-κB activation. Wildtype SIGIRR inhibited NF-κB activation but this effect was completely absent in the mutant lacking an extracellular domain. SIGIRR lacking a TIR domain had limited ability to reduce NF-κB activation while the mutant lacking a C-terminus was unimpaired. Subsequent experiments revealed that the extracellular immunoglobulin domain is involved in preventing the heterodimerization of IL-1R1 with IL-1R accessory protein (IL-1RAcP), a protein involved in determining the specificity of IL-1RI signaling, whereas LPS-induced NF-κB and JNK activation were impaired in the SIGIRR mutant lacking a TIR domain. SIGIRR inhibition of IL-1 and LPS signaling via the TIR domain was shown to be achieved through the sequestering of MYD88 and IRAK1. Thus Wald and colleagues (2003) concluded that interaction of SIGIRR with the IL-1R1/TLR4 complex was necessary for inhibition of IL-1RI/TLR4 signaling. They also highlighted the differing mechanisms utilized by SIGIRR to modulate IL-1R1 and TLR4 signaling. In contrast to Polentarutti and colleagues (2003), they reported that both the intracellular and extracellular domains of SIGIRR were required for IL-1R1 inhibition, whereas TLR4 inhibition was mediated

through the TIR domain only. **Figure 1.4** illustrates the mechanisms through which SIGIRR modulates IL-1R1/TLR4 signaling.

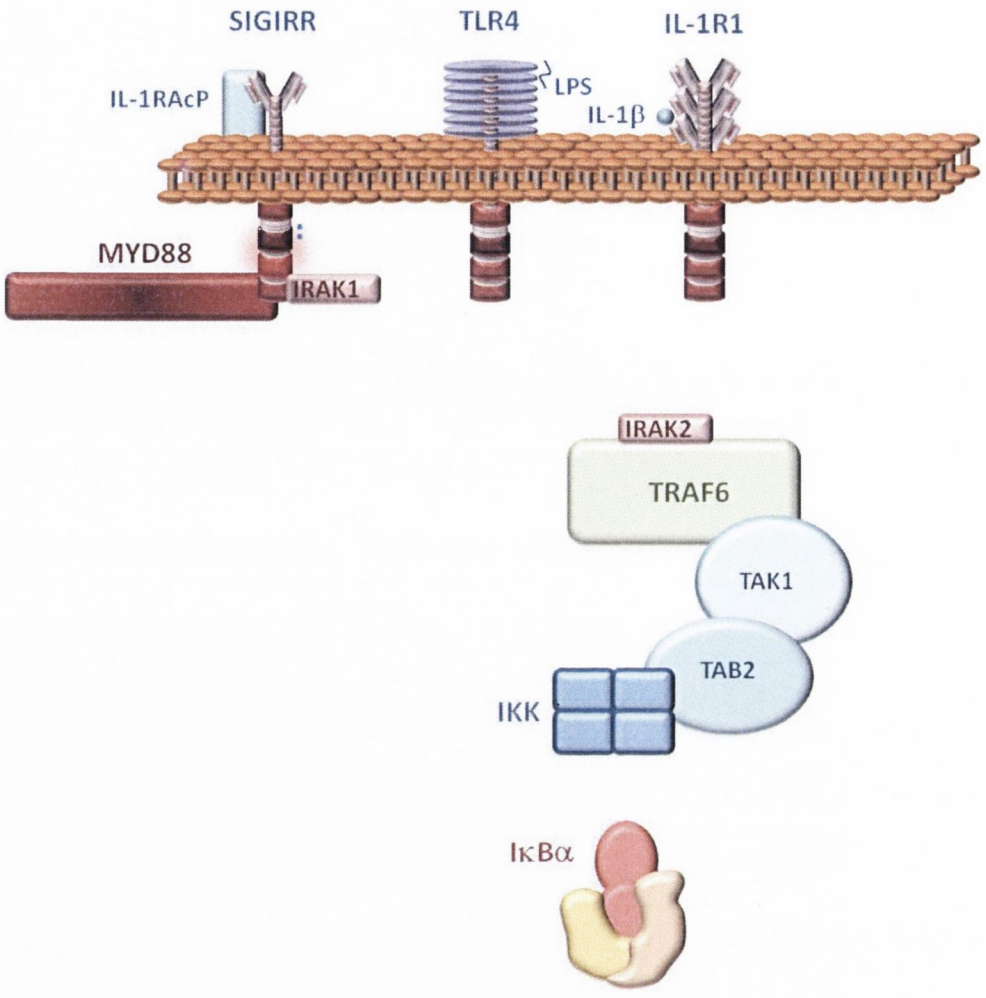


Figure 1.4. SIGIRR negatively regulates IL-1RI/TLR4 signaling pathway

Figure 1.4. summarises the current understanding of how SIGIRR modulates IL-1R1/TLR4 signaling. SIGIRR is activated when IL-1R1 or TLR4 are in their ligand-bound form. SIGIRR extracellular Ig domain interacts with IL-1R1 to prevent its heterodimerisation with IL-1RAcP. This step results in substantial downregulation of IL-1R1 signaling. Although the intracellular TIR domain contributes to this downregulation to a lesser degree, it is the primary component of SIGIRR which is responsible for the negative regulation of TLR4 signaling. It does this by sequestering the adaptor proteins MYD88 and IRAK through homotypic TIR domain interactions. Activation of downstream signal transducers in the absence of these adaptor proteins is severely limited.

Having established a basic mechanism of action for SIGIRR, the focus began to switch to the role it plays in general physiology. SIGIRR is highly expressed in the kidney, particularly on renal tubular epithelial cells and renal dendritic cells. TLR signaling from these cells contribute to the pathology of infective pyelonephritis, a severe urinary tract infection. Lech and colleagues (2007) discovered that SIGIRR suppressed LPS-induced upregulation of CCL2 from renal cells. This effect was found to be cell-specific, with SIGIRR only negatively regulating TLR4 signaling on renal dendritic cells. Although the receptor is located in abundance on tubular epithelial cells, TLR4 signaling in these cells was unimpaired. The researchers determined that this was a result of differential post-translational modifications of SIGIRR in the different cell types. It is apparent from this study that renal tubular epithelial cells act as sensors of infection in the kidney and that modulation of inappropriate inflammatory responses by SIGIRR is restricted to antigen-presenting cells (Lech et al., 2007). A later study examined the role of SIGIRR in limiting tissue injury in the post-ischemic kidney and it was shown that SIGIRR has a modulatory effect on innate immune signaling in the kidney. This was determined by the aggravated upregulation of the inflammatory mediators macrophage inflammatory protein 2 (MIP-2), CCL2 and IL-6 in SIGIRR-deficient mice post-ischemia and was accompanied by increased migration of myeloid cells into the kidney contributing to a greater inflammatory phenotype in SIGIRR-deficient mice. Consistent with the previous study, upregulation of inflammatory mediators was much greater in myeloid cells from lacking in SIGIRR than tubular epithelial cells, confirming the cell-specificity of the receptor (Lech et al., 2009). A study of the role played by SIGIRR in the immune response to kidney transplantation found that in a mouse model of kidney transplants in which the introduced organ is spontaneously accepted, expression of SIGIRR is higher than in naïve organs. In addition, transplants were rejected in SIGIRR-deficient mice with an accompanying increase in dendritic cell maturation (Noris et al., 2009). This study suggested that SIGIRR may represent a future therapeutic target to improve transplant success. These data illustrate the role of SIGIRR in dampening the inflammatory response to infection and ischemia in the kidney and in immune acceptance of organ transplants. Interestingly, it was also suggested that the immunomodulatory actions of SIGIRR may act primarily through suppression of TLR/IL-1R signaling in cells of the myeloid lineage.

Further evidence of the ability of SIGIRR to negatively modulate TLR/IL-1R signaling came from a model of *Mycobacterium tuberculosis* infection in SIGIRR-deficient mice. The mutant mice exhibited rapid death in comparison with wild-type mice, due to necrosis in the liver. Both mutant and wildtype mice had similar levels of cytotoxic and memory T-cells. SIGIRR-deficient mice had a higher rate of infiltration of myeloid cells into the lungs and this was accompanied by elevated levels of pro-inflammatory cytokines e.g. IL-1β and TNF-α. Additional *in vitro* experiments confirmed that dendritic cells infected with *M. tuberculosis* produced greater amounts of several pro-inflammatory cytokines and chemokines. Blocking IL-1β and TNF-α significantly reduced the mortality rate in the SIGIRR-deficient mice, emphasising their pathological role in *M. tuberculosis* toxicity (Garlanda et al., 2009). The increased susceptibility of SIGIRR-deficient mice to a rapid increase in inflammatory mediators emphasises the role SIGIRR plays in dampening excessive innate immune response to infection.

These recent data have indicated that SIGIRR represents a promising new target for the development of small molecule drugs to modulate signaling in the innate immune system. The specific targeting of TLR/IL-1R signaling presents an opportunity to develop novel anti-inflammatory therapies without the broad spectrum effects that restrict long-term use of NSAIDs. In addition, SIGIRR appears to act in a cell-specific manner. It has been shown in the kidney to act primarily on myeloid-lineage cells while leaving constitutive TLR/IL-1R signaling from other cell types unaffected. This feature is particularly important in the context of infectious disease when modulation, rather than suppression, of the innate immune response is more appropriate. Therapeutic targeting of SIGIRR has been hampered by the fact that thus far, an endogenous ligand has yet to be identified. It is clear that many questions remain regarding activation of SIGIRR. Answering these questions may see SIGIRR emerge as the newest target for anti-inflammatory therapies.

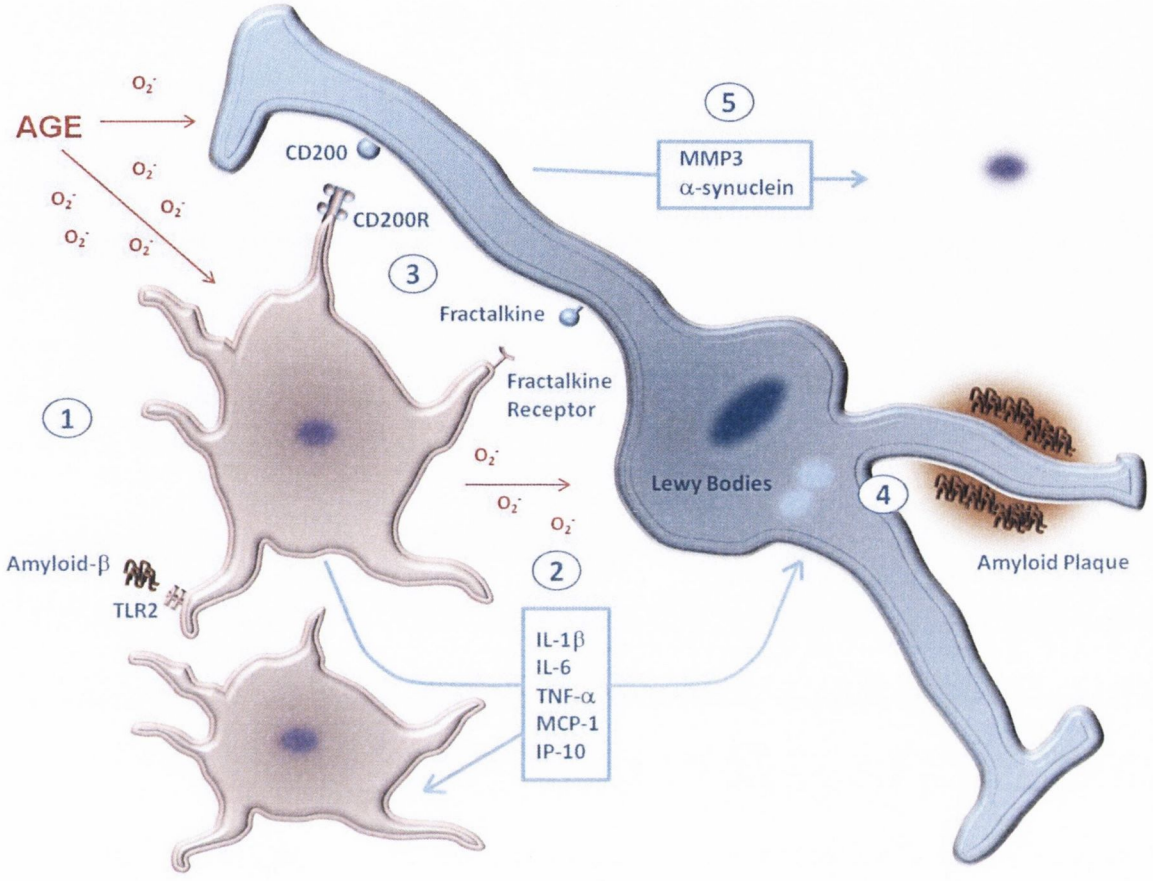


Figure 1.5. Chronic neuroinflammation in aging and neurodegenerative disease

Figure 1.5. illustrates some of the many processes that contribute to chronic neuroinflammation and highlights the interaction between microglia and neurons in these processes. (1) Inflammatory signaling can be initiated by a number of factors such as the increase in ROS associated with age and the interaction of toxic proteins e.g. Aβ on cell surface receptors. Signaling may be modulated by cell surface proteins such as SIGIRR. (2) Activated microglia release a wide variety of cytokines in order to signal to adjacent glia to participate in the inflammatory process. Activated microglia also produce ROS which may accumulate if activation is prolonged. (3) An acute inflammatory response is usually terminated when the noxious insult is removed by the activated microglia. Proteins such as CD200 and Fractalkine, often found on neuronal membranes, interact with their receptors on microglial cell membranes and induce the cells to return to a "deactivated" phenotype. (4) If the inflammatory stimulus is persistent, such as the Lewy bodies or senile plaques associated with PD and AD, microglia can become chronically activated leading to a build-up of toxic factors which exacerbate neurodegeneration. (5) Damaged and dying neurons release a number of factors which further activate microglia, creating vicious cycle of chronic inflammation and neurodegeneration.

1.7. Aims

Although a large and growing number of researchers have examined the role of SIGIRR in modulating inflammation in a variety of peripheral cell types and systems, thus far there has been a dearth of studies examining the effects of SIGIRR on inflammatory signaling in the brain. With the increasing evidence linking dysregulated inflammation with the neurodegeneration and cognitive decline characteristic of age and disease, it is vital to increase our understanding of the endogenous mechanisms that exist to modulate these processes. Endogenous modulators of inflammation represent a ready-made target for potential therapeutics for diseases with an inflammatory component. The overall aim of this study to investigate the modulatory effects of SIGIRR on glia.

One of the most effective means to understand the physiological function of a protein is to analyse the effect of its absence on physiological processes. To this end, the production of pro-inflammatory cytokines and chemokines and the upregulation of markers of glial activation were investigated in cultured mixed glia, astrocytes and microglia prepared form SIGIRR-deficient mice, and compared with responses in wildtype mice. The effects of TLR4, IL-1R1 and TLR2 stimulation were examined using the bacterial endotoxin LPS, the pro-inflammatory cytokine IL-1β, the synthetic TLR2 agonist Pam_3CSK_4 and Aβ respectively. The aim of these experiments was to assess whether the modulatory effect of SIGIRR on TLR and IL-1R1 signaling in peripheral immune cells extended to glia, the immune cells of the CNS.

This investigation was continued in an *in vivo* study to investigate the ability of SIGIRR to modulate various aspects of the inflammatory process in the brain which are associated with aging and Aβ toxicity. This was achieved using intrahippocampal injections of Aβ in young and middle-aged mice wildtype and SIGIRR-deficient mice as models of Aβ toxicity, aging and SIGIRR-deficiency. It was hypothesized that any effects of SIGIRR-deficiency observed in the *in vitro* experiments would be replicated *in vivo*, and that this study would provide further information about the physiological role of SIGIRR in the brain.

Finally, a series of experiments was conducted to investigate possible mechanisms to exploit the potential of SIGIRR as an anti-inflammatory agent in the brain. These included: an investigation of the anti-inflammatory cytokine IL-1F5 as a potential ligand for SIGIRR; the use of a SIGIRR fusion protein (SIGIRR/Fc) to investigate homotypic activation of SIGIRR; an investigation of a TREM-1 blockade as an indirect enhancer of SIGIRR expression and finally an attempt at molecular cloning of SIGIRR in order to drive over-expression of SIGIRR.

Chapter 2: Methods

2.1. Preparation of cultured mixed glia, microglia and astrocytes

2.1.1. Preparation of cultured cortical mixed glia from wildtype and SIGIRR$^{-/-}$ mice

Primary cortical mixed glia were prepared from postnatal 1-day old C57BL/6 and SIGIRR$^{-/-}$ mice, supplied by BioResources Unit in Trinity College, Dublin 2. Dissection instruments were sterilised in 70% ethanol. Mice were decapitated, brains were removed and cortices were dissected free using a sterile forceps. The cortices were cross-chopped using a sterile scalpel and incubated in warm Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich, UK) supplemented with fetal calf serum (10%; Gibco, UK), penicillin (100U/ml; Gibco, UK) and streptomycin (100U/ml; Gibco, UK) (5 min, room temperature). Tissue was triturated using a sterile Pasteur pipette, filtered through a nylon mesh filter (40μm; BD Biosciences, US) and centrifuged (2000rpm, 3 min, 20°C). The pellet was re-suspended in DMEM (1.5ml) and aliquots of re-suspended glia (250μl) were plated onto each well of a 6-well plate (Sarsted, Germany). Glia were left to adhere for 2 h in a humidified incubator, 5% CO_2, 95% air at 37°C, before wells were flooded with warm supplemented DMEM (1.5ml). Media was changed every 3 days and grown for 10-14 days before treatment. Mixed glia prepared according to this protocol have been shown to consist of approximately 70% astrocytes and 30% microglia as assessed by expression of CD11b using fluorescence activated cell sorting (FACS) (Costello et al., 2011).

2.1.2. Preparation of isolated cortical microglia and astrocytes from wildtype and SIGIRR$^{-/-}$ mice

Primary cortical microglia and astrocytes were isolated from postnatal one-day old C57BL/6 and SIGIRR$^{-/-}$ mice. Neonates were decapitated and cortical tissue prepared as described above, however following resuspension, glia were plated into T25 flasks using a plastic Pasteur pipette and left in the incubator to allow the cells to adhere. After 2 h, the flasks were flooded with warm complete DMEM (6ml). After 24h the media was removed and replaced with complete DMEM containing mononuclear phagocyte colony stimulating factor (M-CSF; 20ng/ml, R&D Systems, UK) and granulocyte macrophage colony stimulating factor (GM-CSF; 10ng/ml, R&D Systems, UK). Media was replaced with fresh media supplemented with M-CSF and GM-CSF 4

and 8 days after culturing. On day 10 the flasks (neck and cap) were wrapped with parafilm to make them airtight and placed on an orbital shaker and shaken (2 h, 110rpm, room temperature) in order to detach adherent microglia. The flasks were returned to the hood and each one was tapped 10 times and the supernatants with suspended microglia from all the flasks was poured into a new 50ml Falcon tube, which was centrifuged (2000rpm, 3 min, 20°C). The supernatant was removed and the resulting pellet was resuspended in complete DMEM (2ml). The microglial cells were counted and equalised to a cell density of $1x10^6$ cells/ml. The microglial cells were plated into 6-well plates and incubated for 2 h in 5% CO^2 at 37° C to allow the cells to adhere, then flooded with complete DMEM (1.5ml). The astrocytes remaining in the flasks were washed with sterile 1X PBS and incubated in trypsin-EDTA (1.5ml) at 37°C. The flasks were periodically observed and when there was visible lifting of the cell layer, they were tapped 10 times and the cell suspension from all the flasks was poured into a new 50ml Falcon tube. This was centrifuged (2000rpm, 3 min, 20°C), the supernatants were removed and the pellet was resuspended in complete DMEM (2ml). The astrocytes were counted and equalised to a density of $1x10^6$ cells/ml. The cells were plated into 6-well plates and incubated for 2 h in 5% CO_2 at 37°C to allow the cells to adhere, then flooded with 1.5ml complete DMEM. Isolated astrocytes and microglia prepared according to this protocol have been shown have purity of >90% and >95% respectively as assessed by expression of CD11b using fluorescence activated cell sorting (FACS) (Cowley et al., 2010; Denieffe, Unpublished data).

2.2. Cell treatments and harvesting

All agents used to treat cells were diluted to required concentrations in pre-warmed supplemented DMEM and all solutions were filter-sterilised through a 0.2μm cellulose acetate membrane filter.

- Lipopolysaccaride (LPS; Sigma Aldrich, UK) was diluted to a final concentration of 10-100ng/ml in supplemented DMEM. Cells were treated for 24h with LPS.

- IL-1β (R&D Systems; UK) was prepared as a stock solution in sterile phosphate-buffered saline (PBS) and 0.1% BSA and diluted to the desired concentration of 10ng/ml in supplemented DMEM. Cells were treated for 24 h with IL-1β.

- Pam_3CSK_4 (Invivogen, UK) was prepared as a stock solution in sterile water and was diluted to a final concentration of 10 ng/ml in supplemented DMEM. Cells were treated for 24 h with Pam_3CSK_4.

- Amyloid-β_{1-40} ($A\beta_{1-40}$) and $A\beta_{1-42}$ (Invitrogen; US) were aggregated according to the manufacturer's instructions. $A\beta_{1-42}$ was dissolved in sterile, distilled water to a concentration of 6mg/ml and then further diluted to a 1mg/ml stock solution with PBS (SIGMA, UK). $A\beta_{1-40}$ was dissolved in PBS to a concentration of 1mg/ml. $A\beta_{1-40}$ peptide was aggregated for 24 h at 25°C and the $A\beta_{1-42}$ peptide for 48 h at 37°C. All stock solutions were aliquoted and stored at minus 20°C until required. Cells were incubated with a cocktail of $A\beta_{1-40}$ (4.2μM) and $A\beta_{1-42}$ (5.6μM) for 24 h.

- IL-1F5 (Enzo Life Sciences, UK) was prepared as a stock solution in sterile water. Cells were pre-treated with IL-1F5 (3μg/ml) for 2 h and co-incubated in the presence of IL-1β (10ng/ml; R&D Systems, UK) for a further 24 h.

- SIGIRR/Fc (R&D Systems, UK) was prepared as a stock solution in sterile PBS. Cells were pre-treated with SIGIRR/Fc (0.5-5μg/ml) for 2 h and co-incubated in the presence or absence of LPS (100ng/ml; Enzo Life Sciences, UK), or IL-1β (10ng/ml; R&D Systems, UK) for a further 24 h.

- TREM1Fc (R&D systems, UK) was prepared as a stock solution in sterile PBS. Cells were pre-treated with TREM-1/Fc (2-10ng/ml) for 30 min and co-incubated in the presence or absence of LPS (10-100 ng/ml; Enzo Life Sciences, UK) for a further 6, 12 or 24 h.

Following the indicated treatments; supernatants were removed and placed in 1.5ml tubes (Fisher Scientific; UK) for later cytokine and chemokine analysis. Cells used for polymerase chain reaction (PCR) were harvested by washing in sterile 1x PBS and lysed in RA1 buffer (Macherey-Nagel; US) containing β-mercaptoethanol (1:100 dilution; Sigma Aldrich; UK) and stored at -80°C in RNase free tubes (Medical Supply Company; Ireland) for later analysis of messenger ribonucleic acid (mRNA) expression. Cells used for western immunoblotting were harvested by washing once in sterile 1x PBS and lysed in ice-cold lysis buffer (100µl; 10mM Tris HCl, 50nM NaCl, 10mM $Na_4P_2O_7.H_20$, 50mM NaF, 1% Igepal, 1:100 dilution phosphatase inhibitor cocktail I and II, 1:100 dilution protease inhibitor cocktail; Sigma, UK), scraped off and stored at -80°C in fresh 1.5ml tubes.

2.3. Analysis of mRNA by reverse transcriptase polymerase chain reaction (RT-PCR)

2.3.1. RNA Extraction

mRNA was extracted from cortical and hippocampal tissue from wildtype and SIGIRR$^{-/-}$ mice treated with saline or Aβ and lysates of mixed glia, microglia and astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice treated as described above. Tissue was homogenized with two separate 5s pulses with a sonocator. Cells were scraped from 6-well plates in a cell lysis buffer containing RA1 buffer and 2-mercapto-ethanol (100:1). Tissue homogenate and cell lysates were filtered using NucleoSpin filters, collected in an Eppendorf tubes and centrifuged (11,000 x g, 1 min). Ethanol (70%, 350µl) was added to the filtrate, mixed and loaded onto NucleoSpin RNA II columns. Tubes were centrifuged (8,000 x g, 30s) and the RNA binds to the column. The silica membrane was desalted by adding membrane desalting buffer (350µl) and centrifuged (11,000 x g, 1 min) to dry the membrane. To digest the DNA, DNase reaction mixture (95µl) was added to the column and incubated at room temperature for 15 min. The silica membrane was washed and dried. RNA was eluted by adding RNase free H_20 and centrifuged (11,000 x g, 1 min) and RNA concentration was quantified using a nanoDrop-spectrophotometer (ND-1000 V3.5, Nanodrop Technologies Inc., USA).

2.3.2. Reverse transcription for cDNA synthesis

Total mRNA (1μg) was reverse transcribed into cDNA using high-capacity cDNA archive kit (Applied Biosystems, Darmstadt, Germany) according to the protocol provided by the manufacturer. Briefly, RNA (1μg) was added to fresh tubes containing the appropriate volume of nuclease-free H_20 to make a 25μl volume. A 2x mastermix was prepared containing the appropriate volumes of 10x RT buffer, 25x dNTPs, 10x random primer multiscribe reverse transcriptase (50U/μl). The mastermix (25μl) was added to the RNA and nuclease free H_20. Tubes were incubated for 10 min at 25°C followed by 2 h at 37°C on a thermocycler (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences Ireland)

2.3.3. cDNA amplification by RT- PCR

Real-time PCR primers and probes were delivered as "TaqMan® Gene Expression Assays" for the mouse genes CD40, CD68, CD11b, IL-6, TNF-α, CCL2, CXCL10, CCL3, IL-1R1, TLR2, TLR4, RAGE and SIGIRR (Applied Biosystems, Darmstadt, Germany). Real-time PCR was performed on Applied Biosystems ABI Prism 7300 Sequence Detection System v1.3.1in 96-well format and 25μl reaction volume per well. cDNA samples (200pg/well) were mixed with TaqMan Universal PCR Mastermix (Applied Biosystems, Darmstadt, Germany) and the respective target gene assay. Mouse β-actin RNA (# 4352341E, Applied Biosystems, Darmstadt, Germany) were used as reference. Each sample was measured in a single RT-PCR run. Forty cycles were run with the following conditions: 2 min at 50°C, 10 min at 95°C and for each cycle 15s at 95 °C for denaturation and 1 min at 60°C for transcription.

2.3.4. PCR Quantification

The expression of each target gene was determined using the efficiency-corrected comparative CT method. Target genes in different samples were compared to a reference gene (β-actin) and the relative differences between samples were expressed as a ratio. Values are expressed as relative quantities of specific genes (7300 real-time PCR software, Applied Biosystems, USA).

2.4. Analysis of cytokine concentrations

2.4.1. Preparation of samples

The concentrations of IL-6, TNF-α, CCL2, CXCL10 and CCL3 were assessed by ELISA in supernatants from mixed glia, microglia and astrocytes prepared from 1-day old wildtype and SIGIRR$^{-/-}$ mice.

2.4.2. General ELISA protocol

In all cases 96-well plates were incubated overnight at 4°C with capture antibody (50µl; see Table 2.2 for specific details). Plates were washed 3 times with wash buffer (PBS containing 0.05% Tween-20) and incubated at room temperature for 1h in blocking buffer (200µl; see Table 2.2 for specific details). Plates were washed 3 times with wash buffer and incubated at room temperature for 2h with samples and standards (50µl in duplicate; see Table 2.2 for specific details). Plates were washed 3 times with wash buffer and incubated at room temperature with detection antibody (50µl; see Table 2.2 for specific details). Plates were washed 3 times with wash buffer and incubated in the dark at room temperature for 30 min with horseradish peroxidase-conjugated streptavidin (strep-HRP) (50µl; 1:200 dilution in assay diluent; see Table 2.2 for specific details). Plates were washed 3 times with wash buffer and incubated with substrate solution (50µl; 1:1 H_2O_2:tetramethylbenzidine; R&D Systems, US) in the dark at room temperature until colour developed (approximately 20 min). The reaction was stopped using stop solution (50µl: 1M H_2SO_4) and absorbance was read at 450nm using a 96-well plate reader. Sample concentrations were determined wit reference to a standard curve, which was made up by plotting the concentrations of the standards with their measured absorbances. Cytokine concentration was expressed as pg/ml of supernatant.

2.5. Analysis of Protein expression and phosphorylation

2.5.1. Protein Quantification

Hippocampal tissue was homogenised in lysis buffer (100µl; 10mM Tris HCl, 50nM NaCl, 10mM $Na_4P_2O_7.H_20$, 50mM NaF, 1% Igepal, 1:100 dilution phosphatase inhibitor cocktail I and II, 1:100 dilution protease inhibitor cocktail; Sigma, UK). The protein concentrations were assessed using bicinchoninic acid (BCA) protein assay kit (Pierce, The Netherlands). Standards (0mg/ml-1mg/ml; mBSA), and samples (diluted 1:10) were added to the 96-well plate in triplicate and duplicate respectively (25µl/well). Pierce BCA reagent (200µl/well; 1:50 Reagent B to Reagent A) was added to the plate and samples were incubated for 30 min at 37°C. The optical density was determined by measuring the absorbance at 565nm. Protein concentrations were calculated relative to the standard curve and were equalised in samples using lysis buffer and 4x Tris-glycine sample buffer (0.5M Tris-hydrogen chloride (HCL) pH 6.8, 25% SDS solution, glycerol, bromophenol blue, 1:100 dilution β-mercaptoethanol in dH_20,).

2.5.2. Western Blotting

Samples equalised for protein (30µg) were heated at 70-95°C for 3 min and separated on 7% or 10% Tris-glycine gels (composition; 30% acrylamide; H_20; 4x SPA buffer: 1.5M Tris/Sodium dodecyl sulfate (SDS) 0.2%, pH 8.8, 10% Ammonium persulfate (APS) and Tetramethylethylenediamine (temed)). MagicMark™ XP protein standard (Invitrogen, Ireland) and SeeBlue® Plus2 Prestained Standard 1x (Invitrogen, Ireland) were loaded on the gel to allow direct visualisation of protein standard bands on the blot with and without chemiluminescence respectively. Proteins were transferred to 0.2 or 0.4µM nitrocellulose membrane and blocked in Tris-buffered-saline-0.05% Tween 20 (TBS-T) (composition; 20mM Tris-HCl, 150mM NaCl, 0.05% Tween 20) and 5% dried milk at room temperature. Membranes were incubated overnight at 4°C with primary antibody (see table 2.3), washed, and incubated with the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature (see table 2.3).

Immunoreactive bands were detected using enhanced chemiluminescence. Blots were stripped (Re-blot plus; Chemicon, US) and reprobed for β-actin (see table 2.3; Sigma, UK). Images were captured using the Fijifilm LAS-4000 system and the image reader LAS-4000 package.

2.6. Animals

Male and female, young (3 months) and middle-aged (12-14 months), C57BL/6 mice and SIGIRR$^{-/-}$ mice were supplied by Bio-resources unit, Trinity College, and gifted by Alberto Mantovani (Istituto Clinico Humanitas, Milan) respectively. Animals were maintained in the Bio-resources Unit, Trinity College Dublin, under veterinary supervision and were housed in a controlled environment under a 12 h light-dark cycle at an ambient temperature of 22-23^{0}C. All animals used in these studies had access to normal laboratory chow and water on an *ad libitum* basis. Experiments were performed under license from the Department of Health and Children (Ireland) and with ethical approval from the local ethics committee in Trinity College Dublin, in compliance with the Cruelty to animals act, 1876 and the European Community Directive, 86/609/EC. Every effort was made to minimise stress to the animals at all stages of the study.

2.6.1. Treatment with Amyloid-β and Saline

Wildtype and SIGIRR$^{-/-}$ mice were anaesthetised with Avertin (20μl/g; Sigma Aldrich; Ireland), deep anaesthesia was confirmed by the absence of a pedal reflex. Once anaesthetised, young and middle-aged, wiltype and SIGIRR$^{-/-}$ mice were randomly assigned into treatment groups (n=6-8 animals per group). Young and middle-aged Aβ-treated animals of both genotypes received a cocktail of A$\beta_{1\text{-}40/1\text{-}42}$ (5μl; 2.75μl A$\beta_{1\text{-}42}$, 2.25 A$\beta_{1\text{-}40}$μl) intrahippocampally whereas young and middle-aged control animals of both genotypes received an intrahippocampal injection with sterile saline.

2.6.2. Dissection and preparation of tissue

Animals were sacrificed 4 h post-treatment by decapitation. The brain was rapidly removed and placed on ice, the cerebellum and olfactory bulbs were removed and the brain was bisected along the midline. A quarter of the hippocampus and section of cortex of the right hemisphere were snap-frozen in liquid nitrogen in an RNAse-free tube for later analysis by PCR and Western Blotting. The remaining hippocampal and cortical tissue was sliced bi-directionally, placed in RNAse-free tubes and snap-frozen for later analysis.

2.7. Molecular Biology

2.7.1. Preparation of pENTR221 hSIGIRR plasmid

A DH5alpha T1-resistant *E. coli* stab culture of transformed with pENTR221_hSIGIRR was obtained from Source Bioscience, Germany. Luria Bertani (LB) broth (10 g Peptone, 5 g yeast extract, 5g sodium chloride) was added to 1 L water and autoclaved. The stab colony was used to inoculate 1ml of LB medium. The culture was incubated for 1h with continuous shaking at 200 rpm at 37°C and then grown on pre-warmed agar plates supplemented with either kanamycin or ampicillin at 50 µg/ml overnight in an incubator at 37°C. A single colony was picked from the plate and used to inoculate 5 ml LB medium. The culture was incubated overnight with continuous shaking at 200 rpm at 37°C. The plasmid DNA was extracted from the bacterial culture using a mini-prep kit according to manufacturer's recommendation (Qiagen).

2.7.2. PCR and restriction digestion

The components used for polymerase chain reaction (PCR) were 20 pg DNA template, 1x PCR buffer, 10 p/mol forward and reverse primer, dNTPs (200 µM) and 1 U of DNA polymerase in a 20 µl reaction mix. After the initial denaturation temperature (94°C for 5 min) the PCR reaction cycle was programmed as follows: denaturation (94°C for 60 sec), annealing (65°C for 60 sec) and elongation (72°C for 2 min). This cycle was repeated 30 times to achieve the desired

amplification. Before ending the reaction, the elongation temperature was maintained for 10 min, at the end of 30th cycle. The PCR fragments were purified using a PCR purification kit (Qiagen). The sample was then stored at 4°C (or -20°C longer term) until further use. The restriction digestion was performed in a reaction volume of 20 µl using 1 µg of DNA were incubated with 1 U of restriction enzyme and 1x appropriate buffer at 37°C for 2 hrs. DNA fragments were run on agarose gels (1 x TBE (40 mM Tris-borate and 1 mM EDTA) and 1% agarose) alongside a ladder to determine whether they were of appropriate size. Following restriction digest the fragments were purified using a gel purification kit (Qiagen). The process was repeated to digest 1µg pEGFP_C2 mammalian vector.

2.7.3. Dephosphorylation and ligation

To restrict self-ligation, the digested and purified vector DNA was dephosphorylated by incubating in 1x CIP buffer and 1 U of phosphatase enzyme for 1 hr at 37°C. The reaction was then deactivated by placing in a heatblock set to 70°C for 10 min. After dephosphorylation, the vector was separated in 1% agarose gel and rescued using a gel extraction kit (Qiagen). Ligation reactions included 3 units of gene insert and 1 unit of vector. The ligation reaction was performed with T4 ligase kit (Invitrogen). To ligate 200 ng of insert with 50 ng of cut vector, 1x ligation buffer and 1 U of T4 DNA ligase (total vol 20 µl) were used. The ligation mixture was incubated overnight at 16°C. Heat shock method of transformation was used to obtain recombinant bacterial colonies. The DH5α strain was used to yield plasmid DNA. Tubes containing competent cells were removed from -80°C and thawed on ice for 15-20 min. Next, 1-2 ml of ligation mixture was mixed with 50 µl of competent bacteria and incubated on ice for 15 min. Heat shock was applied for 90 sec by placing the tube in a water bath at 42°C then immediately transferring onto ice for 10 min. To recover the cells, 1 ml LB was added to the transformed bacteria and incubated with continuous shaking at 200 rpm for 60 min at 37°C. The cells were centrifuged at 3,000 x g for 2 min. The pellet was resuspended in 200 µl LB and spread on pre-warmed agar plates supplemented with either kanamycin or ampicillin at 50 µg/ml. Plates were incubated overnight at 37°C. A single colony was used to inoculate in 5 ml of LB medium and left overnight with continuous shaking at 200 rpm at 37°C. The plasmid DNA was

extracted from the bacterial culture by using a plasmid mini-prep or plasmid maxi-prep kit according to manufacturer's recommendation (Qiagen).

2.8. Statistical Analysis

Data are expressed as means +/- standard error of the mean (SEM). A two-tailed Student's t-test for unpaired means, or a one or two way analysis of variance (ANOVA) was performed, where appropriate, to determine whether significant differences existed between means. When a two-way ANOVA indicated significance ($*p < 0.05$), a Bonferroni post-hoc test was employed to determine which conditions were significantly different from each other. Statistical analysis was carried out using Prism software (Graphpad Prism, v 4.0; USA).

List of primers (Applied Biosystems, Darmstadt, Germany)		
Gene name	**Gene description**	**Assay I.D.**
CD40	Cluster of differentiation 40	Mm00441891_m1
CD11b	Cluster of differentiation 11b	Mm00441891_m1
CD68	Cluster of Differentiation 68	Mm03047340_m1
IL-6	Interleukin-6	Mm00446190_m1
TNF-α	Tumour necrosis factor-α	Mm00443258_m1
CCL2	Chemokine (C-C motif) ligand 2	Mm00441242_m1
CXCL10	C-X-C motif chemokine 10	Mm00445235_m1
IL-1R1	Interleukin-1 Receptor 1	Mm00434237_m1
TLR2	Toll-like Receptor 2	Mm00442346_m1
TLR4	Toll-like Receptor 4	Mm00445273_m1
CD14	Cluster of Differentiation 14	Mm00438094_g1
SIGIRR	Single Immunoglobulin IL-1 receptor related	Mm00491700_m1

Table 2.1 Mouse PCR primer assay numbers

Cytokine	Supplier	Block	Capture Antibody	Standard	Detection Antibody
IL-6	BD Biosciences, USA	1% BSA in PBS	Monoclonal rat anti-mouse IL-6 4μg/ml in PBS	Mouse recombinant IL-6 standards	Biotinylated anti-mouse IL-6
TNF-α	BD Biosciences, USA	1% BSA in PBS	Monoclonal anti-mouse TNF-α	Mouse recombinant TNF-α standards	Biotinylated anti-mouse TNF-α
CCL2	BD Biosciences, USA	1% BSA in PBS	Monoclonal anti-mouse CCL2	Mouse recombinant CCL2 standards	Biotinylated anti-mouse CCL2
CXCL10	BD Biosciences, USA	1% BSA in PBS	Monoclonal anti-mouse CXCL10	Mouse recombinant CXCL10 standards	Biotinylated anti-mouse CXCL10
CCL3	BD Biosciences, USA		Monoclonal anti-mouse CCL3	Mouse recombinant CCL3 standards	Biotinylated anti-mouse CCL3

Table 2.2 Summary of ELISA protocols

Target Protein (Source)	Supplier	1° Antibody Dilution	2 ° Antibody Dilution	Protein Band (kDa)
β-Actin (Mouse)	Sigma, UK	1:5000; 1h @ RT	1:5000; 1h @ RT	42
pIκBα (Rabbit)	Cell Signalling, USA	1:1000; o/n @ 4 °C	1:5000; 1h @ RT	40
pIKK (Rabbit)	Cell Signalling, USA	1:1000; o/n @ 4 °C	1:5000; 1h @ RT	87

Table 2.3 Summary of western immunoblotting protocols

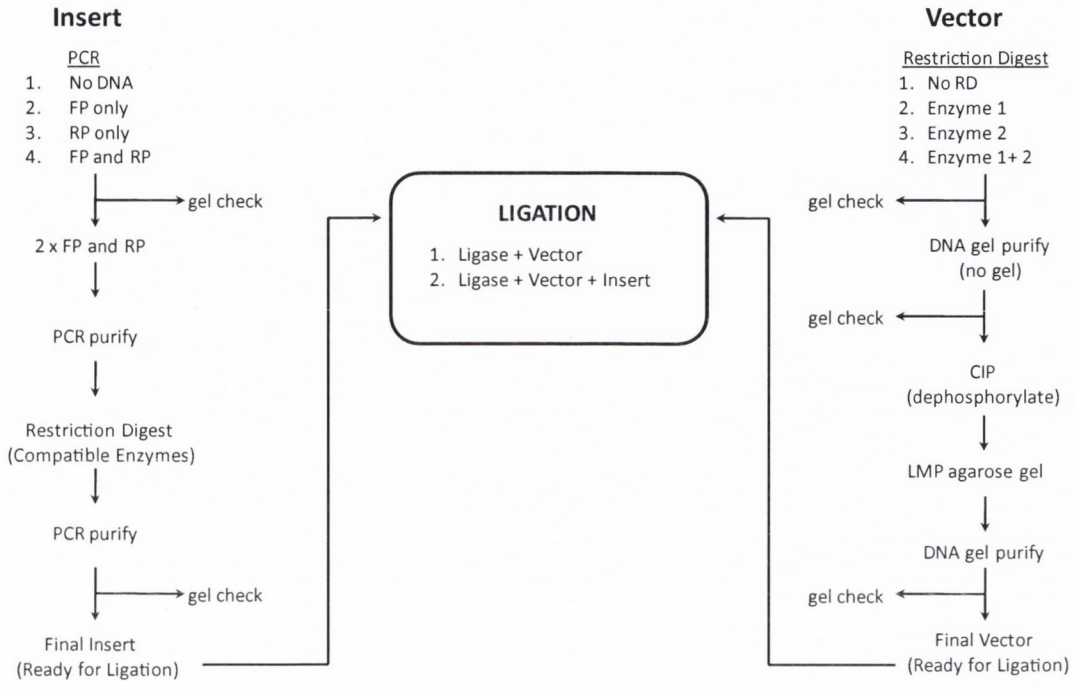


Figure 2.3. Summary of molecular cloning protocol

Chapter 3: Analysis of the effects of LPS and IL-1β on glial cells and SIGIRR-deficient mice

3.1 Introduction

The IL-1/TLR superfamily is a group of cell surface receptors which includes IL-1R1, TLR4 and TLR2. These receptors play a major role in the recognition of pro-inflammatory stimuli by glial cells and the signaling pathways they propagate, particularly the NF-κB pathway, have emerged as possible targets for anti-inflammatory therapies. A member of the IL-1/TLR family, SIGIRR has been identified which appears to have a negative regulatory influence on IL-1/TLR signaling.

There have been few studies examining the role of SIGIRR in modulating pro-inflammatory signaling in the brain. In 2010, Watson and colleagues reported that mixed glia prepared from SIGIRR-deficient mice exhibited increased pro-inflammatory cytokine production in response to the TLR4 agonist, LPS, compared with cells prepared from wildtype mice. It was also noted that this regulatory effect was present only in isolated microglia, not astrocytes (Watson et al., 2010). Mice injected with LPS were also found to have increased microglial activation and pro-inflammatory cytokine expression. A later study reported that hippocampal slices prepared from SIGIRR-deficient mice had depressed LTP caused by enhanced NF-κB signaling. This enhanced signaling was found to be the result of elevated levels of TLR4 and IL-1R1, and their respective endogenous ligands HMGB1 and IL-1α, in the SIGIRR-deficient mice (Costello et al., 2011b).

The aim of this study was to investigate the production of pro-inflammatory cytokines and chemokines and the upregulation of markers of glial activation in mixed glia, astrocytes and microglia prepared form SIGIRR-deficient mice. The effects of TLR4 and IL-1R1 stimulation were examined using the bacterial endotoxin LPS and the pro-inflammatory cytokine IL-1β respectively.

The aims of these experiments were:

- To examine the effect of LPS and IL-1β treatment on markers of microglial activation and cytokine release in mixed glial cells prepared from wildtype and SIGIRR-deficient mice.

- To investigate whether SIGIRR deficiency differentially affected astrocytes and microglia in their response to LPS and IL-1β.

3.2. Methods

Primary cortical mixed glia, microglia and astrocytes were prepared from postnatal 1-day old wildtype and SIGIRR-deficient mice and cultured for 14 days before treatment (see section 2.1 for details). All agents used to treat cells were diluted to required concentrations in pre-warmed supplemented DMEM and all solutions were filter-sterilised through a 0.2μm cellulose acetate membrane filter. LPS was diluted to a final concentration of 100ng/ml in supplemented DMEM. Cells were treated for 24 h in the presence or absence of LPS. IL-1β was prepared as a stock solution in sterile PBS and 0.1% BSA and diluted to the desired concentration of 10ng/ml in supplemented DMEM. Cells were treated for 24 h in the presence or absence of IL-1β. mRNA expression of cytokines and cell surface proteins were assessed by RT-PCR and concentrations of cytokines and chemokines was determined by ELISA (see sections 2.3 and 2.4). Data are expressed as means ± SEM. Students t-test or ANOVA were performed to determine whether significant differences existed between treatment and genotype groups and Bonferroni post-hoc tests were performed where appropriate.

Table 3.1. Caveats

The concentrations of LPS and IL-1β were chosen on the basis of previous work in the lab which had established that these concentrations elicit a robust response from glial cells. However it should be noted that the concentration of IL-1β is not within the range that glial cells would typically be exposed to physiologically, and therefore experiments should be viewed as a study of glial responses to IL-1β rather than an in vitro model of IL-1β driven inflammation. In addition, it may have been useful to test a range of concentrations as genotype related differences may have been masked by high concentrations of cytokine.

All data was collated following 24 hours of treatment. There are differences in the latency of mRNA transcription and cytokine release between different cytokines and this may have affected the results shown here. A time course experiment would have been advisable to determine the optimum time to harvest samples for each cytokine measured.

The data in **Figure 3.2.** are representative of one experiment with each condition replicated four times. These data are repeats of similar experiments published by Watson et al., 2008.

No analysis was undertaken to determine the level of endotoxin present in reagents used. The presence of endotoxin in these reagents may have affected the responses observed from glial cells

3.3 Results

3.3.1 Investigating the effect of LPS on mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice.

LPS, induces pro-inflammatory cytokine release from mixed glia through interaction with TLRs; the effect of LPS may also be enhanced through an increase in TLR expression or a reduction in proteins which modulate TLR activity. In this study, an increase in TLR4 mRNA expression was observed in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with mixed glia from wildtype mice ($^{*}p<0.05$; Students t-test; **Figure 3.2, A**). The data show that LPS induced a reduction in the expression of SIGIRR mRNA in mixed glia prepared from wildtype mice ($^{*}p<0.05$; Students t-test; **Figure 3.2, B**).

LPS increased mRNA expression of IL-6, TNF-α and CCL2 ($^{***}p<0.001$; ANOVA; **Figure 3.3, A**; $^{***}p<0.001$; ANOVA; **Figure 3.3, C**; $^{***}p<0.001$; ANOVA; **Figure 3.3, E**) in mixed glia prepared from wildtype mice and the effect on TNF-α and CCL2 mRNA was significantly greater in mixed glia prepared from SIGIRR$^{-/-}$ mice ($^{+++}p<0.001$; ANOVA; **Figure 3.3, C**; $^{+++}p<0.001$; ANOVA; **Figure 3.3, E**). LPS also increased supernatant concentration of IL-6, TNF-α and CCL2 in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice ($^{***}p<0.001$; ANOVA; **Figure 3.3, B**; $^{***}p<0.001$; ANOVA; **Figure 3.3, D**; $^{***}p<0.001$; ANOVA; **Figure 3.3, F**) and this increase was significantly greater in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with glia from wildtype mice ($^{+++}p<0.001$; ANOVA; **Figure 3.3, B**; $^{++}p<0.01$; ANOVA; **Figure 3.3, D**; $^{+}p<0.05$; ANOVA; **Figure 3.3, F**).

3.3.2 Investigating the effect of LPS on astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice

Astrocytes are the major glial cell in the CNS and have an immune function. TLR4 expression was greater in astrocytes prepared from SIGIRR$^{-/-}$ mice compared with astrocytes from wildtype

mice ($^{*}p<0.05$; Students t-test; **Figure 3.4, A**) however LPS does not affect SIGIRR expression (**Figure 3.4, B**).

The data demonstrate that LPS increased mRNA expression of IL-6, TNFα and CXCL10 in astrocytes prepared from wildtype mice ($^{***}p<0.001$; ANOVA; **Figure 3.5, A**; $^{**}p<0.001$; ANOVA; **Figure 3.5, C**; $^{***}p<0.001$; ANOVA; **Figure 3.5, E**) and the effect was significantly greater in astrocytes prepared from SIGIRR$^{-/-}$ mice ($^{+++}p<0.001$; ANOVA; **Figure 3.5, A**; $^{++}p<0.01$; ANOVA; **Figure 3.5, C**; $^{+}p<0.05$; ANOVA; **Figure 3.5, E**). LPS increased release of IL-6, TNF-α and CCL3 in astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice ($^{***}p<0.001$; ANOVA; **Figure 3.5, B**; $^{***}p<0.001$; ANOVA; **Figure 3.5, D**; $^{***}p<0.001$; ANOVA; **Figure 3.5, F**) however only CCL3 was significantly greater in astrocytes prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA; **Figure 3.5, F**).

3.3.3 Investigating the effects of LPS on microglia prepared from wildtype and SIGIRR$^{-/-}$ mice

Microglia are the main immune cells and cytokine producing cells in the CNS. The present data show that LPS significantly increased the release of IL-6, TNF-α and CCL3 in microglia prepared from wildtype mice in response to LPS ($^{***}p<0.001$; ANOVA; **Figure 3.6, A**; $^{***}p<0.001$; ANOVA; **Figure 3.6, B**; $^{***}p<0.001$; ANOVA; **Figure 3.6, C**). IL-6, TNF-α and CCL3 release was significantly greater in cells prepared from SIGIRR$^{-/-}$ mice compared with those from wildtype mice ($^{+++}p<0.001$; ANOVA; **Figure 3.6, A**; $^{+++}p<0.001$; ANOVA; **Figure 3.6, B**; $^{+++}p<0.001$; ANOVA; **Figure 3.6, C**).

3.3.4 Investigating the effect of IL-1β on glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice.

IL-1β induced an increase the expression of IL-1R1 mRNA in mixed glia prepared from SIGIRR$^{-/-}$ mice compared to cells prepared from wildtype mice ($^{**}p<0.01$; ANOVA; **Figure 3.7,**

A). There was a reduction in the expression of SIGIRR mRNA in mixed glia prepared from wildtype mice following treatment with IL-1β ($^{**}p<0.01$; Students t-test; **Figure 3.7, B**).

IL-1β induced the expression of CD11b mRNA in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice ($^{***}p<0.001$; ANOVA; **Figure 3.8, A**) and this effect was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with glia from wildtype mice ($^{+++}p<0.001$; ANOVA; **Figure 3.8, A**). Mixed glia prepared from SIGIRR$^{-/-}$ mice exhibit increased mRNA expression of CD68 compared with cells from wildtype mice (***p<0.01; ANOVA; **Figure 3.8, B**).

IL-1β increased mRNA expression of IL-6 ($^{**}p<0.01$; ANOVA; **Figure 3.9, A**) in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice and the effect was significantly enhanced in mixed glia prepared from SIGIRR$^{-/-}$ mice ($^{++}p<0.01$; ANOVA; **Figure 3.9, A**). IL-1β increased supernatant concentration of IL-6 in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice ($^{***}p<0.001$; ANOVA; **Figure 3.9, B**) and this increase was significantly greater in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with glia from wildtype mice ($^{+++}p<0.001$; ANOVA; **Figure 3.9, B**). TNF-α mRNA expression was increased in mixed glia prepared from SIGIRR$^{-/-}$ mice ($^{**}p<0.01$; ANOVA; **Figure 3.9, C**) and IL-1β enhanced this effect ($^{+++}p<0.001$; ANOVA; **Figure 3.9, C**). IL-1β had no effect on TNF-α release from mixed glia (**Figure 3.9, D**). IL-1β induced a significant increase in CCL2 and CXCL10 mRNA expression in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice ($^{***}p<0.001$; ANOVA; **Figure 3.9, E**; $^{***}p<0.001$; ANOVA; **Figure 3.9, G**). This effect was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with glia from wildtype mice ($^{+++}p<0.001$; ANOVA; **Figure 3.9, E**; $^{+++}p<0.001$; ANOVA; **Figure 3.9, G**). IL-1β significantly increased CCL2 release from mixed glia prepared from wildtype mice ($^{***}p<0.001$; ANOVA; **Figure 3.9, F**) and this effect was significantly increase in mixed glia prepared from SIGIRR$^{-/-}$ mice ($^{+}p<0.001$; ANOVA; **Figure 3.9, F**). IL-1β failed to increase the expression of CCL3 mRNA in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice (**Figure 3.9, H**).

3.3.5 Investigating the effects of IL-1β on astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice

There was a significant increase in the expression of IL-6 mRNA in astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice in response to IL-1β ($^{***}p<0.001$; ANOVA; **Figure 3.10, A**) and the effect was significantly greater in cells prepared from SIGIRR$^{-/-}$ mice compared with those from wildtype mice ($^{++}p<0.01$; ANOVA; **Figure 3.10, A**). There was a significant increase in IL-6 release from astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice in response to IL-1β ($^{***}p<0.001$; ANOVA; **Figure 3.10, B**) but there was no difference in IL-6 protein release between astrocytes prepared from wildtype or SIGIRR$^{-/-}$ mice following exposure to IL-1β. Astrocytes prepared from SIGIRR$^{-/-}$ mice exhibited increased basal TNF-α mRNA ($^{***}p<0.001$; ANOVA; **Figure 3.10, C**) and IL-1β significantly increased the expression of TNF-α mRNA in astrocytes prepared from SIGIRR$^{-/-}$ ($^{+}p<0.01$; ANOVA; **Figure 3.10, C**) compared with those from wildtype mice. IL-1β increased the expression of CXCL10 mRNA in astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice ($^{*}p<0.05$; ANOVA; **Figure 3.10, D**) although there was no difference in CXCL10 mRNA expression between astrocytes prepared from wildtype or SIGIRR$^{-/-}$ mice following exposure to IL-1β.

3.3.6 Investigating the effects of IL-1β on microglia prepared from wildtype and SIGIRR$^{-/-}$ mice

TLR4 was not increased in microglia prepared from SIGIRR-deficient mice (**Figure 3.11, A**). IL-1β failed to alter the expression of SIGIRR (**Figure 3.11, B**), CD40 (**Figure 3.11, C**) or TNF-α (**Figure 3.11, D**) mRNA or in microglia prepared from wildtype and SIGIRR$^{-/-}$ mice.

Table 3.2. Results summary

Key: R=mRNA; P=protein; ↑=increase; ↓=decrease; - = no change; NA=not analysed; NML=no measureable levels

		Effect on WT[1]		Effect on SIG$^{-/-}$[2]	
	Parameter	**LPS**	**IL-1β**	**LPS**	**IL-1β**
Mixed Glia	**CD68**	NA	**R** -; **P** NML	NA	**R** -; **P** NML
	CD11b	NA	**R** ↑; **P** NA	NA	**R** ↑; **P** NA
	CD40	NA	NML	NA	NML
	IL-6	**R** -; **P** ↑	**R** ↑; **P** ↑	**R** ↑; **P** ↑	**R** ↑; **P** ↑
	TNF-α	**R** ↑; **P** ↑	**R** ↑; **P** NML	**R** ↑; **P** ↑	**R** ↑; **P** NML
	IL-1β	NA	NA	NA	NA
	CCL2	**R** ↑; **P** ↑	**R** ↑; **P** ↑	**R** ↑; **P** ↑	**R** ↑; **P** ↑
	CXCL10	NA	**R** ↑; **P** NML	NA	**R** ↑; **P** NML
	CCL3	NA	**R** NA; **P** -	NA	**R** NA; **P** -
Astrocytes	**IL-6**	**R** ↑; **P** ↑	**R** ↑; **P** ↑	**R** ↑; **P** -	**R** ↑; **P** -
	TNF-α	**R** ↑; **P** ↑	**R** -; **P** NA	**R** ↑; **P** -	**R** ↑; **P** NA
	IL-1β	NA	**NA**	NA	NA
	CCL2	NA	NA	NA	NA
	CXCL10	**R** ↑; **P** NA	**R** ↑; **P** NA	**R** ↑; **P** NA	**R** -; **P** NA
	CCL3	**R** NA; **P** ↑	NA	**R** NA; **P** ↑	NA
Microglia	**CD68**	NA	NML	NA	NML
	CD11b	NA	NML	NA	NML
	CD40	NA	**R** -; **P** NA	NA	**R** -; **P** NA
	IL-6	**R** NA; **P** ↑	**R** NML; **P** NML	**R** NA; **P** ↑	**R** NML; **P** NML
	TNF-α	**R** NA; **P** ↑	**R** -; **P** NML	**R** NA; **P** ↑	**R** -; **P** NML
	IL-1β	NA	NA	NA	NA
	CCL2	NA	**R** NML; **P** NML	NA	**R** NML; **P** NML
	CXCL10	NA	**R** NML; **P** NA	NA	**R** NML; **P** NA
	CCL3	**R** NA; **P** ↑	**R** NA; **P** NML	**R** NA; **P** ↑	**R** NA; **P** NML

[1]Relative to control treated WT; [2]Relative to similarly treated WT

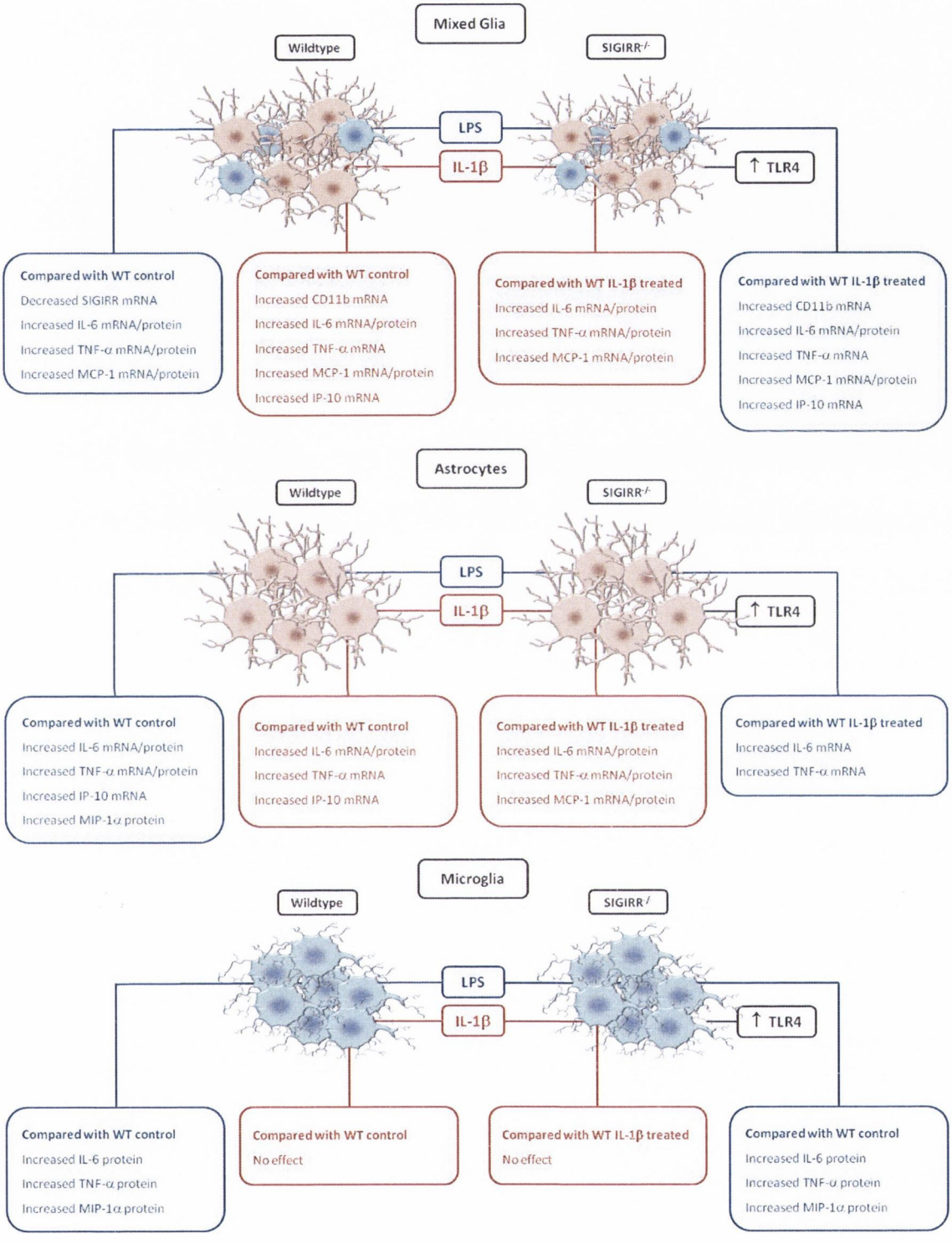


Figure 3.1. Results summary

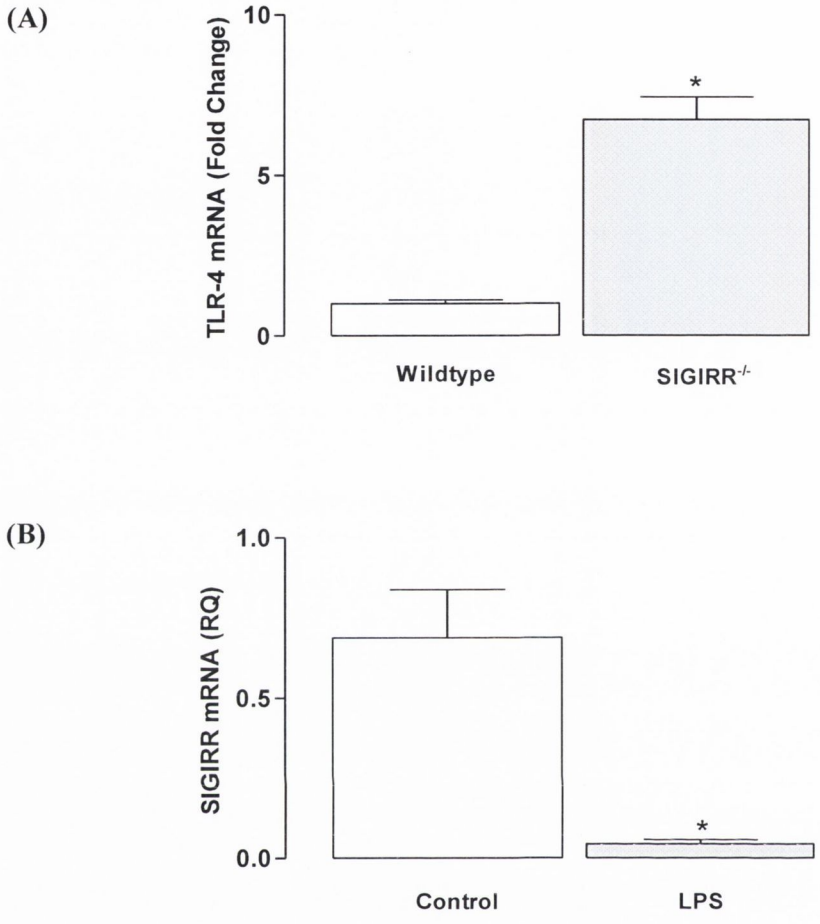


Figure 3.2. TLR4 and SIGIRR expression in mixed glia were modulated by LPS and SIGIRR deficiency

Primary mouse mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice. **(A)** Mixed glia prepared from SIGIRR$^{-/-}$ mice exhibit increased basal TLR4 mRNA compared with wildtype mice (*$p<0.05$; Student's t-test). Primary mouse mixed glia prepared from wildtype mice were treated with LPS (100ng/ml; 24 h). **(B)** Treatment with LPS induced a significant decrease in SIGIRR mRNA (*$p<0.05$; Student's t-test). Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate.

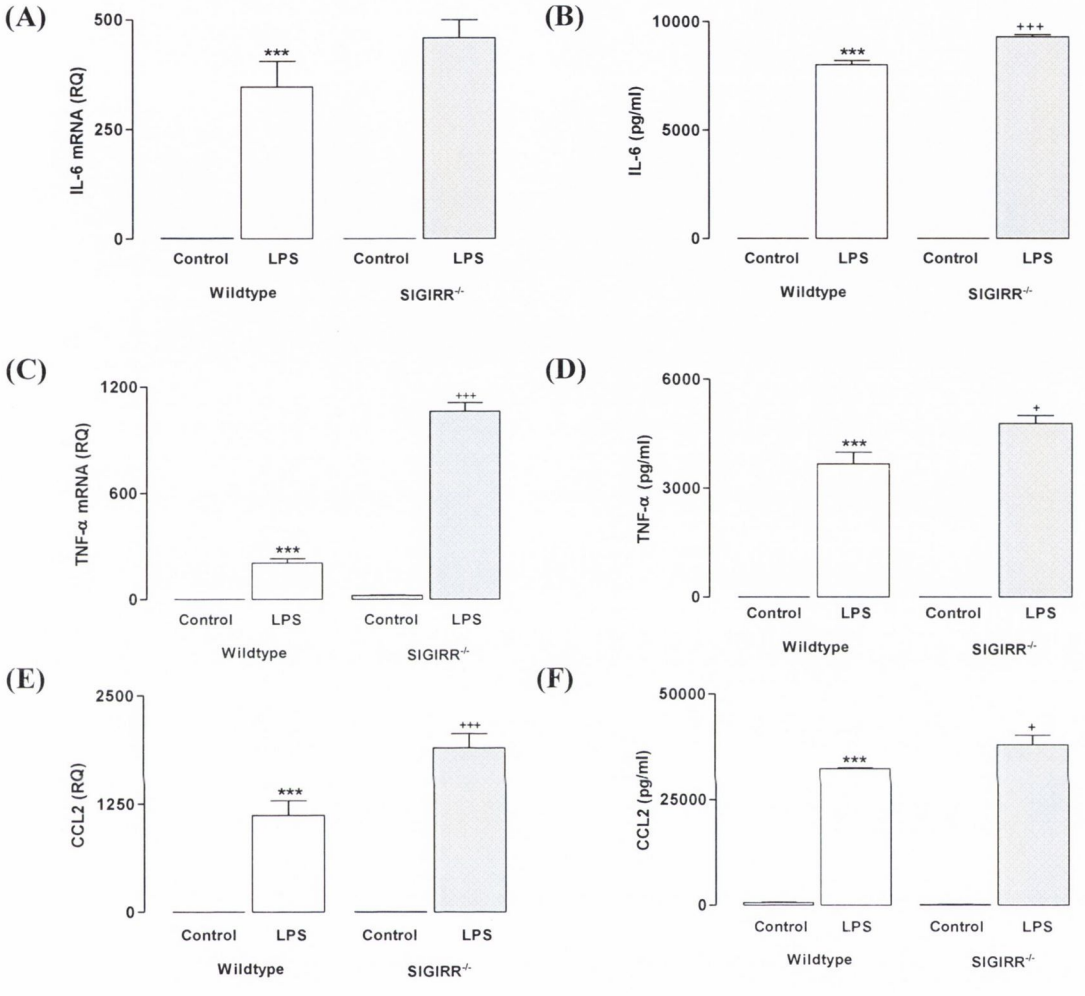


Figure 3.3. LPS induced cytokine production in mixed glia was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice

Primary mouse mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with LPS (100ng/ml; 24 h). LPS induced an increase in mRNA expression of **(A)** IL-6, **(C)** TNF-α and **(E)** CCL2 from mixed glia (***$p<0.001$; ANOVA). LPS-induced mRNA expression of **(C)** TNF-α and **(E)** CCL2 was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA). LPS induced an increase in release of **(B)** IL-6, **(D)** TNF-α and **(F)** CCL2 in mixed glia (***$p<0.001$; ANOVA). LPS-induced cytokine release was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+}p<0.05$, $^{+++}p<0.001$; ANOVA).Values are presented as means (± SEM). The data are representative of 1 experiment with each condition tested in quadruplicate. Data for mRNA is expressed as a ratio to β-actin mRNA and standardised to a control sample.

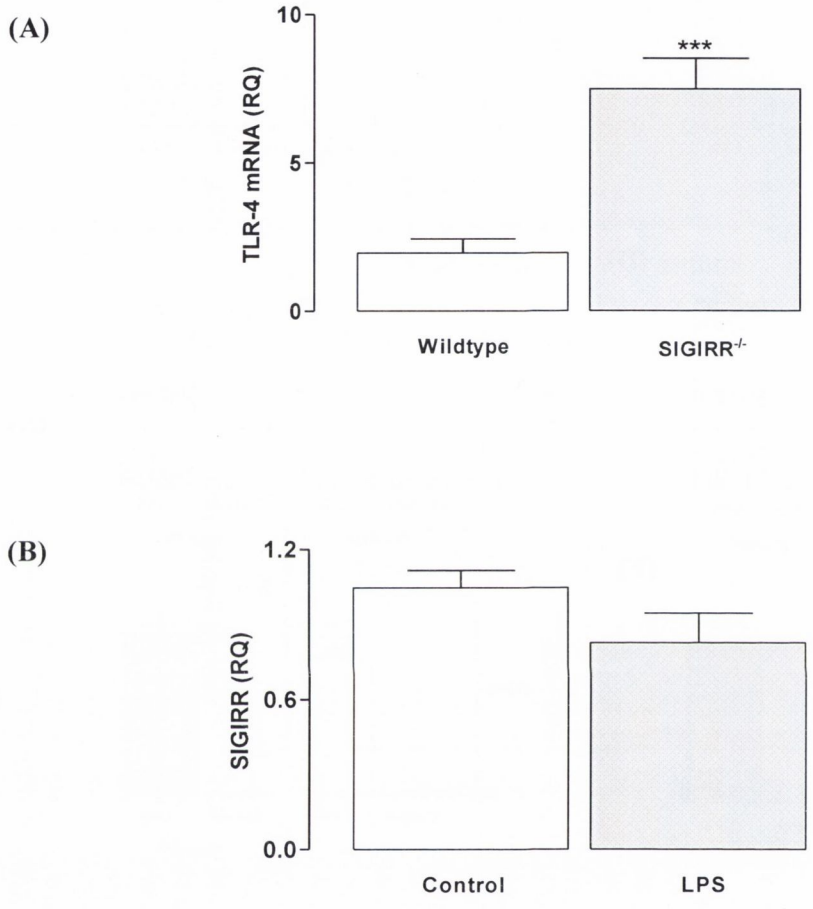


Figure 3.4. TLR4 expression was increased in astrocytes prepared from SIGIRR$^{-/-}$ mice.

Secondary mouse astrocytes were prepared from wildtype and SIGIRR$^{-/-}$ mice. **(A)** Astrocytes prepared from SIGIRR$^{-/-}$ mice exhibit increased basal TLR4 mRNA compared with wildtype mice (***$p<0.001$; Student's t-test). LPS did not affect mRNA expression of SIGIRR in astrocytes. Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 3 separate experiments with each condition tested in duplicate.

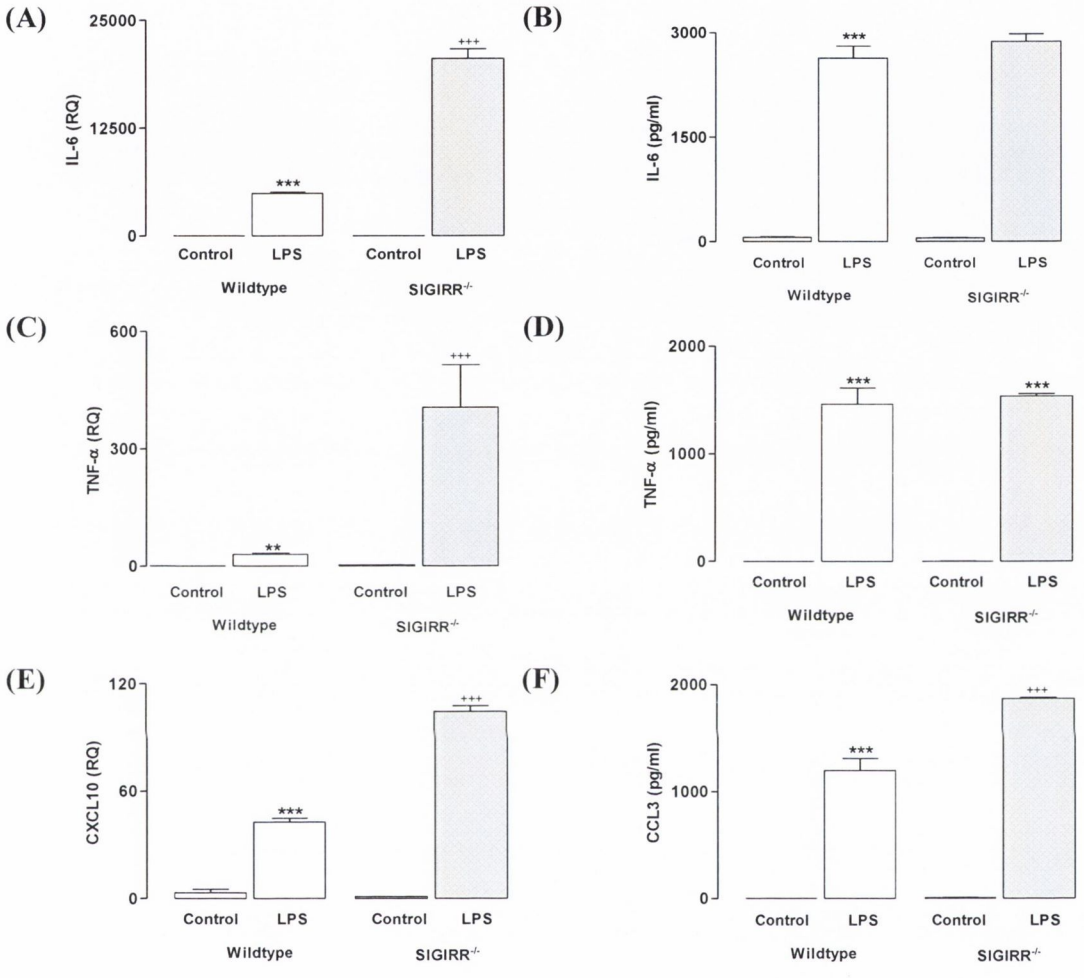


Figure 3.5. LPS induced cytokine mRNA expression in astrocytes was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice.

Secondary mouse astrocytes were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with LPS (100ng/ml; 24 h). LPS induced an increase in mRNA expression of **(A)** IL-6, **(C)** TNF-α and **(E)** CXCL10 from astrocytes (**$p<0.01$, ***$p<0.001$; ANOVA). LPS-induced cytokine mRNA expression was significantly increased in astrocytes prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA). LPS induced an increase in release of **(B)** IL-6, **(D)** TNF-α and **(F)** CCL3 in astrocytes (***$p<0.001$; ANOVA). LPS-induced release of **(F)** CCL3 was significantly increased in astrocytes prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA). Values are presented as presented as means (± SEM). Data for mRNA expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 3 separate experiments with each condition tested in duplicate.

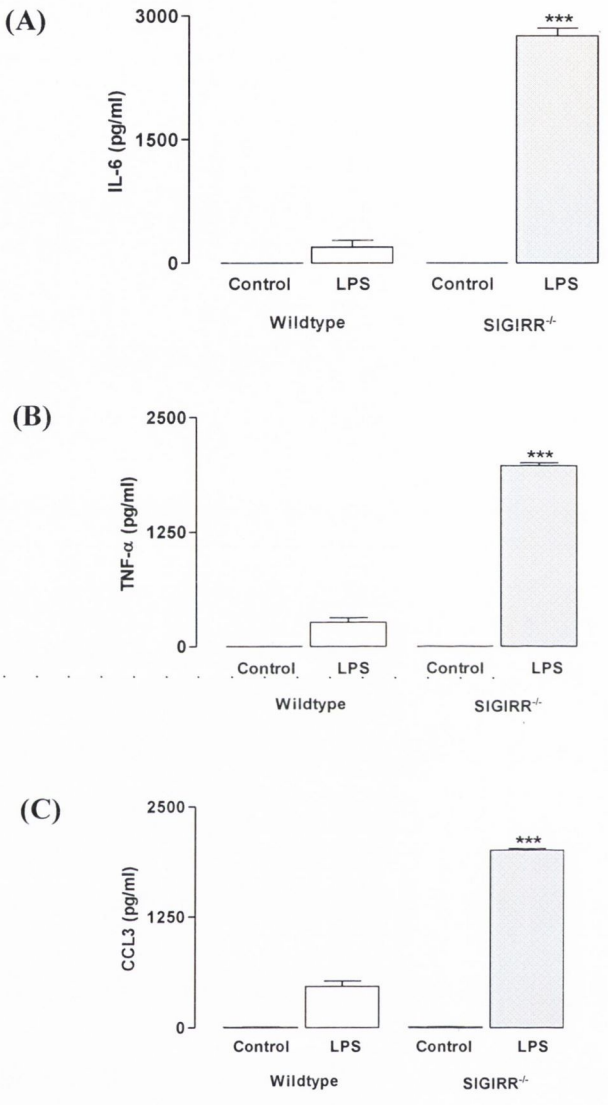


Figure 3.6 LPS-induced cytokine production in microglia was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice.

Secondary mouse microglia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with LPS (100ng/ml; 24 h). LPS induced an increase in release of **(A)** IL-6, **(B)** TNF-α and **(C)** CCL3 in mixed glia (***$p<0.001$; ANOVA). LPS-induced cytokine release was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice (***$p<0.001$; ANOVA). Values are presented as means (± SEM). The data are representative of 3 separate experiments with each condition tested in duplicate.

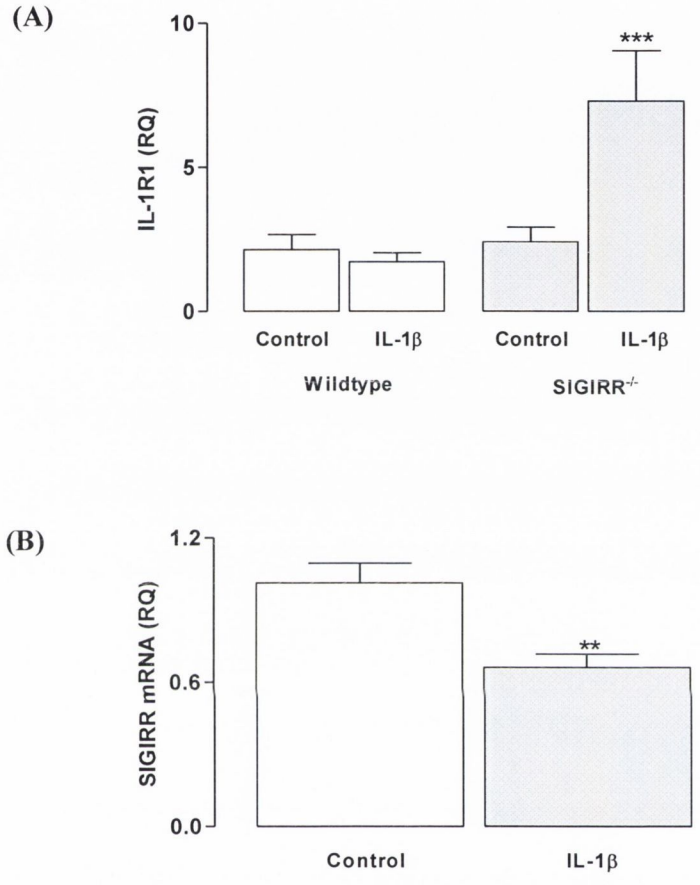


Figure 3.7. IL-1R1 and SIGIRR expression in mixed glia were modulated by IL-1β

Primary mouse mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with IL-1β (10ng/ml; 24 h). **(A)** IL-1β induced an increase in mRNA expression of IL-1R1 in mixed glia prepared from SIGIRR$^{-/-}$ mice (***$p<0.001$; ANOVA). **(B)** Treatment with IL-1β significantly decreased SIGIRR mRNA expression in mixed glia. Values are presented as presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 3 separate experiments with each condition tested in duplicate.

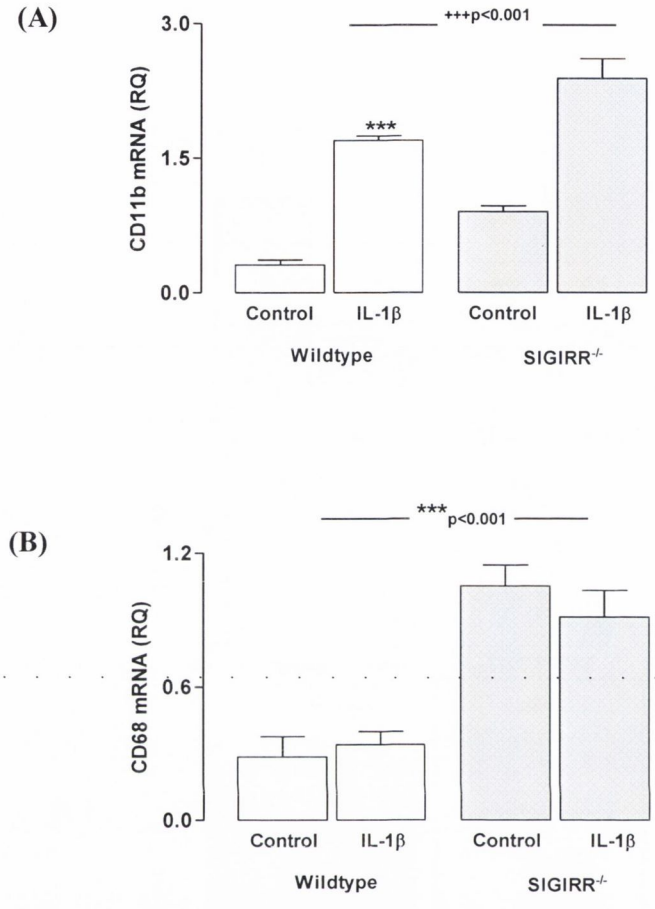


Figure 3.8. Expression of markers of microglial activation was increased in mixed glia prepared from SIGIRR$^{-/-}$ mice.

Primary mouse mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with IL-1β (10ng/ml; 24 h). IL-1β induced the expression of **(A)** CD11b mRNA in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice ($^{***}p<0.001$; ANOVA) and this effect was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with glia from wildtype mice ($^{+++}p<0.001$; ANOVA). Mixed glia prepared from SIGIRR$^{-/-}$ mice exhibit increased mRNA expression of **(B)** CD68 compared with cells from wildtype mice (***p<0.01; ANOVA).Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 3 separate experiments with each condition tested in duplicate.

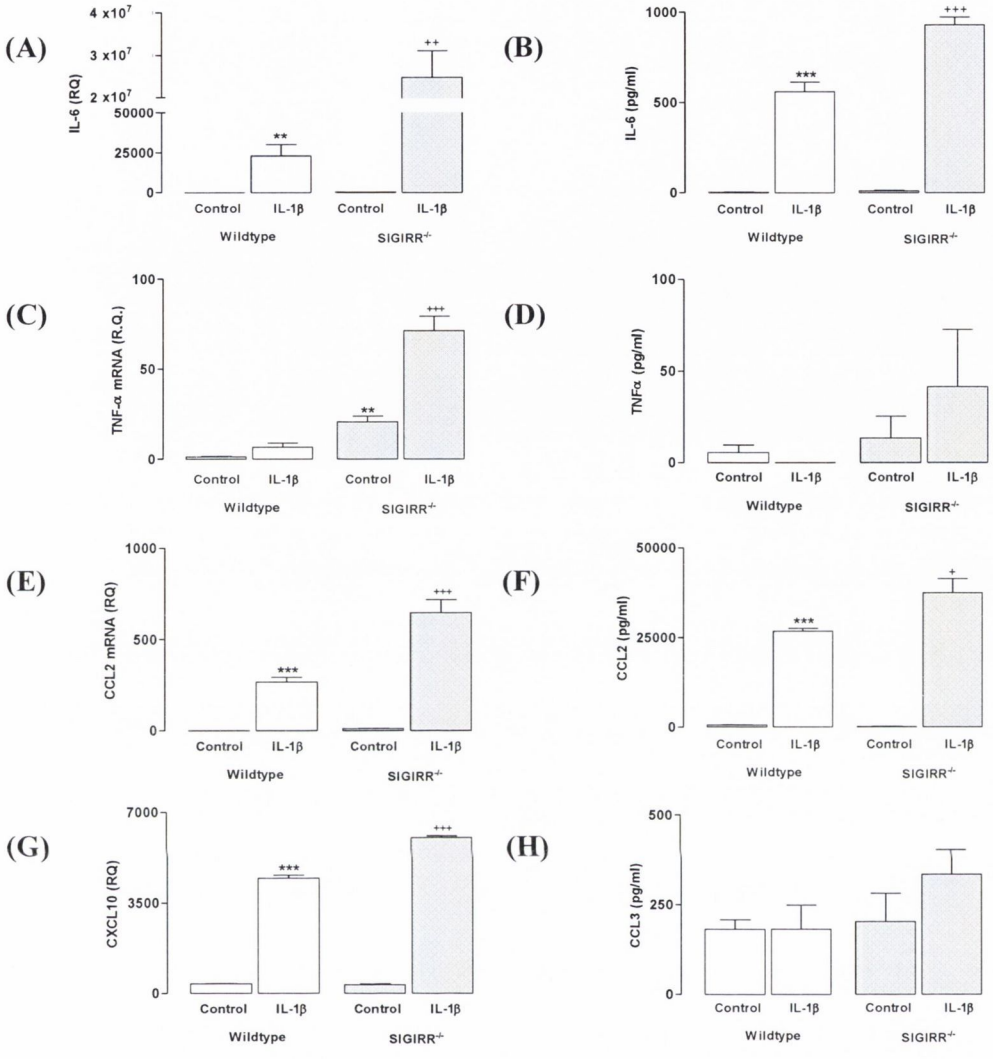


Figure 3.9. IL-1β-induced cytokine production in mixed glia was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice

Primary mouse mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with IL-1β (10ng/ml; 24h). IL-1β induced an increase in mRNA expression of **(A)** IL-6, **(E)** CCL2 and **(G)** CXCL10 from mixed glia (**p<0.01, ***p<0.001; ANOVA). IL-1β-induced mRNA expression of **(A)** IL-6, **(C)** TNF-α, **(E)** CCL2 and **(G)** CXCL10 was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{++}$p<0.001, $^{+++}$p<0.001; ANOVA). IL-1β induced an increase in release of **(A)** IL-6 and **(E)** CCL2 in mixed glia (***p<0.001; ANOVA). IL-1β-induced cytokine release was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+}$p<0.05, $^{+++}$p<0.001; ANOVA).Values are presented as means (± SEM). Data for mRNA is expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 3 separate experiments with each condition tested in duplicate.

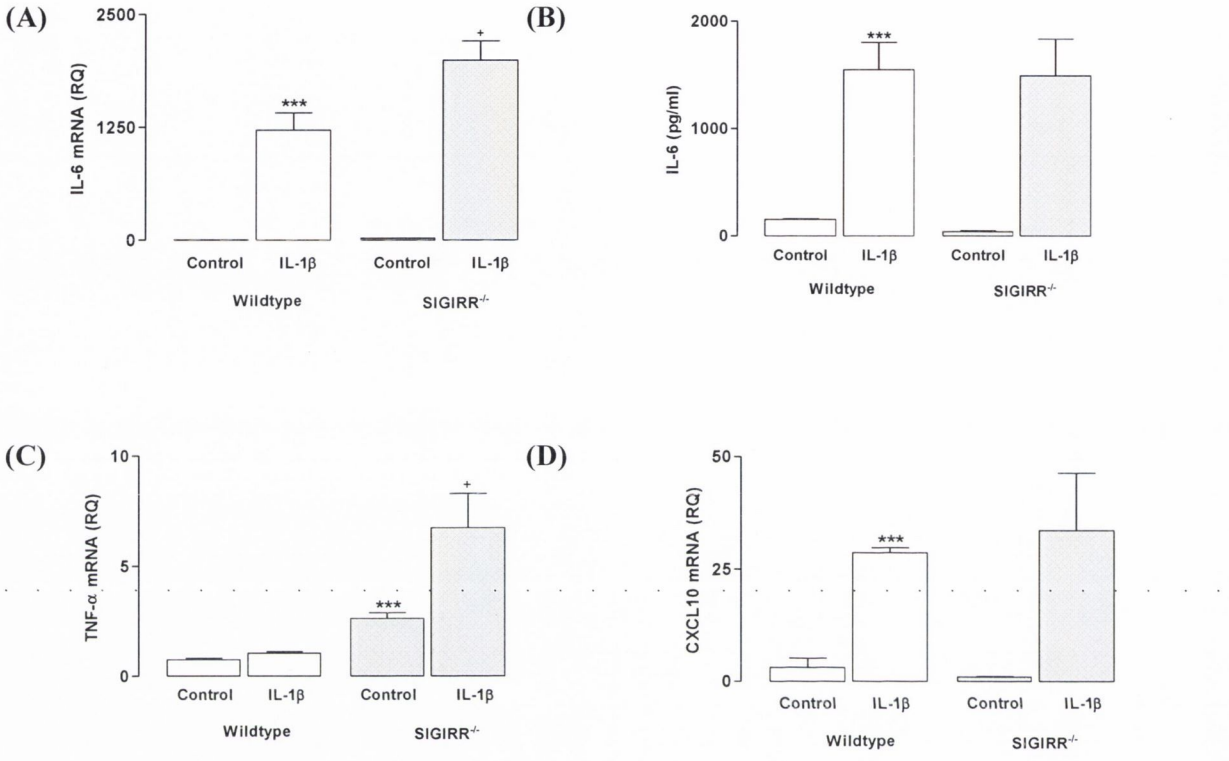


Figure 3.10. IL-1β-induced cytokine mRNA expression in astrocytes was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice.

Secondary mouse astrocytes were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with IL-1β (10ng/ml; 24h). IL-1β induced an increase in mRNA expression of **(A)** IL-6 and **(D)** CXCL10 (***$p<0.001$; ANOVA). IL-1β-induced mRNA expression of **(A)** IL-6 and **(C)** TNF-α was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+}p<0.05$, $^{+++}p<0.001$; ANOVA). IL-1β induced an increase in release of **(B)** IL-6 from mixed glia (***$p<0.001$; ANOVA) and there was no significant difference between cells prepared from wildtype and SIGIRR$^{-/-}$ mice. Values are presented as means (± SEM). Data for mRNA is expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate or triplicate.

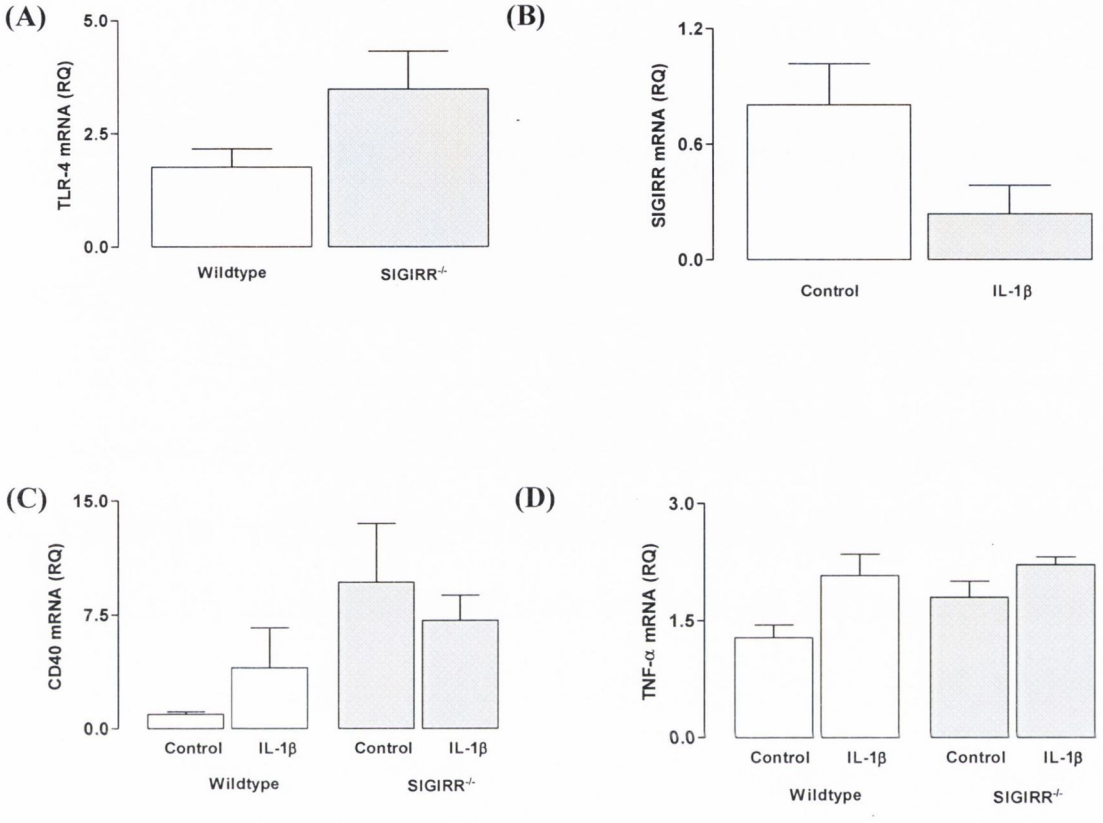


Figure 3.11. IL-1β had no effect on microglia prepared from wildtype or SIGIRR$^{-/-}$ mice

Secondary mouse microglia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with IL-1β (10ng/ml; 24h). IL-1β failed to alter mRNA expression of **(A)** TLR4, **(B)** SIGIRR, **(C)** CD40 or **(D)** TNF-α in microglia from wildtype mice and SIGIRR/- mice. Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate.

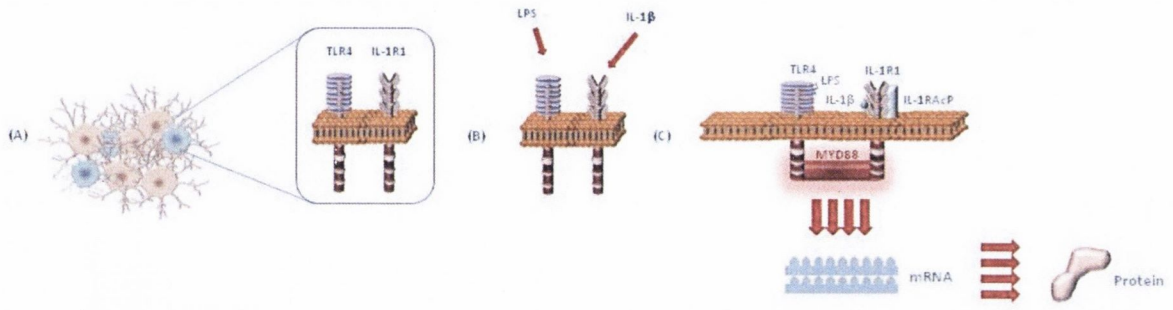


Figure 3.12. Unifying mechanism

(A) Glial cells prepared from SIGIRR-/- mice express higher mRNA levels of the pro-inflammatory receptors TLR4 and IL-1R1. **(B)** The increased levels of receptor expression allow increased binding of ligands such as LPS and IL-1β. **(C)** In the absence of the modulatory effect of SIGIRR, the signaling pathways induced by receptor-ligand interactions are enhanced leading to an upregulation of transcription and translation of genes involved in the inflammatory response.

3.4. Discussion

3.4.1. Overview

The aim of this study was to investigate the role of SIGIRR as a modulator of pro-inflammatory signalling *in vitro*. The role of SIGIRR in the regulation of LPS and IL-1β induced glial activation and cytokine release was investigated by comparing glial cells prepared from mice lacking the gene for SIGIRR with glia prepared from wildtype mice. The hypothesis was that in the absence of SIGIRR, glial cells would exhibit an exaggerated response to exogenously applied pro-inflammatory stimuli and that this would result in enhanced upregulation of markers of glial activation and increased production of pro-inflammatory cytokines. It was further hypothesised that the changes observed in the SIGIRR-deficient mice would be accompanied by an increase in the expression of the receptors for LPS and IL-1β, TLR4 and IL-1R1 respectively. The data demonstrate that mixed glia prepared from SIGIRR-deficient mice exhibited increases in the expression of a variety of markers of glial activation in response to LPS and IL-1β. Cells from SIGIRR-deficient mice secreted greater amounts of pro-inflammatory cytokines and chemokines than those from wildtype mice. Similar responses to LPS and IL-1β were observed in isolated astrocytes and microglia, however only LPS produced a consistent exaggerated effect on cytokine release from microglia prepared from SIGIRR-deficient mice. These changes were accompanied by an increase in the expression of TLR4 in mixed glia and astrocytes prepared from SIGIRR-deficient mice.

3.4.2. The effect of LPS treatment on mixed glia from wildtype and SIGIRR$^{-/-}$ mice

It is established that mixed glia cultured *in vitro* are responsive to a variety of pro-inflammatory stimuli. In this study, LPS induced an increase in the expression of IL-6 and CCL2 mRNA in mixed glia prepared from wildtype mice. This was accompanied by increases in the release of IL-6, CCL2 and TNF-α. These results corroborate previous studies that have shown these molecules to be upregulated in the presence of LPS *in vitro* (Lee et al., 1993, Kong et al., 1997,

Hua and Lee, 2000). The LPS-induced increases were further enhanced in mixed glia prepared from SIGIRR-deficient mice. Watson and colleagues (2010) first reported that pro-inflammatory cytokine production was increased in hippocampal tissue and mixed glia prepared from SIGIRR-deficient mice compared with those from wildtype mice (Watson et al., 2010). Previous studies had reported exaggerated pro-inflammatory cytokine production in dendritic cells (Garlanda et al., 2004, Huang et al., 2006). In addition, mRNA expression of TLR4, the receptor for LPS, was significantly increased in unstimulated mixed glia prepared from SIGIRR-deficient mice compared with cells from wildtype mice. This inverse relationship between SIGIRR and TLR4 expression has been observed before by Watson and colleagues (2010) who found that TLR4 was increased in the hippocampus of SIGIRR deficient mice compared with wildtype mice (Watson et al., 2010). It has also been reported that wildtype mice injected with an anti-SIGIRR antibody exhibited increased TLR4 expression in the cornea. In contrast, TLR4 was found to be decreased in a macrophage cell line over-expressing SIGIRR (Huang et al., 2006). These data suggest that, in addition to sequestering TLR4 binding proteins, SIGIRR may also be involved in the regulation of TLR4 expression. Thus, the exaggerated response to LPS observed in SIGIRR-deficient mice may be due to the combined effect of a lack of an inhibitory signal from SIGIRR, and an increase in the expression of TLR4. In mixed glia prepared from wildtype mice, it was observed that treatment with LPS caused a downregulation of SIGIRR mRNA expression, an effect which has been observed in previous studies in various tissues (Polentarutti et al., 2003, Wald et al., 2003). It may be that SIGIRR is downregulated in order to facilitate pro-inflammatory signalling. This characteristic highlights the potential of SIGIRR as a target for possible anti-inflammatory therapies.

3.4.3. The effect of LPS treatment on astrocytes from wildtype and SIGIRR$^{-/-}$ mice

Having established that mixed glia prepared from SIGIRR-deficient mice exhibit an exaggerated response to LPS, the specific responses of individual cell types were examined. The data corroborate previous studies which characterised astrocytes as having an immune function (Dong and Benveniste, 2001). LPS was shown to induce a significant release of the pro-inflammatory cytokines IL-6 and TNF-α and the chemokine CCL3 and an increase in the mRNA expression of

CXCL10. This pattern of cytokine release from astrocytes has previously been reported by others (Lieberman et al., 1989, Sawada et al., 1992, Quinones et al., 2008). The pattern of cytokine expression exhibited by astrocytes differed somewhat from that observed in mixed glia. mRNA expression of IL-6 and TNF-α mRNA in astrocytes prepared from SIGIRR-deficient mice was increased by LPS to a greater extent than in wildtype mice, however there was no significant difference in IL-6 and TNF-α release between astrocytes prepared from wildtype and SIGIRR-deficient mice. This finding was unexpected, as the mechanism by which SIGIRR modulates LPS signalling is thought to involve recruitment of the TIR-domain-containing signalling proteins proximal to TLR4, and thus occurs upstream of protein translation. These data appear to indicate that in the absence of SIGIRR the transcription of pro-inflammatory genes in astrocytes is upregulated, however the additional mRNA transcripts are not subsequently translated into protein. It is unclear whether or not SIGIRR is having additional effects on translation machinery or if some other unidentified mechanism is involved. This pattern of cytokine modulation was not seen with the release of CCL3, and an exaggerated effect in the SIGIRR-deficient astrocytes similar to that seen in the mixed glial cultures was observed. Although no previous studies had examined its release from SIGIRR-deficient astrocytes, Lech and colleagues (2007) previously reported increased CCL3 expression from colon homogenates of SIGIRR-deficient mice (Lech et al., 2007). The exaggerated upregulation of pro-inflammatory cytokine mRNA in astrocytes prepared from SIGIRR-deficient mice was accompanied by a significant increase in the mRNA expression of TLR4 compared with astrocytes prepared from wildtype mice.

3.4.4. The effect of LPS treatment on microglia from wildtype and SIGIRR$^{-/-}$ mice

SIGIRR acts as a modulator of pro-inflammatory signalling. Therefore, its role in regulating the behaviour of the resident immune cells of the CNS, microglia, was investigated. The present data demonstrate that exposure to LPS caused the release of IL-6, TNF-α and CCL3 from microglia prepared from wildtype mice. This is consistent with many studies which have reported a similar cytokine profile in microglia exposed to LPS (Sawada et al., 1992, Lee et al., 1993, McManus et al., 1998, Nakajima et al., 2003). LPS-induced cytokine release was enhanced in microglia prepared from SIGIRR-deficient mice. These data contrast with the lack of difference observed

in this study in the release of IL-6 and TNF-α between astrocytes prepared from wildtype and SIGIRR-deficient mice and may indicate a more significant role for SIGIRR in the modulation of TLR4 signalling in microglia.

3.4.5. The effect of IL-1β treatment on mixed glia from wildtype and SIGIRR$^{-/-}$ mice

Having established that SIGIRR is an important modulator of TLR4 signalling, its role as a regulator of another member of the IL-1/TLR superfamily was investigated. Mixed glial cells prepared from wildtype and SIGIRR-deficient mice were treated with the pro-inflammatory cytokine IL-1β in order to examine the role of SIGIRR as a modulator of IL-1R1 signaling. The data demonstrate that IL-1β increased CD11b mRNA expression in mixed glia prepared from wildtype mice. CD11b mRNA expression was exaggerated in unstimulated glia prepared from SIGIRR-deficient mice, and following stimulation with IL-1β, compared with cells from wildtype mice. These data suggest that in addition to modulating signaling from activated receptors, SIGIRR may exert a modulatory influence on unstimulated glia, helping to maintain them in a quiescent state. This modulatory effect was further highlighted when the cytokine profile of wildtype and SIGIRR-deficient mixed glia treated with IL-1β was examined. The data show that IL-1β increased expression of CCL2 and CXCL10 consistent with previous studies which indicated that IL-1β has the ability to induce chemokine release from glial cells (Hua and Lee, 2000, Lee et al., 2002). Whereas IL-1β did not affect IL-6 and TNF-α mRNA expression in mixed glia prepared from wildtype mice, it increased expression in cells prepared from SIGIRR-deficient mice. These results were reflected in the cytokine concentrations in the supernatants from the mixed glial cultures, where changes were more pronounced in the absence of SIGIRR. Unlike TLR4, IL-1R1 was not increased in unstimulated mixed glia prepared from SIGIRR-deficient mice compared with cells from wildtype mice, but receptor expression was increased following IL-1β treatment. These results suggest that the modulatory role of SIGIRR is similar in mixed glia for IL-1β and LPS-induced signalling as described previously. However, the effect on IL-1β-induced signaling may be limited to direct modulation of downstream signalling proteins as described previously (Qin et al., 2005). Unlike TLR4, unstimulated IL-1R1 expression is not

elevated in the absence of SIGIRR, indicating that SIGIRR does not affect constitutive expression of IL-1R1. The increase in IL-1R1 expression in mixed glia from SIGIRR-deficient mice in response to IL-1β suggests that SIGIRR may modulate IL-1R1 during inflammatory conditions only, in order to dampen down excessive inflammation.

3.4.6. The effect of IL-1β treatment on astrocytes from wildtype and SIGIRR$^{-/-}$ mice

Astrocytes prepared from SIGIRR-deficient mice appeared to differentially regulate transcription and translation of IL-6 and TNF-α following LPS stimulation. When treated with IL-1β, both IL-6 mRNA expression and supernatant concentration from SIGIRR-deficient cells was increased. The ability of astrocytes to release IL-6 in response to IL-1β is already well established (Aloisi et al., 1992, Norris et al., 1994). Although IL-6 mRNA expression was greater in the astrocytes from SIGIRR-deficient mice, there was no genotype-specific change in supernatant concentration of IL-6. IL-1β also increased TNF-α mRNA in astrocytes prepared from SIGIRR-deficient mice, however TNF-α release was undetectable. A possible explanation for this is that the supernatants were harvested 24 hours post-treatment, hours after the maximum effect on TNF-α release; after 4 hours significant degradation is reported to occur (Lee et al., 1993). These data indicate that a specific pattern of IL-6 production occurs following IL-1R1 and TLR4 activation.

3.4.7. The effect of IL-1β treatment on microglia from wildtype and SIGIRR$^{-/-}$ mice

Cytokine release from isolated microglia in response to IL-1β was not detectable. There is conflicting evidence in the literature with regard to the effect of IL-1β on microglia; McManus and colleagues (1998) reported chemokine release from microglia in response to IL-1β while Lee and colleagues (1993) observed only an increase in gene transcripts (Lee et al., 1993, McManus et al., 1998). CD40 and TNF-α mRNA were expressed in microglia prepared from wildtype and SIGIRR-deficient mice however there were no significant differences between treatment groups

or genotypes. In addition, no change in SIGIRR mRNA was observed in wildtype mice exposed to IL-1β.

These data support the hypothesis that the role of SIGIRR as a modulator of TLR4 and IL-1R1 signaling extends to glial cells. They suggest that control of receptor expression may be a feature of the modulation of TLR4 signaling by SIGIRR. They also suggest that SIGIRR may differentially regulate TLR4 signaling in different cells types, with a greater effect of SIGIRR-deficiency observed in microglia compared with astrocytes in response to LPS.

Chapter 4: Analysis of the effects of Pam_3CSK_4 and Aβ on glial cells from wildtype and SIGIRR-deficient mice.

4.1 Introduction

The evidence presented in the previous chapter indicated that absence of SIGIRR exacerbated the effect of LPS and IL-1β on glial cells. This is consistent with the findings of several studies which demonstrated that SIGIRR modulates signaling through IL-1/TLR.

Although the majority of studies examining the modulatory effect of SIGIRR have focused on its ability to modulate TLR4 and IL-1R1 signaling, there have been some investigations into the relationship between SIGIRR and TLR2. It has been reported that $CD45^+$ renal immune cells prepared from SIGIRR-deficient mice exhibited significantly greater expression of RANTES mRNA and a trend towards an increase in IL-6 and TNF-α mRNA following exposure to Pam_3CSK_4 compared with cells from wildtype mice (Skuginna et al., 2011). It has also been reported that intestinal epithelial cells (IEC) treated with SIGIRR siRNA secreted higher levels of the pro-inflammatory chemokine IL-8 in response to Pam_3CSK_4. The same study reported an attenuation of IL-8 release from IECs over-expressing SIGIRR in response to Pam_3CSK_4.

The aim of this study was to further investigate the production of pro-inflammatory cytokines and chemokines and the upregulation of markers of glial activation in mixed glia, astrocytes and microglia prepared form SIGIRR-deficient mice. The effect of SIGIRR deficiency on TLR2 activation was examined using Pam3CSK4. Because of the evidence suggesting that the toxic peptide Aβ may induce microglial activation through TLR2 and TLR4, the effects of a cocktail of $Aβ_{1-40}$ and $Aβ_{1-42}$ were also observed.

The aim of these experiments was to investigate whether SIGIRR may also be a potential target for modulation of TLR2 and Aβ-induced signaling and microglial activation.

4.2. Methods

Primary cortical mixed glia, microglia and astrocytes were prepared from postnatal 1-day old wildtype and SIGIRR-deficient mice and cultured for 14 days before treatment (see section 2.1 for details). All agents used to treat cells were diluted to required concentrations in pre-warmed supplemented DMEM and all solutions were filter-sterilised through a 0.2μm cellulose acetate membrane filter.

Pam_3CSK_4 was diluted to a final concentration of 100ng/ml in supplemented DMEM. Cells were treated for 24 h in the presence or absence of Pam_3CSK_4. $A\beta_{1-40}$ and $A\beta_{1-42}$ were aggregated according to the manufacturer's instructions. $A\beta_{1-42}$ was dissolved in sterile, distilled water to a concentration of 6mg/ml and then further diluted to a 1mg/ml stock solution with PBS. $A\beta_{1-40}$ was dissolved in PBS to a concentration of 1mg/ml. $A\beta_{1-40}$ peptide was aggregated for 24 h at 25°C and the $A\beta_{1-42}$ peptide for 48 h at 37°C. Cells were incubated in the presence or absence of a cocktail of $A\beta_{1-40}$ (4.2μM) and $A\beta_{1-42}$ (5.6μM) for 24 h. mRNA expression of cytokines and cell surface proteins were assessed by RT-PCR and concentrations of cytokines and chemokines was determined by ELISA (see sections 2.3 and 2.4). Data are expressed as means ± SEM. Students t-test or ANOVA were performed to determine whether significant differences existed between treatment and genotype groups and Bonferroni post-hoc tests were performed where appropriate.

Table 4.1. Caveats

The concentrations of Pam_3CSK_4 and Aβ were chosen on the basis of previous work in the lab which had established that these concentrations elicit a robust response from glial cells. However it should be noted that the concentration of Aβ is not within the range that is typically associated with amyloid-related pathologies e.g. Alzheimer's disease. Therefore be these experiments should be viewed as a study of *in vitro* responses of glial cells to Aβ and not as an *in vitro* model of amyloid pathologies.

All data was collated following 24 hours of treatment. There are differences in the latency of mRNA transcription and cytokine release between different cytokines and this may have affected the results shown here. A time course experiment would have been advisable to determine the optimum time to harvest samples for each cytokine measured.

The data in this chapter are representative of one experiment with each condition replicated 4-6 times. Therefore these data should be considered only as preliminary data which must be repeated in order to properly interperet the implications of the results.

No analysis was undertaken to determine the level of endotoxin present in reagents used. The presence of endotoxin in these reagents may have affected the responses observed from glial cells.

4.3. Results

4.3.1. Investigating the effect of Pam_3CSK_4 on glial cells prepared from wildtype and $SIGIRR^{-/-}$ mice.

Pam_3CSK_4 is a synthetic triacylated lipopeptide (LP) that mimics the acylated amino terminus of bacterial LPs. Pam_3CSK_4 is a potent activator of the proinflammatory transcription factor NF-κB. Activation is mediated by TLR2 which recognizes LPs with three fatty acids, a structural characteristic of bacterial LPs (http://www.invivogen.com/pam3csk4).

Mixed glia prepared from $SIGIRR^{-/-}$ mice exhibit increased TLR2 mRNA expression compared with mixed glia from wildtype mice ($^{***}p<0.001$; Student's t-test; **Figure 4.2**). Pam_3CSK_4 increased the expression of CD40 mRNA in mixed glia prepared from wildtype and $SIGIRR^{-/-}$ mice ($^{***}p<0.001$; ANOVA; **Figure 4.3**), however there was no difference in expression between genotype. Pam_3CSK_4 increased the expression of IL-6, TNF-α and IL-1β mRNA in mixed glia prepared from wildtype and $SIGIRR^{-/-}$ mice ($^{***}p<0.001$; ANOVA; **Figure 4.4, A**; $^{***}p<0.001$; ANOVA; **Figure 4.4, C**; $^{***}p<0.001$; ANOVA; **Figure 4.4, E**) The Pam_3CSK_4 induced increase in TNF-α and IL-1β mRNA was significantly increased in mixed glia prepared from $SIGIRR^{-/-}$ mice ($^{+++}p<0.001$; ANOVA; **Figure 4.4, C**; $^{+++}p<0.001$; ANOVA; **Figure 4.4, E**). Pam_3CSK_4 increased IL-6 and TNF-α release in mixed glia prepared from wildtype and $SIGIRR^{-/-}$ mice (***p<0.001; ANOVA; **Figure 4.4, B**; ***p<0.001; ANOVA; **Figure 4.4, D**) The effect was significantly increased in mixed glia prepared from $SIGIRR^{-/-}$ mice compared with glia from wildtype mice ($^{+++}p<0.001$; ANOVA; **Figure 4.4, B**; $^{+++}p<0.001$; ANOVA; **Figure 4.4, D**).

4.3.2. Investigating the effect of Pam_3CSK_4 on isolated astrocytes and microglia prepared from wildtype and $SIGIRR^{-/-}$ mice.

Isolated astrocytes prepared from $SIGIRR^{-/-}$ mice exhibit increased TLR2 mRNA expression compared with mixed glia from wildtype mice (**p<0.01; Student's t-test; **Figure 4.5**).

Isolated astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice exhibited increased supernatant concentration of IL-6 and TNF-α in response to Pam_3CSK_4 (***p<0.001; ANOVA; **Figure 4.6, A**; ***p<0.001; ANOVA; **Figure 4.6, B**). This effect was significantly enhanced in astrocytes prepared from SIGIRR$^{-/-}$ mice compared with cells from wildtype mice ($^{+++}$p<0.001; ANOVA; **Figure 4.6, A**; $^{+++}$p<0.001; ANOVA; **Figure 4.6, B**). Isolated microglia prepared from wildtype and SIGIRR$^{-/-}$ mice exhibited increased supernatant concentration of IL-6 and TNF-α in response to Pam_3CSK_4 (***p<0.001; ANOVA; **Figure 4.7, A**; ***p<0.001; ANOVA; **Figure 4.7, B**). The effect of Pam_3CSK_4 on IL-6 was significantly enhanced in astrocytes prepared from SIGIRR$^{-/-}$ mice compared with cells from wildtype mice ($^{+++}$p<0.001; ANOVA; **Figure 4.7, A**).

4.3.3. Investigating the effect of Aβ on glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice.

It has been reported that fibrillar $A\beta_{1-42}$ can induce the expression of markers of microglial activation and the release of pro-inflammatory cytokines in mixed glia (Lyons et al., 2007). Although the exact mechanism through which Aβ interacts with glial cells is not clear, it is believed to bind to a number of cell surface receptors including TLR2 and TLR4 (Reed-Geaghan et al., 2009).

Aβ increased the mRNA expression of CD40 in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice (***p<0.001; ANOVA; **Figure 4.8**).

Aβ increased the mRNA expression of IL-6, TNF-α and IL-1β in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice (***p<0.001; ANOVA; **Figure 4.9, A**; ***p<0.001; ANOVA; **Figure 4.9, C**; ***p<0.001; ANOVA; **Figure 4.9, E**) The Aβ induced increase in IL-6 and IL-1β mRNA was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice ($^{+++}$p<0.001; ANOVA; **Figure 4.9, A**; $^{+++}$p<0.001; ANOVA; **Figure 4.9, E**). Aβ increased IL-6 and TNF-α supernatant concentrations in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice (***p<0.001; ANOVA; **Figure 4.9, B**; ***p<0.001; ANOVA; **Figure 4.9, D**) The effect was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with glia from wildtype mice ($^{+++}$p<0.001; ANOVA; **Figure 4.9, B**; $^{+++}$p<0.001; ANOVA; **Figure 4.9, D**).

Table 4.2. Results summary

Key: R=mRNA; P=protein; ↑=increase; ↓=decrease; - = no change; NA=not analysed; NML=no measureable levels

		Effect on WT[1]		Effect on SIG$^{-/-}$[2]	
	Parameter	**Pam3CSK4**	**Aβ**	**Pam3CSK4**	**Aβ**
Mixed Glia	**CD40**	**R** ↑; **P** NA	**R** ↑; **P** NA	**R** -; **P** NA	**R** -; **P** NA
	IL-6	**R** ↑; **P** ↑	**R** ↑; **P** ↑	**R** -; **P** ↑	**R** ↑; **P** ↑
	TNF-α	**R** ↑; **P** ↑	**R** ↑; **P** ↑	**R** ↑; **P** ↑	**R** -; **P** ↑
	IL-1β	**R** ↑; **P** NA	**R** ↑; **P** NA	**R** ↑; **P** NA	**R** ↑; **P** NA
Astrocytes	**IL-6**	**R** NA; **P** ↑	NA	**R** NA; **P** ↑	NA
	TNF-α	**R** NA; **P** ↑	NA	**R** NA; **P** ↑	NA
	IL-1β	NA	NA	NA	NA
Microglia	**CD40**	NA	NA	NA	NA
	IL-6	**R** NA; **P** ↑	NA	**R** NA; **P** ↑	NA
	TNF-α	**R** NA; **P** ↑	NA	**R** NA; **P** -	NA
	IL-1β	NA	NA	NA	NA

[1]Relative to control treated WT; [2]Relative to similarly treated WT

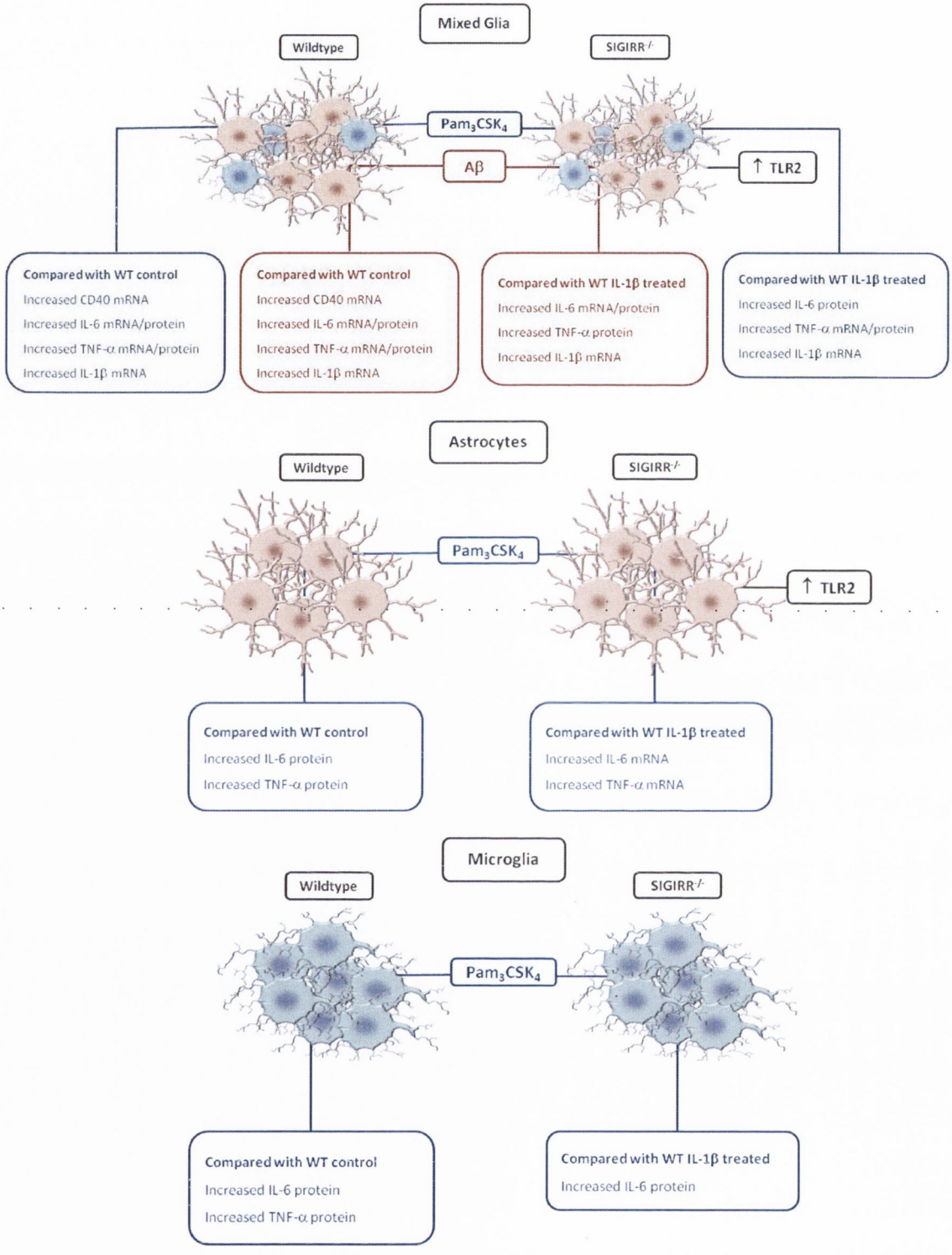


Figure 4.1. Results summary

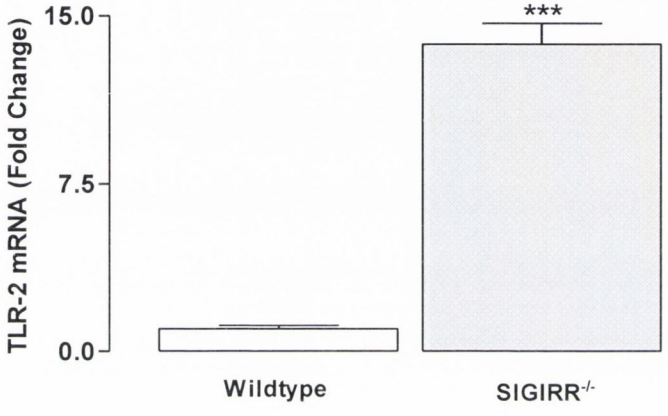


Figure 4.2. TLR2 expression was increased in mixed glia prepared from SIGIRR$^{-/-}$ mice.

Primary mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice. Mixed glia prepared from SIGIRR$^{-/-}$ mice exhibited increased mRNA expression of TLR2 compared with mixed glia from wildtype mice (***$p<0.001$; Student's t-test). Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 1 experiment with 4 replicates.

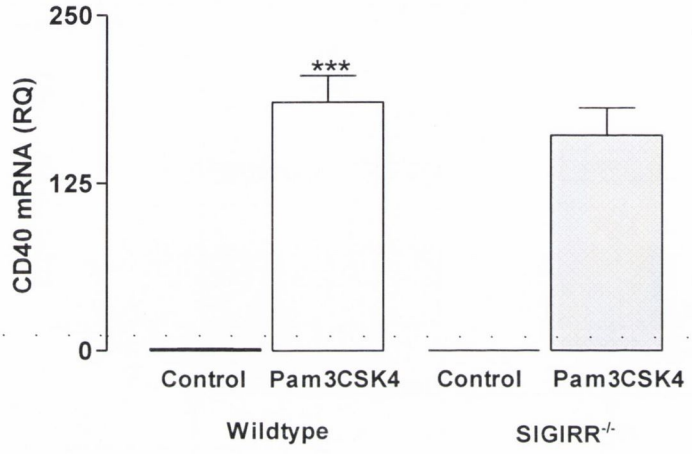


Figure 4.3. Pam_3CSK_4 induced CD40 expression in mixed glia.

Primary mixed glia were prepared from wildtype and $SIGIRR^{-/-}$ mice and treated with Pam_3CSK_4 (100ng/ml; 24h). Pam_3CSK_4 induced an increase in CD40 in mixed glia prepared from wildtype and $SIGIRR^{-/-}$ mice and there was no difference between mixed glia prepared from wildtype and $SIGIRR^{-/-}$ mice (***$p<0.001$; ANOVA). Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 1 experiment with 6 replicates.

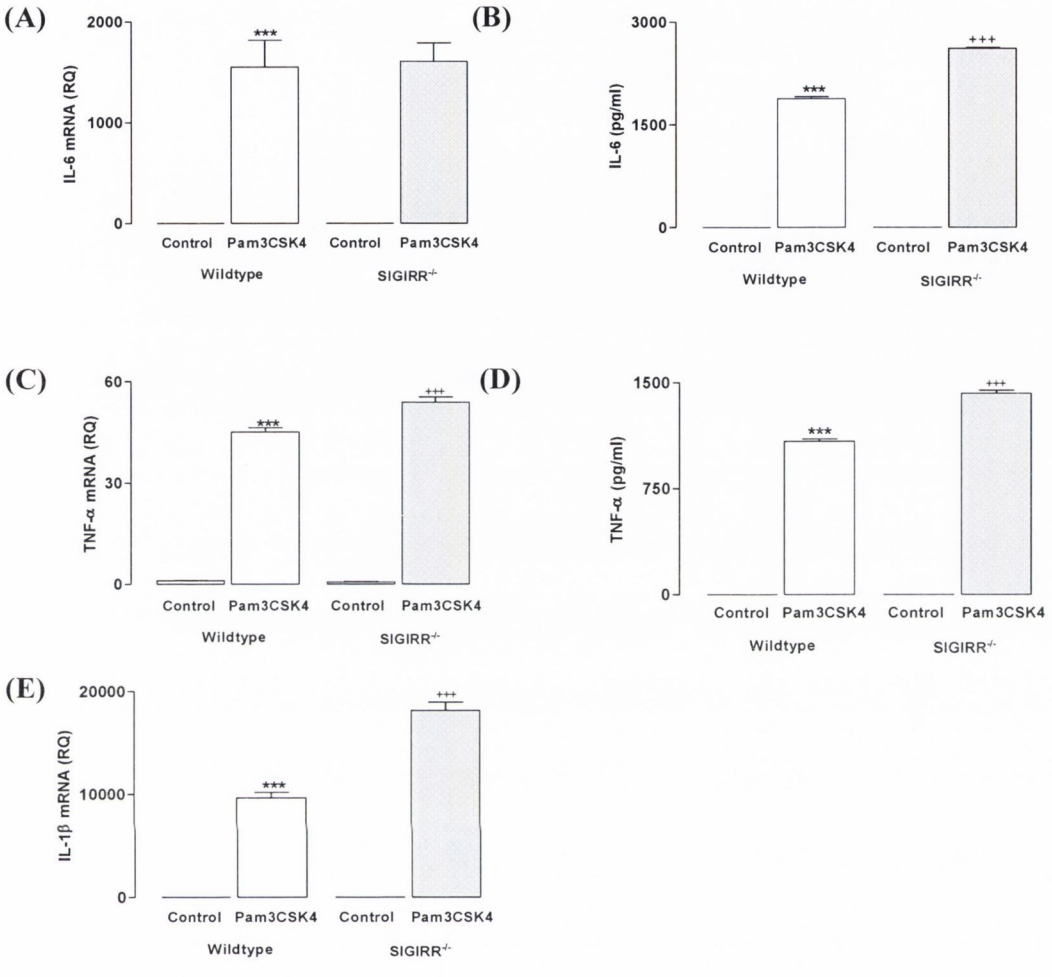


Figure 4.4. Pam_3CSK_4- induced cytokine production in mixed glia was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice

Primary mouse mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with Pam_3CSK_4 (100ng/ml; 24 h). Pam_3CSK_4 induced an increase in mRNA expression of **(A)** IL-6, **(C)** TNF-α and **(E)** IL-1β from mixed glia (***$p<0.001$; ANOVA). Pam_3CSK_4-induced mRNA expression of **(C)** TNF-α and **(E)** IL-1β was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA). Pam_3CSK_4 induced an increase in release of **(B)** IL-6 and **(D)** TNF-α in mixed glia (***$p<0.001$; ANOVA) and cytokine release was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA).Values are presented as means (± SEM). Data for mRNA is expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 1 experiment with 6 replicates.

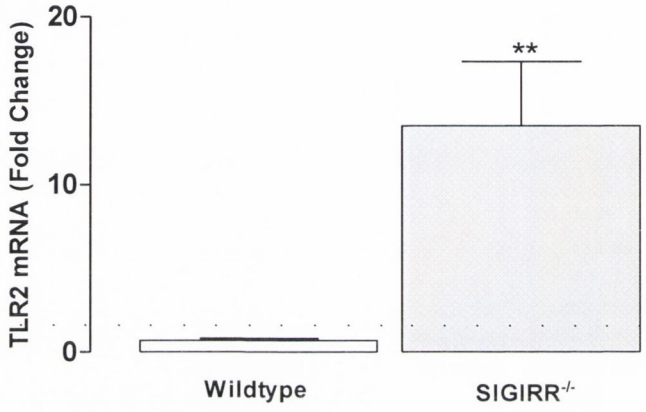


Figure 4.5. TLR2 expression is increased in astrocytes prepared from SIGIRR$^{-/-}$ mice.

Secondary astrocytes were prepared from wildtype and SIGIRR$^{-/-}$ mice. Astrocytes prepared from SIGIRR$^{-/-}$ mice exhibited increased mRNA expression of TLR2 compared with mixed glia from wildtype mice (**$p<0.001$; Student's t-test). Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 1 experiment with 5 replicates.

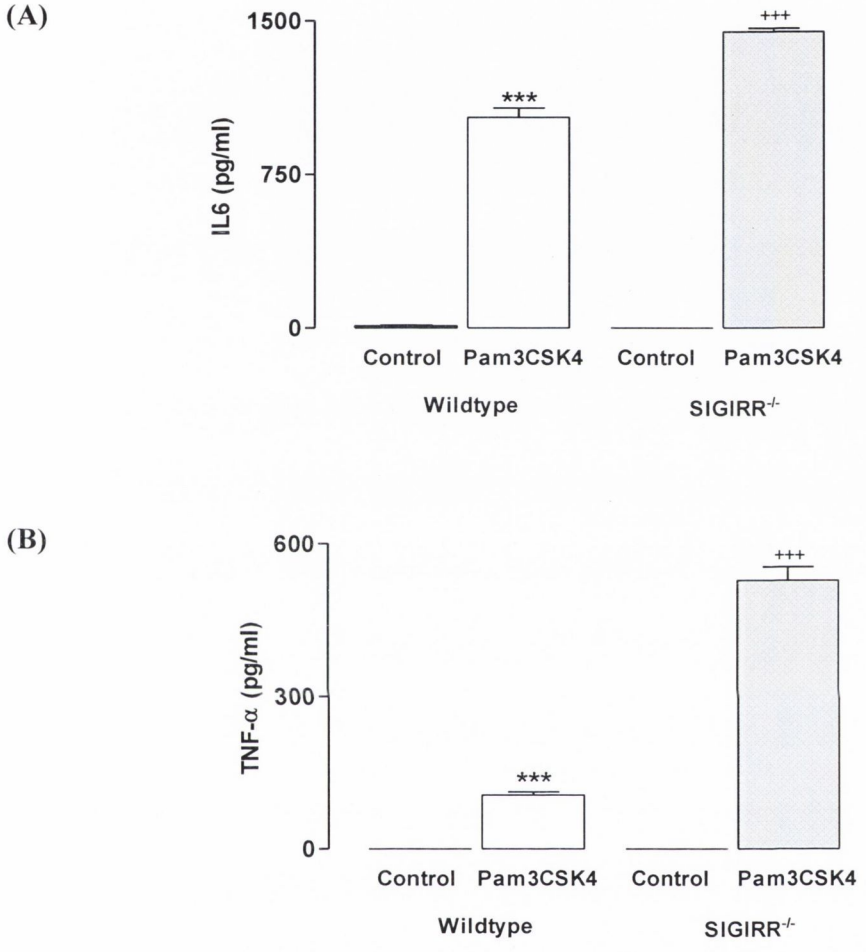


Figure 4.6. Pam_3CSK_4 induced cytokine release from astrocytes was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice

Secondary astrocytes were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with Pam_3CSK_4 (100ng/ml; 24 h). Pam_3CSK_4 induced an increase in the release of **(A)** IL-6 and **(B)** TNF-α in astrocytes prepared from wildtype and SIGIRR$^{-/-}$ mice (***$p<0.001$; ANOVA) and this effect was significantly greater in astrocytes prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA). Values are presented as means (± SEM). The data are representative of 1 experiment with 6 replicates.

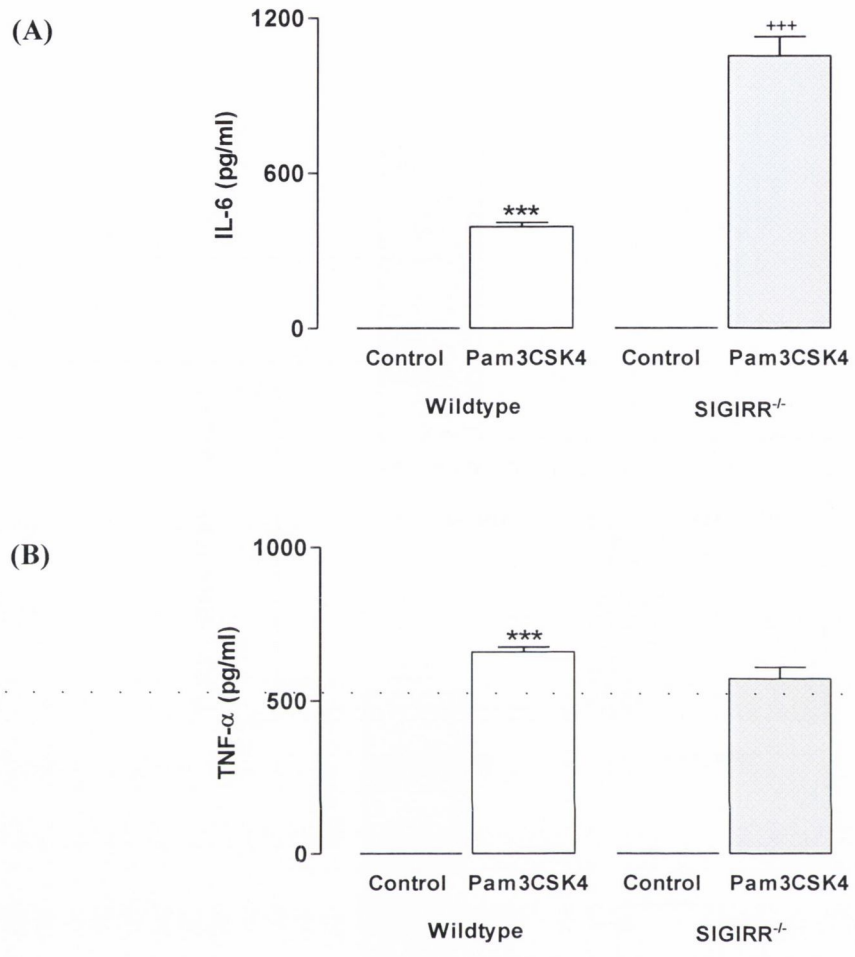


Figure 4.7. Pam_3CSK_4-induced release of IL-6 from microglia was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice

Secondary microglia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with Pam_3CSK_4 (100ng/ml; 24 h). Pam_3CSK_4 induced an increase in the release of **(A)** IL-6 and **(B)** TNF-α in microglia prepared from wildtype and SIGIRR$^{-/-}$ mice (***$p<0.001$; ANOVA). The Pam_3CSK_4-induced increase of **(A)** IL-6 was significantly greater in cells prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA). Values are presented as means (± SEM). The data are representative of 1 experiment with 6 replicates.

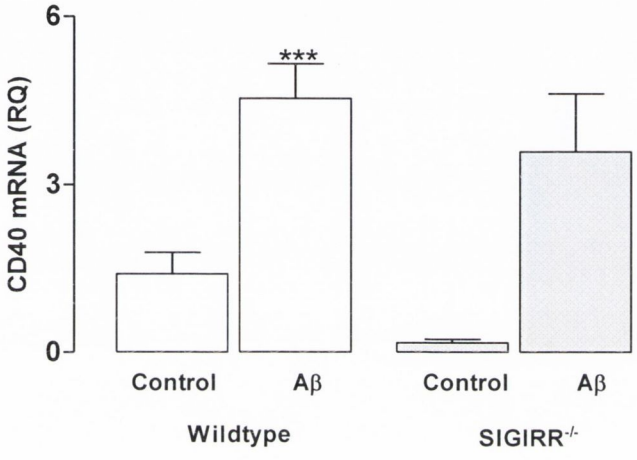


Figure 4.8. Aβ induced CD40 expression in mixed glia.

Primary mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with Aβ ($A\beta_{1-40}$ (4.2μM) and $A\beta_{1-42}$ (5.6μM); 24h). Aβ induced an increase in the mRNA expression of CD40 in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice and there was no difference between mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice (***$p<0.001$; ANOVA). Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 1 experiment with 6 replicates.

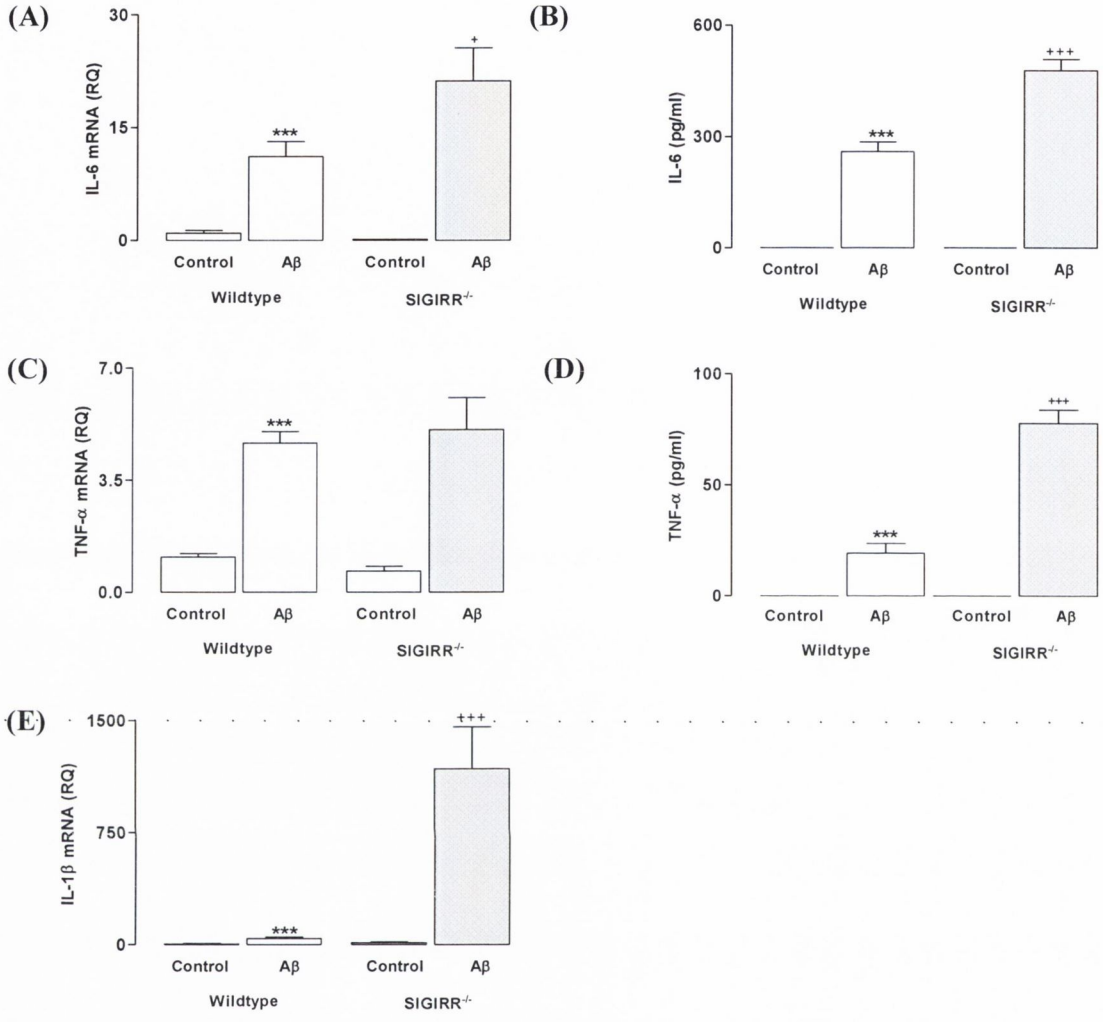


Figure 4.9. Aβ-induced cytokine production in mixed glia was exaggerated in cells prepared from SIGIRR$^{-/-}$ mice

Primary mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with Aβ ($A\beta_{1\text{-}40}$ (4.2μM) and $A\beta_{1\text{-}42}$ (5.6μM); 24h). Aβ induced an increase in mRNA expression of **(A)** IL-6, **(C)** TNF-α and **(E)** IL-1β in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice (***$p<0.001$; ANOVA). The Aβ-induced increase in **(A)** IL-6 and **(E)** IL-1β was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared wildtype mice ($^{+}p<0.05$; $^{+++}p<0.001$; ANOVA). Aβ induced an increase in release of **(B)** IL-6 and **(D)** TNF-α from mixed glia (***$p<0.001$; ANOVA). Aβ induced cytokine release was significantly increased in mixed glia prepared from SIGIRR$^{-/-}$ mice compared wildtype mice ($^{+++}p<0.001$; ANOVA). Values are presented as means (± SEM; n=1) and expressed as a ratio to β-actin mRNA. The data are representative of 1 experiment with 6 replicates.

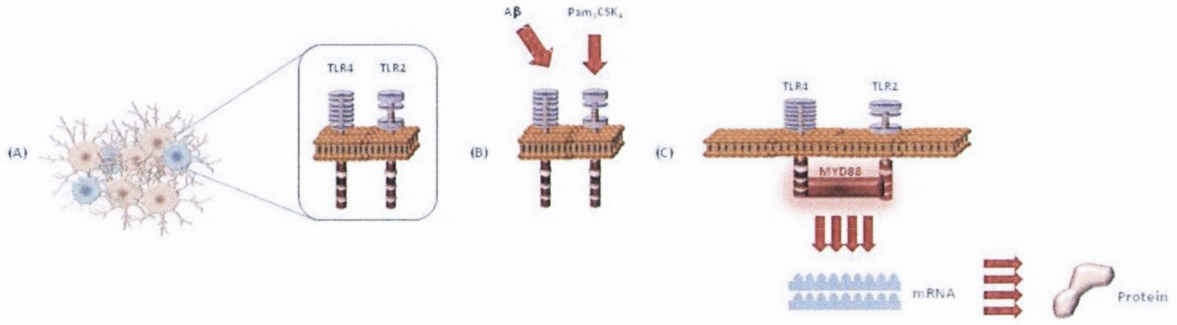


Figure 4.10. Unifying mechanism

(A) Glial cells prepared from SIGIRR$^{-/-}$ mice express higher mRNA levels of the pro-inflammatory receptors TLR2 and TLR4. **(B)** The increased levels of receptor expression allow increased binding of ligands such as Pam_3CSK_4 and Aβ. **(C)** In the absence of the modulatory effect of SIGIRR, the signaling pathways induced by receptor-ligand interactions are enhanced leading to an upregulation of transcription and translation of genes involved in the inflammatory response.

4.4. Discussion

4.4.1. Overview

The role of SIGIRR as a modulator of TLR2 signalling has not been fully established. Activation of TLR2 initiates a signalling cascade, similar to that initiated by TLR4, which results in the activation of NFκB. As this occurs in a MyD88-dependent manner, it can be hypothesised that SIGIRR would modulate TLR2 signalling by recruiting MyD88 via its TIR domain, as occurs during TLR4 signalling. Although it has been observed that SIGIRR acts directly on TLR4 and IL-1R1 in order to modulate their signalling, it is not clear whether it interacts directly with TLR2.

4.4.2. The effect of Pam_3CSK_4 treatment on mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice

Pam_3CSK_4 is a potent activator of the proinflammatory transcription factor NF-κB. It has been shown previously that Pam_3CSK_4 can induce the expression of markers of microglial activation and the release of a variety of pro-inflammatory cytokines from mixed glial cells, and that this effect was exaggerated in cells prepared from a CD200-deficient mice. It was suggested that this was linked to the increased expression of TLR2 in cells prepared from CD200-deficient mice (Costello et al., 2011a). In the present study, an increase in TLR2 mRNA in unstimulated mixed glia prepared from SIGIRR-deficient mice compared with cells from wildtype mice was observed. Previous studies using *in vitro* models of inflammation have found similar increases in TLR2 expression. Kielian et al., (2004) reported that microglia exposed to *S. Aureus* exhibit increase mRNA expression of TLR2 (Kielian et al., 2004). This effect was also observed in macrophages treated with LPS or peptidoglycan (Liu et al., 2001). Mixed glial cells prepared from wildtype and SIGIRR-deficient mice were treated with Pam_3CSK_4 in order to observe any differences in microglial activation and cytokine release in cells lacking SIGIRR compared with those from wildtype mice. Pam_3CSK_4 induced an increase in the expression of CD40 mRNA in

mixed glia prepared from wildtype and SIGIRR-deficient mice, however there was no significant genotype-related difference in CD40 mRNA expression. This was somewhat counter-intuitive, as Watson et al., (2010) had previously shown that CD40 expression in mixed glia induced by the TLR4 agonist LPS, which shares common signalling mechanisms with TLR2, was exaggerated in cells prepared from SIGIRR deficient mice (Watson et al., 2010). It is possible that the greater specificity of Pam_3CSK_4 for its receptor, TLR2, accounts for this difference, as LPS is known to activate microglia via both TLR4 and TLR2 and therefore may act as a more potent activator when the modulatory influence of SIGIRR is absent. Pam_3CSK_4 induced an increase in the expression of IL-6, TNF-α and IL-1β mRNA and in the supernatant concentration of IL-6 and TNF-α from mixed glia prepared from wildtype mice. These data corroborate previous studies in which Pam_3CSK_4 was found to induce pro-inflammatory cytokine release from mixed glial and isolated microglial cultures (Ribes et al., 2009, Costello et al., 2011a). The increase in TNF-α and IL-1β mRNA expression and in IL-6 and TNF-α release was significantly greater in cells prepared from SIGIRR-deficient mice. These data add to the small number of studies that have examined the relationship between SIGIRR and TLR2 and which suggest that SIGIRR does exert a modulatory effect on TLR2 signalling. Skuginna et al., (2011) reported that $CD45^+$ renal immune cells prepared from SIGIRR-deficient mice exhibited significantly greater expression of RANTES mRNA and a trend towards an increase in IL-6 and TNF-α mRNA compared to cells from wildtype mice. It has also been reported that intestinal epithelial cells (IEC) treated with SIGIRR siRNA secreted higher levels of the pro-inflammatory chemokine IL-8 in response to Pam_3CSK_4. The same study reported an attenuation of IL-8 release from IECs over-expressing SIGIRR in response to Pam_3CSK_4. The data presented here support the hypothesis that the affinity of SIGIRR towards TIR domain-containing proteins such as MyD88 enable to it to act as a negative regulator of pro-inflammatory receptors which signal through the NF-κB pathway, including TLR2.

4.4.3. The effect of Pam_3CSK_4 treatment on astrocytes and microglia prepared from wildtype and $SIGIRR^{-/-}$ mice

Astrocytes prepared from SIGIRR-deficient mice exhibited increased TLR2 mRNA expression compared with cells from wildtype mice. It has previously been reported that astrocytic TLR2 expression is induced via the NF-κB pathway, and the absence of an important modulator of NF-κB activation in astrocytes prepared from SIGIRR-deficient mice is likely to contribute to the increased expression of TLR2 in these cells (Phulwani et al., 2008). Pam_3CSK_4 induced an increase in the supernatant concentration of IL-6 and TNF-α, and this effect was exaggerated in astrocytes prepared from SIGIRR-deficient mice. Previous studies have shown that Pam_3CSK_4 is capable of inducing the release of a variety of chemokines and cytokines, including CCL2, CCL3 and TNF-α from isolated astrocytes (McKimmie and Graham, 2010). Pam_3CSK_4 also induced an increase in supernatant concentrations of IL-6 and TNF-α from isolated microglia prepared from wildtype and SIGIRR-deficient mice, and there was an exaggerated production of IL-6 in the cells from SIGIRR-deficient mice. It has previously been reported that Pam_3CSK_4 induced the release of the pro-inflammatory cytokines IL-12 and IL-27 from isolated microglia and that this effect was absent in microglia prepared from TLR2-deficient mice (Holley et al., 2012). It has also been reported that IP injection of Pam_3CSK_4 induced the proliferation of microglia and an increase in IL-6, IL-1 and CCL2 in the mouse cortex. It is likely that the exaggerated production of cytokines in response to Pam_3CSK_4 in astrocytes and microglia prepared from SIGIRR-deficient mice is due to the increased expression of TLR2, and the increase in NF-κB signaling through TLR2 in the absence of SIGIRR. The data support the evidence ascribing a role for astrocytes as sensors of infection through cell surface receptors such as TLR2 (McKimmie and Graham, 2010). They also suggest that SIGIRR may play a role in the modulation of TLR2 signaling in both astrocytes and microglia, in contrast to the modulation of TLR4 signaling by SIGIRR which appears to be specific to microglia.

It is thought that inflammation represents a major driving force behind the pathology of Alzheimer's disease (Akiyama et al., 2000). Insoluble plaques composed of fibrillar Aβ act as a potent inflammatory stimulus to resident immune cells of the CNS (Lyons et al., 2007). Recent studies have provided strong evidence that fibrillar Aβ peptides propagate pro-inflammatory signals within microglia via numerous cell-surface receptors, including the innate immune receptors TLR4 and, in particular, TLR2. This has been demonstrated by Udan et al., (2008), who showed that the Aβ-induced release of TNF-α from a monocyte cell line was attenuated

when the cells were incubated with TLR2 or TLR4 blocking antibodies (Udan et al., 2008). The data presented in Chapter 3 demonstrated the ability of SIGIRR to act as a negative regulator of TLR4 signaling in glial cells, and it was therefore hypothesised that SIGIRR may exert a modulatory effect on Aβ-induced inflammatory signaling. The following experiment sought to establish whether or not the absence of SIGIRR exacerbated the ability of Aβ to induce microglial activation and cytokine release from mixed glial cells.

4.4.4. The effect of Aβ treatment on mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice

Aβ induced an increase in CD40 mRNA expression from mixed glia prepared from wildtype and SIGIRR-deficient mice. It has been reported that CD40L-CD40 interaction is a necessary component APP processing and in Aβ-induced microglial activation. Previous studies have demonstrated that cultured microglia treated with Aβ exhibited increased expression of CD40 mRNA (Tan et al., 2002). No genotype related difference in CD40 mRNA expression was observed. It is possible that Aβ can induce CD40 through NF-κB-independent pathways, which would not be affected by SIGIRR deficiency. Although it has been reported that CD40/CD40L interaction-induced Aβ production occurs primarily in an NF-κB dependent manner, it is unclear if Aβ-induced CD40 expression occurs in the same manner (Ait-Ghezala et al., 2007). Aβ also induced the mRNA expression of IL-6, TNF-α and IL-1β mRNA and release of IL-6 and TNF-α from mixed glia prepared form wildtype and SIGIRR-deficient mice. These data support a number of studies that report Aβ induction of pro-inflammatory cytokine release from glial cells (Lyons et al., 2007; Jiao et al., 2008). The Aβ-induced expression of IL-6 and IL-1β mRNA and IL-6 and TNF-α release was enhanced in cells prepared from SIGIRR-deficient mice compared with wildtype mice. These data provide the first evidence that SIGIRR may have a role in the modulation of Aβ signaling in glial cells. This is most likely due to the modulatory effect of SIGIRR on TLR2 and TLR4 signaling and receptor expression, as described previously.

The data presented here provide evidence that SIGIRR exerts a modulatory effect on Pam_3CSK_4 signaling, and suggest that SIGIRR may act as a negative regulator of TLR2 activation, a role

which has not yet been confirmed in peripheral cells. They suggest that SIGIRR may exert this effect in part through control over the expression of TLR2. The data also present evidence that SIGIRR modulates Aβ-induced cytokine production in glial cells, and suggest that SIGIRR may be a target for the treatment of chronic inflammation associated with AD.

Chapter 5: Analysis of the effect of intrahippocampal injection of Aβ in young and middle-aged, wildtype and SIGIRR-deficient mice

5.1. Introduction

It has been widely documented that chronic inflammation is a contributing factor in the cognitive decline associated with aging and age-related neurological disorders such as AD (Akiyama et al., 2000, Bodles and Barger, 2004, Lynch et al., 2007). Increased microglial activation, levels of pro-inflammatory receptors and cytokines, and increased activation of transcription factors have all been associated with aging (Heyen et al., 2000, Lynch and Lynch, 2002, Lynch et al., 2010). These processes are exacerbated in the AD brain, primarily due to the presence of highly toxic peptides, specifically Aβ (Benzing et al., 1999, Lyons et al., 2007b). It has been previously reported that SIGIRR may exert a modulatory effect on age-related inflammatory changes *in vivo,* however there have been no studies thus far to assess its ability to modulate Aβ-induced changes in the brain (Watson et al., 2010). There is evidence to suggest that TLR2 acts as a receptor for Aβ peptide, and signalling via this receptor may be the principal method by which Aβ induces microglial activation and pro-inflammatory cytokine release (Liu et al., 2012). The data from chapter 4 suggest that SIGIRR exerts a modulatory role on Aβ-induced glial activation and cytokine release *in vitro* and that this may be linked to control over the expression of TLR2. In this chapter, a series of experiments were performed to investigate the relationship between Aβ and TLR2, the inflammatory effects of ageing and Aβ toxicity, and the possibility that the ability of SIGIRR to modulate Aβ-induced inflammatory processes *in vitro* would be replicated *in vivo*. The aims of the study were to;

- Examine the role of TLR2 as a receptor for Aβ.
- Assess the effects of an intrahippocampal injection of Aβ on cytokines, pro-inflammatory receptors, glial activation and NF-κB activation in mouse models of aging and SIGIRR-deficiency.

5.2. Methods

Primary cortical mixed glia were prepared from postnatal 1-day old mice and cultured for 14 days before treatment (see section 2.1 for details). All agents used to treat cells were diluted to required concentrations in pre-warmed supplemented DMEM and all solutions were filter-sterilised through a 0.2μm cellulose acetate membrane filter. $A\beta_{1-40}$ and $A\beta_{1-42}$ (Invitrogen; USA) were aggregated according to the manufacturer's instructions (see section 4.2). Cells were incubated in the presence of a cocktail of $A\beta_{1-40}$ (4.2μM) and $A\beta_{1-42}$ (5.6μM) for 24 h. Anti-TLR2 antibody was prepared as a stock solution in PBS. Cells were pre-treated with anti-TLR2 antibody (5μg/ml) for 2 h before being co-incubated with Aβ for 24 h.

Male and female, young (3 months) and middle-aged (12-14 months), wildtype and $SIGIRR^{-/-}$ mice were anaesthetised with Avertin (20μl/g; Sigma Aldrich; IRE) and deep anaesthesia was confirmed by the absence of a pedal reflex. Young and middle-aged wildtype and $SIGIRR^{-/-}$ mice were randomly assigned to a control group or an Aβ-treated group (n=6-8 animals per group). Control animals received an intrahippocampal injection with sterile saline (5μl) and Aβ-treated animals of both genotypes received a cocktail of $A\beta_{1-40/1-42}$ (5μl; 2.75μl $A\beta_{1-42}$; 2.25μl $A\beta_{1-40}$) intrahippocampally. Animals were sacrificed 4 h post-treatment by decapitation. The brain was rapidly removed, the cerebellum and olfactory bulbs were discarded and the hippocampus and cortices were snap-frozen in liquid nitrogen in an RNAse-free tube for later analysis by PCR (see section 2.3) and WB (see section 2.5).

The Students t-test or ANOVA were performed to determine whether significant differences existed between treatment and genotype groups and Bonferroni post-hoc tests were performed where appropriate.

Table 5.1. Caveats

The concentrations of Aβ were chosen on the basis of previous work in the lab which had established these concentrations elicit a robust response in hippocampal and cortical tissue following intrahippocampal injection. However it should be noted that the concentration of Aβ is not within the range that is typically associated with amyloid-related pathologies e.g. Alzheimer's disease. Therefore be these experiments should be viewed as a study of *in vivo* responses to Aβ toxicity rather than as a model of Alzheimer's disease.
All data was collated following 4 hours of treatment. This time point was chosen based on previous experiments performed in the lab. It should be noted however that phosphorylation events in the NF-κB signaling pathway occur at a mush earlier stage, while cytokine production and the upregulation of markers of microglial activation may occur later. In addition, the difference in timepoint between the *in vitro* and *in vivo* experiments performed must be considered when any attempt is made to compare the results of two experiments.
The animals used in these experiments were maintained in facilities which are not specific pathogen free (SPF). This should be considered with regard to inflammatory processes which may be affected by pathogens present in the animal's environment.
No analysis was undertaken to determine the level of endotoxin present in reagents used. The presence of endotoxin in these reagents may have affected the responses observed from glial cells

5.3. Results

5.3.1. The effect of Aβ and anti-TLR2 antibody on mixed glial cells

Aβ induced a significant increase in IL-6 (***$p<0.001$; ANOVA; **Figure 5.1, A**) and TNF-α (***$p<0.001$; ANOVA; **Figure 5.1, B**) release from mixed glia compared to control treated cells. Pre-treating the cells with anti-TLR2 antibody significantly attenuated the Aβ-induced increase in IL-6 ($^{+++}p<0.001$; ANOVA; **Figure 5.1, A**) and TNF-α ($^{+++}p<0.001$; ANOVA; **Figure 5.1, B**).

5.3.. The effect of age, Aβ and absence of SIGIRR on cytokines in cortical and hippocampal tissue.

Neither age nor Aβ significantly affected IL-6 mRNA in cortical (**Figure 5.2, A**) or hippocampal (**Figure 5.2, B**) tissue from wildtype or SIGIRR-deficient mice.

5.3.3. The effect of age, Aβ and absence of SIGIRR on receptors in cortical and hippocampal tissue

There was a significant increase in TLR2 mRNA in cortical tissue from 3 month-old SIGIRR-deficient mice compared with wildtype mice (*$p<0.05$; ANOVA; Figure **Figure 5.3, A**) however there was no significant effect of age on TLR2 mRNA in either wildtype or SIGIRR-deficient mice. There was a significant effect of genotype (**$p<0.01$; ANOVA; **Figure 5.3, D**) and a significant interaction between age and genotype (*$p<0.05$; ANOVA; **Figure 5.3, D**) on RAGE mRNA in hippocampal tissue from wildtype and SIGIRR-deficient mice. Post-hoc analysis revealed that the age related changes were observed in tissue from SIGIRR-deficient mice only (Bonferroni post-test; ***$p<0.001$; **Figure 5.3, D**).

5.3.4. The effect of age, Aβ and absence of SIGIRR on markers of glial activation in cortical and hippocampal tissue

Neither age nor Aβ significantly affected CD40 mRNA in cortical (**Figure 5.4, A**) or hippocampal (**Figure 5.4, B**) tissue from wildtype or SIGIRR-deficient mice. Both age (*$p<0.05$; ANOVA; **Figure 5.5**) and genotype (**$p<0.01$; ANOVA; **Figure 5.5**) had a significant effect on CD68 protein in hippocampal tissue from wildtype and SIGIRR-deficient mice. There was a significant interaction between age and genotype (**$p<0.01$; ANOVA; **Figure 5.5**).

5.3.5. The effect of age, Aβ and absence of SIGIRR on NF-κB activation in cortical and hippocampal tissue

Neither age nor Aβ significantly affected IKK phosphorylation in cortical (**Figure 5.6, A**) or hippocampal (**Figure 5.6, B**) tissue from wildtype and SIGIRR-deficient mice, however an overall effect of genotype on IκBα phosphorylation in hippocampal tissue was observed (*$p<0.05$; ANOVA; **Figure 5.1, D**).

Table 5.2. Results summary

Summary of *in vitro* experiments

Key: R=mRNA; P=protein; ↑=increase; ↓=decrease; - = no change; NA=not analysed; NML=no measureable levels

	Parameter	Aβ[1]	αTLR-2[2]
Mixed Glia	IL-6	R NA; P ↑	R NA; P ↓
	TNF-α	R NA; P ↑	R NA; P ↓

[1]Relative to control treated WT; [2]Relative to Aβ treated

Summary of *in vivo* experiments

Parameter	Effect of age, Aβ and SIGIRR deficiency
IL-6	No significant effect in cortical or hippocampal tissue
TLR-2	Significantly greater expression in cortical tissue from control treated young SIGIRR$^{-/-}$ mice compared with WT
RAGE	Significantly greater expression in cortical tissue from control treated young SIGIRR$^{-/-}$ mice compared with WT; further increase with age
CD40	No significant effect in cortical or hippocampal tissue
CD68	Significantly greater protein expression in hippocampal tissue from aged SIGIRR$^{-/-}$ mice compared with aged WT
pIKK	No significant effect in cortical or hippocampal tissue
pIκBα	Significantly greater expression in hippocampal tissue from SIGIRR$^{-/-}$ mice compared with WT

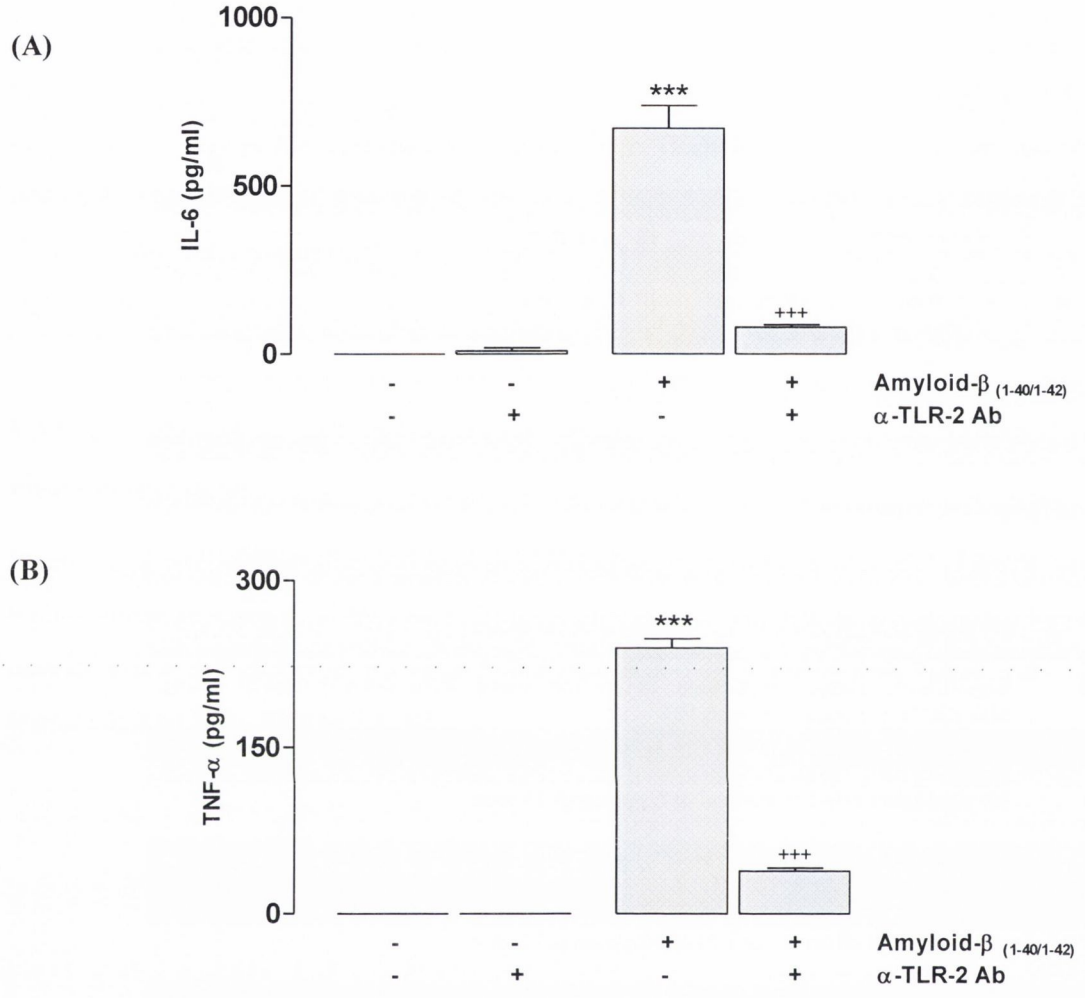


Figure 5.1. Blockade of TLR2 attenuated the Aβ-induced increase in cytokine release from mixed glia

Primary mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with Aβ ($A\beta_{1\text{-}40}$ (4.2μM) and $A\beta_{1\text{-}42}$ (5.6μM); 24h). Aβ induced the release of **(A)** IL-6 and **(B)** TNF-α from mixed glia prepared from wildtype mice (***$p<0.001$; ANOVA). Pre-treatment with αTLR2 antibody (5μg/ml; 2h) significantly attenuated the Aβ-induced increase in cytokine release ($^{+++}p<0.001$; ANOVA). Values are presented as means (± SEM; n=2).

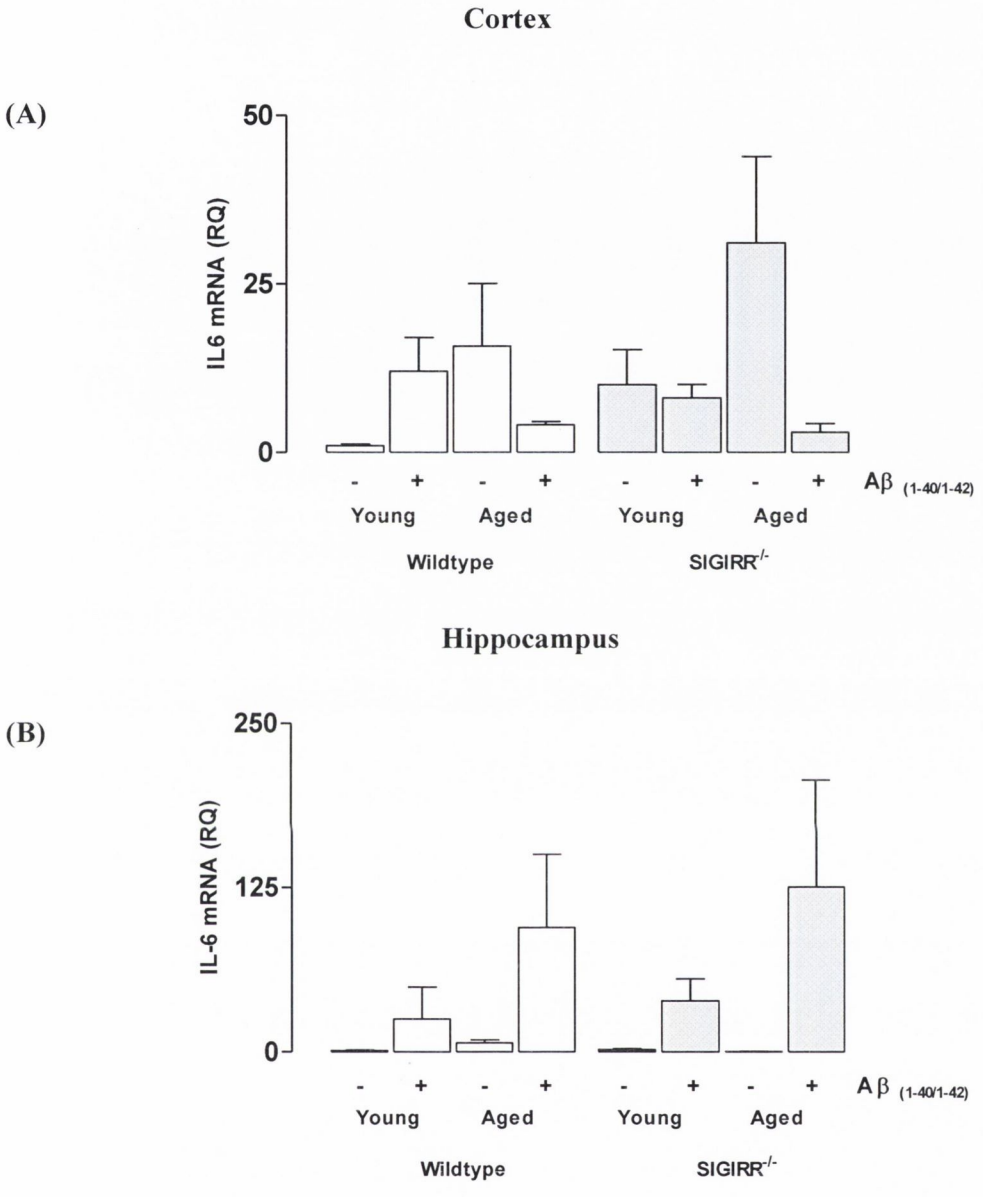


Figure 5.2. There was no effect of age, Aβ or SIGIRR deficiency on mRNA expression of IL-6 in cortical and hippocampal tissue from wildtype and SIGIRR$^{-/-}$ mice.

Wildtype and SIGIRR$^{-/-}$ mice, aged 3months or 12-14 months, were injected intrahippocampally with saline (5µl; 4h) or Aβ (5µl; 2.75µl $A\beta_{1\text{-}40}$ and 2.25µl $A\beta_{1\text{-}42}$; 4h). There was no significant effect of age or Aβ injection on mRNA expression of IL-6 in **(A)** cortical or **(B)** hippocampal tissue. There was no difference in mRNA expression of IL-6 between tissue from wildtype and SIGIRR$^{-/-}$ mice. Values are presented as means (± SEM; n=6) and expressed as a ratio to β-actin mRNA and standardised to a control sample.

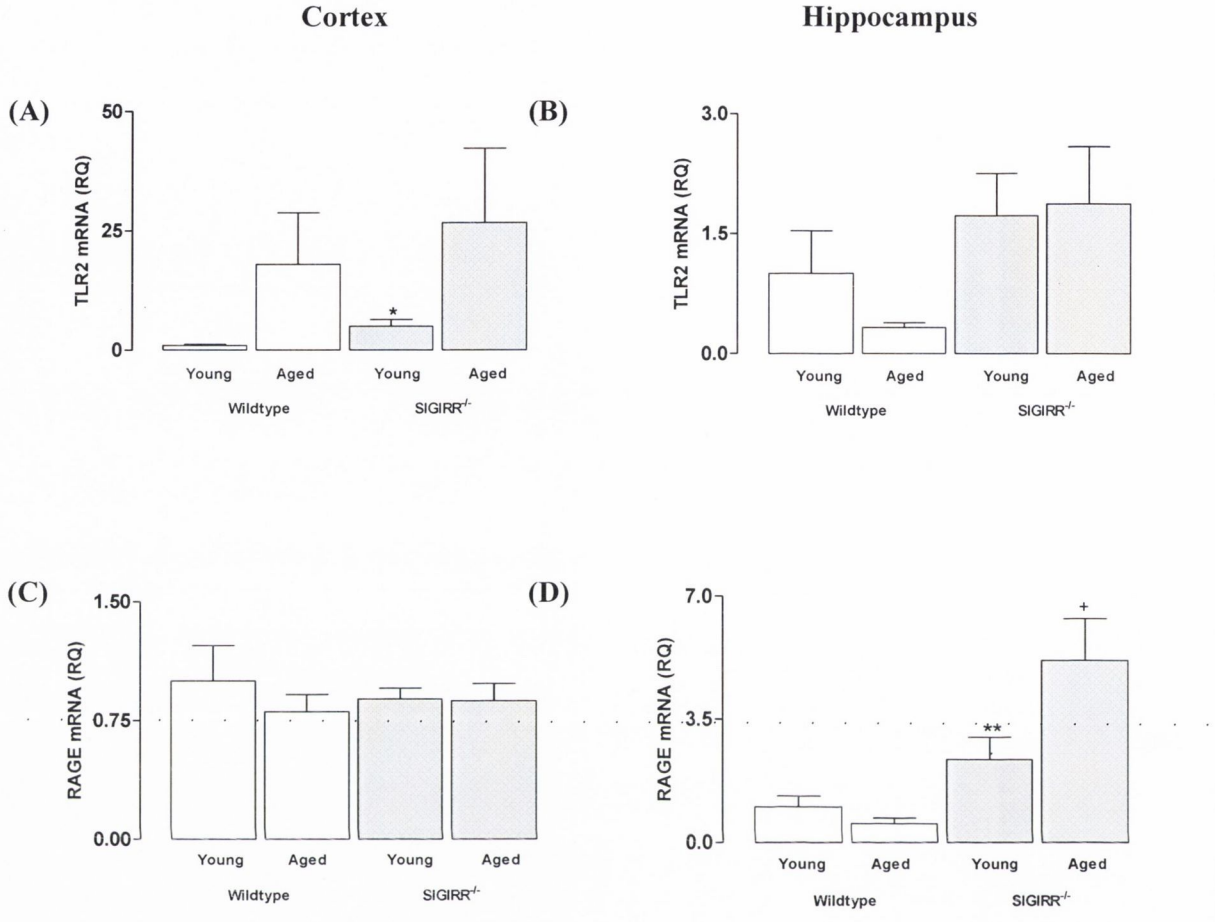


Figure 5.3. mRNA expression of receptors for Aβ was increased in cortical and hippocampal tissue prepared from SIGIRR$^{-/-}$ mice.

Wildtype and SIGIRR$^{-/-}$ mice, aged 3months or 12-14 months, were injected intrahippocampally with saline (5µl; 4h) or Aβ (5µl; 2.75µl $A\beta_{1\text{-}40}$ and 2.25µl $A\beta_{1\text{-}42}$; 4h). There was an increase in the expression of **(A)** TLR2 mRNA in cortical tissue prepared from 3 month old SIGIRR$^{-/-}$ mice compared with tissue from wildtype mice (*$p<0.05$; ANOVA). There was an increase in the expression of **(D)** RAGE mRNA in cortical tissue prepared from 3 month old SIGIRR$^{-/-}$ mice compared with tissue from wildtype mice (**$p<0.01$; Student's t-test). There was a further increase in RAGE mRNA in hippocampal tissue prepared from 12-14 month old SIGIRR$^{-/-}$ mice compared with 3 month old SIGIRR$^{-/-}$ mice ($^{+}p<0.05$; ANOVA). Values are presented as means (± SEM; n=6) and expressed as a ratio to β-actin mRNA and standardised to a control sample.

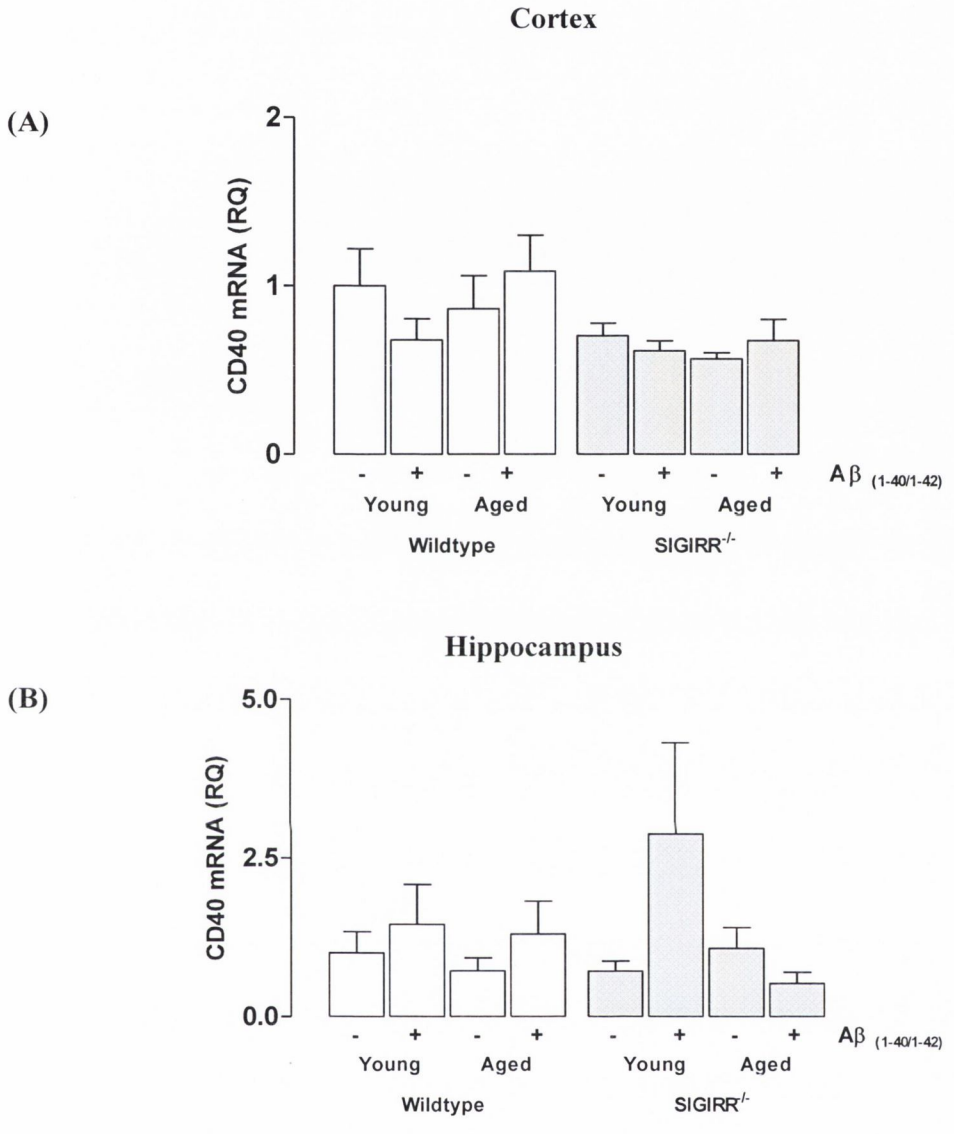


Figure 5.4. There was no effect of age, Aβ or SIGIRR deficiency on mRNA expression of CD40 in cortical and hippocampal tissue from wildtype and SIGIRR$^{-/-}$ mice.

Wildtype and SIGIRR$^{-/-}$ mice, aged 3months or 12-14 months, were injected intrahippocampally with saline (5µl; 4h) or Aβ (5µl; 2.75µl $A\beta_{1\text{-}40}$ and 2.25µl $A\beta_{1\text{-}42}$; 4h). There was no significant effect of age or Aβ injection on mRNA expression of CD40 in **(A)** cortical or **(B)** hippocampal tissue. There was no difference in mRNA expression of CD40 between tissue from wildtype and SIGIRR$^{-/-}$ mice. Values are presented as means (± SEM; n=6) and expressed as a ratio to β-actin mRNA and standardised to a control sample.

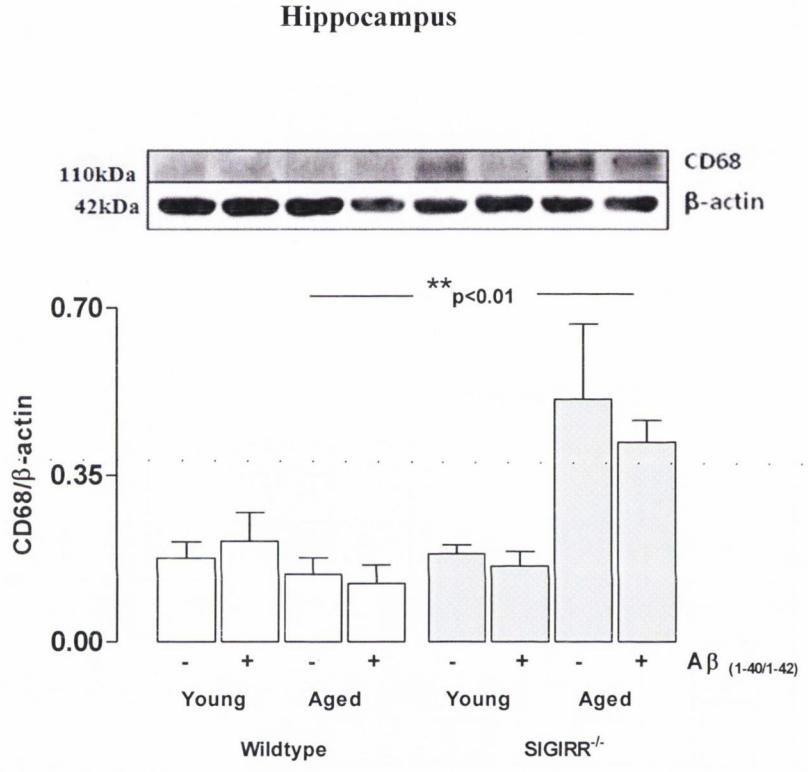


Figure 5.5. CD68 was increased in hippocampal tissue from aged SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice, aged 3months or 12-14 months, were injected intrahippocampally with saline (5µl; 4h) or Aβ (5µl; 2.75µl $A\beta_{1\text{-}40}$ and 2.25µl $A\beta_{1\text{-}42}$; 4h). There was a significant effect of age (*p<0.05; ANOVA) and SIGIRR deficiency (**p<0.01; ANOVA) on CD68 expression in hippocampal tissue prepared from wildtype and SIGIRR$^{-/-}$ mice. There was a significant interaction between age and SIGIRR deficiency (**p<0.01; ANOVA). Values are presented as means (± SEM; n=5) and expressed as arbitrary units.

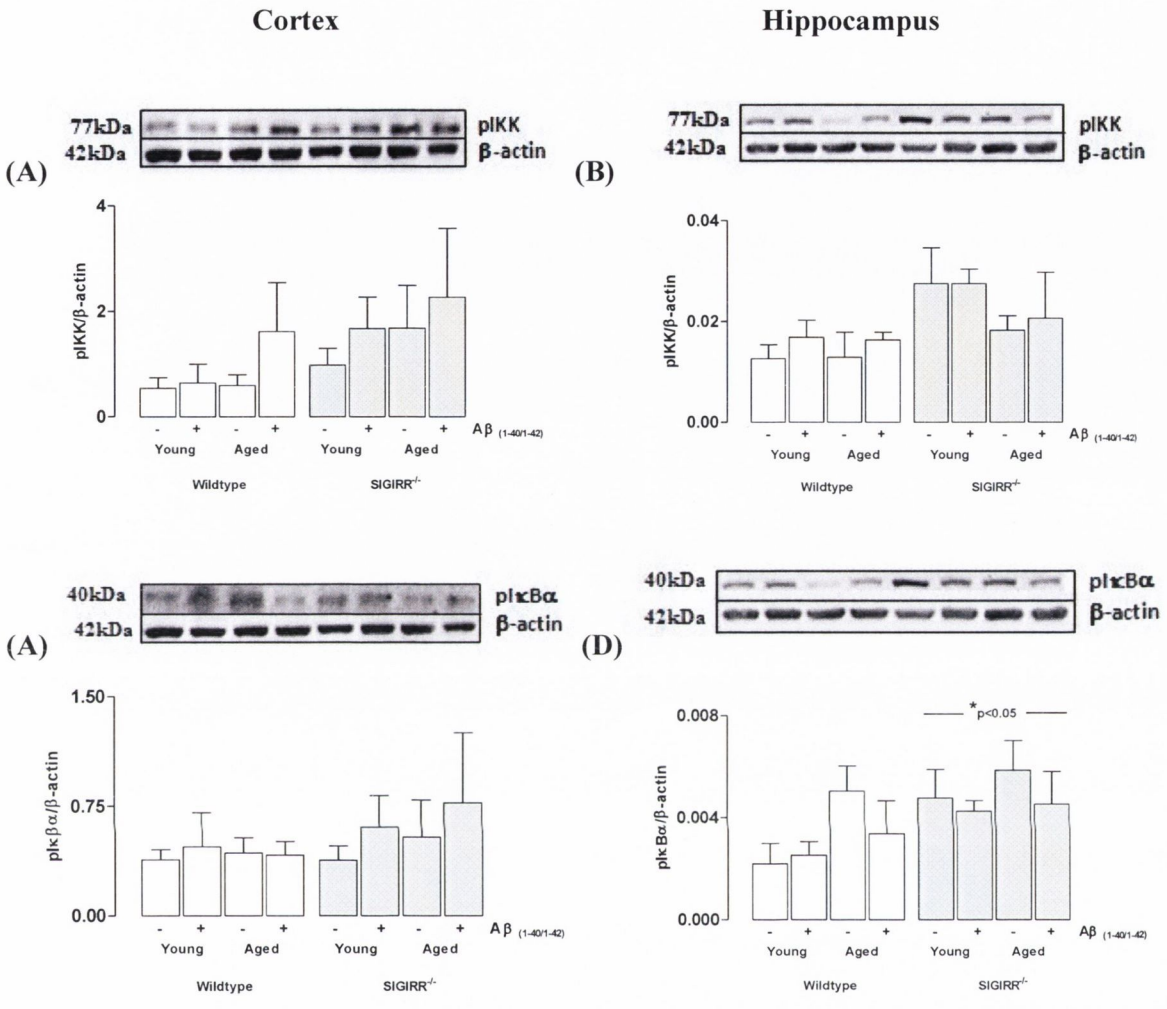


Figure 5.6. Phosphorylation of IκBα was increased in hippocampal tissue from SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice, aged 3months or 12-14 months, were injected intrahippocampally with saline (5μl; 4h) or Aβ (5μl; 2.75μl $Aβ_{1-40}$ and 2.25μl $Aβ_{1-42}$; 4h). There was a significant effect of SIGIRR deficiency on **(D)** pIκBα in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice (*$p<0.05$; ANOVA). There was no significant effect of age, Aβ or SIGIRR deficiency on the phosphorylation of IKK in cortical or hippocampal tissue. Values are presented as means (± SEM; n=6) and expressed as arbitrary units.

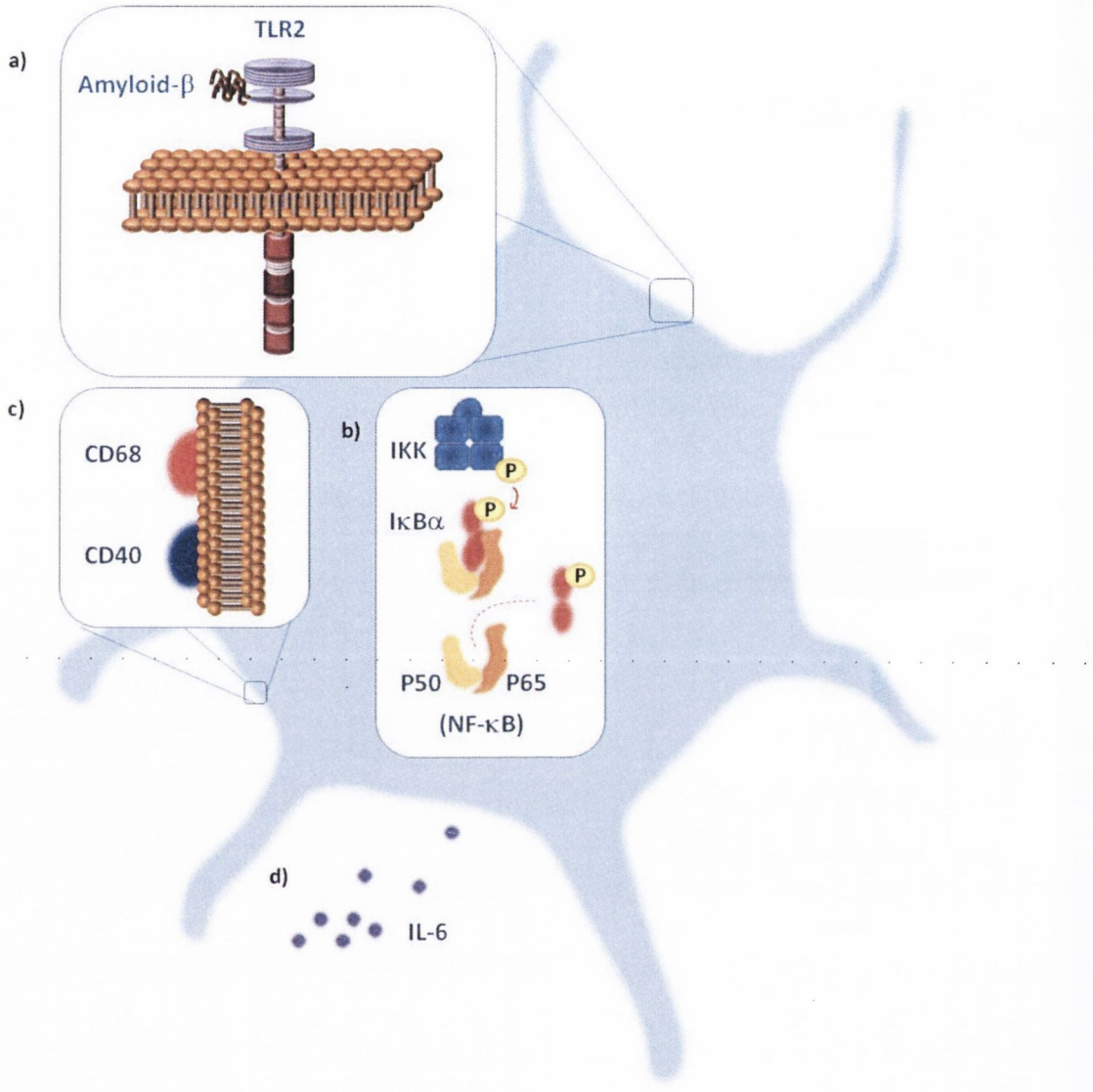


Figure 5.7. Unifying mechanism

Aβ binds to **(A)** TLR2 which activates an intracellular signalling cascade resulting in the activation of **(B)** NF-κB. Activated NF-κB promotes the transcription of markers of microglial activation **(C)** and pro-inflammatory cytokines **(D)**. It was predicted that this process would be exaggerated in mouse models of age and SIGIRR-deficiency.

5.4. Discussion

5.4.1. Overview

These experiments were conducted to investigate the effects of an intrahippocampal injection of Aβ in middle-aged mice and SIGIRR-deficient mice. The role of TLR2 as a receptor for Aβ was also examined, with reference to data acquired in previous experiments indicating a role for SIGIRR in the modulation of TLR2 expression and signalling. The overall aim of the chapter was to establish whether SIGIRR could act as a modulator of Aβ-induced pro-inflammatory signalling *in vivo*, possibly through control over the expression of TLR2.

5.4.2. The effect of blocking TLR-2 on Aβ-induced cytokine production in mixed glia

The first experiment was undertaken in an attempt to corroborate evidence from the literature that TLR2 acts as a receptor for the toxic peptide Aβ and propagates pro-inflammatory signals induced by Aβ (Tahara et al., 2006, Vollmar et al., 2010, Liu et al., 2012). There was a significant increase in the release of the pro-inflammatory cytokines IL-6 and TNF-α from primary cultured mixed glia incubated in the presence of Aβ. This effect has been shown in previous experiments described in chapter 4 and in a number of other studies (Lyons et al., 2007b, Jana et al., 2008, Jiao et al., 2008). In order to link the Aβ-induced increase in pro-inflammatory cytokine release to TLR2 activation, cells were pre-treated with an anti-TLR2 blocking antibody. This had the effect of significantly attenuating Aβ-induced IL-6 and TNF-α release. Jana and colleagues (2008) had provided evidence of TLR2-dependent Aβ signalling in glial cells in a number of experiments in which pre-treatment of mouse primary microglia with an anti-TLR2 neutralising antibody attenuated the Aβ-induced increase in TNF-α, iNOS and CD11b mRNA expression. It was also shown that anti-sense knockdown of TLR2 in microglia attenuated the Aβ-induced increase in IL-6 and TNF-α mRNA (Jana et al., 2008). An Aβ binding assay showed that incubation of THP-1 monocytes with an anti-TLR2 antibody

significantly reduced binding of Aβ to the cells (Reed-Geaghan et al., 2009). Similarly it was shown that microglia prepared from TLR2-deficient mice exhibited decreased production of ROS and reduced activation of p38 MAPK in response to Aβ (Reed-Geaghan et al., 2009). Taken together these data strongly suggest that preventing Aβ from interacting with TLR2 significantly decreases the pro-inflammatory effect of the peptide and suggest that TLR2 is a principal receptor for Aβ.

5.4.3. The effect of aging on glial function

A large number of studies have described the chronically elevated levels of pro-inflammatory signalling associated with the aging brain (Lynch and Johnson, 2012). It has been proposed that the effects of infection and noxious insults are more harmful in the immunosenescent brain and this has been linked to the progressive dysregulation of microglial function with age (Lynch et al., 2010, Lyons et al., 2011). Glial cells in the aged brain are typically hyper-responsive, and pro-inflammatory signalling may persist to the detriment of surrounding tissue. This feature of aging is particularly devastating in the case of neurodegenerative disorders, such as AD, in which toxic Aβ peptides act as persistent inflammatory stimuli to an already hyper-responsive innate immune system (McGeer and McGeer, 2002). Addressing the dysregulation of microglial activation therefore presents one of the most promising tools for the treatment of age-related neurodegenerative disorders.

5.4.4. Hypothesis of *in vivo* study

This study investigated the effects on the brain of exposing middle-aged and SIGIRR-deficient mice to an acute injection of Aβ. It was hypothesised that Aβ would induce an inflammatory response in the brain, characterised by an increase in pro-inflammatory cytokine and receptor expression, increased markers of microglial activation, and increased activation of intracellular pro-inflammatory signalling proteins. It was predicted that these effects would be exaggerated in middle-aged animals due to the more pro-inflammatory environment and heightened sensitivity

of glial cells typical of the aged brain. It was further hypothesised that animals deficient in SIGIRR would also exhibit an exaggerated inflammatory response due to the lack of a modulatory effect of SIGIRR on pro-inflammatory signalling. Furthermore it was predicted that the complementary effects of age and SIGIRR deficiency would yield the greatest inflammatory response to Aβ.

5.4.5. The effect of age, Aβ injection and SIGIRR deficiency on cytokine production in cortical and hippocampal tissue

The evidence most indicative of an inflammatory response includes the production of pro-inflammatory cytokines. Although there was an apparent increase in IL-6 mRNA expression in the cortex of middle-aged wildtype and SIGIRR-deficient mice (15-fold and 3-fold respectively) compared with young mice, these changes were not statistically significant due to a high degree of variability. Elevated levels of IL-6 have previously been reported in the middle-aged rodent brain and it is thought to contribute to the pro-inflammatory signalling and neuronal damage associated with age (Dugan et al., 2009, Panarsky et al., 2012). Variability also appears to account for the lack of statistical significance in the apparent 10-fold increase in IL-6 mRNA expression in the cortex of young SIGIRR-deficient mice compared with young wildtype mice, and the 2-fold increase in the cortex of middle-aged SIGIRR-deficient mice compared with middle-aged wildtype mice. Although there have been no reported differences in IL-6 expression in the brains of young SIGIRR-deficient mice compared to wildtype mice, an age related increase in IL-6 expression in SIGIRR-deficient mice has been shown previously (Watson et al., 2010). It is possible that increasing the number of animals in this study would have reduced the overall variation within groups. It may also be that the animals were not old enough to exhibit a significant increase in pro-inflammatory cytokines. Indeed in later experiments, SIGIRR-deficient mice aged 20-24 months exhibited robust increases in IL-6 mRNA and other pro-inflammatory cytokines (Carney, unpublished data). Intrahippocampal injection of Aβ induced an apparent 25-fold increase in IL-6 mRNA in hippocampal tissue from young wildtype mice and a further 4-fold increase in middle-aged mice, with marginally higher expression in tissue prepared from SIGIRR-deficient mice, but because of variation these changes did not prove

significant. However, overall the data are consistent with a previous study examining the effects of acute 4-hour intracerebroventricular injection of $A\beta_{1-42}$ in rats (Lyons et al., 2007). It has previously been reported that intracerebral injections with $A\beta_{1-40}$ induced an increase in IL-6 which peaked 24 hours post-injection (Rosales-Corral et al., 2004). Cortical injections of $A\beta_{25-35}$ have also been reported to induce IL-6 in rat hippocampus several days post-injection (Diaz et al., 2012). The differences in both Aβ species and experimental timeline should be noted in both studies described, and one possibility is that the difference in IL-6 measured may be the result of the chronic exposure to Aβ as opposed to the acute, 4-hour treatment presented in this study.

5.4.6. The effect of age, Aβ injection and SIGIRR deficiency on pro-inflammatory receptor expression in cortical and hippocampal tissue

The hypothesis that SIGIRR may exert a modulatory role on Aβ toxicity *in vivo* is based, in part, on *in vitro* evidence presented in chapter 4 of its ability to regulate the expression of, and signalling through, TLR2. As indicated here *in vitro* and in other studies *in vivo*, TLR2 acts as a receptor for Aβ (Richard et al., 2008, Vollmar et al., 2010). Here it was hypothesised that brain tissue from SIGIRR-deficient mice would have greater levels of TLR2 mRNA than wildtype mice, and that any exaggerated pro-inflammatory effects of Aβ in SIGIRR-deficient mice may be, in part, a result of this increase. It was found that TLR2 expression in cortical tissue from SIGIRR-deficient mice was increased compared with tissue from wildtype mice. This finding corroborates the *in vitro* results presented in chapter 4, provides further evidence that SIGIRR plays a role in regulating TLR2 expression and is consistent with previous findings indicating that pro-inflammatory receptors TLR4, CD14 and IL-1R1 were increased in hippocampal tissue from SIGIRR-deficient mice compared with wildtype mice (Watson et al., 2010, Costello et al., 2011b). Expression of another pro-inflammatory receptor, RAGE, was also greater in the hippocampus of SIGIRR-deficient mice and this was increased further with age. Although RAGE is not a member of the TLR family of proteins, it is a pattern recognition receptor capable of binding to similar ligands as TLR4 such as HMGB1, and can initiate pro-inflammatory responses via NF-κB (Bierhaus et al., 2001). Importantly, RAGE is also believed to play a role in

Aβ recognition (Yan et al., 1996). Although it is not known whether SIGIRR interacts with RAGE as it does with TLRs, the expression of RAGE is thought to be increased by activation of the receptor itself in a positive-feedback cycle (Li and Schmidt, 1997, van Beijnum et al., 2008). HMGB1 has been shown to be increased in the hippocampus of SIGIRR-deficient mice, and this may increase basal activation of the receptor and drive the increased expression of RAGE seen in SIGIRR-deficient mice (van Beijnum et al., 2008, Costello et al., 2011b). Together these data indicate that in the absence of SIGIRR there is an upregulation of a variety of pro-inflammatory receptors in the brain. It remains unclear whether SIGIRR directly modulates the expression of pro-inflammatory receptors and the associated signalling events, or whether the increase in receptor expression is a consequence of the more pro-inflammatory environment that exists as a result of the lack of modulation by SIGIRR on pro-inflammatory signalling.

5.4.7. The effect of age, Aβ injection and SIGIRR deficiency on markers of microglial activation in cortical and hippocampal tissue

With an increase in pro-inflammatory receptor expression, it might be predicted that there would be a concurrent increase in microglial activation in SIGIRR-deficient mice as a result of increased receptor-ligand interaction. Although Aβ induced a 2.5 fold increase in CD40 expression in hippocampal tissue from young SIGIRR-deficient mice, this did not reach statistical significance due to high variability. It has been previously reported that CD40 mRNA is increased in hippocampal tissue from SIGIRR-deficient mice in response to LPS challenge (Watson et al., 2010). However, no previous study had examined the effects of age on CD40. CD68 is also an indicator of microglial activation and here an increase in hippocampal tissue from middle-aged SIGIRR-deficient mice was observed. An increase in CD68 has been previously been reported in the hippocampus of wildtype mice and in other regions of the brain (Wong et al., 2005, Lynch et al., 2010). In this study, changes in CD68 were only detected in tissue from SIGIRR-deficient mice. This may be due to the age of the animals, as the increase in CD68 mRNA reported by Wong and colleagues occurred in the brains of 24 month-old mice compared with 12 month-old mice in the present study. The increased basal inflammatory state that exists in the brain of adult SIGIRR-deficient animals mimics the conditions seen in middle-

aged animals, and result in increased microglial activation compared with adult wildtype mice. Interestingly, Aβ failed to exacerbate the age-related increase in CD68. CD68 is thought to be indicative of lysosomal activity and phagocytosis, and these processes are also believed to be increased in senescent microglia (Wong et al., 2005, Lynch, 2009). It has been reported that microglia from aged rats exhibited increased uptake of quantum dots compared to microglia from young rats, indicating increased phagocytic activity (Lynch et al., 2010). It may be that increased phagocytic activity in the hippocampus of middle-aged, SIGIRR-deficient mice results in the breakdown of Aβ, attenuating any inflammatory effect it may have exerted on the microglia.

5.4.8. The effect of age, Aβ injection and SIGIRR deficiency on NF-κB signaling in cortical and hippocampal tissue

Any increase in microglial activation associated with Aβ, age, or SIGIRR-deficiency may be a result of increased pro-inflammatory signalling through cell surface receptors. It has been shown in this chapter that TLR2 and RAGE expression is increased in brain tissue from SIGIRR-deficient mice and that this is accompanied by an increase in CD68. Although a number of pro-inflammatory signalling pathways may have been involved in this process, the demonstrated ability of SIGIRR to modulate the NF-κB pathway suggests that it is the primary medium through which these changes are affected (Wald et al., 2003). Consistent with this, there was a significant increase in the phosphorylation of IκBα in the hippocampus of SIGIRR-deficient mice compared with wildtype mice. IκBα is a regulatory protein that maintains the p50 and p65 subunits of NF-κB in an inactive state. Phosphorylation of IκBα causes its dissociation from p50/p65 and results in the activation of NF-κB. Watson and colleagues (2010) previously demonstrated that basal IκBα phosphorylation was higher in the hippocampus of SIGIRR-deficient mice compared with wildtype mice. The increased phosphorylation of IκBα appears to be a general effect of SIGIRR-deficiency, but neither age nor Aβ exerted any additional effect. There was an apparent increase in the phosphorylation of IKK in the hippocampus of SIGIRR-deficient mice however this did not reach significance. Comparing the data presented here with

data reported by Costello and colleagues (2011) that indicate an increase in IRAK1 expression in the hippocampus of SIGIRR-deficient mice, it appears that SIGIRR deficiency induces an upregulation in NF-κB signalling in the hippocampus, which corroborates a number of *in vitro* studies which have described similar change (Wald et al., 2003; Li et al., 2003).

The data presented in this chapter provide further evidence that TLR2 acts as a ligand for Aβ and indicate that SIGIRR-deficiency is associated with an upregulation of the receptors, TLR2 and RAGE, with which Aβ interacts. These increases are accompanied by an increase in microglial activation, as evidenced by the exaggerated expression of CD68. It remains unclear whether SIGIRR-deficiency exacerbates pro-inflammatory changes in middle-aged animals. Robust age-related increases in microglial activation and cytokine release were observed in 20-24 month old SIGIRR-deficient mice compared with young mice (Carney, unpublished data). In contrast to the in vitro data presented in chapter 4, there was no significant effect of exogenously administered Aβ on pro-inflammatory markers in the brain highlighting the difficulty of extrapolating from an *in vitro* situation into an *in vivo* situation. In light of the evidence suggesting that the physiological role of SIGIRR is to act as a constitutive modulator of endogenous signalling, it may be more appropriate to assess the effect of chronic Aβ exposure, perhaps in a mouse in which APP is overexpressed.

Chapter 6: Analysis of the effects of IL-1F5, SIGIRR/Fc and TREM-1/Fc on stimulated glial cells

6.1. Introduction

SIGIRR exerts a negative regulatory effect on TLR/IL-1R signalling in an indirect ligand-dependant manner, interacting with intracellular TIR-domain containing proteins upon binding of TLR/IL-1R ligands to their specific receptors (Wald et al., 2003). However it remains unclear whether SIGIRR may be directly induced to exert its modulatory effects in the absence of, or complementary to, TLR/IL-1R ligand binding in order to enhance its anti-inflammatory effects. As yet there has been no identified ligand for SIGIRR, and it is currently classified as an orphan receptor. Among the potential candidates is the anti-inflammatory cytokine and member of the IL-1 family of proteins IL-1F5. IL-1F5 is structurally similar to IL-1Ra, a negative regulator of IL-1 signalling, and there is evidence to suggest that it has a similar negative regulatory role in IL-1 signalling (Dunn et al., 2003, Blumberg et al., 2007). IL-1F5 has been shown to attenuate IL-1β-induced IL-6 in mouse and rat cultured glial cells and rat hippocampus, possibly through the induction of IL-4. It was also revealed that that the ability of IL-F5 to attenuate LPS-induced IL-1β release from mouse glial cells was absent in glia prepared from SIGIRR$^{-/-}$ mice (Costelloe et al., 2008). These data suggested that IL-1F5 exerted its actions in a SIGIRR-dependent manner, and identified it as a potential ligand for the hitherto-orphan receptor.

The formation of functional homodimers is a feature common to many members of the TLR/IL-1R family, including TLR4, and is often necessary to induce signalling cascades (Toshchakov et al., 2011). Although it is not known if SIGIRR homodimerizes under normal physiological conditions, its structural similarities to other member of the family suggests that it may be a conserved feature of the TLR/IL-1 superfamily of proteins. It was therefore hypothesised that a recombinant mouse SIGIRR fusion protein (SIGIRR/Fc) may be capable of modulating glial cell activation, through the binding and activation of endogenous SIGIRR, or through some other mechanism.

TREM-1 is a member of the Ig family of proteins and is involved in the propagation of pro-inflammatory signalling in myeloid lineage cells. TREM-1 is believed to work synergistically with TLR4 and, in 2011, Huang and colleagues demonstrated that TREM-1 activation leads to the downregulation of SIGIRR in the corneas of mice infected with *Pseudomonas aeruginosa*.

Conversely, it was discovered that when a TREM-1 fusion protein (TREM-1/Fc) was used to block TREM-1 signalling in the infected tissue, SIGIRR expression was enhanced in comparison to untreated controls (Wu et al., 2011). Here it is hypothesised that glial cells treated with TREM-1/Fc will exhibit an attenuated response to LPS, and that this is linked to increased SIGIRR expression.

In the absence of a clear mechanism by which SIGIRR is activated, the most effective way to enhance its modulatory ability is to directly increase SIGIRR expression through gene transfection. This approach has been used in a number of studies to elucidate the mechanism of action of SIGIRR and to examine its role in the modulation of cytokine production from various peripheral cell types (Drexler et al., 2010, Khan et al., 2010). One of the aims of this study was to attempt to overexpress SIGIRR in a microglial cell line using molecular cloning techniques.

In this chapter, a series of experiments were performed to establish a mechanism of directly or indirectly activating or increasing the expression of SIGIRR. This chapter describes;

- An investigation of the anti-inflammatory cytokine IL-1F5 as a potential ligand for the direct activation of SIGIRR
- An investigation of SIGIRR/Fc as a modulator of TLR/IL-1R signalling
- An investigation of TREM-1/Fc as an indirect enhancer of SIGIRR expression
- An attempt at molecular cloning of SIGIRR in order to drive its over-expression

6.2. Methods

Primary cortical mixed glia, microglia and astrocytes were prepared from postnatal 1-day old wildtype and SIGIRR-deficient mice and cultured for 14 days before treatment (see section 2.1 for details). All agents used to treat cells were diluted to required concentrations in pre-warmed supplemented DMEM and all solutions were filter-sterilised through a 0.2μm cellulose acetate membrane filter.

LPS (Sigma, UK) was diluted to a final concentration of 10-100ng/ml in supplemented DMEM. Cells were treated for 24 h with LPS. IL-1β (R&D Systems; UK) was prepared as a stock solution in PBS and 0.1% BSA and diluted to the desired concentration of 10ng/ml in supplemented DMEM. Cells were treated for 24 h with IL-1β. SIGIRR/Fc (R&D systems, UK) was prepared as a stock solution in sterile PBS. Cells were pre-treated with SIGIRR/Fc (0.5-5μg/ml) for 2 h and co-incubated in the presence or absence of LPS (100 ng/ml; Enzo Life Sciences, UK), or IL-1β (10ng/ml; R&D Systems, UK) for a further 24 h. TREM-1/Fc (R&D systems, UK) was prepared as a stock solution in sterile PBS. Cells were pre-treated with TREM-1/Fc (2-10ng/ml) for 30 min and co-incubated in the presence or absence of LPS (10-100ng/ml; Enzo Life Sciences, UK) for a further 6, 12 or 24 h. mRNA expression of cytokines and cell surface proteins were assessed by RT-PCR and concentrations of cytokines and chemokines was determined by ELISA (see sections 2.3 and 2.4). Molecular cloning of human SIGIRR was undertaken as described in section 2.8 and figure 2.1.

Data are expressed as means ± SEM. Students t-test or ANOVA were performed to determine whether significant differences existed between treatment and genotype and Bonferroni post-hoc tests were performed where appropriate.

Table 6.1. Caveats

The concentration of IL-1F5 was chosen on the basis of previous published work which had established that this concentration was capable of attenuating LPS-induced cytokine release in glial cells from wildtype mice. There was no available data on the activity of SIGIRR/Fc and therefore a concentration curve experiment was performed. However the concentrations analysed should have been selected based on a logarithmic scale in order to properly determine the most effective range of SIGIRR/Fc. The concentration of TREM-1/Fc was chosen on the basis of activity levels reported by R&D Systems in a similar cell types.
All data was collated following 24 hours of treatment. There are differences in the latency of mRNA transcription and cytokine release between different cytokines and this may have affected the results shown here. A time course experiment would have been advisable to determine the optimum time to harvest samples for each cytokine measured.
The data in **Figure 6.10.** are representative of one experiment with each condition replicated three times and should be regarded as preliminary data which must be repeated in order to confirm the results
No analysis was undertaken to determine the level of endotoxin present in reagents used. The presence of endotoxin in these reagents may have affected the responses observed from glial cells

6.3. Results

6.3.1. Investigating the effect of IL-1F5 on IL-1β-treated glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice.

There was a significant effect of IL-1β treatment on IL-6 mRNA (***$p<0.001$; ANOVA; **Figure 6.2, A**) and release (***$p<0.001$; ANOVA; **Figure 6.2, B**) in mixed glia prepared from wildtype and SIGIRR-deficient mice, and a significant effect of genotype on IL-6 (**$p<0.01$; ANOVA; **Figure 6.2A**; ***$p<0.001$; ANOVA; **Figure 6.2, B**). The IL-1β-induced increase in IL-6 was exacerbated in mixed glia prepared from SIGIRR-deficient mice compared with wildtype mice; there was a significant interaction between treatment and genotype (**$p<0.01$; ANOVA; **Figure 6.2, A;** ***$p<0.001$; ANOVA; **Figure 6.2, B**). IL-1F5 significantly attenuated the IL-1β-induced increase in IL-6 release in cells prepared from wildtype mice, but that this effect was absent in cells prepared from SIGIRR-deficient mice ($^{+++}p<0.001$; ANOVA; **Figure 6.2, B**).

There was a significant effect of IL-1β treatment on TNF-α mRNA (**$p<0.01$; ANOVA; **Figure 6.2, C**) in mixed glia from wildtype and SIGIRR$^{-/-}$ mice as well as significant differences between genotype (**$p<0.01$; ANOVA; **Figure 6.2, C**). TNF-α mRNA was increased to a greater extent in cells prepared from SIGIRR-deficient mice as revealed by a significant interaction between IL-1β treatment and genotype (*$p<0.05$; ANOVA; **Figure 6.2, C**). IL-1F5 did not affect supernatant concentration of TNF-α in glia from either genotype (**Figure 6.2, D**).

IL-1β also affected chemokine expression in mixed glia prepared from wildtype and SIGIRR-deficient mice. There was a significant effect of IL-1β treatment on CXCL10 (***$p<0.001$; ANOVA; **Figure 6.2, E**) and CCL2 (***$p<0.001$; ANOVA; **Figure 6.2, F**) mRNA expression. Analysis of CCL2 mRNA also revealed a significant interaction between IL-1β treatment and genotype, with a significant increase in CCL2 mRNA in the cells prepared from SIGIRR-deficient mice compared to wildtype mice (***$p<0.001$; ANOVA; **Figure 6.2, F**). There was no effect of IL-1F5 on CXCL10 or CCL2 (**Figure 6.2, E; Figure 6.2, F).**

IL-1β is increased IL-1R1 mRNA expression in mixed glia prepared from wildtype and SIGIRR-deficient mice (*$p<0.05$; ANOVA; **Figure 6.3, A**). Although there was no significant difference in IL-1R1 mRNA between cells from the two genotypes when unstimulated, there was a significant interaction between IL-1β treatment and genotype (**$p<0.01$; ANOVA; **Figure 6.3, A**). IL-1F5 had no effect on cells prepared from wildtype mice, and failed to attenuate the IL-1β-induced increase in IL-1R1 in cells prepared from SIGIRR-deficient mice. Neither IL-1β nor IL-1F5 had any effect on SIGIRR expression in mixed glia prepared from wildtype mice (**Figure 6.3, B**).

6.3.2. Investigating the effect of SIGIRR/Fc on LPS and IL-1β-treated glial cells.

The evidence suggests that the modulatory effect of SIGIRR relies on its ability to associate with IL-1R1 and TLR4 inhibiting receptor-mediated signalling (Qin et al., 2005). Here the possibility that SIGIRR activation by a fusion protein (SIGIRR/Fc) might exert a modulatory effect on LPS-induced or IL-1R1-induced effects was assessed by incubating LPS-treated or IL-1β-treated cells, obtained from wildtype mice, in the presence and absence of SIGIRR/Fc.

LPS increased CD11b mRNA in mixed glia (***$p<0.001$; ANOVA; **Figure 6.4**). SIGIRR/Fc significantly attenuated this increase at a concentration of 5μg/ml only ($^{++}p<0.01$; ANOVA; **Figure 6.4**).

LPS significantly increased IL-6 mRNA (***$p<0.001$; ANOVA; **Figure 6.5, A**) and supernatant concentration (***$p<0.001$; ANOVA; **Figure 6.5, B**) from mixed glia Pre-treatment with SIGIRR/Fc at a concentration of 1μg/ml attenuated the LPS-induced increase in IL-6 concentration ($^{+}p<0.05$; ANOVA; **Figure 6.5, B, Inset**), however it had no significant effect on IL-6 mRNA. LPS significantly increased TNF-α mRNA (***$p<0.001$; ANOVA; **Figure 6.5, C**) and supernatant concentration (***$p<0.001$; ANOVA; **Figure 6.5, D**) of TNF-α from mixed glial cells. Although pre-treatment with SIGIRR/Fc had no effect on TNF-α mRNA expression, LPS-induced TNF-α release was significantly attenuated by SIGIRR/Fc at concentrations of 1μg/ml ($^{++}p<0.01$; ANOVA; **Figure 6.5, D**), 2.5 μg/ml ($^{+}p<0.05$; ANOVA; **Figure 6.5, D**) and 5

μg/ml ($^{+}p<0.05$; ANOVA; **Figure 6.5, D**). LPS significantly reduced SIGIRR mRNA expression in mixed glia ($^{**}p<0.01$; ANOVA; **Figure 6.6**). SIGIRR/Fc did not affect the LPS-induced change.

There was no significant effect of IL-1β or SIGIRR/Fc on mRNA expression of CD11b (**Figure 6.7, A**). IL-1β significantly increased CD40 ($^{**}p<0.01$; ANOVA, **Figure 6.7, B**) mRNA but SIGIRR/Fc exerted no modulatory effect on the IL-1β-induced change. IL-1β also induced a significant increase in IL-6 mRNA (***p<0.001; ANOVA; **Figure 6.8, A**) and supernatant concentration (***p<0.001; ANOVA; **Figure 6.8, B**). Pre-treatment with SIGIRR/Fc significantly attenuated the increase in IL-6 mRNA at concentrations of 0.5 ($^{++}p<0.01$; ANOVA; **Figure 6.8, A**; **Inset**) and 1μg/ml ($^{+}p<0.05$; ANOVA; **Figure 6.8, A**; **Inset**). The IL-1β-induced IL-6 release was significantly attenuated by SIGIRR/Fc at a concentration of 2.5μg/ml ($^{+}p<0.05$; ANOVA; **Figure 6.8, B**; **Inset**).

6.3.3. Investigating the effect of TREM-1/Fc on LPS-treated glial cells

LPS increased IL-6 (***p<0.001; ANOVA; **Figure 6.9, A**) and TNF-α (***p<0.001; ANOVA; **Figure 6.9, B**) release from mixed glia. The increase in TNF-α was attenuated following pre-treatment with TREM-1/Fc at a concentration of 6ng/ml ($^{++}p<0.01$; ANOVA; **Figure 6.9, B**). The data in **Figure 6.10** confirm that LPS induced a decrease in SIGIRR mRNA in mixed glia (*p<0.05; Student's t-test; **Figure 6.10**). Although TREM-1/Fc had no effect on basal levels of SIGIRR mRNA, it reversed the LPS-induced decrease at a concentration of 6ng/ml (*p<0.05; ANOVA; **Figure 6.10**).

LPS induced an increase in TNF-α release (***p<0.001; ANOVA; **Figure 6.11, A**) and a decrease in SIGIRR mRNA (***p<0.001; ANOVA; **Figure 6.11, B**) in isolated microglia. TREM-1/Fc, at a concentration of 6ng/ml, attenuated the LPS-induced increase in TNF-α ($^{+}p<0.05$; ANOVA; **Figure 6.11, A**) but had no effect on SIGIRR mRNA.

6.3.4. Molecular cloning of human SIGIRR gene into mammalian vector.

Figure 6.12, A, shows a simplified vector map of the commercial bacterial cloning vector pENTR221. The map illustrates the presence of a T7 promoter sequence, the region containing a resistance gene for the selection antibiotic kanamycin, and the multiple cloning site (MCS) containing the sequence for human SIGIRR. The full sequence of the human SIGIRR insert, with start and stop codons highlighted, is represented in **Figure 6.12, B.**

Figure 6.13, A, outlines the basic protocol for the excision, amplification and purification of the SIGIRR insert from the commercial cloning vector pENTR211. It also illustrates the preparation of the mammalian vector pEGFP_C2 through restriction digestion using the enzymes ECOR1 and BAMH1, dephosphorylation using calf-intestinal alkaline phosphatase (CIP) and purification. **Figure 6.13, B,** shows an agarose gel picture illustrating the various gel checks performed during the purification of the insert. **Figure 6.13, C,** shows an agarose gel picture illustrating the various gel checks performed during the purification of the vector.

Table 6.2. Results summary

Key: R=mRNA; P=protein; ↑=increase; ↓=decrease; - = no change; NA=not analysed; NML=no measureable levels

		Effect on WT		Effect on SIG-/-	
	Parameter	**IL-1β[1]**	**IL-1F5[2]**	**IL-1β[1]**	**IL-1F5[2]**
Mixed Glia	**IL-6**	R ↑; P ↑	R ↓; P↓	R ↑; P ↑	R -; P -
	TNF-α	R ↑; P NML	R -; P NML	R ↑; P NML	R -; P NML
	CCL2	R ↑; P NA	R -; P NA	R ↑; P NA	R -; P NA
	CXCL10	R ↑; P NA	R -; P NA	R ↑; P NA	R -; P NA
	IL-1R1	R -; P NA	R -; P NA	R ↑; P NA	R -; P NA
	SIGIRR	R -; P -	R -; P -	NA	NA

[1]Relative to control-treated cells; [2]relative to IL-1β-treated cells

	Parameter	**LPS[1]**	**SIGIRR/Fc[2]**	**IL-1β[1]**	**SIGIRR/Fc[3]**
Mixed glia	**CD11b**	R ↑; P NA	R ↓; P NA	R ↑; P NA	R -; P NA
	CD40	NML	NML	R ↑; P NA	R -; P NA
	IL-6	R ↑; P ↑	R -; P↓	R ↑; P ↑	R ↓; P↓
	TNF-α	R ↑; P ↑	R -; P↓	NML	NML
	SIGIRR	R ↓; P NA	R -; P NA	NA	NA

[1]Relative to control treated; [2]relative to LPS-treated; [3]relative to IL-1β-treated

	Parameter	**LPS[1]**	**TREM-1/Fc[2]**
Mixed Glia	**IL-6**	R NA; P↑	R NA; P-
	TNF-α	R NA; P↑	R NA; P↓
	SIGIRR	R ↓; P NA	R ↑; P NA
Microglia	**IL-6**	R NA; P↑	R NA; P-
	TNF-α	R NA; P↑	R NA; P↓
	SIGIRR	R ↓; P NA	R -; P NA

[1]Relative to control treated; [2]relative to LPS treated

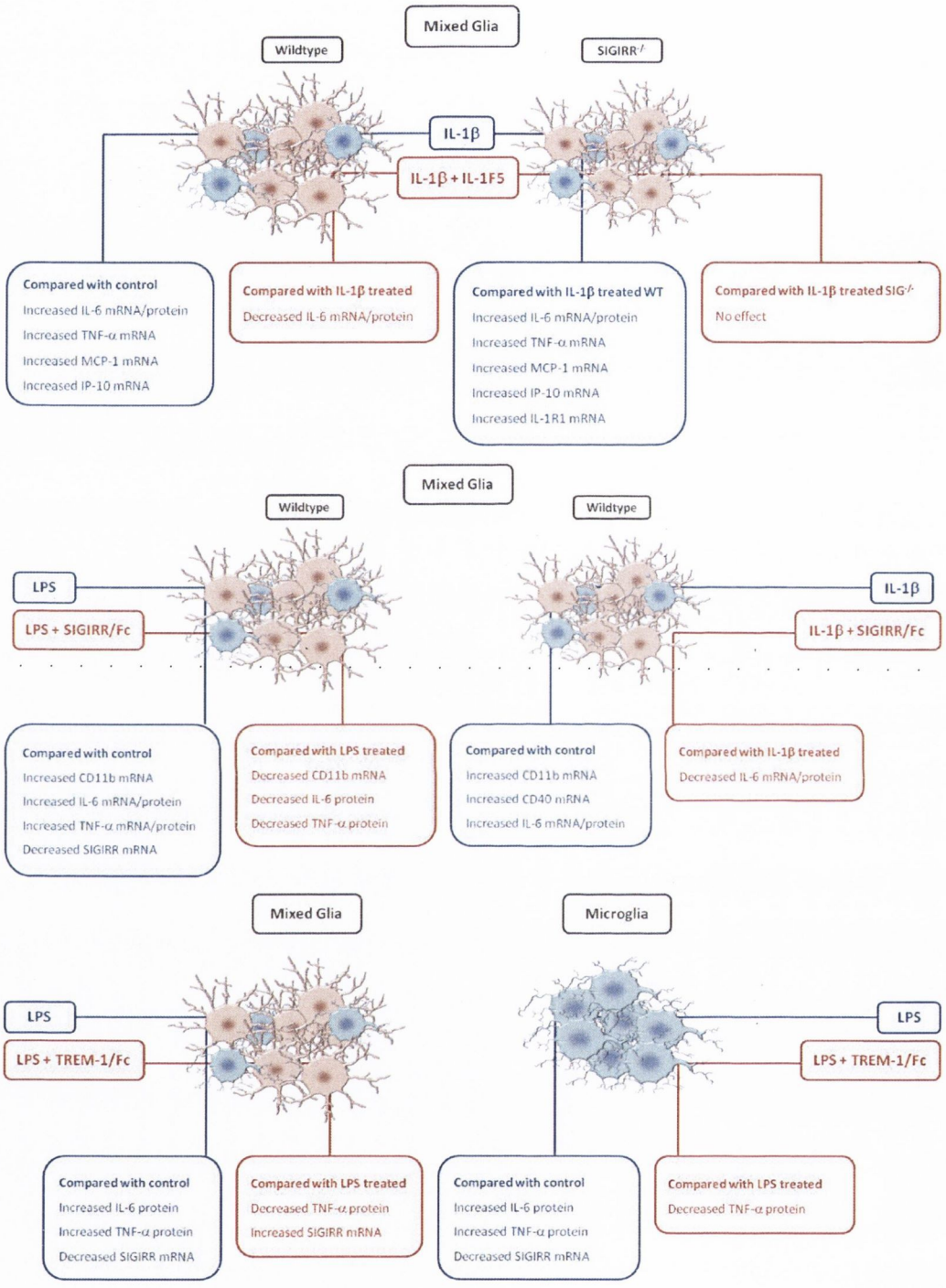


Figure 6.1. Results summary

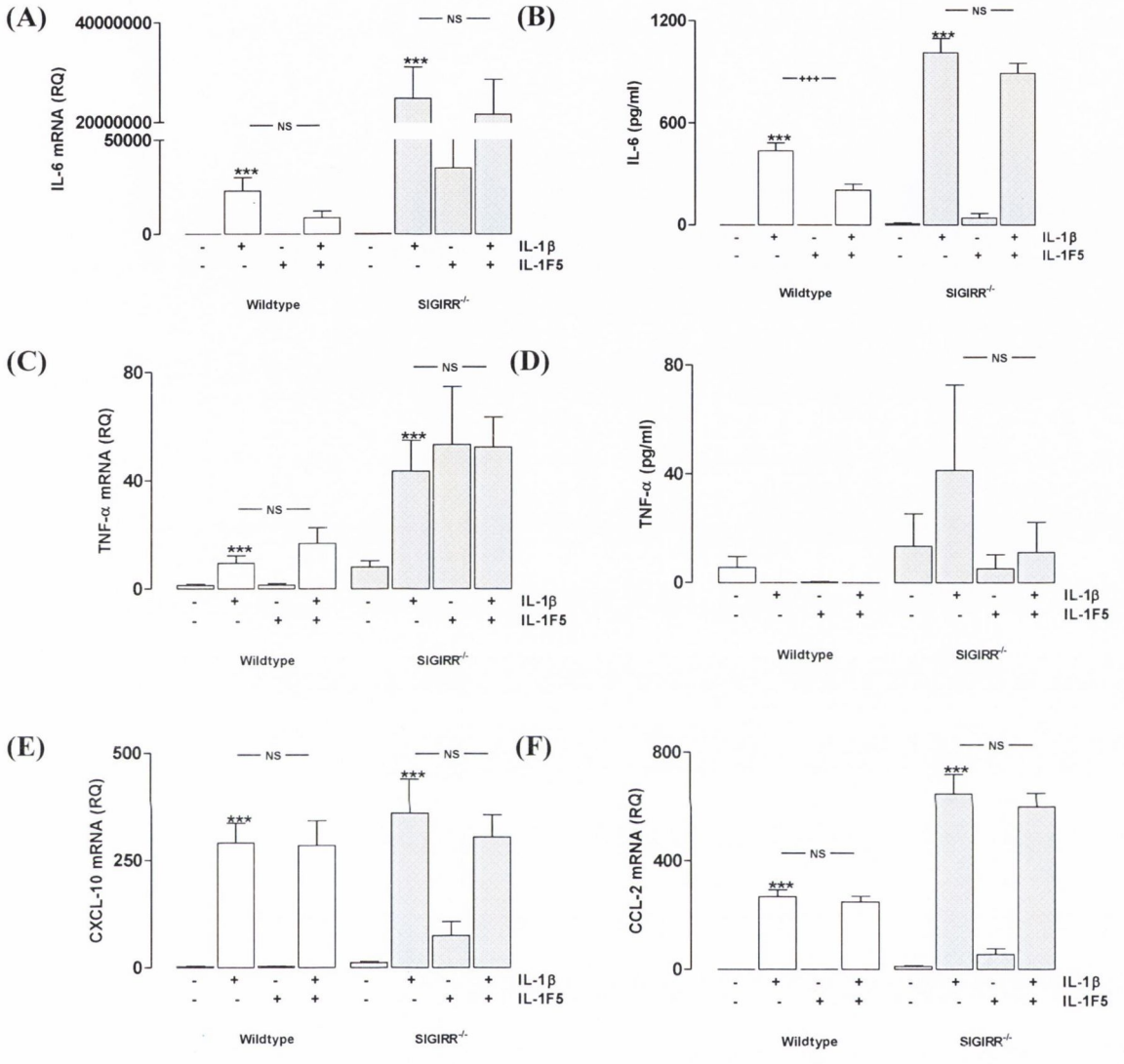


Figure 6.2. IL-1F5 attenuated IL-6 release in mixed glia prepared from wildtype mice but not SIGIRR$^{-/-}$ mice

Primary mouse mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with IL-1β (10ng/ml; 24h). There was a significant effect of IL-1β on mRNA expression of **(A)** IL-6, **(C)** TNF-α, **(E)** CXCL10 and **(F)** CCL2 and on the release of **(B)** IL-6 in mixed glia prepared from wildtype and SIGIRR$^{-/-}$ mice (***$p<0.001$; ANOVA). Pre-treatment with IL-1F5 (3μg/ml; 2h) significantly attenuated the IL-1β-induced increase in **(B)** release of IL-6 in mixed glia from wildtype mice but not SIGIRR$^{-/-}$ mice ($^{+++}p<0.001$; ANOVA). Values are presented as means (± SEM). Data for mRNA is expressed as a ratio to β-actin mRNA and standardised a control sample. The data are representative of 3 separate experiments with each condition tested in duplicate.

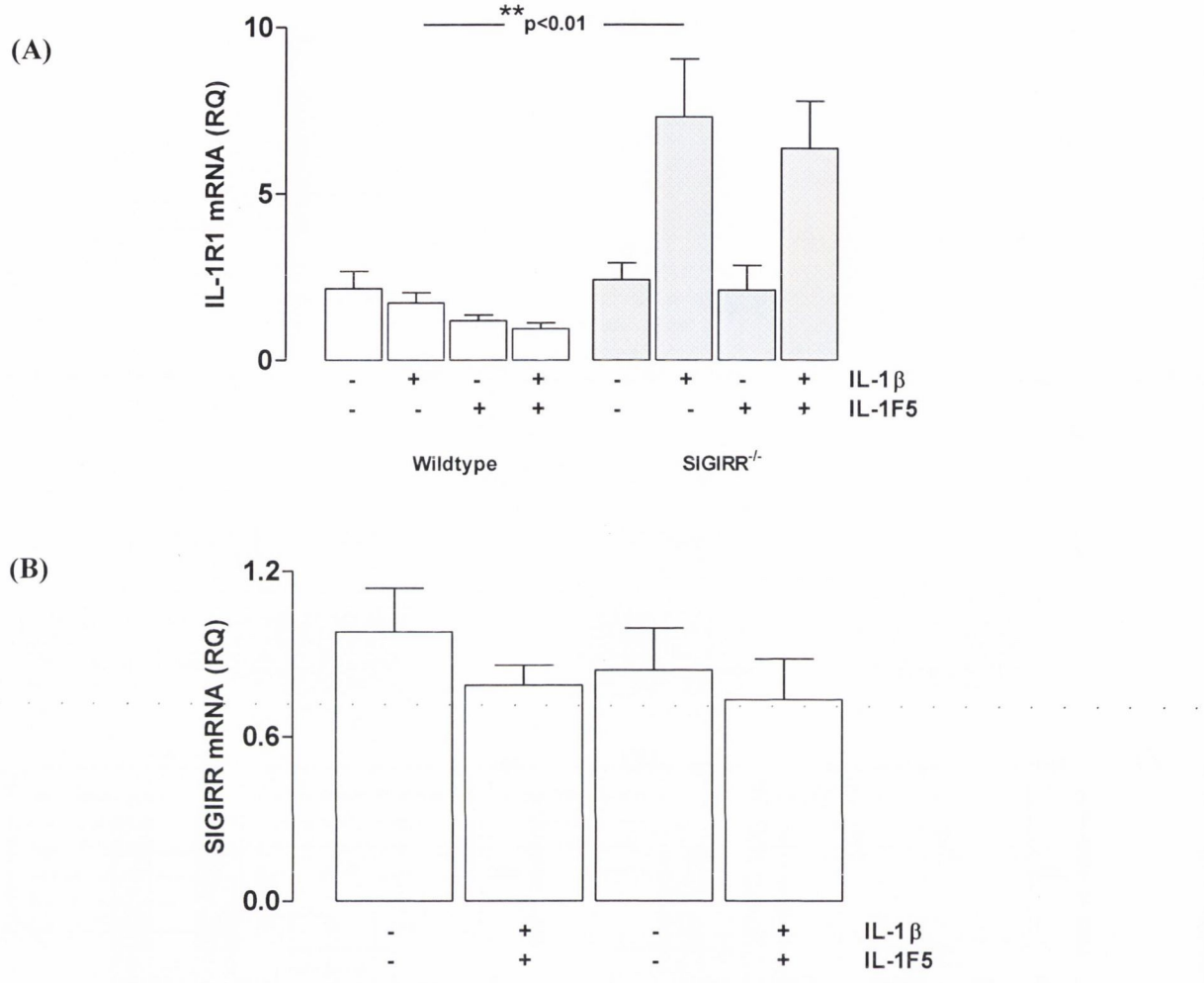


Figure 6.3. IL-1F5 had no effect on mRNA expression of IL-1R1 or SIGIRR in mixed glia from wildtype and SIGIRR$^{-/-}$ mice

Primary mouse mixed glia were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with IL-1β (10ng/ml; 24h). There was a significant effect of IL-1β on mRNA expression of **(A)** IL-1R1 in mixed glia prepared from SIGIRR$^{-/-}$ mice (**p<0.01; ANOVA). Pre-treatment with IL-1F5 (3μg/ml; 2h) had no effect on mRNA expression of IL-1R1 or **(B)** SIGIRR. Values are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 3 separate experiments with each condition tested in duplicate.

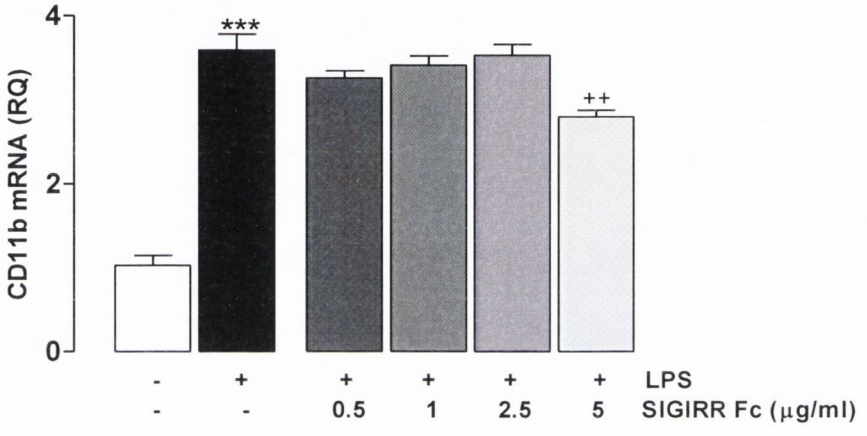


Figure 6.4. SIGIRR/Fc attenuated the LPS-induced increase in mRNA expression of CD11b from mixed glia

Primary mouse mixed glia were prepared from wildtype mice and treated with LPS (100ng/ml; 24h). LPS induced a significant increase in mRNA expression of CD11b in mixed glia (***$p<0.001$; ANOVA). Pre-treatment with SIGIRR/Fc (5μg/ml; 2h) attenuated the LPS-induced increase in mRNA expression of CD11b ($^{++}p<0.01$; ANOVA). Values are presented as presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate.

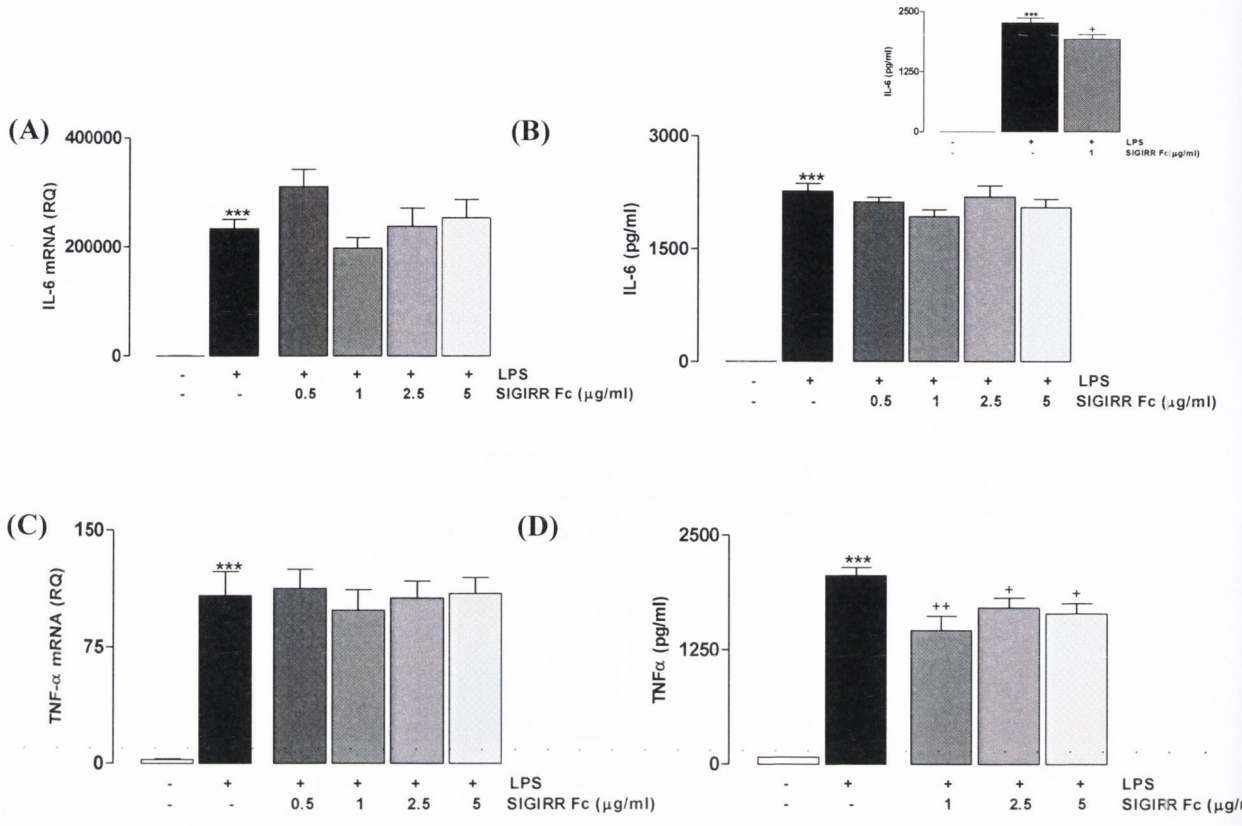


Figure 6.5. SIGIRR/Fc attenuates the LPS-induced increase in cytokine release from mixed glia

Primary mouse mixed glia were prepared from wildtype mice and treated with LPS (100ng/ml; 24h). LPS induced an increase in the mRNA expression of **(A)** IL-6 and **(C)** TNF-α and in the release of **(B)** IL-6 and **(D)** TNF-α from mixed glia (***$p<0.001$; ANOVA). Pre treatment with SIGIRR/Fc (1µg/ml; 2h) attenuated the LPS-induced increase in **(B; Inset)** IL-6 release ($^{+}p<0.05$; ANOVA). Pre treatment with SIGIRR/Fc (1, 2.5, 5µg/ml; 2h) attenuated the LPS-induced increase in **(D)** TNF-α release ($^{++}p<0.01$; ANOVA). Values are presented as presented as means (± SEM; n=2). Data for mRNA is expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate.

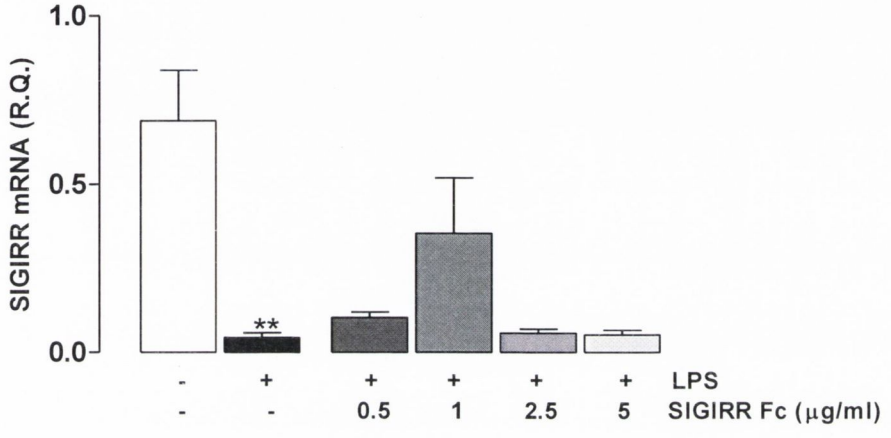


Figure 6.6. SIGIRR/Fc had no effect on the mRNA expression of SIGIRR in mixed glia.

Primary mouse mixed glia were prepared from wildtype mice and treated with LPS (100ng/ml; 24h). LPS induced a significant decrease in the mRNA expression of SIGIRR in mixed glia ($^{**}p<0.01$; ANOVA). Pre-treatment with SIGIRR/Fc failed to reverse this decrease at any concentration. Values for RT-PCR are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate.

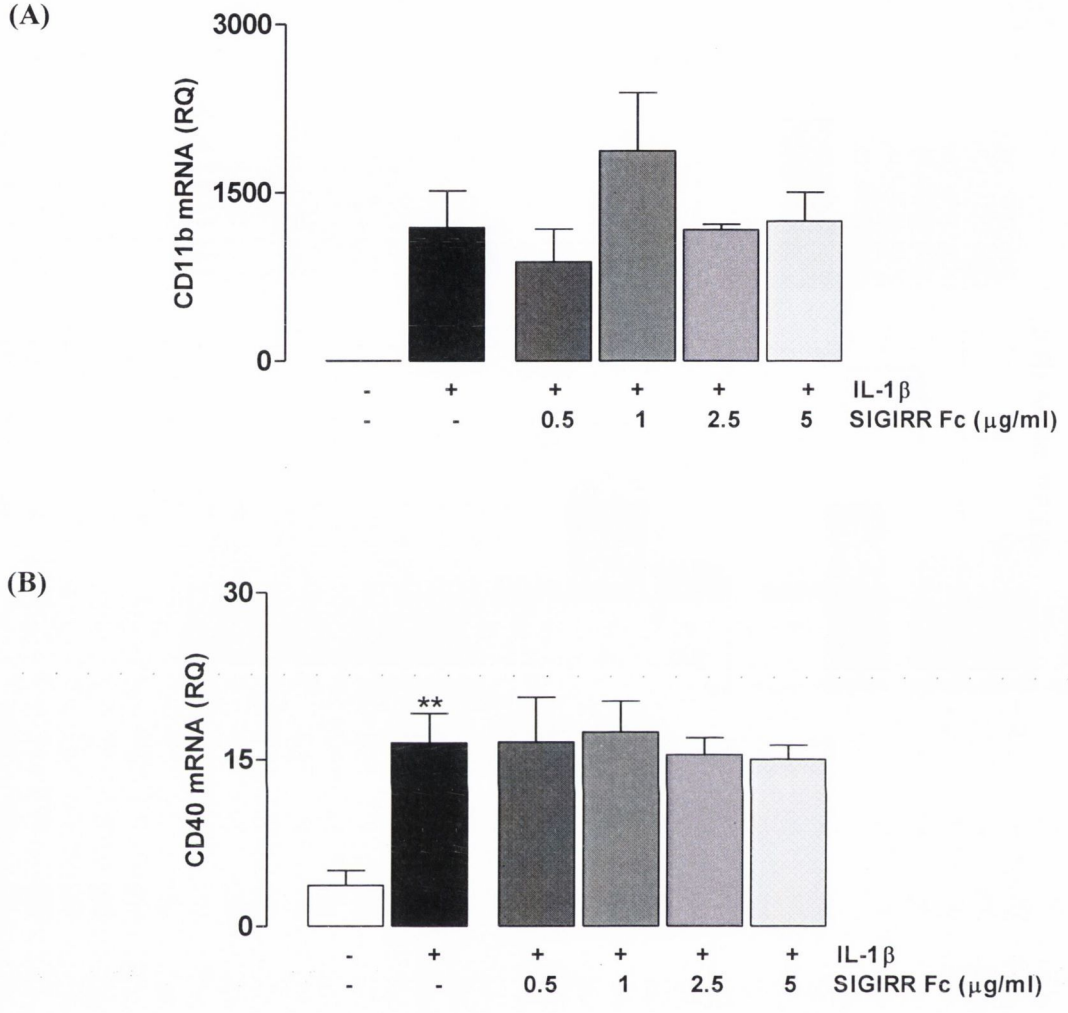


Figure 6.7. SIGIRR/Fc failed to attenuate the IL-1β-induced increase in mRNA expression of CD40 from mixed glia.

Primary mouse mixed glia were prepared from wildtype mice and treated with IL-1β (10ng/ml; 24h). There was no significant effect of IL-1β or IL-1F5 on mRNA expression of **(A)** CD11b. IL-1β induced an increase in mRNA expression of **(B)** CD40 in mixed glia ($^{**}p<0.01$; ANOVA). Pre-treatment with SIGIRR/Fc failed to attenuate this increase at any concentration. Values for RT-PCR are presented as means (± SEM; n=2) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate.

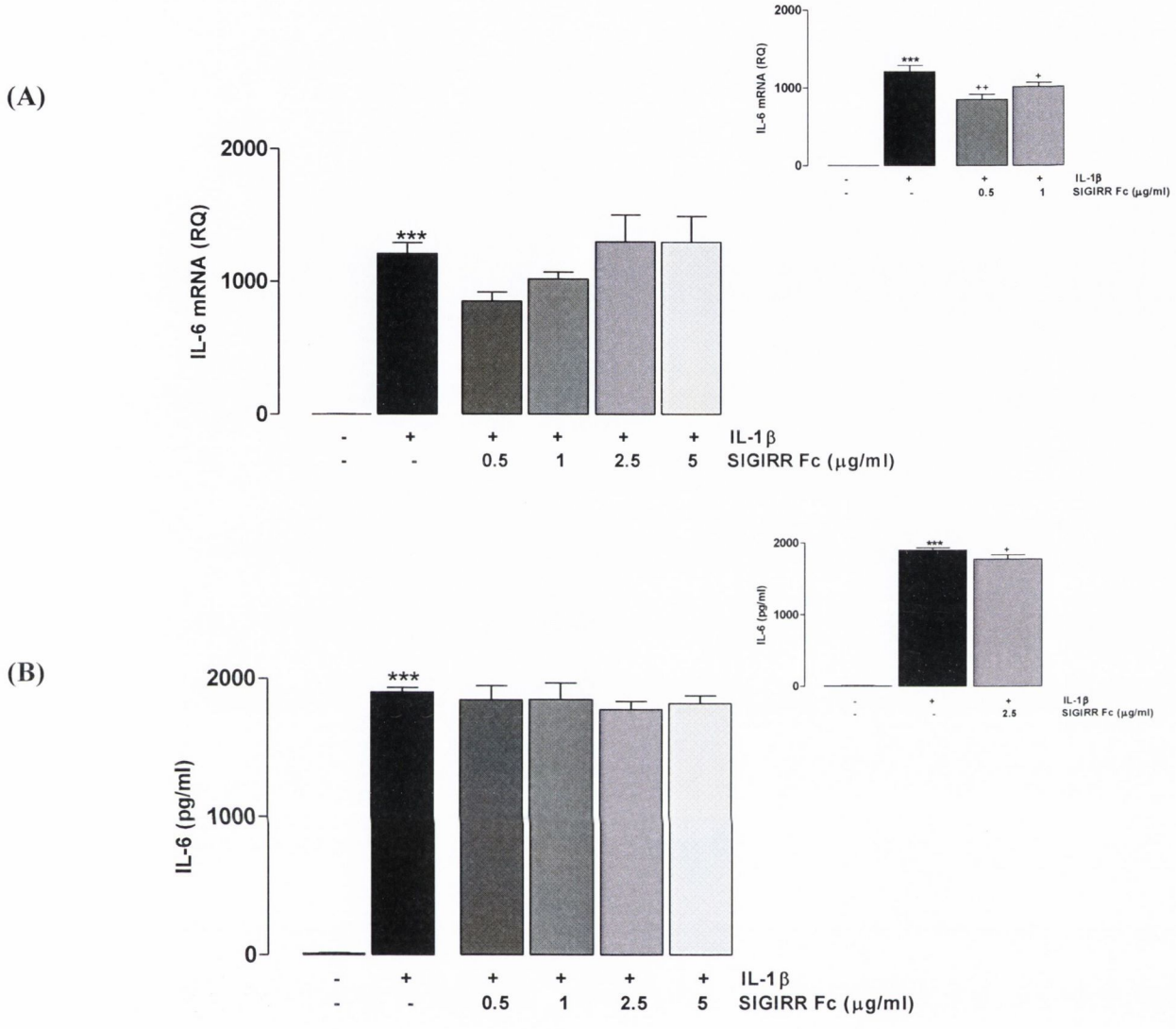


Figure 6.8. SIGIRR/Fc attenuated the IL-1β-induced increase in IL-6 from mixed glia

Primary mouse mixed glia were prepared from wildtype mice and treated with IL-1β (10ng/ml; 24h). IL-1β induced an increase in **(A)** mRNA expression of IL-6 and **(B)** release of IL-6 from mixed glia (***$p<0.001$; ANOVA). Pre-treatment with SIGIRR/Fc (0.5, 1μg/ml; 2.5μg/ml; 2h) attenuated the LPS-induced increase in **(A; Inset)** IL-6 mRNA expression and **(B; Inset)** IL-6 release ($^{+}p<0.05$; $^{++}p<0.01$; ANOVA). Values are presented as means (± SEM; n=2). Data for mRNA are expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate.

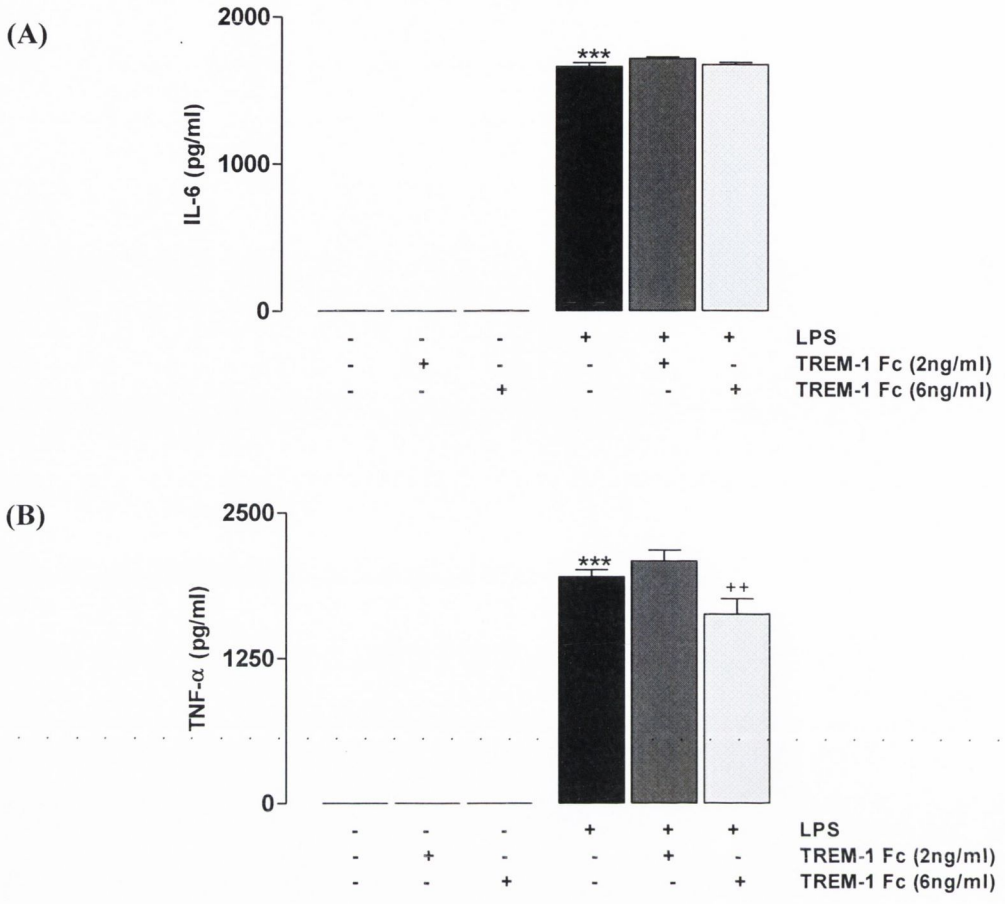


Figure 6.9. TREM-1/Fc attenuated the LPS-induced increase in TNF-α from mixed glia

Primary mouse mixed glia were prepared from wildtype mice and treated with LPS (100ng/ml; 24h). LPS induced an increase in release of **(A)** IL-6 and **(B)** TNF-α from mixed glia (***$p<0.001$; ANOVA). Pre-treatment with TREM-1/Fc had no effect on release of **(A)** IL-6. Pre-treatment with TREM-1/Fc (6ng/ml; 30min) attenuated the LPS-induced increase in release of **(B)** TNF-α ($^{++}p<0.01$; ANOVA). Values are presented as presented as means (± SEM). The data are representative of 2 separate experiments with each condition tested in duplicate or triplicate.

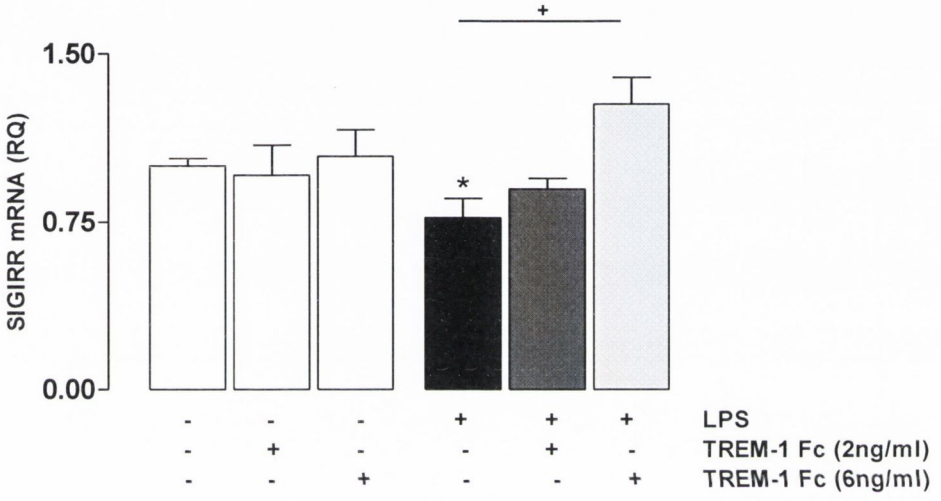


Figure 6.10. TREM-1/Fc reversed the LPS-induced decrease in mRNA expression of SIGIRR in mixed glia.

Primary mouse mixed glia were prepared from wildtype mice and treated with LPS (100ng/ml; 24h). LPS induced a significant decrease in SIGIRR mRNA in mixed glia (*$p<0.05$; ANOVA). Pre-treatment with TREM-1/Fc (6ng/ml; 30min) significantly increased mRNA expression of SIGIRR compared with cells treated with LPS alone ($^{+}p<0.05$; ANOVA) Values for RT-PCR are presented as means (± SEM) and expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 2 separate experiments with each condition tested in duplicate or triplicate.

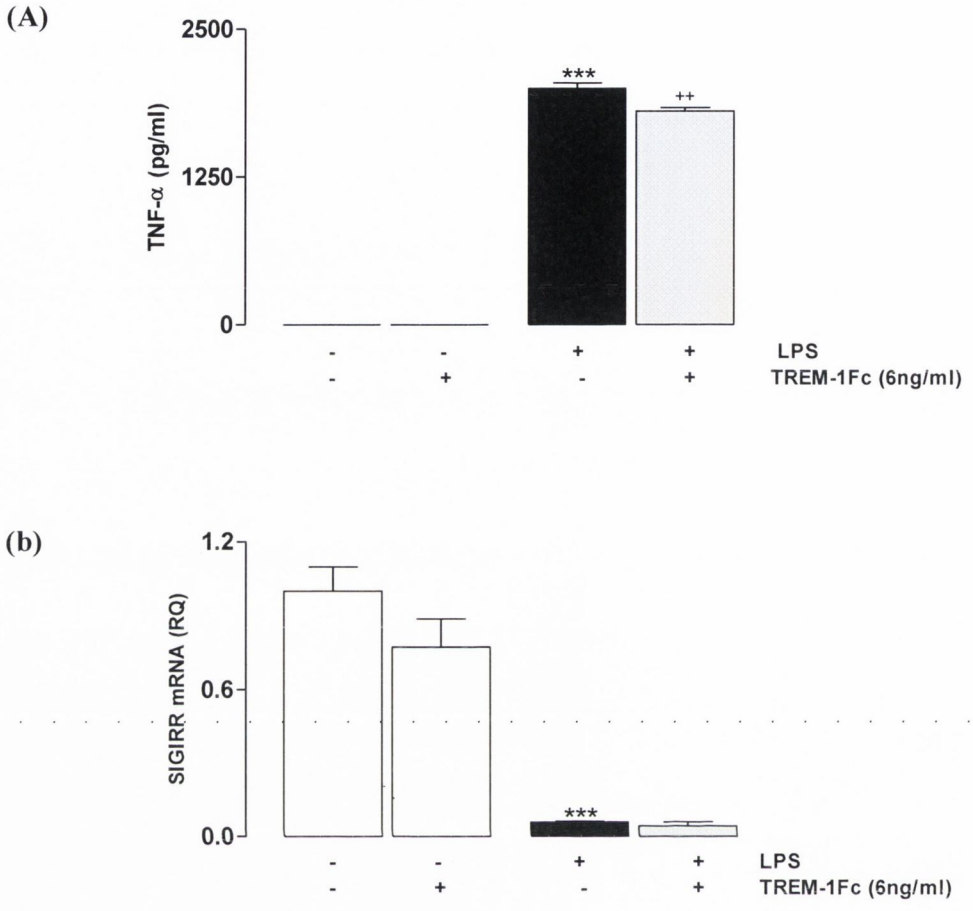
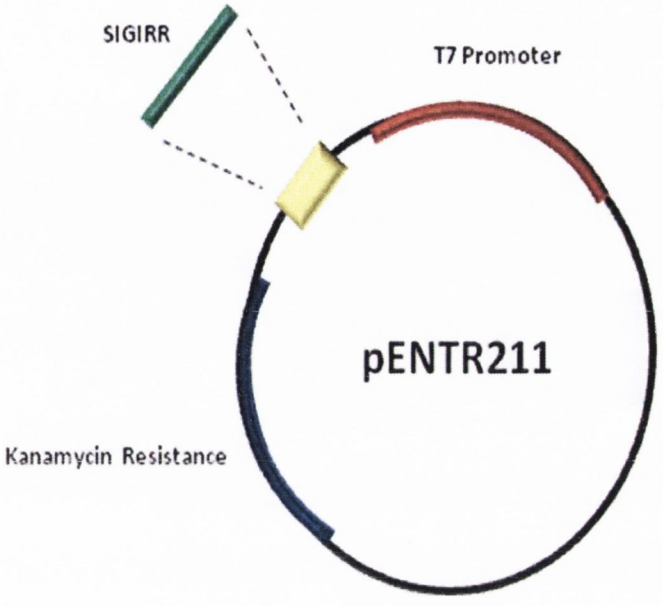


Figure 6.11. TREM-1/Fc attenuates the LPS-induced increase in release of TNF-α from microglia

Secondary microglia were prepared from wildtype mice and treated with LPS (100ng/ml; 24h). LPS induced an increase in release of **(A)** TNF-α and a reduction in the mRNA expression of **(B)** SIGIRR in microglia (***$p<0.001$; ANOVA). Pre-treatment with TREM-1/Fc (6ng/ml; 30min) attenuated the LPS-induced increase in TNF-α release but had no effect on SIGIRR mRNA expression ($^{++}p<0.01$; ANOVA). Values are presented as means (± SEM). Data for mRNA are expressed as a ratio to β-actin mRNA and standardised to a control sample. The data are representative of 1 experiment with each condition tested in triplicate.

(A)



(B)

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Figure 6.12. Original SIGIRR Insert from Commercial Cloning Vector pENTR211

(A) Vector map for pENTR211 showing multiple cloning site for human SIGIRR, kanamycin resistance gene and T7 promotor. **(B)** Sequence of human SIGIRR insert with start and stop codons.

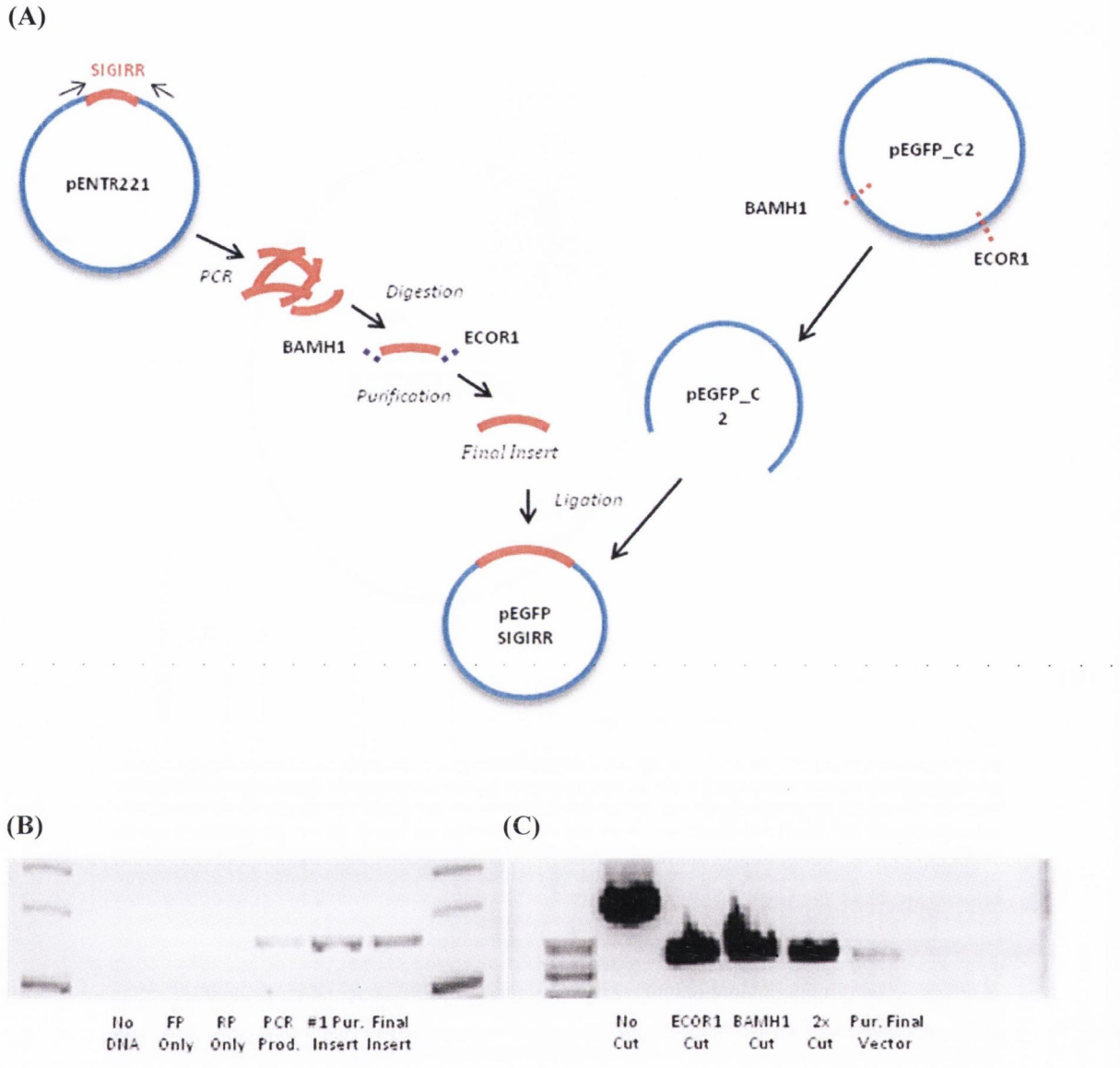

Figure 6.13. pEGFP_C2_mSIGIRR Cloning

(A) Schematic showing protocol for purification of SIGIRR insert and vector preparation for cloning*. **(B)** Agarose gel picture showing gel check stages of cloning protocol for SIGIRR insert. **(C)** Agarose gel picture showing gel check stages of cloning protocol for pEGFP_C2 vector.* Protocol schematic courtesy of Gillian Muirhead.

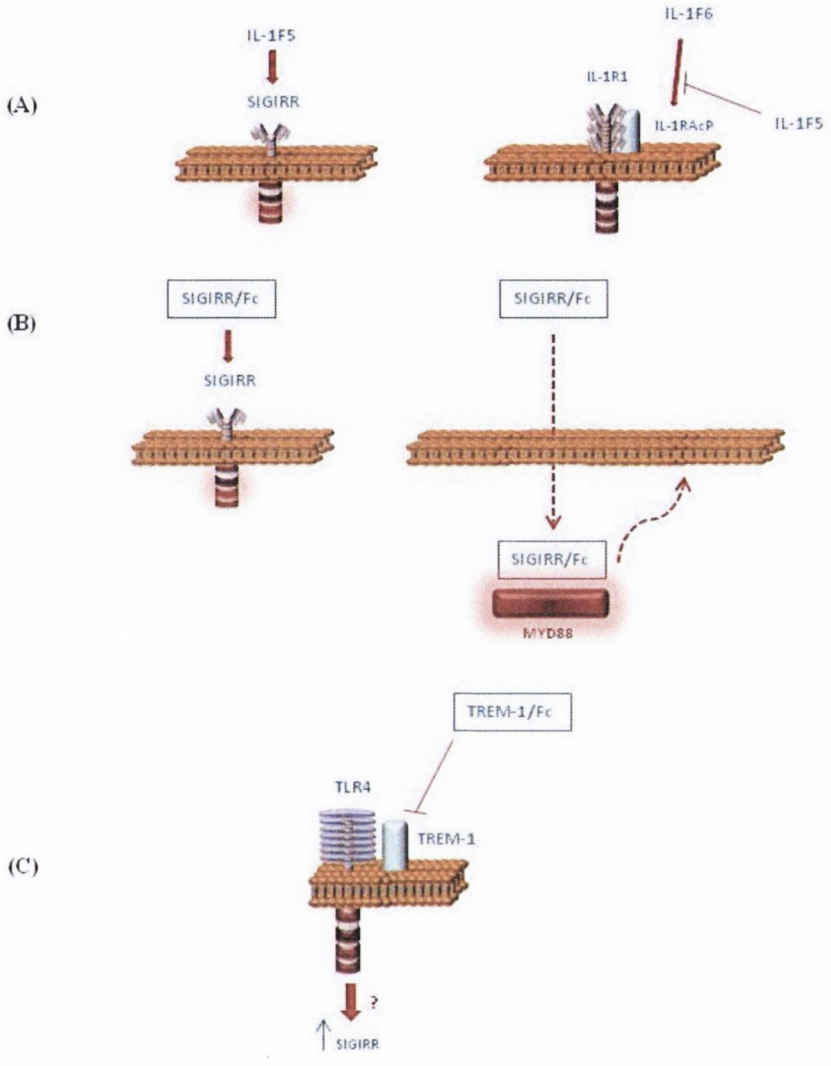

Figure 6.14. Unifying mechanism

(A) It has been proposed that IL-1F5 acts as a ligand for SIGIRR. IL-1F5 may also inhibit IL-1R1 mediated pro-inflammatory signaling by disrupting the interaction of IL-1RAcP with IL-1R1. **(B)** SIGIRR/Fc may act directly on SIGIRR to activate the receptor through homotypic interaction. SIGIRR/Fc may be internalised by the cell and interact with IR domain containing proteins or be recycled to the cell surface. **(C)** TREM-1/Fc blocks TREM-1 and prevents it from enhancing pro-inflammatory signaling through TLR-4 and increases SIGIRR expression. It is unclear whether the increased SIGIRR expression is a direct effect of TREM-1 blockade or whether it occurs secondary to the reduction in signaling through TLR4.

6.4. Discussion

6.4.1. Overview

The aim of this study was to attempt to establish a mechanism to further examine the anti-inflammatory properties of SIGIRR. A number of experiments were performed to investigate methods of directly and indirectly activating endogenous SIGIRR and/or increasing the expression of SIGIRR in glial cells. Specifically, IL-1F5, SIGIRR/Fc and TREM-1/Fc were assessed in an effort to upregulate SIGIRR or activate the receptor. In addition, molecular cloning techniques were employed with a view to creating a microglial cell line which constitutively overexpressed SIGIRR with the objective of modulating inflammatory responses in microglia.

6.4.2. The effect of IL-1F5 on IL-1β-induced cytokine production from mixed glia

The first set of experiments sought to investigate the reported anti-inflammatory effects of the cytokine IL-1F5 and to determine whether these effects were SIGIRR-dependent. Based on the proposition that IL-1F5 may be a ligand for SIGIRR, it was hypothesised that IL-1F5 would attenuate the release of pro-inflammatory cytokines from cultured mixed glia in response to IL-1β and that this effect would be absent in cells prepared from SIGIRR-deficient mice (Costelloe et al., 2008). The data from chapter 3 demonstrate that mixed glia prepared from wildtype and SIGIRR-deficient mice respond to IL-1β by increasing the mRNA expression and release of cytokines and chemokines which corroborated evidence in the literature (Hua and Lee, 2000, Lee et al., 2002). The data also showed that IL-1β-induced cytokine and chemokine production was enhanced in cells prepared from SIGIRR-deficient mice. These findings were replicated by the data presented in this chapter. The mRNA expression of IL-6 and TNF-α, CCL2 and IL-1R1 was increased in cells prepared from SIGIRR-deficient mice compared with wildtype mice

following treatment with IL-1β. Furthermore, IL-1β-induced IL-6 release from glia and pre-treatment with IL-1F5 attenuated the IL-1β-induced increase in IL-6 release in cells prepared from wildtype mice but this effect was absent in mixed glia prepared from SIGIRR-deficient mice. Costelloe and colleagues (2008) reported similar findings, and observed that the ability of IL-1F5 to attenuate an LPS-induced increase in IL-1β was lost in cells prepared from SIGIRR-deficient mice. These findings would seem to support the hypothesis that IL-1F5 exerts its effects in a SIGIRR-dependant manner but a binding study determined that IL-1F5 bound weakly to SIGIRR, suggesting that the cytokine does not act as a ligand but rather through some indirect mechanism (Watson, personal communication). It is important to note that IL-1F5 failed to attenuate IL-1β-induced increases in TNF-α, CXCL10, CCL2 and IL-1R1. These molecules were all markedly increased in cells prepared from SIGIRR-deficient mice compared with wildtype mice and therefore it might be predicted that they would be attenuated by IL-1F5 if it exerts its effects directly through SIGIRR. TNF-α and CXCL10 are primarily induced via innate and classical immune pathways respectively, though their induction may also be secondary to the release of other factors triggered by IL-1β. In contrast, IL-6 is produced directly via IL-1β signalling (Luster et al., 1985, Dumitru et al., 2000).

IL-1F6 is another member of the IL-1 family, structurally similar to IL-1F5, and is thought to signal through IL-1RAcP, which is required for IL-1 induced activation of IL-1R1. IL-1F6 has been shown to robustly increase the secretion of IL-6 from Jurkat cells in an IL-1RAcP-dependant manner (Towne et al., 2004). It has previously been shown that SIGIRR inhibits IL-1 signalling in part through the inhibition of IL-1RAcP (Qin et al., 2005). Interestingly, IL-1F5 appears to act as a functional antagonist to IL-1F6, potently attenuating IL-1F6-induced activation of NF-κB (Debets et al., 2001). It is possible that both SIGIRR and IL-1F5 inhibit IL-1β signalling by interacting with IL-1RAcP to prevent it from associating with IL-1R1. IL-1F5 and SIGIRR may act synergistically to inhibit IL-1RAcP and induce a modest attenuation on IL-1β-induced IL-6 release. In the absence of SIGIRR, IL-1F5 alone may not sufficiently inhibit IL-1RAcP to have such an effect. This would account for the apparent SIGIRR-dependency of IL-1F5 without requiring it to act as a ligand for SIGIRR.

6.4.3. The effect of SIGIRR/Fc on LPS and IL-1β-induced cytokine production from mixed glia

The next set of experiments sought to investigate possible anti-inflammatory actions of SIGIRR/Fc. It was considered that activation of SIGIRR with a fusion protein may attenuate LPS and/or IL-1β-induced changes. LPS increased the mRNA expression of CD11b, IL-6 and TNF-α, and the supernatant concentration of IL-6 and TNF-α. These data replicate the findings in Chapter 3 and are similar to data previously published (Hua and Lee, 2000; Kong et al., 1997; Lee et al., 1993). Pre-treating the cells with SIGIRR/Fc attenuated the LPS-induced increase in CD11b mRNA, IL-6 and TNF-α protein, and the IL-1β-induced increase in IL-6 mRNA and protein. To my knowledge these are the first data describing the effects of exogenous application of SIGIRR on cultured cells. Since it has been established that the extracellular domain of SIGIRR can inhibit IL-1R1 signalling by disrupting the binding of IL-1R1 to IL-1RAcP, it is possible that exogenously applied SIGIRR/Fc inhibits IL-1β-induced cytokine release through this method (Li et al., 2005). However this would not explain the ability of SIGIRR/Fc to attenuate LPS-induced cytokine release, since SIGIRR modulates TLR4 through interaction with intracellular TIR domains. One possibility is that SIGIRR/Fc crosses the membrane to exert this effect. SIGIRR/Fc has a molecular weight of 39 kDa, and non-polar molecules of this size may cross the membrane via membrane transport proteins. However, SIGIRR is estimated to have an isoelectric point (pI) of 5.5, which would confer a negative charge on the protein at physiological pH. Therefore it is likely that an active method of transporting SIGIRR/Fc across the cell membrane would be required. LPS has been shown to increase phagocytosis of both rat and mouse secondary microglial cells as measured by fluorescent staining and bead uptake assays (Neher et al., 2011, Fricker et al., 2012). One possibility is that SIGIRR/Fc may be phagocytosed, perhaps by opsonised phagocytosis through fragment crystallisable region (Fc) receptors on the microglial cell membrane. The Fc tag on SIGIRR/Fc is derived from IgG_1, which binds to FcγR1 with a high affinity and induces phagocytosis (Indik et al., 1995). Indeed it has been shown that the rate of Fc receptor-mediated phagocytosis of IgG-coated erythrocytes was enhanced following exposure to LPS (Abd-el-Basset and Fedoroff, 1994). Bitonti and colleagues (2004) demonstrated that an erythropoietin fusion protein (EpoFc) was capable of

being transported across epithelial cells using the endogenous FcRn-IgG transport pathway (Bitonti et al., 2004). If internalised, SIGIRR/Fc may be recycled to the cell membrane, or exist in a soluble form, and be available to bind to intracellular TIR-domain containing signalling proteins thus mimicking the function of endogenous SIGIRR.

6.4.4. The effect of TREM-1/Fc on LPS-induced cytokine production from mixed glia

The recombinant TREM-1 fusion protein, TREM-1/Fc, demonstrated an ability to attenuate the LPS-induced increase in TNF-α from mixed glia and microglia in a dose-dependent manner. However, the effect was modest, decreasing the LPS-induced change by about 15%. The effect was also confined to TNF-α, with no modulatory influence on the LPS-induced change in IL-6. Previous studies have reported an anti-inflammatory effect of TREM-1/Fc, with mouse models of sepsis showing significantly improved survival rates following TREM-1/Fc treatment (Tong et al., 2011). In addition, TREM-1/Fc attenuated the expression of TNF-α, IL-6 and IL-1β from a THP-1 monocytic cell line and reduced monocytic activation in patients with sepsis (Wong-Baeza et al., 2006, Tong et al., 2011). In 2011, Wu and colleagues investigated the role of TREM-1 in the progression of corneal disease induced by *Pseudomonas aeruginosa* infection in mice. This group reported that mice which received an injection of TREM-1/Fc exhibited reduced corneal disease, with an accompanying decrease in pro-inflammatory markers such as IL-1β and IL-6.

The present data also show that LPS induced a downregulation of SIGIRR mRNA, but that pre-treatment with TREM-1/Fc reversed this effect. This is consistent with the data suggesting that LPS-induced changes might be SIGIRR-dependent. Interestingly, it has been reported that TREM-1/Fc promoted the expression of SIGIRR in corneal tissue from mice with *P. aeruginosa* infection corneal infection. The present data support this evidence. The sequence of events following TREM-1/Fc treatment remains unclear however, and a time-course study is required to determine whether the expression of SIGIRR precedes the attenuation of the pro-inflammatory markers, and this would be consistent with a possible role for SIGIRR in the process. It may be

that, following the initial LPS-induced downregulation of SIGIRR, the blockade of TREM-1 by TREM-1/Fc diminishes continuous production of pro-inflammatory cytokines, allowing SIGIRR expression to recover. In any case, TREM-1/Fc failed to increase the expression of basal levels of SIGIRR in mixed glia and therefore does not represent an effective indirect mechanism of exploiting the anti-inflammatory effects of SIGIRR.

IL-1F5, SIGIRR/Fc and TREM-1/Fc all exhibit a modest ability to attenuate glial activation and cytokine release. Previous data indicated that IL-1F5's inability to act as a direct ligand for SIGIRR, with evidence of poor binding affinity between the two molecules. The limited effect of IL-1F5 on modulation of LPS and IL-1β-induced cytokines, suggest that IL-1F5 and SIGIRR may cooperate to exert a modulatory effect on IL-1R1, possibly by acting on IL-1RAcP, rather than by a direct interaction. Similarly, the mechanism of action of SIGIRR/Fc remains a subject for speculation and further studies are required to determine the nature of its modulatory effect on TLR4 and IL-1R1 signalling. However, the limited attenuation of glial activation and cytokine release achieved by SIGIRR/Fc, and the failure to a dose with consistent effects dose, suggests that activation of SIGIRR by SIGIRR/Fc is not a viable is not a viable mechanism to enhance SIGIRR-mediated effects. Although the anti-inflammatory effects of TREM-1/Fc warrant further study, it appears to be an ineffective stimulator of SIGIRR expression.

6.4.5. Transfection of BV2 cells with vector containing *sigirr* gene

In light of the limited success of exogenously-applied agents in targeting SIGIRR and inducing anti-inflammatory effects, a more direct mechanism of increasing SIGIRR expression was sought. A number of authors have used SIGIRR overexpression in a variety of cell types to demonstrate the modulatory effect of SIGIRR in pro-inflammatory signalling and to investigate the mechanisms of this effect. Qin and colleagues (2005) transfected HEK293 cells with a construct containing an N-terminus FLAG-tagged SIGIRR insert and reported that the cells overexpressing SIGIRR exhibited significantly reduced levels of NF-κB activation in response to TLR4 and IL-1R1 stimulation compared with control cells. A similar attenuating effect on IL-1R1 and TLR4-induced NF-κB activation and cytokine release was observed in HT29 cells,

macrophages, dendritic cells and RAW264.7 cells transfected with SIGIRR (Wald et al., 2003; Drexler et al., 2010; Huang et al., 2006). In the present study, an insert containing the human SIGIRR gene was successfully amplified and purified from the bacterial vector pENTR221. The SIGIRR insert, and the mammalian vector pEGFP_C2, were incubated with the restriction enzymes BAMH1 and ECOR1 in order to produce the complementary single-stranded "sticky ends" necessary to enable ligation. A number of protocols were employed to promote the ligation of the SIGIRR insert into pEGFP_C2 however they proved unsuccessful. There are a number of reasons why the ligation may have failed, including the presence of contaminating DNA ligase inhibitors, or restriction endonucleases, which were not filtered out during the purification process. It may also be that pEGFP_C2 is an inappropriate vector for the ligation of the SIGIRR insert, and there are a number of other mammalian expression vectors which may prove more effective.

The investigation of SIGIRR as a target for possible anti-inflammatory therapies is made difficult by the nature of SIGIRR as an orphan receptor and the lack of an endogenous ligand, or a synthetic agonist. If the beneficial effects of SIGIRR are to be effectively exploited, it seems likely that research will have to be focused on methods of driving its overexpression, whether through the indirect actions of a second protein, or through the use of gene expression techniques.

Chapter 7: Conclusion

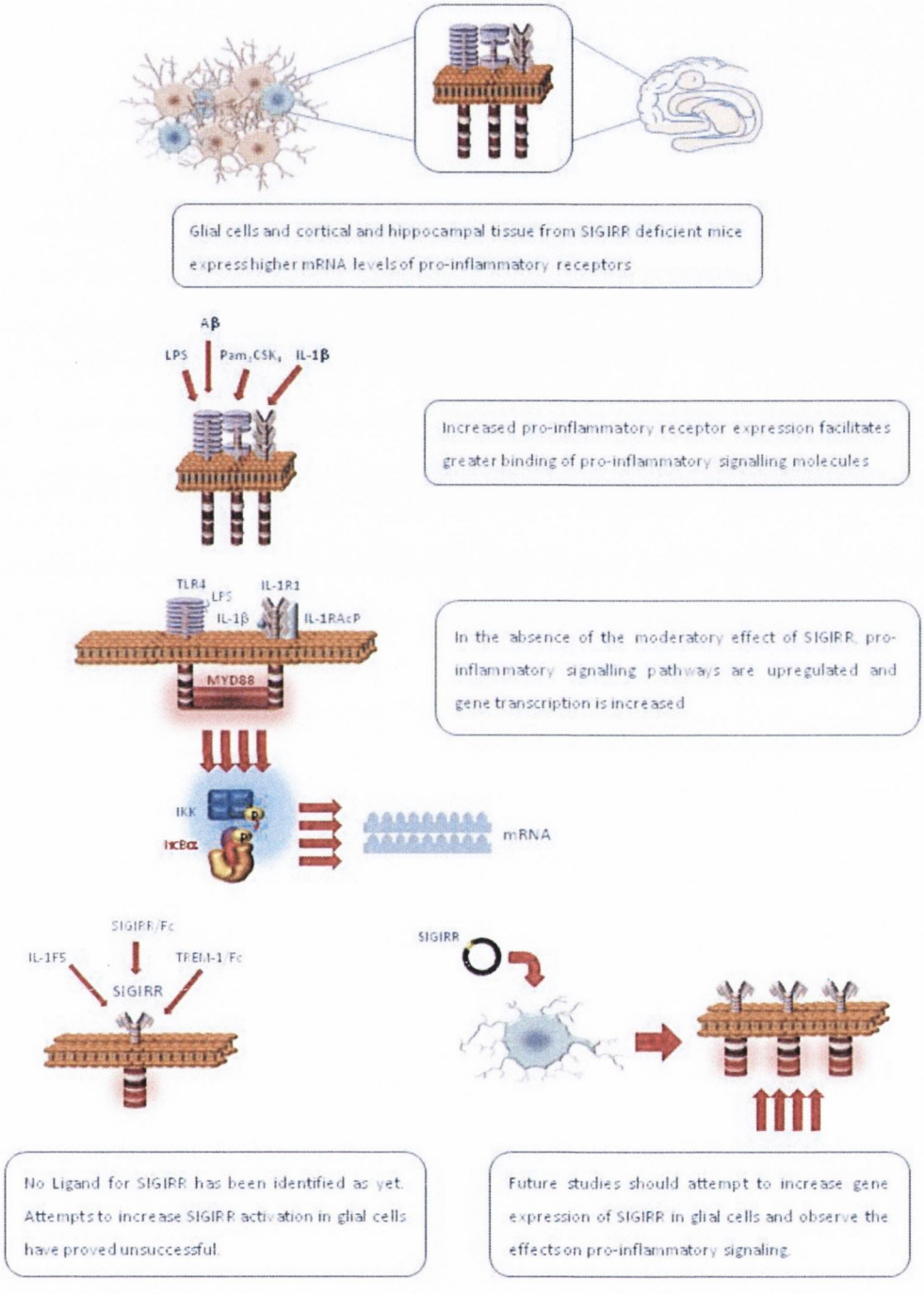

Figure 7.1. Unifying mechanism

7.1. Overview

The objective of this study was to investigate the modulatory effects of SIGIRR on glia, particularly microglia. A functional role for SIGIRR has been described in a variety of cell types throughout the periphery, particularly in the kidney and intestine, where it acts as a modulator of inflammatory signalling. SIGIRR has been ascribed an important role in the suppression of an array of inflammatory diseases such as rheumatoid arthritis, lupus erythematosus and sepsis. However, as yet there have been few studies which have examined the role of SIGIRR as a moderator of inflammation in the brain. Although the expression of SIGIRR in the brain is low compared with most peripheral tissues, it has been detected in the cortex, hippocampus and thalamus, on both neurons and glial cells. In light of increasing evidence implicating activated microglia in the pathogenesis of many neurodegenerative diseases and in the damaging effects of aging, it is important to investigate novel endogenous suppressors of pro-inflammatory signalling in microglia. Evidence that SIGIRR is involved in the inflammatory response of peripheral cells of myeloid lineage lead to the hypothesis that SIGIRR may represent a hitherto unknown modulator of inflammatory signalling in glial cells.

7.2. SIGIRR deficiency and glial cell function

The first two chapters of this thesis describe experiments that sought to characterise the response of glial cells prepared from SIGIRR-deficient mice to a variety of pro-inflammatory stimuli. Observing the differences in the expression of markers of microglial activation and pro-inflammatory cytokines between SIGIRR-deficient cells and cells which express SIGIRR allows the inference of a functional role for SIGIRR in the regulation of these processes. The exaggerated response of glia prepared from SIGIRR-deficient mice to LPS support and expand on the findings by Watson and colleagues (2010) that SIGIRR is involved in the suppression of pro-inflammatory signalling through TLR4 on glial cells. In the present study, it was established for the first time that SIGIRR is also capable of modulating pro-inflammatory signalling through IL-1R1 and TLR2 as evidenced by the exaggerated response of glial cells to IL-1β and

Pam_3CSK_4 respectively. Upon stimulation of glial cells with each of these factors, the absence of SIGIRR consistently led to an exaggerated production of the pro-inflammatory cytokines IL-6 and TNF-α. The upregulation of these cytokines is known to be driven by the transcription factor NF-κB, and these data support the evidence from the literature describing the modulation of this pathway by SIGIRR. There were some inconsistencies in the effects of SIGIRR deficiency on markers of microglial activation however. It had previously been reported by Watson and colleagues (2010) that CD40 and ICAM1 mRNA expression was exaggerated in mixed glia prepared from SIGIRR-deficient mice following LPS stimulation, and in the data presented in this thesis, IL-1β was shown to induce an exaggerated expression of CD11b and CD68 in glia from SIGIRR deficient mice. However, there was no genotype difference observed in the expression of markers of microglial activation following Pam_3CSK_4 treatment. This may reflect the varying ability of SIGIRR to suppress microglial activation through the different receptors, although further work is required to substantiate this hypothesis. Although TLR2 utilises the same inflammatory signalling pathway as TLR4 and IL-1R1, Pam_3CSK_4 is a much more specific agonist for TLR2 than LPS is for TLR4; it is accepted that LPS is capable of activating a number of receptors, and therefore the effects of LPS may be more profound. Similarly, although IL-1β is a specific IL-1R1 agonist, SIGIRR is capable of modulating IL-1R1 through a number of mechanisms additional to those thought to be used in the suppression of TLR2 signalling. This may account for the seemingly increased glial activation seen in response to IL-1β in cells from SIGIRR-deficient animals.

Inconsistencies were also apparent in the differing responses of isolated microglia and astrocytes to the various stimuli. Isolated microglia, but not astrocytes, from SIGIRR-deficient mice exhibited exaggerated production of pro-inflammatory cytokines in response to LPS. This finding was unsurprising, in light of the role ascribed to microglia as the primary immune cell of the brain. However the pattern of response was not replicated following Pam_3CSK_4 treatment, with both cell types exhibiting an exaggerated response. One possible explanation for this is the phenomenon of astrocytic TLR2 hypersensitivity, in which TLR2 on astrocytes is upregulated and sensitised following "priming" by microglia-derived cytokines, particularly TNF-α (Phulwani et al., 2008, Henn et al., 2011). The process of culturing astrocytes (see section 2.1.2.) involves initial co-culture with microglia and treatment with GM-CSF, which is capable of

inducing the production of TNF-α (Scian et al., 2011). Thus, the period in which astrocytes are in co-culture with microglia may induce TLR2 hypersensitivity on the astrocytes, which can persist for days after the cells have been isolated and the priming stimulus has been removed (Henn et al., 2011).

One of the interesting finding in this study is that basal expression of TLR2 and TLR4 was increased in glia from SIGIRR-deficient mice. This seems to suggest that SIGIRR may exert control over TLR expression. It has been reported that inhibition of TLR endocytosis enhances TLR-mediated NF-κB signalling (Husebye et al., 2006). It may be that SIGIRR promotes the ubiquitin-dependent degradation of TLRs, which would contribute to the suppression of TLR signalling. The TIR domain of SIGIRR may allow it to interact with ubiquitin protein ligases, such as Triad3A, which are known to promote the degradation of TLRs, although how SIGIRR-Triad3A interaction might promote the activity of the ligase is unclear (Chuang and Ulevitch, 2004, Fearns et al., 2006).

This study extensively characterises the response of glial cells to TLR and IL-1R stimulation in the absence of SIGIRR. It provides evidence that control of pro-inflammatory receptor expression may be one means by which SIGIRR modulates pro-inflammatory signalling. The data add evidence to the proposition that SIGIRR acts as a modulator of TLR2 signalling, a role which remains to be unequivocally identified in the periphery. The study supports the hypothesis that the previously-described ability of SIGIRR to act as a negative regulator of TLR/IL-1R signalling extends to the brain.

Future Studies: *Although the data presented in this thesis suggest that SIGIRR can modulate inflammatory processes such as cytokine release and activation of glia, they do not indicate the exact mechanism through which this is achieved. Evidence from the literature would suggest that inhibition of NF-κB through the sequestering of MyD88 is the most likely mechanism, however it is possible to activate NF-κB in an MyD88-independent manner. Future experiments could analyse the phosphorylation of components of the NF-κB signalling pathway in glia from SIGIRR-deficient mice in response to various stimuli. These experiments could be repeated in the presence of a MyD88 blocking peptide or following MYD88 gene silencing to determine whether*

SIGIRR exerts control over MyD88-independent pro-inflammatory signalling. A time-course treatment of glia from wildtype and SIGIRR-deficient mice would be useful to elucidate whether downregulation of SIGIRR by inflammatory stimuli occurs prior to, or subsequent to, cytokine release. An important future study would be an investigation into the possibility that SIGIRR modulates TLR recycling. This may involve studying the interaction of SIGIRR with Triad3A.

7.3. The role of SIGIRR as a modulator of inflammation *in vivo*

This study presents the first investigation into the role of SIGIRR as a suppressor of Aβ-induced inflammatory signalling in glial cells. A robust exaggeration in TNF-α and IL-6 release was reported in glia prepared from SIGIRR-deficient mice compared with wildtype mice in response to Aβ. However, this did not occur in young and middle-aged SIGIRR-deficient mice in response to an intrahippocampal injection of Aβ. Although there were indications that SIGIRR-deficiency was associated with higher basal levels of pro-inflammatory receptors, upregulation of microglial activation and NF-κB signalling in the brain, there was a marked difference in the potency of Aβ as an inflammatory stimulus in the absence of SIGIRR *in vitro* compared with *in vivo*. Although discrepancies between *in vitro* and *in vivo* results are common, it is reasonable to speculate on why they occurred in this case. It is worth noting that CD68 was upregulated in the SIGIRR-deficient animals, which may indicate an increase in phagocytosis. If microglial cells in the SIGIRR-deficient animals were actively phagocytosing Aβ, the diminished amount of available peptide would probably have a less potent stimulatory effect on microglial cell surface receptors. However, as the increase in CD68 was associated with age, this would only account for the lack of a predicted response in the middle-aged animals. It seems likely that assessment of tissue 4h after a single intrahippocampal injection of Aβ is not the most appropriate model to analyse the ability of SIGIRR to modulate Aβ-induced inflammation and possible toxicity.

It is worth considering the available evidence related to the modulatory effect of SIGIRR in peripheral tissues. Tissue expression studies have revealed that SIGIRR is particularly highly expressed in the organs of the digestive tract (Polentarutti et al., 2003). The epithelial cells and dendritic cells of the digestive tract express TLRs, and because the intestine is the largest

reservoir of human *flora*, they are constantly exposed to PAMPs. There are a number of endogenous mechanisms which maintain the epithelial cells in a hyporesponsive state and evidence from the literature suggests that one of the main physiological functions of SIGIRR is the tonic regulation of TLR signalling from intestinal epithelial cells and dendritic cells (Garlanda et al., 2007, Davies et al., 2010, Khan et al., 2010). This is consistent with the possibility that SIGIRR is more effective as a modulator of chronic low-level inflammatory conditions, where TLRs are constitutively active, rather than as a regulator of inflammatory changes induced by an acute noxious insult. This role would appear to be supported by the *in vivo* data presented in this thesis, in which the upregulation of pro-inflammatory receptor expression, microglial activation and NF-κB signalling are associated with the overall effect of SIGIRR deficiency, rather than in response to Aβ. For this reason it may have been more interesting to observe the effect of SIGIRR-deficiency in a model of old-age, when the inflammatory changes are more chronic. In the case of AD, Aβ peptides accumulate and are formed into plaques over a period of many years, and chronic inflammation is a feature even in early pre-clinical AD (Akiyama et al., 2000). Therefore it is likely that a longer exposure to Aβ would have produced a more robust inflammatory response in SIGIRR-deficient animals.

Future Studies: *Any future investigations into the role of SIGIRR in the modulation of Aβ toxicity would be improved with the use of sub-cutaneously implanted osmotic mini-pumps which can be used to deliver a steady infusion of Aβ into the brain over a longer period of time (typically 28 days) (Miller et al., 2009). This experimental set-up would more closely replicate the chronic inflammatory stimulus induced by Aβ particularly if infusion was carried out in animals of different ages. It would enable the examination of the effects of SIGIRR deficiency on inflammatory processes induced by chronic Aβ. A more ambitious experimental set-up could involve the use of a viral vector to drive the overexpression of SIGIRR in an APP/PS1 mouse model of Alzheimer's disease. This system could determine the efficacy of SIGIRR as a modulator the chronic inflammation associated with endogenous amyloid plaque deposition.*

7.4. SIGIRR as a target for anti-inflammatory therapeutics

When discussing the modulation of inflammatory signalling as a therapeutic strategy for any disease it is important to always keep in mind the cliché description of inflammation as "a double-edged sword". The existence of an inflammatory environment is, in almost every case, a reaction to the presence of a noxious insult. Abrogation of the inflammatory response would allow potentially damaging insults to remain indefinitely, and the devastating effects of immunodeficiency disorders highlight the need for a healthy inflammatory response. However, the sheer number of disorders with an inflammatory basis necessitates the study of targeted and limited suppression of inflammation. Corticosteroids have been in use in the treatment of inflammatory diseases for decades. Non-steroidal anti-inflammatory drugs (NSAIDs) are used in a similar manner and early studies indicated that long-term administration of NSAIDs reduced the cognitive deficits associated with neurodegenerative diseases (McGeer et al., 1996). However, these therapies have proved inadequate for the treatment of chronic neuroinflammatory disorders. The broad-spectrum actions induce a myriad of side-effects during long-term use and serve to highlight the danger of non-specific immunosuppression. The lack of effectiveness of NSAIDs in the treatment of AD is unsurprising, since anti-inflammatory therapy cannot be expected to overcome the problems associated with profound neuronal loss. It is important to keep this in mind when discussing the advantages and disadvantages of SIGIRR as a target for drug therapy in the CNS.

The BBB adds a particular obstacle to the delivery of targeted immunosuppressants to the CNS. The importance of specific, limited immune-suppression has driven research into endogenous regulators of specific inflammatory processes, and CD200 has emerged as an encouraging potential target for drug therapy. The complementary pattern of expression of CD200 in the CNS, primarily located on neurons, and CD200R, on microglia, confers a degree of specificity on CD200-induced suppression of inflammatory signalling that SIGIRR would appear to lack. In contrast, the near-ubiquitous expression of SIGIRR on most tissues of the body would incur the risk of widespread side-effects to any potential SIGIRR-based therapy, particularly in the

digestive tract where SIGIRR is thought to have a role in the maintenance of homeostasis. In contrast, the effects of CD200-based therapies are likely to be restricted to cells of the myeloid lineage (Wright et al., 2001). Indeed, even in the absence of such concerns, the data presented in this thesis demonstrate the difficulty in exploiting the anti-inflammatory effects of SIGIRR. A number of studies have demonstrated that SIGIRR failed to interact with putative ligands of the IL-1 family and data presented in this thesis appear to rule out the previously considered IL-1F5 as a ligand. The fact that SIGIRR is almost certainly an orphan receptor immediately precludes the design of mimetic ligand-based drugs. In addition, the modest anti-inflammatory effect induced by SIGIRR/Fc suggests that homotypic interaction is not an effective means of activating SIGIRR. It would appear that the only effective means to exploit the anti-inflammatory effects of SIGIRR is to boost the expression of the receptor. However, this requires the identification of mechanisms which enhance the expression of SIGIRR. Data presented here indicate that one such proposed mechanism, blockade of TREM-1, had only a modest effect on SIGIRR expression, at least under the experimental conditions used here, although it may still prove to be an effective therapy in the treatment of corneal inflammation (Wu et al., 2011). The most effective method of SIGIRR overexpression is likely to be through the use of a viral vector to deliver a package of SIGIRR DNA with a promoter to drive the overexpression of the protein. The advantages of this method are manifold; the cells own machinery is used to increase production of the protein; viral vectors are capable of crossing the BBB; the vector can be designed to target specific cells types, such as microglia, leaving peripheral cells unaffected. However, although viral vectors were shown to be effective in the treatment of neurodegeneration in a mouse model over a decade ago, gene therapy is still in its infancy, and much basic research into examining and understanding the functional characteristics of SIGIRR must be done before gene therapy can be applied to induce the upregulation of SIGIRR (Blomer et al., 1998). Although proteins such as CD200 appear to represent a more practical target for CNS anti-inflammatory therapy, SIGIRR may still be useful in the treatment of chronic inflammatory diseases. It has been reported that SIGIRR is an important modulator of the innate immune response to organ transplantation (Noris et al., 2009). This implies that SIGIRR may be useful in the suppression of immune rejection of stem cell transplants which could greatly improve the success rate of such treatments (Zhao et al., 2011).

Future Studies: *The most important future study for the advancement of our understanding of SIGIRR is the development of an appropriate vector for the transfection of microglia with SIGIRR. Observing the effects of SIGIRR overexpression in microglia would further our understanding of how SIGIRR modulates microglial activation. It would also be the first step towards developing a viral vector which could be used to drive SIGIRR expression in animal models of neurodegenerative diseases.*

The data presented in this thesis support the hypothesis that SIGIRR acts as a modulator of inflammation in immune cells of the brain. Although the expression of SIGIRR in the brain is low in comparison to peripheral tissues, and its role may be limited in comparison to other modulators of microglial activation, these data suggest that SIGIRR may represent a future drug target in the treatment of CNS inflammation.

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