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Anti-inflammatory effects of Single-Ig Interleukin-1 (IL-1)-related receptor (SIGIRR) in the brain; Interaction with IL-1F5

Melanie Watson

Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College

2009

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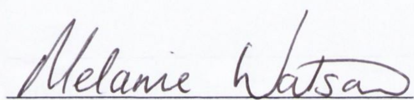

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III. Abstract

It is well established that inflammatory changes in the brain are associated with ageing and neurodegenerative disorders. Central to these changes is activation of microglial cells with the accompanying increases in the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) and activation of downstream signalling cascades. The IL-1 ligand and receptor super-family have been shown to play pivotal roles in CNS-mediated inflammation. However, recent data show that single-Ig IL-1-related receptor (SIGIRR), an orphan receptor, is structurally and functionally different from the other members and is a negative regulator of Toll-like receptor/IL-1 receptor (TLR/IL-1R) signalling. The ligand and downstream signalling events induced by SIGIRR are largely unknown. Interestingly, a newly characterised member of the IL-1 ligand super-family, termed interleukin-1F5 (IL-1F5), which shares significant homology with IL-1 receptor antagonist (IL-1ra) and exhibits anti-inflammatory actions, does not bind to any of the IL-1Rs suggesting it may be a ligand for an orphan receptor. The aims of this study were to characterise the expression of SIGIRR in the brain, investigate the anti-inflammatory actions of SIGIRR and IL-1F5 on lipopolysaccharide (LPS), amyloid-β (Aβ) and age-associated inflammatory changes, and to establish if the anti-inflammatory effects of IL-1F5 are dependent on its interaction with SIGIRR.

The data reveal that the expression profile of SIGIRR in the brain is cell-type specific; it is highly expressed on astrocytes but not expressed on microglial cells. The findings from this study show that SIGIRR has anti-inflammatory actions in the CNS, since age and LPS-treatment induced exacerbated inflammation in the hippocampus of SIGIRR$^{-/-}$ mice; this was typified by up-regulated microglial activation and enhanced production of chemokines and the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. Accompanying the heightened inflammatory profile was exacerbated sickness behaviour in SIGIRR$^{-/-}$ mice.

IL-1F5 attenuated LPS-induced expression of IL-1β, IL-6 and TNF-α in mixed glial cells and astrocytes but not in isolated microglial cells. Furthermore IL-1F5 attenuated LPS- and Aβ-induced and age-associated inflammatory changes in rat

hippocampus, dampened microglial activation and induced a significant increase in interleukin-4 (IL-4) mRNA expression. Importantly the data reveal that IL-1F5 was co-localised with SIGIRR in mixed glia and the antagonistic effects of IL-1F5 on LPS-induced signalling were not observed in supernatants prepared from SIGIRR$^{-/-}$ mice. The specific interaction of IL-1F5 with SIGIRR was examined and one major finding of this study is that IL-1F5 binds to the novel orphan receptor SIGIRR.

The evidence presented here establishes that IL-1F5 through its interaction with SIGIRR, and up-regulation of IL-4, mediates an anti-inflammatory signalling cascade in the CNS which is capable of antagonising multiple models of inflammation.

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VII. Abbreviations

The following abbreviations have been used:

Aβ	Amyloid-β
AcP	Interleukin-1 receptor accessory protein
AcPb	Interleukin-1 receptor accessory protein b
AcPL	Accessory protein like gene
AD	Alzheimer's Disease
ANOVA	Analysis of variance
APC	Allophyscocyanin
AP-1	Activator protein 1
APP	Amyloid-precursor protein
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCA	Bicinchonic acid
BCG	Bacillus Calmette-Guerin
BMDMs	Bone-marrow-derived macrophages
BP	Band Pass
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CD	Cluster of differentiation molecule
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CpG-DNA	DNA containing cytosine and guanine motifs
CSF	Cerebrospinal fluid
dATP	Deoxyadenosine triphosphate
DCs	Dendritic cells
dCTP	Deoxycytidine triphosphate
DG	Dentate gyrus
DGLA	Dihomo-gamma-linolenic acid
dGTP	Deoxyguanosine triphosphate

dH_20	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DSS	Dextran sulfate sodium
dTTP	Deoxythymidine triphosphate
DTT	Dithiotheitol
EAE	Experimental allergic encephalomyelitis
EC	Entorhinal cortex
E. coli	Escherichia coli
ECL	Enhanced chemiluminesence
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentanoic acid
EPSP	Excitatory post-synaptic potential
ERK-1	Extracellular regulated kinase-1
ERK-2	Extracellular regulated kinase-2
EST	Expression sequence tag
EtBr	Ethidium Bromide
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
FPR2	Formyl peptide receptor 2
FITC	Fluorescein
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage colony stimulating factor
h	Hour
HIV	Hunan immunodeficiency virus
I4R	Interleukin-4 receptor motif
ICAM	Intracellular adhesion molecule-1
ICE	Interleukin-1 converting enzyme

ICV	Intracerebroventricular
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IκB	Inhibitory kappa B
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1ra	Interleukin-1 receptor antagonist
IL-1RacPL	Interleukin-1 receptor accessory protein-like
IL-1RI	Type I Interleukin-1-receptor
IL-RII	Type II Interleukin-1 receptor
IL-1RI$^{-/-}$	Interleukin-1receptor knockout
IL-1Rrp2	Interleukin-1 recepor related protein 2
IL-4$^{-/-}$	Interleukin-4 knockout
IL-4R	Interleukin-4-receptor
IL-4Rα	Interleukin-4-receptor-α
IL-18R	Interleukin-18 receptor
IL-33	Interleukin-33
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IP-10	IFN-γ-inducible protein-10
IRAK	Interleukin-1 receptor associated kinase
IRS-1	Insulin-receptor substrate 1
IRS-2	Insulin-receptor substrate 2
JAK	Janus kinase
JAK-1	Janus kinase-1
JNK	c-Jun NH_2-terminal kinase
KB	Kilo base
KC	CXCL1 chemokine
kDa	Kilo dalton
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide

LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Mononuclear phagocyte colony stimulating factor
MF	Mossy fibres
MHC I	Major histocompatability complex I
MHC II	Major histocompatability complex II
mIL-6R	Membrane-bound Interleukin-6 receptor
MIP-1α	Macrophage inflammatory protein-1α
MIP-1β	Macrophage inflammatory protein-1β
MIP-2	Macrophage Inflammatory protein-2
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
M. tuberculosis	Mycobacterium tuberculosis
MyD88	Myeloid differentiation primary response gene 88
MyD88s	Myeloid differentiation primary response gene 88 short
NFT	Neurofibrillary tangles
NFκB	Nuclear factor kappa B
NGS	Normal goat serum
NK cells	Natural killer cells
NMDA	N-methyl D-aspartate
NO	Nitric oxide
O/N	Overnight
P.aeruginosa	Pseudomonas aeruginosa
PAMPS	Pathogen-associated molecular patterns
Pam3Cys	Lipopeptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PI-3-K	Phosphoinositide-3-kinase
P-ERK	Phosphorylated extracellular regulated kinase
P-JNK	Phosphorylated c-Jun NH_2-terminal kinase
PMA	Phorbol myristic acid
Poly (I:C)	Polyinosinic:polycytidylic acid
PP	Perforant path
P-p38	Phosphorylated p38
PRR	Pattern recognition receptor
P-STAT6	Phosphorylated signal transducer and activator of transcription 6
P-Tau	Phosphorylated tau
ROS	Reactive Oxygen species
RNA	Ribonucleic acid
RNase	Ribonuclease
RNS	Reactive Nitrogen species
RQ	Relative quantity
RT	Room temperature
RT-PCR	Real time PCR
SAPK	Stress-activated protein kinase
SC	Schaffer collateral
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SIGIRR	Single Ig IL-1-related receptor
SIGIRR-/-	Single Ig IL-1-related receptor knockout
sIL-6R	Soluble Interleukin-6 receptor
SLE	Systemic lupus erythematosus
S/S	Supersignal
STAT	Signal transducer and activator of transcription
STAT-6	Signal transducer and activator of transcription-6
Strep-HRP	Streptavidin-Horseradish peroxidase linked
TBE	Tris borate EDTA

TBS-T	Tris-buffered saline containing Tween-20
T-ERK	Total-extracellular regulated kinase
Th1	T helper-1
Th2	T helper-2
TIGIRR-1	Three Ig IL-1-related receptor-1
TIGIRR-2	Three Ig IL-1-related receptor-2
TIR	Toll/Interleukin-1 receptor
Tir8	Toll-Interleukin-1-receptor 8
T-JNK	Total c-Jun N-terminal kinase
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TNFB	Tumour necrosis factor binding protein
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
TRAF 6	Tumour necrosis factor-receptor associated factor 6
UV	Ultraviolet
Y	Tyrosine
γc	Gamma-chain of Interleukin-4 receptor
γ-irradiation	Gamma-irradiation

Chapter 1

Introduction

1. Introduction

1.1. The Hippocampus

1.1.1. History

The ancient Egyptians believed that the heart was the most important organ of the body, the seat of the mind and the centre of intellectual activities, this view was also held by the great philosopher Aristotle who believed the brain was a mere cooling system for the body (Goss, 1998). It was the Greek physician Alcmaeon who was one of the first to conclude that the brain is the central organ of sensation and thought (Blits, 1999). It is now accepted that the central nervous system (CNS) orchestrates functions of all major organs in the body. Scoville and Milner (1957) were the first to suggest that the medial temporal lobe was the area of the brain associated with memory, following extensive study of an amnesic patient called "Henry M" who had substantial bilateral damage to the medial temporal lobe. Since this is a large region of the CNS, that includes the hippocampus, the amygdala and the adjacent cortex it was difficult to determine exactly which region was important for memory. In 1978 a post-mortem of a patient with amnesia demonstrated damage to the CA1 region of the hippocampus (Zola-Morgan *et al.*, 1986) providing evidence that the hippocampus is the area of the brain associated with memory.

1.1.2. Structure of the hippocampus

The term "hippocampus" is a Greek word for "seahorse" and was first coined by an Italian anatomist Arantius (1587) who likened the structure of the hippocampus to a seahorse, although it later transpired that Arantius was referring to a hippocampal subregion, the dentate gyrus (Walther, 2002). The hippocampus is located in the medial temporal lobe of each cerebral hemisphere in an area far beneath the cortices. It is composed of pyramidal cells arranged in a parallel laminar organisation and is divided into the CA1 and CA3 regions, the dentate gyrus and the subiculum. The neuronal connections within these regions follow this laminar format, are uni-directional and form a well-characterised closed loop that originates in the entorhinal cortex (EC) (Figure 1.1.). The EC neuronal inputs lead to sequential activation of four main neuronal pathways. The first of these

neuronal pathways is the perforant path, which projects to the dentate gyrus granule cells and also to the CA3 pyramidal cells. This results in activation of the mossy fibres of the dentate gyrus granule cells which project to and cause activation of the CA3 pyramidal cells. The Schaffer collaterals of the CA3 pyramidal cells become activated and project to the CA1 pyramidal cells, from here the neurons project to the subicular complex and back to the EC, which forms the principal output from the hippocampus to the neocortex (Andersen *et al.*, 1969).

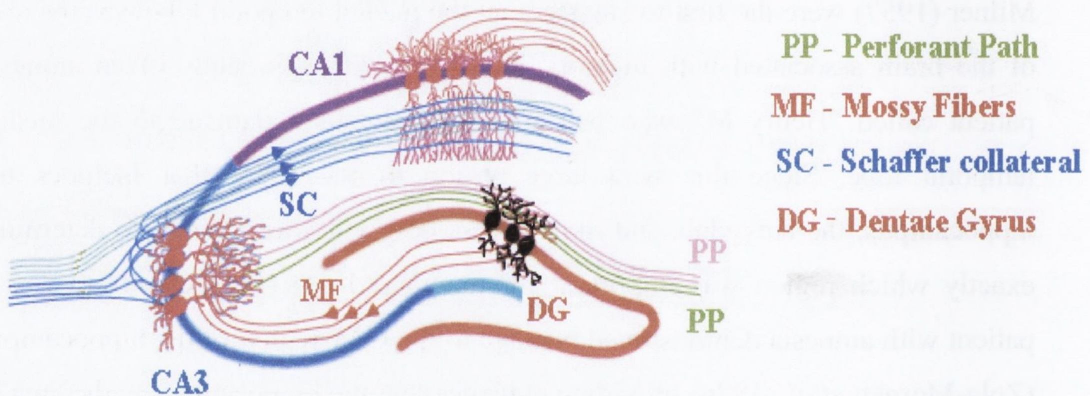


Figure 1.1. The neuronal circuitry of the hippocampus

(Adapted from www.cellscience.com/Reviews5/Storm5.jpg)

1.1.3. Functions of the hippocampus

The first studies on the role of the hippocampus in memory suggested that it was mainly involved in memory processes such as spatial memory (O'Keefe and Nadel, 1978). It is now know that the hippocampus is also important for long term memory processes such as declarative and recognition memory (Suzuki and Clayton, 2000). O'Keefe and Dostrovsky (1971) characterised spatial memory as the ability of the hippocampus to mediate a neural representative of physical space called a "cognitive map", this was based on the findings that the firing rates of certain neuronal cells in the hippocampus increased when rats entered a novel environment and these were termed place cells, which were identified as the pyramidal cells found in the CA3 and CA1 regions of the hippocampus (O'Keefe, 1979). More recently, Eichenbaum and colleagues (1999) likened the cognitive map to a "Cartesian map" in that it provides metrics for the representation of distances and angles in the environment and place cells reflect the occurrence of the rat at a particular coordinate position within this map.

It has now become increasingly apparent that among the memory functions of the hippocampus, it plays an imperative role in information processing and the regulation of behaviour (Bannerman *et al.*, 2004). Crusio (2001) stated that activation of the hippocampus altered behaviour, he proposed that upon entry into a novel environment, the hippocampus becomes activated and initiates exploratory behaviour, allowing the animal to collect information about the environment. In support of this Moser and colleagues (1994) observed potentiation of dentate synapses in response to exploratory learning. Furthermore other studies described activation of hippocampal neurons coinciding with foraging and exploratory behaviour (Kubie *et al.*, 1990; Nitz and McNaughton, 2004). The hippocampus has also been implicated with emotional-related behaviour such as fear conditioning in rats (Phillips and LeDoux, 1992). The determinations of the various disparate functions of the hippocampus have come a long way since the studies by Scoville and Milner on the patient "Henry M." It is now widely accepted that the hippocampus is involved in multifarious CNS processes, from the different forms of learning and memory to information processing, exploratory and anxiety-related behaviour. Interestingly Bannerman and colleagues (2004), suggested that these diverse processes may be associated with different hippocampal sub-regions. They suggested the hippocampus is

divided into dorsal and ventral regions, with the dorsal region having a preferential role in learning and memory and the ventral region being associated with anxiety-related behaviour.

1.2. Ageing

Ageing is a complex process and throughout history a proper 'theory of ageing' was difficult to formulate. In the 19th century there was a boom in the interest of gerontological thinking prompting Adolphe Quetelet to state "A man is born, grows up and dies according to certain laws that have never been properly investigated". This was true at the time, but more recently there has been a progressive increase in the literature on the processes involved in ageing. Harmon (1956) was the first to conclude that the accumulation of endogenous oxygen free radicals generated in cells by aerobic metabolism resulted in damage to DNA, proteins and lipids and this contributed to the ageing process. This hypothesis of ageing was called the 'free-radical theory' of ageing. Since then various other studies have shown that ageing is associated with increased generation of oxidative species and a decline in the robustness of defence and repair, leading to oxidative damage (Cutler, 1991; Bowling *et al.*, 1995; Beckman and Ames 1998).

There is accumulating evidence to suggest that, with age, there are alterations of the immune system leading to elevated inflammation and/or a decline in immune function and that these are factors which contribute to unsuccessful ageing (Renshaw *et al.*, 2002; Nikolich-Zugich, 2005; Licastro *et al.*, 2005). Indeed Franceschi and colleagues (2000) proposed that a new definition of the ageing process, is a global reduction in the ability to cope with stressors and a concomitant progressive increase in pro-inflammatory status; this was called 'inflamm-aging'. Studies have shown that the phagocytic activity of the innate immune cells, macrophages, are altered with age (Plowden *et al.*, 2004), and that the expression and functionality of toll-like receptors (TLRs) on macrophages are down-regulated with age (Renshaw *et al.*, 2002). Others have shown that ageing is associated with increased pro-inflammatory cytokine production (Licastro *et al.*, 2005; Johnson, 2006). Furthermore T cell function is modified with age, T cell receptor and co-stimulatory signalling pathways are blunted and the secretion of cytokines by stimulated naïve T cells is reduced (Nikolich-Zugich, 2005).

1.2.1. Ageing and the CNS

Although the detrimental effects of ageing are evident in all tissues, it is now widely accepted the CNS is particularly affected by age. Indeed ageing is accompanied by learning and memory problems (Driscoll *et al.*, 2003). Consistent with the role of the hippocampus in learning and memory, there is an abundance of evidence to suggest that the area of the CNS most affected by ageing is the hippocampus and inflammatory changes in this area have contributed to deficits in long term potentiation (LTP) the cellular model of learning and memory (Lynch, 1998; McGahon *et al.*, 1999; Miller and O'Callaghan, 2005; Griffin *et al.*, 2006). Studies have shown behavioural deficits associated with ageing, Liu and colleagues (2005), showed age-related impairments in the behaviour of animals in the hole-board , T-maze and water maze tasks that correlated with inflammatory changes in the hippocampus. Indeed there is now evidence to suggest that the deficits in LTP and behaviour with normal ageing are due to increased inflammatory changes in the hippocampus (Lynch and Lynch, 2002; Maher *et al.*, 2005; Griffin *et al.*, 2006). Furthermore inflammation linked to ageing is considered the major risk factor for neurodegenerative diseases (Streit, 2004; Licastro *et al.*, 2005). According to Heininger (2000), ageing provides the biological background which sets the stage on which the patho-physiological processes in Alzheimer's disease (AD) occur.

1.3. Alzheimer's disease

AD is a neurodegenerative disorder that primarily affects hippocampal and neocortical brain regions resulting in a progressive loss of cognitive memory function and ultimately dementia (Streit, 2004; Griffin *et al.*, 2006; Simard *et al.*, 2006). The hallmarks of AD include the presence of extracellular amyloid-β (Aβ) plaques, tau phosphorylation (p-tau) and intracellular neurofibrillary tangles (NFT) (Selkoe, 2003). Amyloid-β (Aβ) peptides are produced as a result of cleavage of Aβ precursor protein (APP) by β- and γ-secretases producing hydrophobic peptides of between 39-43 residues (Yankner *et al.*, 1996). These processes occur with normal ageing and indeed senile plaques, as well as NFT, are widely distributed in the normal aged brain (Heininger, 2000). However, in AD, APP is overexpressed resulting in higher levels of hydrophobic Aβ (Streit, 2004). It is thought that these Aβ deposits are responsible for causing tau

phosphorylation and NFT formation leading to neuronal death and dementia in a theory called the amyloid cascade hypothesis (Hardy and Selkoe, 2002).

However, there is now a school of thought which exposes the view that AD is a result of both the amyloid cascade and neuroinflammation (Allan and Rothwell, 2001; Saastre *et al.*, 2006; Wyss-Coray, 2006). Studies have shown that, accompanying the accumulation of Aβ peptide in amyloid plaques is subsequent microglial-mediated inflammation (Kim and de Vellis, 2005; Rogers *et al.*, 2007). Microglial cells are the immune cells of the CNS and activation of these cells results in the release of neurotoxic substances and pro-inflammatory cytokines which lead to neuronal damage (McGreer and McGreer, 2003). Griffin (1998) suggested that these agents then establish a self-propagating ‘cytokine cycle’ which drives the progression of the disease and plaque formation. Animal models and *in vitro* studies support this hypothesis; studies have shown that treatment of rats and incubation of glial cells with Aβ results in the production of pro-inflammatory cytokines (Minogue *et al.*, 2003; Lyons *et al.*, 2007).

1.4. The immune system

To protect the body from any form of injury the immune system has evolved into the ‘innate immune system’ which is responsible for rapid, immediate, relatively generic, defence and the ‘adaptive immune system’ that responds specifically and requires plasticity and memory to mount a heightened more aggressive immune response (Becher *et al.*, 2000). At the local level, the key cells of the innate immune system are tissue phagocytes (monocytes, macrophages, neutrophils and dendritic cells (DCs)) and, at the systemic level, the key cells are the granulocytes (natural killer cells (NKs), mast cells, eosinophils and basophils) which launch an all-purpose general response. The adaptive immune system involves the cloning of lymphocytes in peripheral immune organs, such as the thymus, spleen and lymph nodes to mount an attack against specifically identified proteins. B lymphocytes produce antibodies to govern the humoral immune response and T lymphocytes possess homing receptors to govern the cell mediated immune response (Akiyama *et al.*, 2000). Functionally the two branches of the immune system are distinct but they work cooperatively; the macrophages of the innate immune system phagocytose antigen and present these

to T cells thus activating the adaptive immune system. On the other hand the adaptive immune system uses the effector mechanisms of the innate immune system to eliminate the insult.

1.4.1. The inflammatory response

Inflammation is the innate immune system's first line of defence to foreign challenge or tissue injury. It functions to identify and eliminate foreign agents, establish a physical barrier against the spread of infection, activate the adaptive immune response and promote restoration and tissue repair (Nathan, 2002). The inflammatory response is initiated following recognition of pathogen-associated molecular patterns (PAMPS); these are molecular structures shared by groups of microbial pathogens such as bacterial proteins, nucleic acids and cell wall components of gram positive and gram negative bacteria and yeast (Xu *et al.*, 2000). Recognition is accomplished via germ-line encoded pattern-recognition receptors (PRR) which are evolutionarily-conserved. They allow the innate immune system to distinguish self molecules from the pathogen-associated non-self structures and also provides the information concerning the type of invading pathogen and the appropriate effector mechanisms (Xu *et al.*, 2000). Upon receptor recognition, the macrophages phagocytose the invading organism and launch an all-purpose inflammatory response characterized by increased blood flow and extravasation of plasma proteins to the site of damage, rapid recruitment of phagocytic cells, neutrophils and NKs. They also facilitate the maturation, differentiation and migration of DCs. Dendritic cells are the sentinel cells that leave the site of damage and activate lymphocytes thus inducing the adaptive immune response leading to invasion of circulating lymphocytes from the vessels into injured tissues to remove the insult (Spector and Willoughby, 1963; Plowden *et al.*, 2004).

1.4.2. Toll-like receptors

Identification of the Toll-like receptor (TLR) family and its involvement in the immune response was a critical finding in the progression of the study of immunology. TLRs are a subset of pattern recognition receptors that are members of the Toll/Interleukin-1 receptor (TLR/IL-1R) family. The two families are sub-divided based on differences in their extra-cellular domains. The TLR family

members contain leucine-rich repeat motifs in their extracellular domain, whereas members of the IL-1R superfamily are characterised by Ig-like structures in their extracellular domain that bind to specific IL-1 related cytokines (Verstrepen *et al.*, 2008). All members of the TLR/IL-1R superfamilies except for IL-1RII share a conserved cytoplasmic signalling domain, which is referred to as Toll/IL-1R (TIR) domain (Kopp and Medzhitov, 1999; Xu *et al.*, 2000; Mantovani *et al.*, 2001). The TIR domain consists of 135-160 residues that form a central five-stranded parallel beta-sheet that is surrounded by a total of five helices on both sides (Xu *et al.*, 2000). The TIR domain of the TLR/IL-1Rs is crucial for homo- and hetero- dimerization with other receptors, co-receptors and signalling molecules in the signal transduction process (Xu *et al.*, 2000).

The TLR family consists of 13 members which recognise microbes, microbial products, yeast cell wall components, lipopolysaccharide (LPS), flagellin, viral double-stranded RNA and bacterial DNA containing cytosine and guanosine motifs linked with a phosphodiester bridge (CpG-DNA) (Verstrepen *et al.*, 2008). The interaction between any of these ligands with their respective receptor results in the secretion of anti-bacterial peptides, defensins and cytokines and recruitment of cells of the adaptive immune system to induce a general inflammatory response, and a specific immune response, to remove the pathogens (Renshaw *et al.*, 2002). According to their ligands, TLRs can be categorised into two main groups: TLRs that are responsive to lipid-based PAMPS and TLRs that are responsive to nucleic acid-based PAMPS (Brentano *et al.*, 2005). TLR1 and TLR2, and TLR2 and TLR6, recognise the cell wall components of Gram-positive bacteria and yeast such as peptidoglycan, lipoprotein, leptospira (Qureshi and Medzhitov, 2003). TLR4 recognises gram-negative bacteria in particular LPS the 'molecular signature' of gram negative bacteria and endogenous ligands such as several heat shock proteins, beta defensin 2 and hyaluronic acid (Qureshi and Medzhitov, 2003). TLR3, TLR5 and TLR9 recognise double-stranded RNA (poly I:C), bacterial flagellin and unmethylated CpG-DNA respectively (Hayashi *et al.*, 2001, Alexopoulou *et al.*, 2001). TLR7 and TLR8 bind uracil-rich single-stranded RNA present in viruses and TLR11 recognises uropathogenic bacteria (Jurk *et al.*, 2002). The ligands for TLR10, TLR12 and TLR13 have not been identified (Tsan and Gao, 2004; Uenishi and Shinkai, 2008).

1.4.3. Lipopolysaccharide structure and signalling

LPS is a complex glycolipid and PAMP, produced from the cell wall of gram-negative bacteria and is a non-specific activator of the immune system (Lacosta *et al.*, 1999). LPS is an amphipathic molecule consisting of three distinct chemical structures: the hydrophobic lipid A moiety, which anchors it to the outer membrane; a well-conserved surface-exposed hydrophilic oligosaccharide, designated the core; and a hypervariable polysaccharide, the O-antigen (Steeghs *et al.*, 2001).

The LPS response is initiated by binding to four conserved protein, LPS binds to the LPS binding protein (LBP) a 60kDa glycoprotein which extracts single molecules from LPS micelles and transfers them to the surface receptor CD14, a 55-kD glycosyl-phosphatidylinositol-anchored cell membrane receptor on monocytes and macrophages (Sweet and Hume, 1996). The CD14 receptor has no cytoplasmic domain and cannot mediate LPS signalling, so the LPS/CD14 complex activates two proteins that comprise the essential signalling complex of the LPS response, TLR4 a type I integral membrane glycoprotein and MD-2 a small cysteine-rich glycoprotein. MD-2 binds to the ectodomain of TLR4 in the endoplasmic reticulum and then transits to the cell surface in an active TLR4-MD-2 complex (Kennedy *et al.*, 2004). Activation of the TLR4-MD-2 intracellular signalling cascades is facilitated by the recruitment of four functional adaptor molecules: myeloid differentiation primary response gene 88 (MyD88), MyD88-adaptor-like (MAL), TIR domain-containing adaptor-inducing IFN-β (Trif) and TRIF-related adaptor molecule (TRAM) to the receptor complex (Miggin & O'Neill, 2006).

Activation of TLR4 predominantly results in recruitment of MyD88-dependent signalling pathways, as MyD88 is the central adaptor molecule interacting with all TLRs except TLR3 (Yamamoto *et al.*, 2002). However it should be noted that TLR4 can also signal using a MyD88-independent pathway through the recruitment of the adaptor molecules TRIF and TRAM resulting in type 1 IFN induction (Fitzgerald *et al.*, 2003). In the MyD88-dependent pathway, the adaptor molecule MAL is first recruited to the TLR4-MD2 complex. MAL has two main roles in TLR4 signalling, firstly it acts as a bridging adaptor between TLR4 and MyD88 (Kawai & Akira, 2006). MyD88 recruits members of the IL-1R-associated kinase (IRAK) family this results in IRAK phosphorylation and

dissociation from MyD88 (Xu *et al.*, 2000). Activated IRAK associates with another adaptor molecule, necrosis factor–receptor associated factor 6 (TRAF6) resulting in its activation, followed by formation and activation of a ternary complex composed of TGF-β-associated kinase-1 (TAK-1), TAK-1-binding protein-1 (TAB-1), TAB-2 and TAB-3. Activated TAK-1 complex then activates the Iκκ complex consisting of Iκκα, Iκκβ and Iκκγ which catalyzes IκB phosphorylation. IκB is then destroyed by the proteasome pathway, allowing NF-κB to translocate into the nuclei, resulting in pro-inflammatory cytokine production (Kawai & Akira, 2006). The second role of MAL is to directly interact TRAF6 through a TRAF interaction motif resulting in the activation of the mitogen activated protein kinase pathway (MAPK) p38 MAPK and c-Jun NH_2-terminal kinase (JNK) and transactivation of the p65 subunit of NF-κB (Mansell *et al.*, 2004). Activation of these transcription factors leads to pro-inflammatory gene transcription and protein translation resulting in the production of inflammatory molecules, such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) (Tetsuka *et al.*, 1996), which forms the basis of the innate inflammatory response.

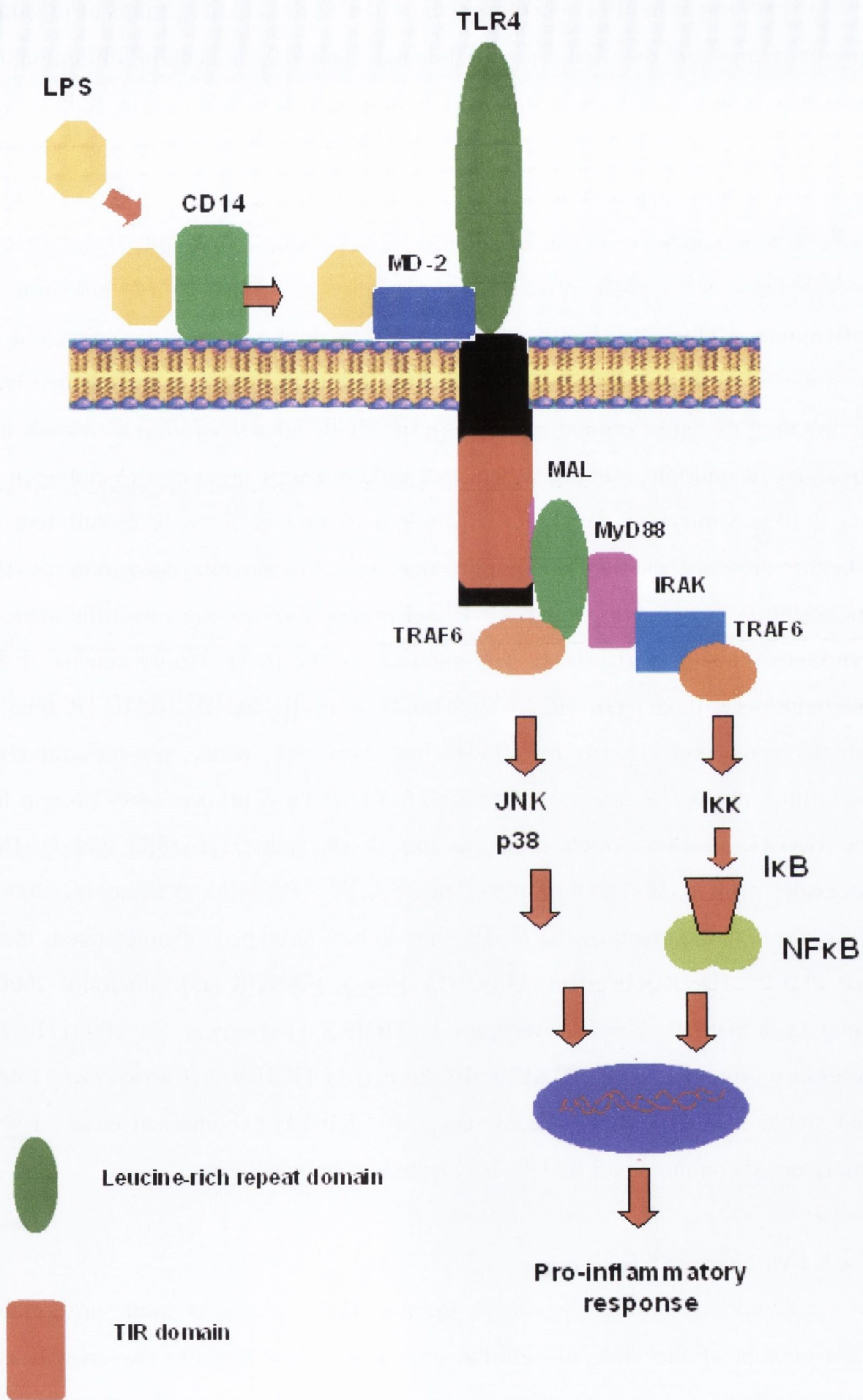


Figure 1.2. LPS signalling cascade

1.4.4. The Interleukin-1 receptor family

Pattern-recognition receptors such as the TLRs identify groups of invading microbes and are the first line of defence launching a general inflammatory response upon the first encounter with a pathogen. However a more orchestrated response requires specific activation of cells of both the innate and adaptive immune systems and communication between the intricate networks of immune cells is undertaken via powerful soluble factors called cytokines. These diverse polypeptides are rapidly produced in response to tissue injury, infection or inflammation (Xiao *et al.*, 2007) and have been shown to alter the behaviour of immune cells (Becher *et al.*, 2000). The IL-1R subfamily superfamily is involved in binding to, and responding to, specific IL-1 related cytokines, which are involved in multiple immunological and inflammatory processes (Verstrepen *et al.*, 2008). Similar to the TLRs, upon ligand binding there is recruitment of adaptor proteins to the receptor complexes, downstream activation of the transcription factors NFκB and AP-1, and production of other pro-inflammatory cytokines (Huang *et al.*, 2004). The members of the IL-1R family consist of the Interleukin-1R type I (IL-1RI) which binds to IL-1α and IL-1β, IL-1R type II which binds both IL-1α and IL-1β but does not induce pro-inflammatory signalling, the IL-1R accessory protein (AcP) which is an accessory protein for the IL-1RI, IL-18R which binds to the IL-18 ligand (IL-18R) and IL-18R accessory protein (IL-18RAcP, also termed AcPL) (O'Neill and Dinarello, 2000). Recently new members of the IL-1R superfamily have been characterised, these are T1/ST2, IL-1R-related protein 2 (IL-1Rrp2) (O'Neill and Dinarello, 2000), three Ig domain IL-1-related receptor-1 (TIGIRR-1) (Born *et al.*, 2000), IL-1R accessory protein-like (IL-1RAPL) also known as TIGIRR-2 (Carrie *et al.*, 1999) and single Ig-interleukin-1-related receptor (SIGIRR) (Thomassen *et al.*, 1999) (more details on the novel IL-1Rs are located in section 1.8.)

1.4.5. Cytokines and disease

Cytokines stimulate early inflammatory reactions and are major determinants of the state of cellular activation. They regulate the growth and differentiation of specific lymphocytes and recruit and activate phagocytes to the sites of infection (Feghali and Wright, 1997). Cytokines elicit their actions by binding to specific membrane receptors on target cells activating intracellular

signalling cascades, which ultimately lead to gene transcription and synthesis of proteins essential for the inflammatory response (Feghali and Wright, 1997). It is now accepted that different types of cytokines are involved in counterbalancing the effects of one another. Cytokines that enhance inflammation are *pro-inflammatory cytokines* and cytokines that seek to inhibit inflammation are *anti-inflammatory cytokines.* IL-1β, IL-6 and TNF-α are pro-inflammatory cytokines that promote inflammation, whereas IL-4, IL-10 and IL-13 are anti-inflammatory cytokines that suppress the activity of pro-inflammatory cytokines (Dinarello, 2000). The direction in which the pendulum of cytokine activation swings, whether it be pro- or anti- inflammatory is critical to the life and death of neurons and a shift of this balance towards pro-inflammatory cytokines has been associated with development of neurodegenerative diseases (Venters *et al.*, 2000).

Inflammation is a beneficial host response and is crucial to maintaining homeostasis when activated in a regulated manner for a defined period of time. However, it is now recognized that sustained, excessive or inappropriate inflammation can be more detrimental than the initial inflammatory stimulus (Nathan, 2002). While the actions of cytokines in the initiation and regulation of inflammatory responses were originally thought to be confined to only peripheral tissues (Hulshof *et al.*, 2002), it is now known that cytokines play fundamental roles in the inflammatory response in peripheral tissues but also in the CNS, thus cytokines are both immuno-regulators and neuro-modulators (Szelenyi, 2001). It has been suggested that, inflammation in the CNS contributes to the aetiology of a number of neurodegenerative disorders (Lonergan *et al.*, 2002). Cytokines appear to be at the centre of the process of neurodegeneration, indeed studies have shown that the dynamic balance between the effects of pro-inflammatory and anti-inflammatory cytokines determines the outcome of disease (Dinarello, 1988). Significantly altered expression of various cytokines in the brain is observed following viral or bacterial infections (Beadling and Slifka, 2004), ischemia (Yu and Lau, 2000) and in a variety of neurodegenerative disorders such as Alzheimer's disease (Akiyama *et al.*, 2000).

1.5. The CNS defence system

Originally it was believed that an immune response could not be mounted in the CNS, as the very means by which it would protect itself would be destructive to the billions of post-mitotic neurons contained within the CNS. Similarly the so-called 'immune-privileged' state of the CNS was supposedly maintained by the highly complex structure of the vasculature, termed the blood brain barrier (BBB), which contains tight junctions that isolates the CNS from the periphery, the absence of lymphatic drainage that would allow direct communication between the CNS and the immune system, the lack of true antigen-presenting cells required for T cell activation and the assumption that immune cells are restricted access to the CNS (Carson and Sutcliffe, 1999). However it is now widely accepted that the CNS is constantly surveyed by the immune system and that there is bidirectional communication between the immune and nervous systems (Carson and Sutcliffe, 1999).

1.5.1. Inflammation in the CNS

The CNS is home to professional antigen-presenting cells, macrophages and dendritic cells located in the meninges and choroid plexuses (McMenamin, 1999) and perivascular macrophages surrounding cerebral vessels at the BBB (Thomas, 1999) suggesting that these immune cells survey all portals of entry for pathogens into the CNS ready to mount an immune response. Similarly, the BBB is not such an insurmountable barrier as immunocompetent T-cells and leukocytes have been shown to enter the CNS and resolve infections (Carson and Sutcliffe, 1999). Moreover CD14 and TLR have been found expressed in the circumventricular organ; this suggests that these receptor complexes might bind to Gram-negative bacterial products such as LPS and initiate LPS signalling and transcription of pro-inflammatory cytokines during endotoxaemia within these organs (Lacroix *et al.*, 1998). Recently the CNS itself has been implicated in mediating the balance between neuronal function and self defence. The CNS parenchyma is host to the resident macrophages, microglia and astrocytes, these cells functioning cooperatively to govern a resident innate immune system and activation of these cells leads to increased release of neurotoxic agents and pro-inflammatory cytokine production.

1.5.2. Microgial cells

Microglial cells are widely regarded as the resident mononuclear phagocytes of the central nervous system as they share many similar properties including chemotaxis, phagocytosis and antigen presentation (Szczepanik *et al.*, 2001). They were first recognised by Franz Nissl who named them "Staebchenzellen" (rod cells) for their rod shaped nuclei (Nissl 1899). However the Spanish neuroanatomist Pio del Rio Hortego (1927) was the first to define microglia as a specific class of brain cell using silver carbonate staining. Despite intense study, the precise origin and cell lineage of microglia are still precisely unknown. One school of thought is that microglial progenitors are derived prenatally from peripheral early embryonic macrophage-like mesodermal precursor cells that colonise the CNS (Chan *et al.*, 2007). Another view still commonly held is that microglia arise from circulating blood monocytes which take up residence in the CNS during the embryonic, fetal and perinatal stages (Kim *et al.*, 2005).

Microglia were first characterised as cells with 'wavy processes beset with spines' due to the presence of delicate spines and thread-like branched processes on the surfaces (Rio Hortega, 1927). However, subsequent studies revealed that microglial cells had the capacity to rapidly respond to their microenvironment, undergoing several changes in function such as in migratory, phagocytic and antigen presenting capacities (Laurenzi *et al.*, 2001). Accompanying these microglial functional changes were distinct morphological changes that occur in a graded fashion, leading to the classification of microglia into four types based on their activation status: resting ramified microglia, amoeboid phagocytic microglia, activated non-phagocytic microglial cells and activated microglial cells (Sasaki *et al.*, 1993).

Microglia serve as sentinel cells involved in immunosurveillance and they are also the first line of defence as they respond to non-specific 'danger' signals by direct glial-immune cell interactions and by CNS-specific inflammatory mediators (Carson and Sutcliffe, 1999). Under "normal" physiological conditions resting ramified microglia are distributed ubiquitously throughout the entire CNS comprising 5-20% of the glial population. This form of microglial cell displays a quiescent phenotype, with a small cellular body and highly ramified morphology including multiple long branching processes extending from it. These processes

are constantly moving and surveying the surrounding environment, sensitive to even minor physiological perturbations (Chan *et al.*, 2007). Ramified microglia are unable to phagocytose cells and are incapable of endocytic activity (Aloisi *et al.*, 2000). These resting microglial cells constitutively express a variety of surface molecules such as CD11a, CD11b and CD11c but display little or no other immunomolecules and thus are extremely poor antigen presenting cells (Akiyama and McGeer, 1990). The purpose of this state is to maintain a constant level of available microglia to detect and fight infection, while maintaining an immunologically silent environment.

Following traumatic brain injury, deprivation of oxygen and nutrients in ischemia, neurotoxic injury such as excitotoxic damage, viral infections and immunological challenge, the brain responds in a well-defined manner by causing graded activation, migration to the damaged sites and proliferation of microglia in a process called 'gliosis' (Stoll *et al.*, 1999; Acarin *et al.*, 2000; Benveniste *et al.*, 2004). Ramified microglia become progressively activated they retract cellular processes and transform into small rounded cells with thick shortened branches called ameboid microglia. This shape allows the microglia free movement throughout the neural tissue, which allows it to fulfil its role as a scavenger cell. Ameboid microglia are able to phagocytose debris, but do not fulfill the same antigen-presenting and inflammatory roles as activated microglia (Kreutzberg *et al.*, 1996). Activated non-phagocytic microglial cells are an intermediate phase of microglial state, microglia appear as "bushy" ameboids and they undergo rapid proliferation in preparation for full activation. The cells are capable of presenting antigen and mediating inflammatory signalling but are unableto phagocytose foreign material (Kreutzberg *et al.*, 1996).

Activated phagocytic microglial cells are the most responsive form of microglia, these cells retract cellular processes and their cell bodies swell and take on a large, ameboid shape, they upregulate and express distinct phenotypic markers, become motile and adopt full immune effector functions. Activated phagocytic microglia perform several innate immune functions including phagocytosis, antigen presentation, and production of pro-inflammatory cytokines, oxygen radicals and nitric oxide (NO) (Aloisi, 2001). In addition to being the principal source of pro-inflammatory cytokines, microglia are also the main target of cytokines, mediating microglial crosstalk leading to further

microglial activation and modulating the glial response to brain injury (Acarin *et al.*, 2000). Activated microglia are also involved in the recruitment and accumulation of leukocytes into the CNS. This is achieved by up-regulation of cell adhesion molecules including intracellular adhesion molecule-1 (ICAM-1) which is induced on cerebral vascular endothelial cells and mediates recruitment, adherence and transendothelial migration of leukocytes across the BBB (Wong *et al.*, 1999). It has been reported that chemokines can facilitate the directional movement of inflammatory leukocytes to the site of damage in the brain (Strack *et al.*, 2002). Up-regulation of the chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1β and monocyte chemoattractant protein-1 (MCP-1) were detected in human microglia following Aβ treatment (Lee *et al.*, 2002), MCP-1 mRNA expression was localised to macrophages and reactive microglia following middle cerebral artery occlusion (MCO) (Gourmala *et al.*, 1997) and IFN-γ-inducible protein-10 (IP-10) was increased in microglia and astrocytes following LPS treatment (Ren *et al.*, 1998). Upon activation, microglia express numerous surface receptors when activated, including major histocompatibility complex (MHC) class I and class II glycoproteins, and co-stimulatory molecules such as CD40 and CD80 which promote the CNS inflammatory response (Carson and Sutcliffe, 1999; Aloisi *et al.*, 2000). MHC I and II are molecules expressed on the surface of phagocytic cells such as macrophages, neutrophils and monocytes and B cells. They function to recognise self and non-self antigens and present these fragmented pieces of antigen to T cells inducing T cell activation (Watts, 1997). MHC II are upregulated with age (Griffin *et al.*, 2006) and in various CNS pathologies (Streit *et al.*, 1999). CD40 is a receptor expressed on microglial cells which binds to its ligand on T cells, activation of the CD40 pathway leads to the induction of pro-inflammatory cytokines, chemokines as well as other co-stimulatory molecules (Stout and Suttles, 1996).

1.5.3. Astrocytes

Astrocytes function along with microglial cells to govern an innate immune response in the CNS. Astrocytes as implied by their name are typically star-shaped cells characterised by the presence of several branched processes radiating in all directions from a central soma forming a ‘star-like’ structure. These processes are covered with feathery sheet-like outfoldings of the

plasmalemma terminating in small swellings called ‘endfeet’. Astrocytes have large nuclei relative to the volume of perikaryon and they are derived from the neuroectoderm which is the primitive neuroepithelial cells of the neural tube (Morest & Silver, 2003). Astrocytes contain characteristic intermediate filaments called glial filaments, which are largely polymers of glial fibrillary acidic protein (GFAP), two major classes of astrocytes have been distinguished on the basis of morphology and distribution: fibrous astrocytes and protoplasmic astrocytes. Fibrous astrocytes have many glial filaments, they extend long cylindrical often unbranching processes through nerve fibres and they are mainly located in white matter. Protoplasmic astrocytes have fewer glial filaments; they extend short thick branched sheet-like processes through the nerve cell bodies and are found mainly in grey matter (Miller & Raff, 1984).

Astrocytes are often referred to as the ‘supporting cells’ of the CNS for their role in supporting neurons, microglia and vascular endothelium. The structure of astrocytes reflects their supporting functions, their processes are interwoven and present ubiquitously in the parenchyma of the entire CNS and provide an ideal framework for the support of the intricate network of neural processes; the axons, synaptic endings, dendrites and spines. Furthermore large numbers of processes are intertwined a the outer and inner surfaces of the CNS, where they form the outer and inner glial limiting membranes. The outer glial limiting membrane is found beneath the pia matter and the inner glial limiting membrane is situated beneath the ependymal lining the ventricles of the brain and central canal of the spinal cord. Many of the processes of astrocytes end in expansions on blood vessels called ‘perivascular feet’ where they form an almost complete covering on the external surface of capillaries and contribute to the maintenance of the blood-brain barrier (Hirano & Llena, 2006).

Astrocytes also impart other important roles in the CNS aside from supporting cells, axon terminals at many sites are separated from other nerve cells and their processes by an envelope of astrocytic processes, where astrocytes serve as electrical insulation preventing axon terminals from influencing neighbouring unrelated neurons. Astrocytes are also involved in ion homeostasis, neurotransmitter re-uptake, glycogen storage, neuronal development, phagocytosis of degenerating synaptic axon terminals and neurons and maintaining the functional integrity of synapses (Ridet *et al*., 1997; Temburni &

Jacob, 2001). In addition to these housekeeping roles, astrocytes play an important, but complex, role in the regulation of CNS immune responses and have a physiological function essential for the generation of immune responses within the CNS. Studies have shown that astrocytes become activated in a process termed 'reactive gliosis' in response to many CNS pathologies such as stroke, trauma, tumour growth and neurodegenerative disease (Pekny & Nilsson, 2005). During reactive gliosis there is hypertrophy of the cellular processes and up-regulated expression of GFAP and vimentin (another intermediate filament protein) by reactive astrocytes all participating in the formation an intermediate filament network. Reactive astrocytes also up-regulate the expression of cell surface proteins, they respond to lymphocyte-derived growth factors, present antigen to T cells and secrete a wide variety of factors such as the pro-inflammatory cytokines IL-1α, IL-6 and TNF-α, chemokines and adhesion molecules that regulate the activity of immune cells entering the nervous system in disease (Lieberman *et al.*, 1989; Frohman *et al.*, 1989; Gourmala *et al.*, 1997; Pekny & Nilsson, 2005). Importantly astrocytes also produce a variety of protective factors such as growth factors and neurotrophins (Lieberman *et al.*, 1989; Rudge *et al.*, 1992).

1.5.4. Role of cytokines in the CNS

Neuronal homeostasis and survival is critically dependent on glial functions, which can exert both neuroprotective and neurotoxic influences (Giulian *et al.*, 1993). Cytokines, secreted by activated microglia and astrocytes in response to injury or infection form a cytokine network within the CNS and this network can exert direct actions on neuronal functions contributing to neuronal injury (Benveniste *et al.*, 1992). These actions include disruption of ion homeostasis, excessive neuronal activation, seizures, release and impaired uptake of neurotransmitters such as glutamate, intracellular entry of calcium and release of NO and free radicals (Murray *et al.*,1997; Panegyres and Hughes, 1998; Vereker *et al.*, 2001). Accompanying the detrimental effects of cytokines on neuronal function, cytokines also induce fever, behavioural changes such as increased sleep, anhedonia, lethargy and anxiousness and this constellation of non-specific symptoms is collectively referred to as 'sickness behaviour' (Dantzer, 2004). More recently, further changes initiated by cytokines have been

identified, including activation of genes that initiate or execute apoptosis in glial cells, endothelial cells and neurons (Martin *et al*., 2002; Wang *et al*., 2005). There is evidence that specific cytokines can modulate most, if not all, of these cell functions, and probably have multiple actions on several cells or systems involved in neurodegeneration. Cytokines produced within the CNS also appear to influence cognition, learning, and memory, thus it is pathophysiologically important to control the extent and duration of cytokine activation.

1.6. Pro-inflammatory cytokines

1.6.1. Interleukin-1

Interleukin-1 (IL-1) is a key mediator of host defence responses to disease and injury, and has been implicated in acute brain injury such as stroke or head trauma, and chronic CNS diseases (Dinarello, 1988). The IL-1 family of cytokines includes three potent mediators of inflammatory and immune responses, IL-1α, IL-1β and IL-18 and the endogenous IL-1 receptor antagonist (IL-1ra). The genes of the IL-1 family all contain conserved amino acid motifs, sequence alignments and conserved secondary-structure features, all consisting of a single structural domain formed from 12 β-strands connected by loop regions, arranged in a so-called β-trefoil configuration (Dunn *et al*., 2003).

IL-1α and IL-1β share less than 30% homology and bind to the same receptor so they were thought to have identical biological actions; it is now known that IL-1α is not a potent player in neural-immune interaction (Gosselin and Rivest, 2007). IL-1β is inactive in its immature form (Mosley *et al*., 1987), is the most rapidly expressed cytokine in response to brain damage (Davies *et al*., 1999) and is the prototypic pro-inflammatory cytokine capable of modulating cerebral functions during systemic and localised inflammatory insults (Gosselin and Rivest, 2007). IL-1β is released from several cell types within the CNS including astrocytes, microglial cells and vascular endothelium (Dinarello, 1988). The biologically inactive 31-kDa precursor form, pro-IL-1β, is subsequently cleaved into an active 17-kDa form by IL-1 converting enzyme (ICE) also known as caspase-1, which is a member of a large family of cysteine proteases 1 (Thornberry *et al*., 1992).

Under normal physiological conditions, IL-1β is present in the CNS in trace amounts but is markedly increased in the brain of aged animals (Griffin *et al.*, 2006), in response to stress (Vereker *et al.*, 2001) and in neurodegenerative disorders (Allan and Pinteaux, 2003). IL-1β has been shown to inhibit one form of synaptic plasticity long term potentiation (LTP) in the hippocampus and decrease glutamate release (Kelly *et al.*, 2003). LTP is a cellular model of learning and memory first described by Bliss and Lomo (1973) who reported that trains of high frequency stimulation applied to any of the three synaptic pathways increased the amplitude of the excitatory postsynaptic potentials (EPSPs) in target hippocampal neurons. It has been consistently shown that increased IL-1β concentration in hippocampus results in impairment in LTP (Katsuki *et al.*, 1990; Minogue *et al.*, 2003; Lonergan *et al.*, 2004). Upregulated expression of IL-1β has been shown to induce a variety of effects including activation of macrophages and T cells and signalling cascades (Dinarello, 1988). IL-1β induces COX-2 and iNOS (Serou *et al.*, 1999) and these lead to the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS) causing damage to lipids and proteins that leads to neuronal cell death. Similarly prostaglandins and thromboxanes, are synthesized from arachidonic acid through the COX pathway and are important regulators of inflammation and immune responses (Marnett *et al.*, 1999). IL-1β causes damage to the BBB (Quagliarello *et al.*, 1991), release of neurotoxins such as NO from the vascular endothelium (Bonmann *et al.*, 1997) and causes the upregulation of adhesion molecules involved in the invasion of leukocytes (Wong and Dorovini-Zis, 1992).

1.6.2. Interleukin-1 receptor and downstream signalling

The responses to IL-1β are initiated by binding mainly to IL-1RI, which is 80 kDa in molecular weight found on T-cells, fibroblasts (Dripps *et al.*, 1991). The type II receptors are 68 kDa in molecular weight and are found on B cells and have a greater affinity for IL-1β (Dripps *et al.*, 1991). Studies have shown that astrocytes and neurons express IL-1 receptors (Ban *et al.*, 1991; Tomozawa *et al.*, 1995; Pousset *et al.*, 2001) and these receptors are localized in several brain areas including the hypothalamus and the hippocampus where they are especially found in dentate gyrus (Ban *et al.*, 1991; Bluthé *et al.*, 2000). The constitutive expression of these IL-1 receptors is low, but expression is rapidly up-regulated in

response to injury. Studies have shown that IL-1RI and IL-1RII gene expression is up-regulated in response to focal cerebral ischaemia (Wang *et al.*, 1997) and IL-1RI, IL-1RII and AcP mRNA expression also increases in mouse primary glial cultures exposed to LPS (Parker *et al.*, 2002). Recently, studies have shown that only IL-1RI is capable of transducing an IL-1 signal; IL-1RII has only 29 amino acids in its cytoplasmic domain and cannot transduce a signal (Gosselin and Rivest, 2007), but it is capable of binding to IL-1β and may therefore act as a decoy receptor (Symons *et al.*, 1995). Binding of IL-1β to IL-1RI, results in recruitment of a second subunit of the IL-1RI, AcP, and subsequent formation of a complex with the adapter molecule MyD88 and IRAK. This results in activation of TRAF-6, leading to activation of NFκB and the stress-activated kinases JNK and p38 and subsequently the transcription factor activator protein (AP-1). A well-known consequence of NFκB and AP-1 is transcriptional activation of most pro-inflammatory genes (Gosselin and Rivest, 2007).

1.6.3. Interleukin-6

Interleukin-6 (IL-6) is a multifunctional cytokine expressed by neurons (Peng *et al.*, 2005), microglia (Benveniste, 1998) and astrocytes (Van Wagoner and Benveniste, 1999). IL-6 mediates its effects through binding to the IL-6 receptor, this complexes with the signal transducing receptor gp130 and leads to downstream IL-6 signalling (Van Wagoner and Benveniste, 1999). The IL-6 receptor is present in a membrane bound (mIL-6R) and soluble form (sIL-6R) and interestingly this soluble receptor can act as an agonist of IL-6 signalling leading to an increase in IL-6 sensitivity (Rose-John and Heinrich, 1994). Evidence suggests that IL-6 has diverse roles in the CNS, exhibiting both neuroprotective and neurotoxic effects. IL-6 was shown to enhance neuronal survival following trauma (Hirota *et al.*, 1996) and excitotoxicity (Pizzi *et al.*, 2004) and pre-treatment of cerebral granule neurons with IL-6 reduced glutamate-induced neuronal damage (Peng *et al.*, 2005). Furthermore IL-6 preserved myelin basic protein (MBP) production and oligodendrocytes in hippocampal slices exposed to excitotoxic insults (Pizzi *et al.*, 2004). In contrast to the neuroprotective effects of IL-6, studies involving the overexpression of IL-6 in the CNS of transgenic mice caused reactive astrocytosis, increased activation of microglial cells and an increase in CNS pro-inflammatory cytokines IL-1β, TNF-α and IL-6 (Di Santo *et*

al., 1996). This suggests that IL-6 may be a 'pro-inflammatory cytokine'. Similarly studies using IL-6 knockout mice, showed that these mice had compromised immune responses, in particular the numbers of activated glial cells and brain macrophages were reduced (Penkowa *et al.*, 1999). These studies highlight the imperative role for IL-6 in initiating, orchestrating and amplifying the inflammatory response. IL-6 has also been implicated as having a contributory role in neurodegenerative diseases. Under normal physiological conditions IL-6 is present at low levels, however IL-6 is increased and overexpressed during brain injury and disease (Van Wagoner and Benveniste, 1999), in response to permanent focal cerebral ischemia (Wang *et al.*, 1995), following treatment with LPS (Lee *et al.*, 1993), in response to the pro-inflammatory cytokines IL-1β and TNF-α (Lee *et al.*, 1993) and in both acute and chronic neurodegenerative disorders (Benveniste, 1998). In summary, IL-6, when tightly regulated, may have protective influences but over-expression and unregulated expression of IL-6 can lead to neuronal damage.

1.6.4. Tumour necrosis factor (TNF)-α

TNF-α is a pro-inflammatory cytokine and often referred to as the 'master regulator' of the immune response. It is produced by activated microglia cells (Nakajima *et al.*, 2004) and astrocytes (Brenner *et al.*, 1993) and is synthesized as a membrane-bound precursor (26kDa) and undergoing proteolytic cleavage to produce the soluble active form (17kDa) (Solomon *et al.*, 1997). It is believed to be the key initiator of immune-mediated inflammation and is induced following LPS treatment (Nakijama, 2004) and in response to ischemia (Shohami *et al.*, 1999); TNF-α is cytotoxic to neurons (Venters *et al.*, 2000) and studies inhibiting TNF activity using TNF binding protein (TBP), a specific anti-TNFα antibody (that binds to TNFα and interferes with its binding to the cell-surface receptor), reduced oedema, BBB impairment and neurological dysfunction following closed head injury (Shohami *et al.*, 1996). Evidence supports the concept that TNF-α is involved in neurodegenerative diseases, and has been linked with the pathogenesis of Alzheimer's disease (Tobinick *et al.*, 2006) while its concentration is increased in striatum and cerebrospinal fluid of patients with Parkinson's disease (Mogi *et al.*, 1994).

1.7. Anti-inflammatory cytokines

1.7.1. Interleukin-1 receptor antagonist

IL-1 receptor antagonist (IL-1ra) competes with IL-1β for binding to IL-1RI (Greenfeder *et al.*, 1995). Unlike IL-1β that binds to the IL-1RI at two receptor binding sites, IL-1ra can only bind to IL-1RI at one receptor binding site (Greenfeder *et al.*, 1995) and, upon binding, IL-1ra cannot associate with AcP and it is unlikely to induce any intracellular IL-1 response (Dunn *et al.*, 2003). IL-1ra has been shown to block most of the *in vitro* and *in vivo* effects of IL–1β, including inflammatory and pyrogenic responses (Bluthé *et al.*, 1995). The production of IL-1ra is stimulated by adherent IgG, other cytokines and bacterial or viral components indicating a role for IL-1ra in chronic inflammatory and infectious diseases (Arend *et al.*, 1998). Studies on the functional consequences of over-expression of IL-1ra have demonstrated an important role for IL-1ra as an endogenous inhibitor in ischemic brain injury (Betz *et al.*, 1995). Studies in IL-1ra knockout mice have revealed the functional cost of an absence of IL-1ra; intraperitoneal injection with LPS was more lethal in IL-1ra knockout mice than in wildtype mice, indicating that IL-1ra is important in host defence against endotoxin-induced injury (Arend *et al.*, 1998). Analysis of the expression profiles of IL-1ra following infection reveals a role for IL-1ra as an acute phase protein in the host defence; for example high levels of IL-1ra were present in the plasma of patients after surgery with adult sepsis (Rogy *et al.*, 1994). In a similar way, IL-1ra mRNA expression was increased in the hypothalamus, hippocampus and cerebellum in response to chronic infusion with IL-1β (Ilyin and Plata-Salaman, 1996) and increased in focal areas of the rat brain following ischemia (Wang *et al.*, 1997). These expression patterns emphasise the role of IL-1ra as an endogenous antagonist of the IL-1 system and are consistent with an anti-inflammatory role in the brain. Indeed the role of IL-1ra as a neuroprotectant was illustrated in experiments involving the administration of recombinant IL-1ra into the brain of rodents which inhibited brain damage caused by cerebral ischemia, brain injury or excitotoxins (Relton and Rothwell, 1992).

1.7.2. Interleukin-4

IL-4 is a 20kDa anti-inflammatory cytokine produced by T helper type 2 (Th2) cells. IL-4 promotes the differentiation of antigen-stimulated naïve T cells to Th2 cells, which produce IL-4 and other anti-inflammatory cytokines such as IL-5, IL-10 and IL-13 (Nelms *et al.*, 1999). IL-4 inhibits the release of IL-1β and up-regulates IL-1ra (Vannier *et al.*, 1992) and has been reported to inhibit the expression and release of LPS-induced pro-inflammatory cytokines by macrophages (Zhou *et al.*, 1994). IL-4 also suppresses the induction of microglial cell surface molecules like MHC class II complexes which are essential for their functions as antigen presenting cells (Chao *et al.*, 1993; Suzumura *et al.*, 1994) and prevents the LPS-induced chemoattractant receptor formyl peptide receptor-2 on microglial cells (Iribarren *et al.*, 2003). Similarly it was shown to have immune-modulatory activity against Aβ-induced microglial activation and the subsequent production of the pro-inflammatory cytokines IL-6 and TNF-α (Szczepanik *et al.*, 2001; Lyons *et al.*, 2007). These studies highlight the capacity of IL-4 to dampen microglial activation and decrease production and release of secretory products. In addition to these effects studies have shown that IL-4 has the ability to downregulate and remove activated microglia thus controlling inflammation in the CNS. IL-4 also modulates astrocyte functions and has been shown to inhibit iNOS, NO and TNF-α production by astrocytes (Brodie *et al.*, 1998), suggesting an immunosuppressive role of IL-4 in the CNS. Studies on the functional consequences of an absence of IL-4 in IL-4-deficient or IL-4 knockout mice, have demonstrated an important role for IL-4 in modulating the severity of the expression of diseases. A study in which Experimental Allergic Encephalomyelitis (EAE), an inflammatory autoimmune disease of the CNS and the principal animal model of MS, was induced in IL-4-deficient C57BL/6 mice showed that these mice developed earlier onset and more severe form of clinical disease. The increase was associated with an increased expression of mRNA for the pro-inflammatory cytokines IFN-γ, IL-1 and TNF-α in spinal cord tissue (Falcone *et al.*, 1998). Similarly, studies examining the functional effects of treating animals affected with EAE with IL-4 indicated a therapeutic effect. In a study by Shaw and colleagues (1997) T cells transduced with a retroviral gene construct to express IL-4 were transferred into EAE mice. This resulted in

amelioration of clinical EAE, delayed onset of the disease and a lower average disease score than mice which received IL-10-producing cells and control mice receiving untransduced cells.

1.7.3. Interleukin-4 receptor and downstream signalling

IL-4 mediates its effects through interaction with the IL-4 receptor (IL-4R), which is expressed on haematopoietic, endothelial, and epithelial cells (Nelms *et al.*, 1999). It has also been shown that IL-4 receptors are present on cultured glial cells and neurons (Nolan *et al.*, 2005) and astrocytes (Brodie *et al.*, 1998). The IL-4R is a member of the haematopoietin receptor superfamily and is composed of 140kDa IL-4Rα chain that interacts with a second chain the gamma common (γc) chain, the cytoplasmic domain of the IL-4α receptor contains five tyrosine residues and a short proline-rich sequence termed the 'box-1 motif' (Nelms *et al.*, 1999). Phosphorylation of these tyrosine residues on the IL-4Rα cytoplasmic chain is responsible for activating specific downstream signalling events. The central tyrosine residue (Y497) on the IL-4Rα chain, which is also on the insulin receptor, is critical for activating pathways involved in regulating proliferation of cells and is termed the insulin IL-4 receptor motif (I4R) (Keegan *et al.*, 1994). The gene regulation domain in which the tyrosine residues (Y575), (Y603) and (Y631) reside, is critical for activating IL-4 responsive genes (Nelms *et al.*, 1999).

Binding of IL-4 to its receptor results in heterodimerization of the 140kDa IL-4Rα chain with a second chain the gamma common (γc) chain, results in tyrosine phosphorylation of the Janus-family (JAK) tyrosine kinases, JAK-1 and JAK-3. Activation of these kinases leads to the tyrosine phosphorylation of the IL-4Rα chain itself, in particular the five tyrosine residues and I4R motif. The I4R motif, once phosphorylated, interacts with a 170kDa phosphoprotein called insulin receptor substrate (IRS)-2 due to similarity with the insulin receptor substrate (IRS-1) (Keegan *et al.*, 1994). Phosphorylated IRS-2 molecules act like docking proteins and interact with phosphoinositide-3-kinase (PI-3K) and the adaptor molecule, Grb-2 (Nelms *et al.*, 1999). Activation of PI-3K by triggering activation of downstream kinases such as Akt kinase plays a role in cell survival (Franke *et al.*, 1997) while phosphorylation of Grb-2 leads to activation of the mitogen-activated protein kinases (MAPK) extracellular-regulated kinase (ERK)-

1 and (ERK)-2, which translocate to the nucleus and activate the expression of genes such as c-*fos*. When the tyrosine residues (Y575), (Y603) and (Y631) are phosphorylated by JAK-1 and JAK-3, activation of a group of molecules essential for gene expression occurs, these are the signal transducers and activator of transcription (STAT) molecules. STAT-6 is the primary STAT molecule activated in response to IL-4 stimulation (Nelms *et al.*, 1999). Once phosphorylated, the STAT-6 molecule forms homodimers with other STAT-6 molecules and the dimerized STAT-6 complexes translocate to the nucleus and binds specific DNA motifs in the promoter region of IL-4 responsive genes.

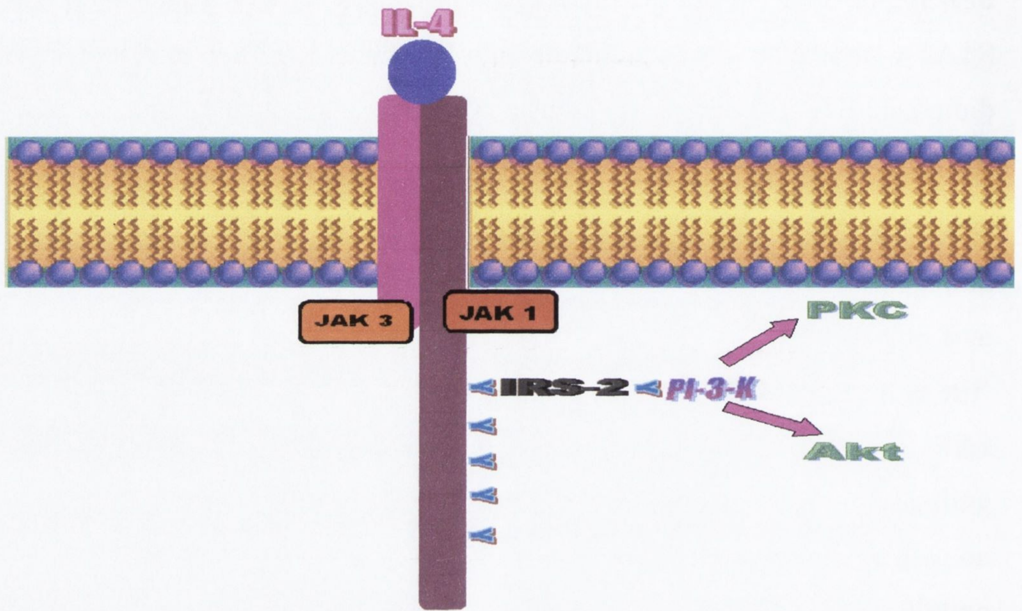
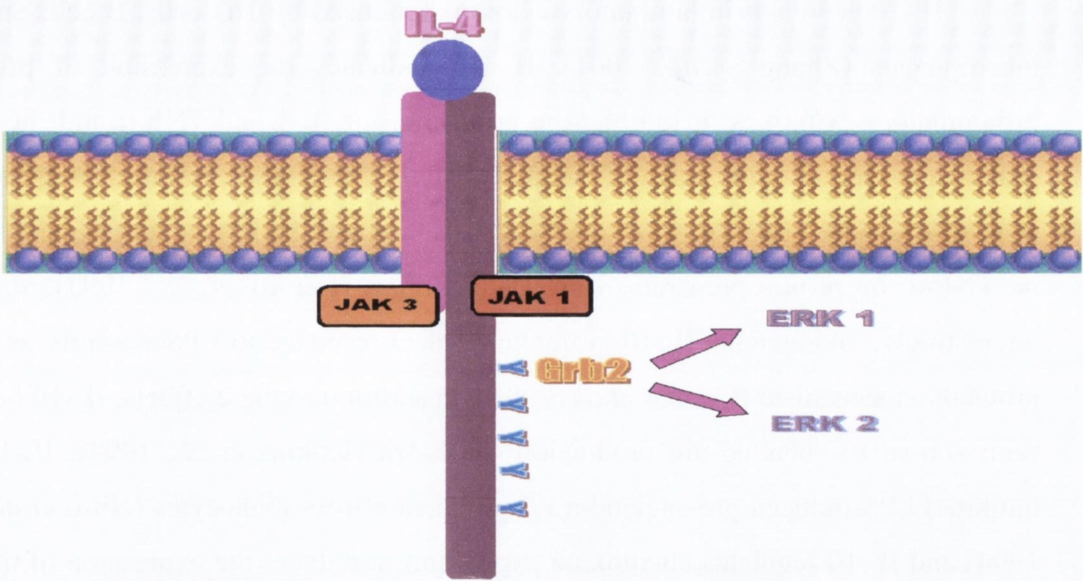


Figure 1.3. IL-4 signalling leading to cellular growth and survival



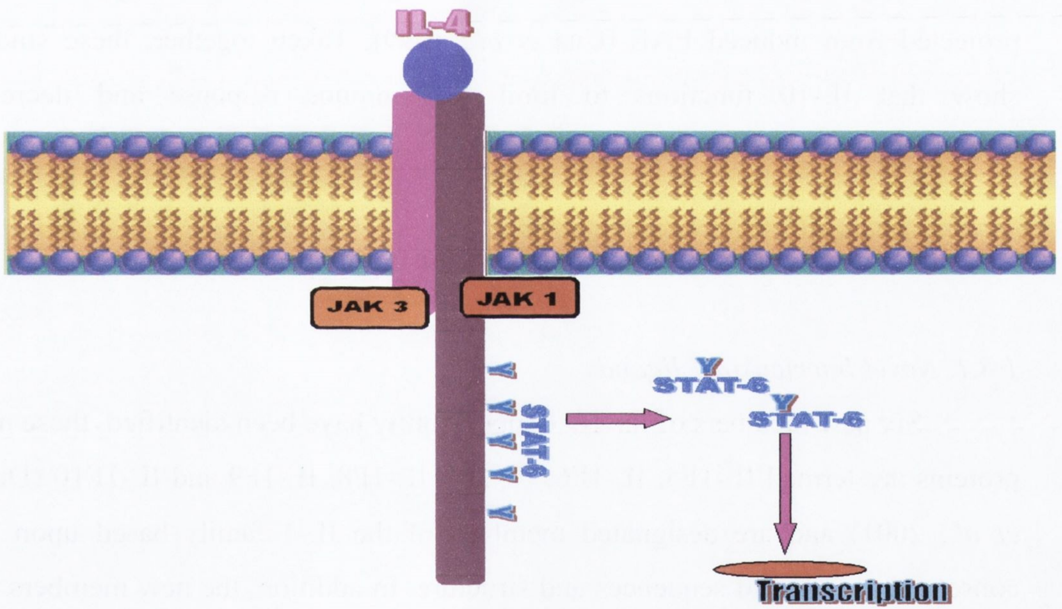


Figure 1.4. IL-4 signalling leading to IL-4 gene expression

1.7.4. Interleukin-10

IL-10 is an anti-inflammatory cytokine produced by Th2 cells, B cells and macrophages (Zhang *et al.*, 2001). IL-10 modulates the expression of pro-inflammatory cytokines; it inhibits the production of IL-1 and TNF-α and these effects are central to its anti-inflammatory activities as these cytokines often act synergistically (Fiorentino *et al.*, 1991). IL-10 abrogates the LPS-induced increase in TNF-α in serum prepared from Balb/c mice (Gerard *et al.*, 1993) and, interestingly, endogenous IL-10 is upregulated in response to LPS possibly as a protective mechanism (Connor *et al.*, 2005). In addition to these effects, IL-10 has been shown to enhance the production of IL-1ra (Jenkins *et al.*, 1994). IL-10 inhibited LPS-induced prostaglandin E2 production from monocytes (Niiro *et al.*, 1994) and IL-10 regulates chemokine expression; it reduces the expression of the chemokine MIP-2 thus dampening the inflammatory response (Berkman *et al.*, 1995). It has been suggested that IL-10 acts as a negative regulator of CNS auto-immune pathologies such as EAE, indeed transgenic mice expressing IL-10 were protected from induced EAE (Cua *et al.*, 1999). Taken together, these studies show that IL-10 functions to limit the immune response and decrease inflammation.

1.8. Novel Interleukin-1 ligand and receptor family members

1.8.1. Novel Interleukin-1 ligands

Six new members of the IL-1 super-family have been identified, these new proteins are termed IL-1F5, IL-1F6, IL-1F7, IL-1F8, IL-1F9 and IL-1F10 (Dunn *et al.*, 2001) and are designated members of the IL-1 family based upon the conserved amino acid sequences and structure. In addition, the new members are all clustered in the same region of human chromosome 2q that contains the other members of the family (except for IL-18 which maps to chromosome 11q) (Smith *et al.*, 2000). More recently, IL-33 (IL-1F11) has been identified as another IL-1 cytokine on the basis of its structural and functional similarities to other IL-1 family members (Schmitz *et al.*, 2005). The IL-1 ligands are all believed to have arisen from a common ancestral gene that underwent multiple duplications (Dunn *et al.*, 2001). The phylogenetic similarity between each member of the IL-1 family is depicted in the Figure 1.5. The new IL-1 family members are expressed on a

variety of organs including the skin, spleen, heart, kidney and brain and are expressed on several cell types for example keratinocytes (Barton *et al.*, 2000) and in monocytes, macrophages and DCs (Barton *et al.*, 2000). Sequence and structural similarities along with these expression profiles suggest a role for the novel IL-1 family members in immunity and inflammation. The exact functions and characteristics of these newly identified members of the IL-1 family has not yet been fully elucidated, however preliminary studies have indicated potential functions for these cytokines.

Little is known about the regulation of expression of IL-1F10, however IL-1F6, IL-1F8 and IL-1F9 are all up-regulated in response to LPS in monocytes (Towne *et al.*, 2004). In contrast IL-33 transcription is modestly up-regulated in response to LPS in DCs and macrophages but is markedly up-regulated in response to TNF-α and IL-1β in fibroblasts and keratinocytes (Schmitz *et al.*, 2005). Upon release IL-33 and IL-1F7 have been shown to be processed in a similar manner to IL-1β by caspase-1 *in vitro* (Kumar *et al.*, 2002; Schmitz *et al.*, 2005). Whereas IL-1F6, IL-1F8, IL-1F9 and IL-1F10 lack signal peptides, have no caspase cleavage sites and appear to be released without processing (Kumar *et al.*, 2000; Smith *et al.*, 2000; Lin *et al.*, 2001).

Similar to other members of the IL-1 ligand family, recent studies have shown that IL-1F6, IL-1F8 and IL-1F9 are capable of inducing pro-inflammatory effects. IL-1F6, IL-1F8 and IL-1F9 were shown to activate NFκB, increase IL-6 and IL-8 concentration and induce the phosphorylation of JNK and ERK in Jurkat, HepG2 and BA/F3 cell lines and in keratinocytes (Towne *et al.*, 2004). Interestingly these effects were dependent on their interaction with IL-1Rrp2 and AcP (Debets *et al.*, 2001; Towne *et al.*, 2004). Furthermore IL-1F8 was shown to stimulate the production of pro-inflammatory cytokines and NO by synovial fibroblasts and articular chondrocytes (Magne *et al.*, 2006). Transgenic expression of IL-1F6 in basal keratinocytes lead to skin abnormalities characterized by acanthosis, hyperkeratosis, the presence of a mixed inflammatory cell infiltrate and increased cytokine and chemokine expression (Blumberg *et al.*, 2007). IL-1F7 binds to the IL-18R with low affinity but does not result in the formation of a ternary complex with AcPL and does not produce IL-18-like signalling activities (Sims *et al.*, 2001; Kumar *et al.*, 2002; Bufler *et al.*, 2004). IL-1F10 has been reported to bind to soluble IL-1R but the significance of this interaction is

unknown (Lin *et al.*, 2001). IL-33 binds to ST2 which is primarily expressed on T cells and activates the NFκB and MAPK signalling pathways and induces Th2-associated cytokines IL-5 and IL-13 and reduces IFN-γ from Th1 cells (Schmitz *et al.*, 2005). Collectively these studies suggest pro-inflammatory pathways of activation of the novel IL-1 family members, however, the only exception is IL-1F5, which antagonised NFκB activation by IL-1F9 (Debets *et al.*, 2001).

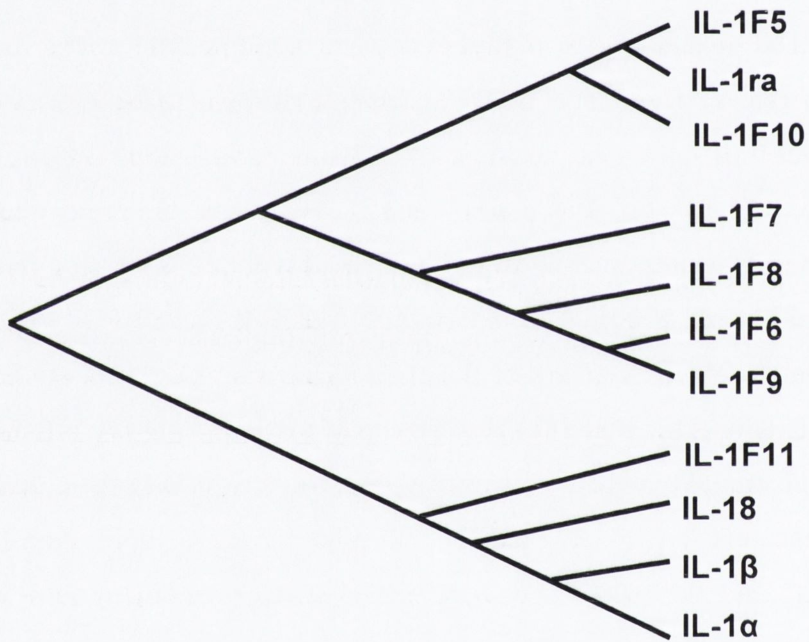


Figure 1.5. The phylogenetic similarities between members of the IL-1 family (Adapted from Barton *et al.*, 2000)

1.8.2 Novel Interleukin-1 receptors

Concomitant with the elucidation of novel members of the IL-1 ligand superfamily, newly characterised members of the IL-1 receptor superfamily were identified; these are T1/ST2, IL-1Rrp2 (O'Neill and Dinarello, 2000), TIGIRR-1 (Born *et al.*, 2000), TIGIRR-2 (Carrie *et al.*, 1999), and SIGIRR (Thomassen *et al.*, 1999). These receptors are members of the TLR/IL-1R superfamily based on the presence of a TIR domain. The TLR/IL-1R family is sub-divided based on differences in their extra-cellular domains. The TLR family members contain leucine-rich repeat motifs in their extracellular domain, whereas members of the IL-1R superfamily are characterised by an Ig-like structures in their extracellular domain depicted in Figure 1.6. (Verstrepen *et al.*, 2008).

Studies on the signalling events associated with the novel IL-1 receptors have revealed interesting results possibly related to their functions. ST2 resembles IL-1RI and IL-18R complexes in its structure (Yanagisawa *et al.*, 1993) and activates JNK, p38 and the transcription factor AP-1 (Brint *et al.*, 2002). However, due to the fact that ST2 was unable to bind to IL-1α, IL-1β and IL-1ra (Yanagisawa *et al.*, 1997), it was originally believed that ST2 was an orphan receptor. More recently studies have revealed that ST2 binds to IL-33 inducing Th2-associated cytokines and MAPK signalling pathways (Schmitz *et al.*, 2005). In relation to IL-1Rrp2, it was shown to mediate the inflammatory effects of IL-1F9. Although IL-1F5 blocked the effects of IL-1F9 in Jurkat cells transfected with IL-1Rrp2, which suggests that IL-1F5 might be acting on this receptor, no antagonism of the binding of IL-1F9 by IL-1F5 was reported and there were no binding studies performed (Debets *et al.*, 2001). In addition to this, IL-1Rrp2 appears to mediate the activation of NFκB by IL-1F6, IL-1F8 and IL-1F9 in cell lines and an antibody for AcP blocked NFκB activation (Towne *et al.* 2004). Furthermore IL-1Rrp2 mRNA was up-regulated in response to LPS and cerebral ischemia suggesting expression is regulated by inflammation and ischemic insults (Andre *et al.*, 2005). TIGIRR-1 and TIGIRR-2 are closely related members of the IL-1R superfamily sharing approximately 63% amino acid sequence homology and an interesting feature of these members is that they have an extra-long carboxyl-terminal tail of roughly 100 amino acids (Sims, 2002). Both receptors are encoded on the X chromosome (Born *et al.*, 2000), and mutations in TIGIRR-2, which is highly expressed in the CNS, have been linked with X-linked mental

retardation (Carrie *et al.*, 1999). TIGIRR-1 is expressed in brain structures involved in hippocampal memory system and it presumably has a role in brain development and/or cognitive functions (Sims, 2002). In a study using chimeric models of TIGIRR-1 and TIGIRR-2 with the extracellular and transmembrane domains of IL-1RI and Acp, TIGIRR-1 and TIGIRR-2 were unresponsive to the cytokines IL-1 and IL-18 and were unable to induce the activation of NFκB (Born *et al.*, 2000). Taken together these findings suggest that TIGIRR-1 and TIGIRR-2 may not have active roles in immune responses despite sequence and structural similarity to the other members of the IL-1R/TLR superfamily but may have a role in cognitive functions. Of increasing interest is the finding that SIGIRR does not induce IL-1-like signalling (Thomassen *et al.*, 1999) and is a negative regulator of TLR/IL-1R signalling (Wald *et al.*, 2003) suggesting SIGIRR has a completely different mode of action to the other receptors (Thomassen *et al.*, 1999).

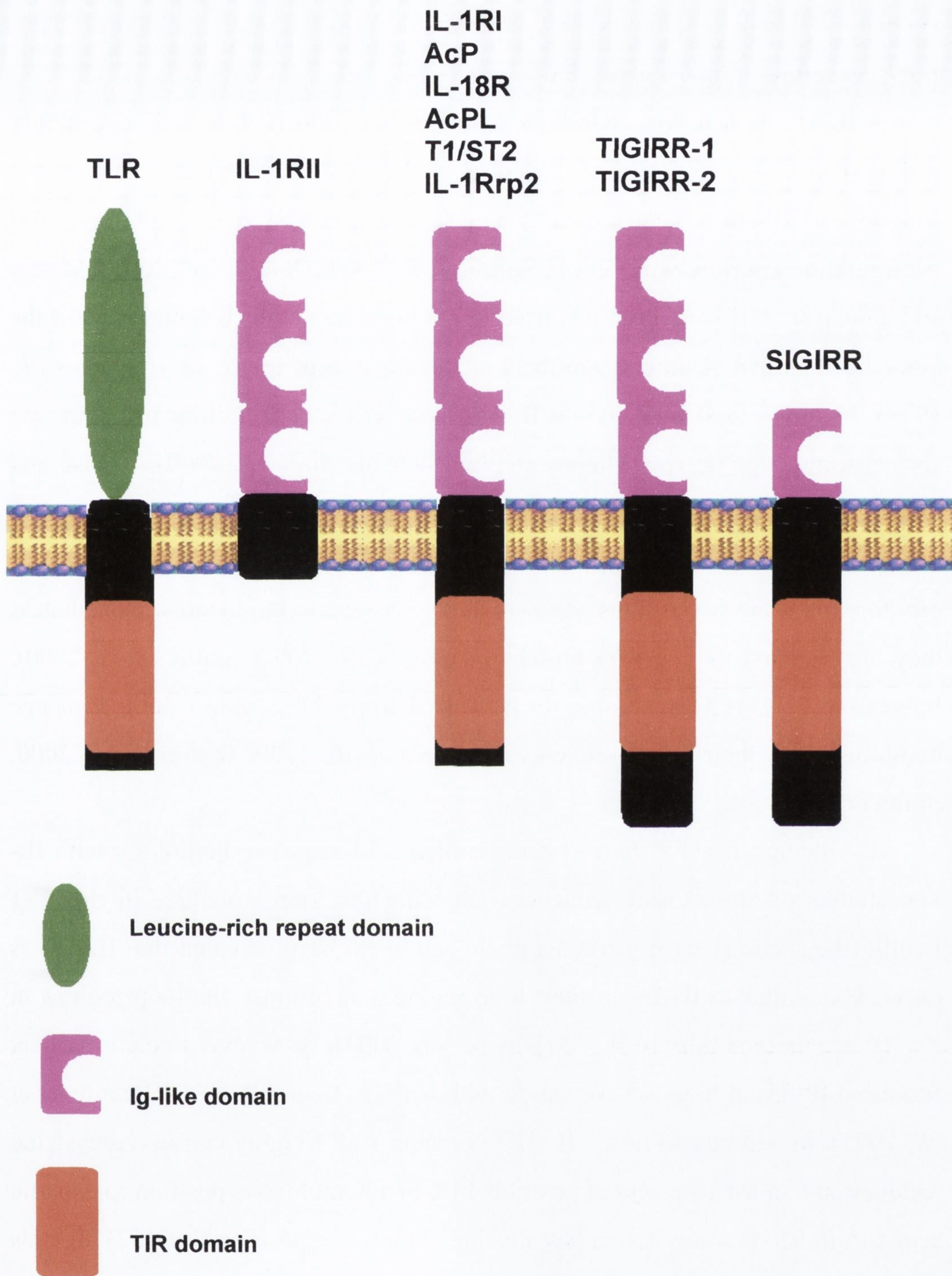


Figure 1.6. Members of the TLR/IL-1R superfamily

1.9. Interleukin-1F5

1.9.1. Structure of Interleukin-1F5

IL-1F5 is a newly characterised member of the IL-1 ligand superfamily which shares the well defined structure of the other IL-1 family members; it consists of 12 β-strands connected by loop regions arranged in a so-called β-trefoil configuration (Barton *et al.*, 2000; Smith *et al.*, 2000; Dunn *et al.*, 2001). Mouse and human IL-1F5 have been localised to chromosome 2 which contains all of the other IL-1 family members (Smith *et al.*, 2000) except for IL-18 (Nolan *et al.*, 1998). In mouse and humans the IL-1F5 gene is located in close proximity on chromosome 2 to IL-1ra (Mulero *et al.*, 1999; Smith *et al.*, 2000). Mouse and human IL-1F5 genes are synthesised as 156 and 155 amino acids long proteins which are 90% identical (Barton *et al.*, 2000) and contain no signal sequence or pro-domain at the N-terminus and no putative N-glycosylation sites. Nonetheless they are secreted as a 17kDa protein (Barton *et al.*, 2000; Smith *et al.*, 2000). Interestingly IL-1F5 is similar to IL-1ra, sharing 52% amino acid sequence homology over their entire sequences (Mulero *et al.*, 1999; Barton *et al.*, 2000; Smith *et al.*, 2000).

Although IL-1F5 shares overall amino acid sequence homology with IL-1ra, studies of amino acid sequences encoding the loop structures in the IL-1 family (the section that imparts agonistic activities) have revealed that IL-1F5 is only 24% similar to IL-1ra in their loop regions. In contrast, the loop regions in IL-1F5 are more similar to IL-1β (Dunn *et al.*, 2003). IL-1ra has a deletion of six residues (49-55) in loop 4-5 compared with both IL-1β and IL-1F5 (Greenfeder *et al.*, 1995). In addition to this, IL-1F5 contains 3 of 4 highly conserved cysteine residues and an aspartic acid at position 148, in a homologous position to aspartic acid 145 in IL-1β while IL-1ra has a lysine at the same position (Lys152) and this is partially responsible for its lack of agonist activity (Mulero *et al.*, 1999). This region was determined to impart agonist activity in IL-1β, as when loop 4-5 from IL-1β is inserted into IL-1ra it confers partial agonist properties (Greenfedar *et al.*, 1995) suggesting that IL-1F5 might not be an antagonist like IL-1ra but rather an agonist for a receptor and it is therefore possible that IL-1F5 through its own receptor may impart a signal that blocks inflammatory pathways indirectly (Dunn *et al.*, 2003).

1.9.2. Expression of Interleukin-1F5

Studies on the expression profile of the novel members of the IL-1 family have revealed that they are not widely expressed and regulation of their expression is stimulus-dependent (Kumar *et al.*, 2000). Despite the similarities in amino acid sequence homology, the expression of IL-1F5 is much less abundant and less widespread than IL-1ra (Barton *et al.*, 2000). IL-1F5 is however highly expressed in lymphoid organs (Smith *et al.*, 2000), placenta (Busfield *et al.*, 2000) and skin (Mulero *et al.*, 1999), and moderately expressed in fetal and adult brain (Mulero *et al.*, 1999). Consistent with its role in inflammation, IL-1F5 is expressed in activated monocytes and macrophages and DCs (Barton *et al.*, 2000; Smith *et al.*, 2000; Mulero *et al.*, 1999; Dunn *et al.*, 2001). The expression of IL-1F5 in other immune cells is less clear; some studies have shown it is expressed in activated B cells and DCs (Smith *et al.*, 2000), whereas others have found no expression in NK cells, lymphocytes or DCs (Barton *et al.*, 2000). On the other hand it has been reported that IL-1F5 is also highly expressed in epithelial cells, such as keratinocytes and is increased in psoriatic skin (Debets *et al.*, 2001; Blumberg *et al.*, 2007) but is not expressed in fibroblasts, endothelial cells or melanocytes (Debets *et al.*, 2001).

Importantly Barton and colleagues (2000), showed that the trophoblastic cell line JEG-3 secreted IL-1F5 as a 17kDa protein and in Cos-7 cells transfected with IL-1F5, IL-1F5 was found in the supernatant and not the cytoplasmic fraction. Similarly IL-1F5 was found secreted in the supernatants and lysates of 293-T cells transfected with IL-1F5 (Debets *et al.*, 2001). Considering that IL-1F5 lacks a secretory signal peptide (Mulero *et al.*, 1999; Kumar *et al.*, 2000; Smith *et al.*, 2000), and can therefore not be released through the conventional endoplasmic reticulum-to-Golgi pathway of secretion, this suggests that IL-1F5 is released through an alternative secretory pathway. IL-1α and IL-1β lack signal peptides and they are released by exocytosis of preterminal endocytic vesicles (Andrei *et al.*, 1999); it is possible that IL-1F5 is secreted through these processes. Alternatively it was suggested that IL-1F5 may be released from cells undergoing necrosis or apoptosis in line with its role in inflammation (Kumar *et al.*, 2000). Additionally, in view of the fact that IL-1F5 lacks a pro-domain at its N-terminus (Kumar *et al.*, 2000) and is released as a 17kDa protein, it might be suggested that it is released without processing in its active form. In support of this, studies have

shown that, although IL-1F5 lacks this pro-domain, its coding regions match with that of cleaved IL-α, IL-1β and IL-1ra suggesting it still retains its tertiary structure and is therefore still active (Kumar *et al.*, 2000).

IL-1F5 is up-regulated in immune cells in response to LPS and phorbol myristic acid (PMA) (Mulero *et al.*, 1999; Barton *et al.*, 2000; Smith *et al.*, 2000) and in response to IL-1β and TNF-α (Debets *et al.*, 2001). Since IL-1F5 is highly expressed in activated monocytes and macrophages (Mulero *et al.*, 1999; Barton *et al.*, 2000; Smith *et al.*, 2000) and since microglial cells share similarities with these cell types, it is reasonable to suggest that microglial cells may be the source of IL-1F5 in the CNS.

1.9.3. Functions and mechanism of actions of Iinterleukin-1F5

Its expression in lymphoid tissue and its similarity to IL-1ra suggest that IL-1F5 plays a role in modulating inflammation. A receptor for IL-1F5 has not been identified; it was originally believed that IL-1F5 may bind to and signal through one of the Toll/IL-1R super family members but, co-precipitation assays using Fc fusions of the IL-1 receptors (IL-1RI, AcP, IL-1Rrp1, IL-1Rrp2, AcpL and T1/ST2) with IL-1F5 demonstrated it could not bind to these receptors (Smith *et al.*, 2000). Born and colleagues (2000) showed that IL-1F5 could not bind to TIGIRR or AcpL, and Dunn and colleagues (2003) showed by superimposition and docking studies that IL-1F5 does not bind to IL-1RI.

Mulero and colleagues (1999) showed that both IL-1ra and IL-1F5 were induced in the monocytic cell line, THP-1 cells by phorbol esters and LPS suggesting an associative role for IL-1F5 as an antagonist. Consistent with the hypothesis that IL-1F5 may have an antagonist role, Barton and colleagues (2000) showed that IL-1F5 did not induce IL-6 production in fibroblasts or endothelial cells or IFN-γ production in the myelomonocytic cell line KG-1 cells and similarly, IL-1F5 did not activate NFκB through the classical IL-1Rs (Debets *et al.*, 2001). However unlike IL-1ra, IL-1F5 did not attenuate IL-6 production from fibroblasts and endothelial cells in response to LPS, or reduce IFN-γ production in response to IL-18 in KG-1 cells (Barton *et al.*, 2000). Further studies by Debets and colleagues (2001) showed that IL-1F5 antagonised the production of NFkB by IL-1F9 in Jurkat cells transfected to express IL-1Rrp2 and demonstrated that the potency of IL-1ra to antagonise IL-1α signalling was about 3 orders of

magnitude less than IL-1F5 suggesting IL-1F5 is a potent antagonist. In contrast Towne and colleagues (2004) reported that IL-1F5 could not activate NFκB in Jurkat T cells, human hepatocellular carcinoma cell line, HepG2 and the mouse B cell line, BA/F3 cells transfected with IL-1Rrp2 or an empty vector, and IL-1F5 could not antagonise the inflammatory effects induced by IL-1F6, IL-1F8 and IL-1F9. Whereas Blumberg and colleagues (2007) showed that the combination of IL-1F6 transgene and IL-1F5 deficiency resulted in heightened inflammation in the skin suggesting that IL-1F5 is an antagonist of IL-1F6 signalling at IL-IRrp2. Results from our previous study revealed an anti-inflammatory role for IL-1F5 in the CNS but not in peripheral cells; we demonstrated that IL-1F5 was capable of attenuating the inflammatory effects of IL-1β and LPS on LTP, pro-inflammatory cytokine production and MAPK activation *in vitro* and *in vivo*. Results from our previous studies showed that the anti-inflammatory actions of IL-1F5 were dependent on its induction of IL-4 and through its interaction with SIGIRR (Costelloe *et al*., 2008). It must be concluded that the current literature on the actions of IL-1F5 are inconsistent; some studies failed to pinpoint a role for IL-1F5 (Barton *et al*., 2000), others suggested it is an antagonist (Debets *et al*., 2001; Blumberg *et al*., 2007) and we have previously reported that IL-1F5 might be an agonist for the previously characterised orphan receptor SIGIRR (Costelloe *et al*., 2008).

Similarly the receptor that mediates the actions of IL-1F5 is unknown, although studies have suggested IL-1F5 acts as an antagonist at IL-1Rrp2 (Debets *et al*., 2001; Blumberg *et al*., 2007), studies have shown that it does not bind to this receptor (Smith *et al*., 2000). Dunn and colleagues (2003) suggested due to the similarity between IL-1F5 and IL-1β, it is possible that IL-1F5 binds to one of the orphan receptors. In support of this, results from our previous study demonstrated a role for SIGIRR in IL-1F5-mediated signalling, but there are no studies to date demonstrating the binding of IL-1F5 to SIGIRR.

1.10. Single immunoglobulin Interleukin-1-related receptor

1.10.1. Structure of SIGIRR

SIGIRR (Single Ig Interleukin-1 related receptor) also known as TIR8 (Toll Interleukin-1 receptor 8) is a newly characterised member of the IL-1R superfamily defined as having a single extracellular Ig-containing domain and an intracellular TIR domain (Thomassen *et al.*, 1999). SIGIRR has been characterised in detail in mouse and human (Thomassen *et al.*, 1999; Polentarutti *et al.*, 2003; Garlanda *et al.*, 2004) and more recently in rat (The UniProt Consortium, 2008). SIGIRR is located on chromosome 7 at the F5 region in the mouse, SIGIRR is located on chromosome 1 at the q41 region in the rat and SIGIRR is located on chromosome 11 at the p15 region in the human. Interestingly most other members of the IL-1R family in human are clustered on chromosome 2 (Yoo *et al.*, 1994), except for AcP which is located on chromosome 3 (Dale *et al.*, 1998), TIGIRR-1 and TIGIRR-2 which are located on the X chromosome (Born *et al.*, 2000) and now SIGIRR which is located on chromosome 11. However, as these receptors are not clustered on the mouse genome this suggests there does not seem to be any functional significance of this clustering (Subramaniam *et al.*, 2004).

There is only one SIGIRR transcript which is 3.5kb long found in mouse (Polentarutti *et al.*, 2003; Garlanda *et al.*, 2004; Lech *et al.*, 2007) and rat (The UniProt Consortium, 2008). Mouse SIGIRR shares 94% sequence alignment with rat SIGIRR (Hubbard *et al.*, 2007). There are four different SIGIRR transcripts found in humans which are 4.4 kb, 2.4kb, 1.5kb and 0.9 kb long (Thomassen *et al.*, 1999). SIGIRR protein is 409, 408 and 410 amino acids long in mouse, rat and human respectively and the peptide sequences of mouse and human SIGIRR are 82% identical (Thomassen *et al.*, 1999; The UniProt Consortium, 2008). Studies have shown SIGIRR is a single pass membrane spanning protein with no signal peptide (Thomassen *et al.*, 1999; Lech *et al.*, 2007). In contrast to other members of the IL-1R family, SIGIRR is composed of a single Ig domain in its extracellular region and this is 117 amino acids long in mouse (Thomassen *et al.*, 1999) and 118 amino acids long in rat and human (Polentarutti *et al.*, 2003; The UniProt Consortium, 2008). The extracellular domain of mouse and rat SIGIRR contains five putative glycosylation sites (Thomassen *et al.*, 1999; The UniProt

Consortium, 2008) and human SIGIRR contains four glycosylation sites (Thomassen *et al.*, 1999). The molecular weight of the mouse glycosylated protein is 75-90 kDa (Lech *et al.*, 2007) and human glycosylated protein is about 50-80 kDa (Thomassen *et al.*, 1999) but the rat glycosylated SIGIRR has not been characterised to date. The cytoplasmic domain of SIGIRR contains a TIR domain and is 271 amino acids long in mouse and human (Thomassen *et al.*, 1999; Polentarutti *et al.*, 2003) and 270 amino acids long in rat (The UniProt Consortium, 2008). The cytoplasmic domain of SIGIRR is unique to the IL-1R family for two reasons. Firstly SIGIRR has two amino acid residues different to the IL-1R in the conserved region (Cys_{222} replaces a Ser and Leu_{305} replaces a Tyr), these residues are believed to be essential for IL-1R signalling (Thomassen *et al.*, 1999). Secondly the cytoplasmic domain of SIGIRR extends that of the typical members by more than 77 amino acids (Thomassen *et al.*, 1999) and although TIGIRR-1 and TIGIRR-2 exhibit this extra long carboxyl-terminal tail their amino acid sequences have no homology to the SIGIRR tail (Sims, 2002). This unusually long cytoplasmic domain is reminiscent of the Drosophillia Toll receptor, which contains two functional sub-domains; the first region is involved in activation of the receptor and the second region is involved in regulating this active domain and making it inaccessible to signalling (Norris *et al.*, 1995).

SIGIRR structure (units)	Mouse	Rat	Human
Chromosomal location	1q41	7F5	11p15
RNA transcripts (kb)	3.5	3.5	4.4, 2.4, 1.5, 0.9
Protein length (amino acids)	409	408	410
Ig domain length (amino acids)	117	118	118
Glycosylation sites (number)	5	5	4
Molecular weight of glycosylated protein (kDa)	75-90	-	50-80
Transmembrane domain length (amino acids)	21	21	21
Cytoplasmic domain length (amino acids)	271	270	271
TIR domain length (amino acids)	148	148	148
Abreviations: kb = kilo base, kDa = kilo Dalton			

Table 1.1. Structure of mouse, rat and human SIGIRR

Mouse SIGIRR

MAGVCDMAPNFLSPSEDQALGLALGREVALNCTAWVFSRPQCPQPSVQWLKDGLAL
GNGSHFSLHEDFWVSANFSEIVSSVLVLNLTNAEDYGTFTCSVWNVSSHSFTLWRAGP
AGHVAAVLASLLVLVVLLLVALLYVKCRLNMLLWYQDTYGEVEMNDGKLYDAYVS
YSDCPEDRKFVNFILKPQLERRRGYKLFLEDRDLLPRAEPSADLLVNLSRCRRLIVVLS
DAFLSRPWCSQSFREGLCRLLELTRRPIFITFEGQRREPIHPALRLLRQHRHLVTLVLWK
PGSVTPSSDFWKELQLALPRKVQYRPVEGDPQTRLQDDKDPMLIVRGRAAQGRGMES
ELDPDPEGDLGVRGPVFGEPPTPLQETRICIGESHGSEMDVSDLGSRNYSARTDFYCLV
SEDDV

Rat SIGIRR

MAGVCDRVPNFLSPSEDQALGPALGSAVALNCTAWVFSRPQCPQPSVQWLKDGLAL
GNGSHFSLHQDFWVSDNFSEIVSSVLVFNLTKAEDYGTFTCSAWNVSSHSFTLWRAGP
AGHVAAVLASLLVLVVLLLVALLYVKCRLNVLLWYQDTYGEVEMNDGKLYDAYVS
YSDRPEDRKFVNFILKPQLERRRGYKLFLEDRDLLPRAEPSADLLVNLSRCRRLIVVLS
DAFLRRPWCSQSFREGLCRLLELTRRPIFITFEGQRREPIHPALRLLRQHRHLVTLLLWK
PGSVTPSSDFWKELQLALPRKVQYRPVEGDPQTRLQDDKDPMLIVRGRAAQGRAMES
ELDPDPEGDLGVRGPVFGEPPTPLHESKVSIGEGHASEMDVSDLGSRNYSARTDFYCL
VSEDDV

Human SIGIRR

MPGVCDRAPDFLSPSEDQVLRPALGSSVALNCTAWVVSGPHCSLPSVQWLKDGLPLGI
GGHYSLHEYSWVKANLSEVLVSSVLGVNVTSTEVYGAFTCSIQNISFSSFTLQRAGPTS
HVAAVLASLLVLLALLLAALLYVKCRLNVLLWYQDAYGEVEINDGKLYDAYVSYSD
CPEDRKFVNFILKPQLERRRGYKLFLDDRDLLPRAEPSADLLVNLSRCRRLIVVLSDAF
LSRAWCSHSFREGLCRLLELTRRPIFITFEGQRRDPAHPALRLLRQHRHLVTLLLWRPG
SVTPSSDFWKEVQLALPRKVRYRPVEGDPQTQLQDDKDPMLILRGRVPEGRALDSEV
DPDPEGDLGVRGPVFGEPSAPPHTSGVSLGESRSSEVDVSDLGSRNYSARTDFYCLVSK
DDM

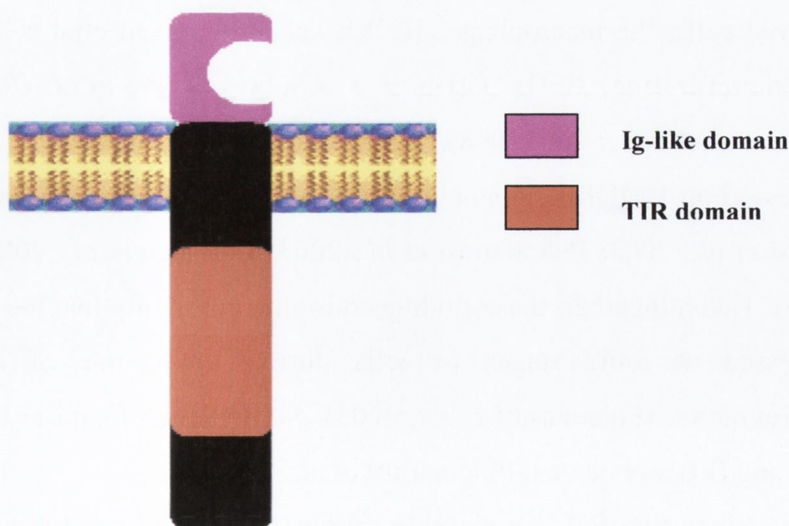


Table 1.2. Amino acid sequences of mouse, rat and human SIGIRR

1.10.2. Expression of SIGIRR

SIGIRR mRNA is ubiquitously expressed in peripheral tissues such as oesophagus, stomach, duodenum, jejunum and ileum, colon, rectum, small intestine, uterus, placenta, ovary, prostate, testis, spleen, thymus (Thomassen *et al*., 1999) and highly expressed in the kidney, liver and gastrointestinal tract (Polentarutti *et al*., 2003; Wald *et al*., 2003; Garlanda *et al*., 2004). SIGIRR is also expressed in the brain (Polentarutti *et al*., 2003; Andre *et al*., 2005; Dimcheff *et al*., 2006; Costelloe *et al*., 2008) and has been found in a variety of species including mouse, rat and human (Thomassen *et al*., 1999, Polentarutti *et al*., 2003, Wald *et al*., 2003, The UniProt Consortium, 2008) and also in cow, horse, pig, dog and chicken (Turin *et al*., 2008). SIGIRR appears to be expressed on the cellular surface as it was found in the membrane fractions of COS-7 cells transfected with SIGIRR and localised to the basolateral and luminal membranes of tubular cells in the kidney (Thomassen *et al*., 1999; Lech *et al*., 2007).

Interestingly although the expression of SIGIRR is widely distributed, within organs the expression of SIGIRR is compartmentalised to specific cell types. SIGIRR is highly expressed on epithelial cells and moderately expressed on splenocytes, fibroblasts, endothelial cells and DCs (Polentarutti *et al*., 2003; Wald *et al*., 2003; Garlanda *et al*., 2004; Lech *et al*., 2007). Curiously, there is no consensus regarding the expression of SIGIRR on monocytes and macrophages. Some studies have found moderate to high expression of SIGIRR on monocytes, myeloid cells, the macrophage GG2EE cell line, mixed glial cells and microglia (Polentarutti *et al*., 2003; Garlanda *et al*., 2004; Andre *et al*., 2005; Lech *et al*., 2007; Costelloe *et al*., 2008), other studies have shown that SIGIRR is not expressed on BMDMs, monocytes, macrophages or macrophage-like RAW cells (Wald *et al*., 2003; Polentarutti *et al*., 2003; Garlanda *et al*., 2004; Huang *et al*., 2006). Taken together, these findings raise the possibility that the receptor may be expressed at some stages in cells during the course of myelomonocytic differentiation (Polentarutti *et al*., 2003). SIGIRR was found to be not expressed on T and B lymphocytes (Polentarutti *et al*., 2003).

Since SIGIRR is a member of the IL-1R family and due to their roles in local and systemic inflammation reactions, the expression and activation of these family members is tightly regulated (Polentarutti *et al*., 2003). Some studies have demonstrated that, in response to LPS treatment, the expression of SIGIRR

mRNA and protein is downregulated (Polentarutti *et al.*, 2003; Wald *et al.*, 2003; Garlanda *et al.*, 2004; Huang *et al.*, 2006). Lech and colleagues (2007) showed that LPS downregulated SIGIRR mRNA expression in tubular epithelial cells but increased SIGIRR mRNA expression in spleen monocytes. While LPS had no effect on SIGIRR mRNA expression in mixed glial cells (Andre *et al.*, 2005). Polentarutti and colleagues (2003) showed that, although LPS decreased the expression of SIGIRR, IL-1β, IFN-γ, TNF-α, IL-4 or CpG-DNA had no effect on SIGIRR expression. One study proposed that the down-regulation of SIGIRR RNA in response to LPS was due to the fact that SIGIRR RNA was being rapidly consumed and translated into SIGIRR protein and proposed that, because of this expression profile, SIGIRR plays an important role in the inflammatory response (Huang *et al.*, 2006). Interestingly, the expression of SIGIRR has been shown to be downregulated with age (Lech *et al.*, 2007); considering that ageing is associated with increased inflammation (Lynch and Lynch, 2002), this further corroborates the hypothesis that the expression of SIGIRR is highly regulated depending on the inflammatory status of the cell.

Up until now no soluble form of SIGIRR had been identified, however in a newly released patent (Sim, J.E. unpublished), a soluble form of SIGIRR is described which still retains its biological activity.

1.10.3. Functions of SIGIRR

The first study to characterise SIGIRR was by Thomassen and colleagues (1999). They identified SIGIRR as a novel member of the IL-1R superfamily, but they stated that it was structurally and functionally distinct from other members in the IL-1R or the TLR families. It was concluded that SIGIRR represented a novel subtype of the IL-1R superfamily (Thomassen *et al.*, 1999). Since its advent into this role, there has been a growing body of evidence to support the fact that SIGIRR acts very differently to other family members and a common *leitmotif* is that SIGIRR is a negative regulator of inflammation (Wald *et al.*, 2003, Garlanda *et al.*, 2004, Qin *et al.*, 2005).

Thomassen and colleagues (1999) showed that SIGIRR could not activate IL-1R-like signalling because it lacked two essential amino acids in its highly conserved TIR domain. They demonstrated, using a NFκB reporter assay, that neither full length SIGIRR, the intracellular domain of SIGIRR, chimeric IL-1R-

SIGIRR constructs, nor chimeric Acp-SIGIRR constructs could activate the IL-8 promoter or NFκB in response to IL-1α. Likewise Polentarutti and colleagues (2003) showed that forced over-expression of HEK293 cells transfected with different expression constructs encoding full length and truncated SIGIRR could not activate NFκB signalling. Moreover Wald and colleagues (2003) showed that over-expression of SIGIRR inhibited IL-1 and IL-18 signalling in multiple cell types. Importantly, it was the construction of SIGIRR knockout (SIGIRR$^{-/-}$) mice that consolidated an inhibitory function for SIGIRR on TLR/IL-1R signalling.

SIGIRR$^{-/-}$ mice were shown to be more susceptible to LPS and IL-1β-induced inflammation than wildtype mice exhibiting an exaggerated inflammatory response often leading to death (Wald *et al.*, 2003; Garlanda *et al.*, 2004; Lech *et al.*, 2007). Consistent with the expression profile of SIGIRR, there was increased activation of the chemokines CXCL1 (KC), MIP-2 and IP-10 in the lung and KC, MCP-1 in the kidneys in response to IL-1 and LPS, (but not TNF-α) in SIGIRR$^{-/-}$ mice compared with wildtype mice (Wald *et al.*, 2003; Lech *et al.*, 2007). Similarly, in myeloid cells prepared from SIGIRR$^{-/-}$ mice there was increased KC and MCP-1 expression in response to LPS (Lech *et al.*, 2007). Accompanying the exacerbated chemokine expression in SIGIRR$^{-/-}$ mice was a parallel increase in pro-inflammatory cytokines and downstream signalling. Garlanda and colleagues (2004) showed DCs but not macrophages prepared from SIGIRR$^{-/-}$ mice showed increased responsiveness to LPS including increased IL-6, IL-12 and IL-10 concentrations. Additionally, Huang and colleagues (2006) showed that RAW cells transfected with SIGIRR down-regulated IL-1β, IL-12, IL-18 and IFN-γ expression. Studies also showed that SIGIRR is capable of directly attenuating signalling downstream from both TLR and IL-IR. Primary kidney epithelial cells and splenocytes extracted from SIGIRR$^{-/-}$ mice had enhanced NFκB and prolonged JNK activition in response to IL-1 and LPS (Wald *et al.*, 2003). While there was increased NFκB and JNK activation in kidney cells prepared from SIGIRR$^{-/-}$ mice in response to IL-1 and LPS but not in response to TNF-α (Qin *et al.*, 2005). Lech and colleagues (2007), showed that spleen macrophages but not tubular epithelial cells obtained from SIGIRR$^{-/-}$ mice had increased NFκB activation in response to LPS treatment. Similarly there was increased activation of NFκB, IκB and JNK activation in SIGIRR$^{-/-}$ colon epithelial cells in response to IL-1 or LPS (but not TNF-α) (Xiao *et al.*, 2007) and Huang and colleagues (2006)

showed that RAW cells transfected with SIGIRR reduced IL-1 and LPS-induced NFκB signalling. Taken together these studies demonstrate a definitive role for SIGIRR as a negative regulator of LPS/IL-1 signalling and suggest that SIGIRR is not involved in regulating TNF signalling. Indeed O'Neill (2003) suggested that SIGIRR does not regulate TNF signalling because the receptor for TNF is not in the TLR/IL-1R superfamily.

In addition to a role of SIGIRR in attenuating TLR4 and IL-IRI signalling, some studies have shown that SIGIRR is also capable of downregulating activation of inflammation by other TLR ligands, in particular lipopeptide (Pam3Cys), polyinosinic:polycytidylic acid (Poly (I:C)), CpG-DNA which bind to TLR1, TLR3 and TLR9 respectively. In myeloid cells prepared from SIGIRR$^{-/-}$ mice there was increased MCP-1 expression in response to Pam3Cys, Poly (I:C) and CpG-DNA (Lech *et al.*, 2007). Furthermore, in DCs prepared from SIGIRR$^{-/-}$ mice there was increased IL-6, IL-12 and IL-10 concentrations and in SIGIRR$^{-/-}$ kidney cells there was increased NFκB and JNK activition in response to CpG-DNA (Garlanda *et al.*, 2004; Qin *et al.*, 2005). Importantly splenocytes obtained from SIGIRR$^{-/-}$ mice and cells in the colonic epithelium of SIGIRR$^{-/-}$ mice have increased cellular proliferation in response to CpG-DNA (Wald et *al.*, 2003; Xiao *et al.*, 2007), Conversely treatment of kidney cells prepared from SIGIRR$^{-/-}$ mice with peptidoglycan, flagellin and poly(I:C) did not induce an increase in NFκB and JNK activation (Qin *et al.*, 2005) and treatment of RAW cells transfected with SIGIRR did not reduce with poly (I:C)-induced NFκB activation (Huang *et al.*, 2006). Taken together these findings suggests that SIGIRR is also a negative regulator of TLR9 signalling and it is possible that SIGIRR could also negatively regulate the other TLR1 and TLR3. More importantly these studies provided evidence suggesting that the inhibitory effects of SIGIRR may expand broader than just that of the TLR/IL-1R superfamily.

In support of this concept there is now ample evidence for a pivotol role of SIGIRR in anti-microbial immunity. Garlanda and colleagues (2004) showed SIGIRR$^{-/-}$ mice had increased weight loss, intestinal bleeding and more severe damage of intestinal muscosa with erosion and inflammatory cell recruitment in dextran sulfate sodium (DSS)-induced colitis. Huang and colleagues (2006), showed that BALB/c mice infected with *Pseudomonas aeruginosa* (*P. aeruginosa*) combined with an anti-SIGIRR antibody exhibited increased corneal

disease with more severe stromal swelling and destruction, additionally they found increased expression of T helper-1 cell- (Th1), but not Th2-associated cytokines, suggesting that SIGIRR can negatively regulate Th1-cell-induced cytokine production. In addition, SIGIRR$^{-/-}$ mice were more susceptible to Mycobacterium tuberculosis (*M. tuberculosis*) infection, exhibiting increased inflammation exemplified by liver necrosis, abnormally elevated levels of pro-inflammatory cytokines and exaggerated mortality. Furthermore Xiao and colleagues (2007) showed that SIGIRR is also a negative regulator of inflammation induced by commensal bacteria in the colon. They reported up-regulation of chemokines, pro-inflammatory cytokines and increased activation of NFκB, IκB and JNK in response to commensal bacteria in the colon mucosa of SIGIRR$^{-/-}$ mice compared with wildtype mice. Importantly they showed, similar to others (Garlanda *et al.*, 2004), that SIGIRR$^{-/-}$ mice were hyper-responsive to DSS-induced colitis demonstrated by increased leukocyte trafficking, pro-inflammatory cytokine production and higher mortality rates than wildtype mice and that this was due to the presence of commensal bacteria in the colon (Xiao *et al.*, 2007). Moreover SIGIRR$^{-/-}$ mice had enhanced colitis-associated tumour production and progression than wildtype mice. SIGIRR$^{-/-}$ mice had a higher incidence of tumours, severe rectal bleeding and diarrhoea, increased inflammatory cell trafficking, increased colonic cell proliferation due to activation of NFκB and IL-6-mediated STAT-3 expression.

The studies described so far illustrate a role for SIGIRR in the defence against microbial-induced inflammation, however more recently a study by Lech and colleagues (2008) showed that SIGIRR is also capable of attenuating autoimmunity, in a model of systemic lupus erythematosus (SLE). There was increased B lymphocyte proliferation, DC activation, production of pro-inflammatory cytokines and increased auto-antigen production in SIGIRR$^{-/-}$ mice with SLE. Consequently this enhanced auto-immunity-induced lung injury and lupus nephritis in SIGIRR$^{-/-}$ mice with SLE. Collectively these findings suggest that SIGIRR may be a negative regulator of innate TLR-mediated-immunity and autoimmunity.

1.10.4. Mechanisms of actions of SIGIRR

Despite the abundance of evidence for the role of SIGIRR in dampening TLR/IL-1R-mediated inflammation (Thomassen *et al.*, 1999; Wald *et al.*, 2003; Garlanda *et al.*, 2004; Lech *et al.*, 2007), microbial-induced inflammation (Garlanda *et al.*, 2004; Huang *et al.*, 2006; Xiao *et al.*, 2007) and auto-immunity (Lech *et al.*, 2007) a definite understanding of the anti-inflammatory signalling mechanisms occupied by the receptor are unknown. SIGIRR is an orphan receptor that does not induce IL-1-like signalling and consistent with this fact, Thomassen and colleagues (1999) showed by plasmon resonance analysis that SIGIRR was unable to bind to the ligands IL-1α, IL-1β or IL-1ra. Whether SIGIRR is able to bind to any ligand is debatable, some studies have suggested that the single Ig domain lacks a ligand binding site and is too short to wrap around a ligand (Thomassen *et al.*, 1999), whereas others have suggested SIGIRR might bind to a ligand and launch a negative signalling pathway (O'Neill, 2003). Others still have suggested that since SIGIRR has been shown to interact with various TLR/IL-1R (Wald *et al.*, 2003; Qin *et al.*, 2005; Huang *et al.*, 2006; Lech *et al.*, 2007), then it may impart its inhibitory effects through interacting with these receptors. Indeed studies have shown that SIGIRR can interact with IRAK and TRAF-6, possibly through interaction with its TIR domain, and sequester these signalling molecules and prevent TLR/IL-1R signalling events (Wald *et al.*, 2003; Qin *et al.*, 2005). However this evidence is based upon immunoprecipitation of SIGIRR with these molecules and does not represent the receptor in its active form. Further studies are required to provide a definitive mode of action for SIGIRR in regulating immunity.

1.11. Aims of research project

Considering that SIGIRR is a negative regulator of TLR/IL-1R signalling (Wald *et al.*, 2003), it was hypothesised that this regulatory role of SIGIRR might extend to the CNS and to other mediators of inflammation. Therefore the first aim of this study was to determine if a deficiency in SIGIRR would exacerbate LPS- and Aβ-induced and age-associated inflammation in the CNS and to establish the functional consequences in the hippocampus associated with this heightened inflammation. The role and exact function of IL-1F5 in the inflammatory response lacks clarity, some studies have suggested it is neither an agonist or antagonistic, whereas others have shown antagonism by IL-1F5 (Barton *et al.*, 2000; Debets *et al.*, 2001). For this reason, the second aim of this study was to determine the anti-inflammatory role of IL-1F5 on inflammatory changes in the CNS using three models which display pro-inflammatory changes, in particular LPS-, Aβ- and age-associated inflammation. Furthermore this study sought to elucidate the mechanism by which IL-1F5 mediates its effects, including examination of the downstream signalling pathways activated by IL-1F5. Finally, this study aimed to determine if the anti-inflammatory effects of IL-1F5 were mediated through interaction with SIGIRR.

Chapter 2

Materials and Methods

2.1. Animals

2.1.1. Animal groups

Groups of young (2-4 months; weighing 20g-40g) and aged (10-12 months; weighing 30-40g) male C57BL/6 (wildtype) and SIGIRR$^{-/-}$ mice were used in this study. SIGIRR$^{-/-}$ mice were generated by homologous recombination of the SIGIRR gene as previously described (Garlanda *et al.*, 2004), genotyped and backcrossed to the C57BL/6 strain; these were a gift from Dr. A Mantovani, Department of Immunology and Cell Biology, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy. C57BL/6 mice were supplied by Harlan (UK). Mice were housed in groups of 5 in an SPF environment in the BioResources Unit, Trinity College, Dublin 2, Ireland.

Groups of young (2-4 months; weighing 250g-300g) and aged (22-24 months; weighing 700g-900g) male Wistar rats were also used in this study. Rats were supplied by Bantham and Kingham, UK. Young rats were housed in groups of 4 or 6 per cage and aged rats were housed in groups of 2 or 3 per cage.

All animals were maintained under veterinary supervision at an ambient temperature of 22°–23°C and under a 12-hour light-dark cycle and food (standard laboratory chow) and water were available *ad libitum*. All animal experimentation was performed under a license granted by the Minister for Health and Children, Ireland, with approval from the local ethical committee and in compliance with the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC, and every effort was made to minimize stress to the animals.

2.2. Animal treatments

2.2.1. Treatment of wildtype and SIGIRR$^{-/-}$ mice

The effects of lipopolysaccharide (LPS) on young (2-4 months) wildtype and SIGIRR$^{-/-}$ mice was examined. Mice were randomly divided into control and experimental groups (*n*=6 mice per group). The control group received an intraperitoneal (ip) injection of sterile saline (200μl). The experimental group received an ip injection of LPS (200μl; 50μg/mouse diluted in sterile saline; LPS was obtained from *Escherichia Coli* (E. coli) serotype 0111.B4; Alexis Biochemicals, UK). Following treatments, wildtype and SIGIRR$^{-/-}$ mice were

assessed in the 'hole board' behavioural test. In another study, the effects of Aβ $_{(1-42)}$ were assessed in aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice. Mice were anaesthetised by an ip injection of urethane (1.2g/kg; 33%w/v; the depth of anaesthesia was determined by absence of the pedal reflex, if needed, a top-up dose of urethane was given (maximum 1.5g/kg). Mice were randomly divided into two treatment groups, control and experimental (*n*=6 mice per group). The control group received an intracerebroventricular (icv) injection of sterile saline (5μl), whilst the experimental group received an icv injection of Aβ $_{(1-42)}$ (5μl; 1mg/ml diluted in sterile saline, Biosource International Incorporated, USA). For the icv injections, scalp fur was trimmed and the animals head was positioned in a stereotaxic frame (Harvard Apparatus Ltd.). A midline incision was made with a scalpel to reveal the skull. The periosteum was scraped clear, bregma identified and a 0.3mm gauge needle was used to form a small hole (to facilitate an icv injection) in the skull at 1mm posterior and 0.34mm lateral to bregma. A syringe was inserted to a depth of 2.5mm. Aβ $_{(1-42)}$ was aggregated according to the manufacturer's instructions and was administered via a single icv injection. In all cases, mice were killed for immediate tissue extraction three hours' post-injections.

2.2.2. Treatment of young and aged rats

The anti-inflammatory actions of IL-1F5 were tested in three rat models which display neurodegenerative changes; LPS- and Aβ-treated rats and aged rats. In all cases, rats were anesthetised by ip injection of urethane (1.5g/kg, 33% w/v; the depth of anaesthesia was determined by the absence of a pedal reflex, if needed, a further top-up dose was administered (to a maximum of 2.5g/kg). In the first study, young (2-4 months) rats were randomly divided into four treatment groups (*n*=6 rat per group). For the icv injections, bregma was identified and a dental drill was used to make a small hole in the skull (to facilitate the icv injections) at 2.5mm posterior and 0.5mm lateral to bregma, a syringe was inserted at a depth of 3.5mm. Rats were either injected icv with IL-1F5 (5μl; 30ng/ml diluted in sterile saline, a gift from L O'Neill, School of Biochemistry and Immunology, Trinity College, Dublin) or ip with LPS (200μl; 100ug/kg diluted in sterile saline) or treated with a combination of LPS and IL-1F5; this final group received IL-1F5 icv and 5 minutes later were injected ip with LPS.

In the second study, rats were randomly divided into four treatment groups (n=6 rat per group). Rats were injected icv with either IL-F5 (5μl; 30ng/ml diluted in sterile saline) or $A\beta_{(1\text{-}42)}$ (5μ l; 1mg/ml; Biosource International Incorporated, USA) or treated with a combination of $A\beta_{(1\text{-}42)}$ and IL-1F5.

In the final study, young (2-4 months) and aged (22-24 months) rats were randomly divided into two treatment groups, control and experimental (n=6 mice per group). The control group received an icv injection of sterile saline (5μl), whilst the experimental group received an icv injection of IL-1F5 (5μl; 30ng/ml diluted in sterile saline). In all cases, rats were killed for immediate tissue extraction three hours' post-injections.

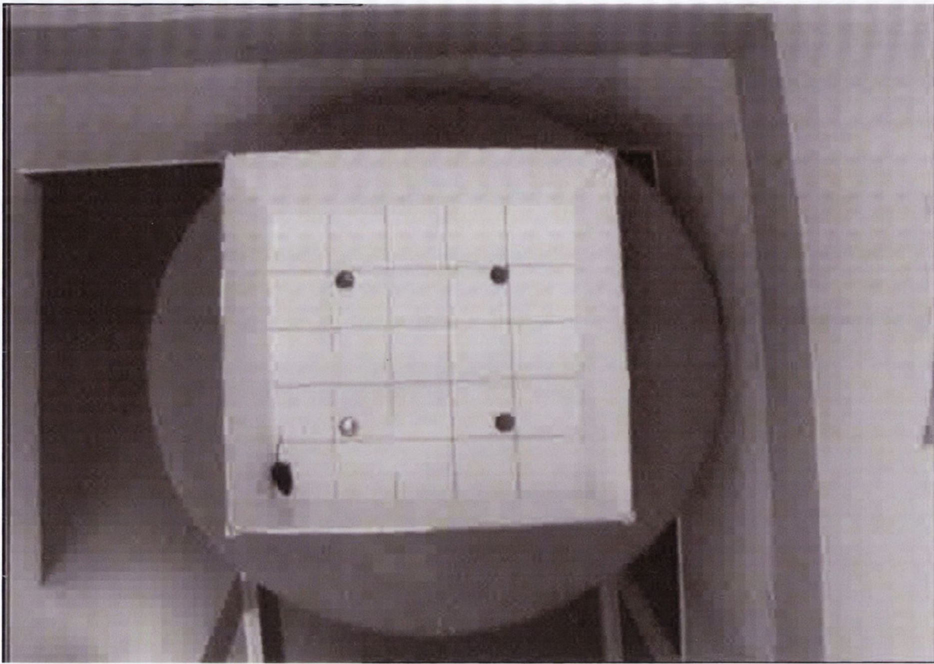
2.3. Behavioural analysis

2.3.1. The hole-board test

Wildtype and SIGIRR$^{-/-}$ mice were examined in the 'hole-board test' which is designed to measure *sickness behaviour* exemplified by changes in exploratory behaviour, anxiety-induced behaviour and locomotory activity. Thirty minutes prior to the behavioural tests, wildtype and SIGIRR$^{-/-}$ mice were either injected ip with sterile saline (200μl) or LPS (200μl, 50μg/mouse; diluted in sterile saline in a different room). Following the injections, the test was carried out in a noise-, light- and temperature-controlled room. Individual mice were released into the same side of the outer corner of a square (60cm width×60cm length×35cm height) hole-board arena (Figure 2.1) for 2 minutes. The arena was made of wood painted white and was divided into a 5x5 grid of equally-sized squares using blue marker. Individual mice were videotaped with a camera mounted on the ceiling circa 2m above the centre of the floor of the arena. Activity was recorded using a video camera and advanced motion-recognition software package (Mediacruise Software, Canopus Corporation, UK) that detects and analyses the movements of mice. The video image of the hole-board arena was partitioned into 25 equal-sized squares; 16 border squares and 8 centre zone squares with a circular hole in each of the central corner squares (Figure 2.1) positioned around a central square.

Exploratory behaviour was examined by measuring the number of times the mice explored the holes in the hole-board arena termed 'head dipping', the

number of rearings which are thought to occur in response to a novel environment and the frequency of entries into the central zone. Anxiety-induced behaviour was measured by the distance the mice stayed to the zone border and the time spent in the various parts of the arena (e.g. the outer zone versus the central zone). Locomotorary activity was calculated by measuring the total distance, maximum distance and mean velocity travelled by the mice.



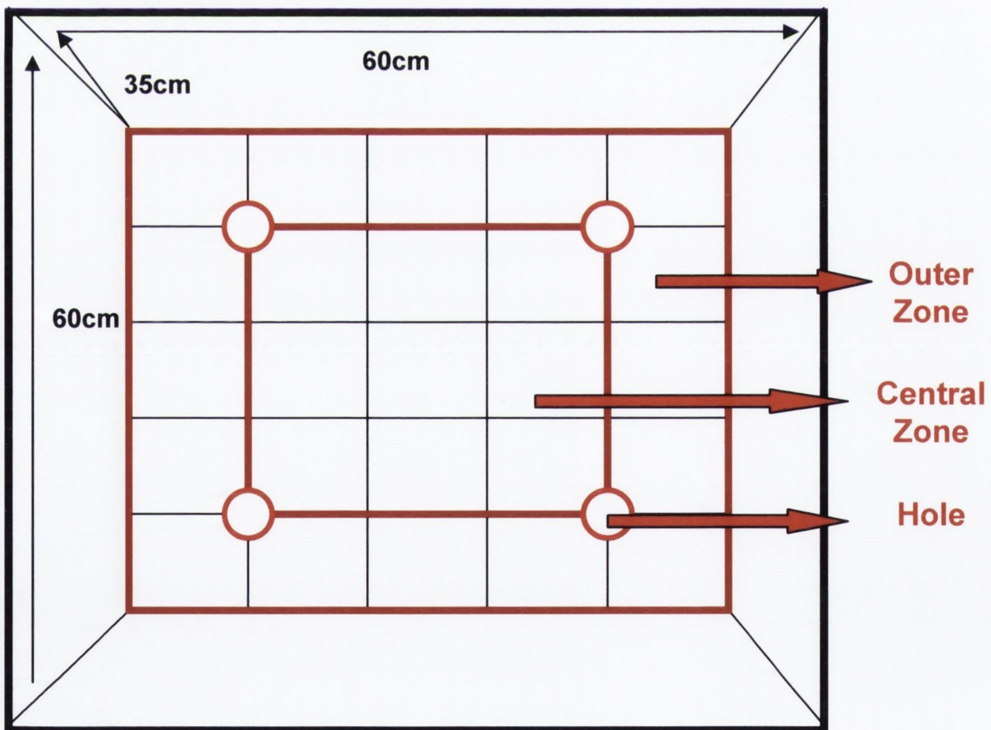


Figure 2.1. Hole-board test arena

2.4. Preparation of tissue

2.4.1. Dissections and preparation of tissue

In all studies, the mice and rats were euthanized by cervical dislocation and decapitated three hours post-treatments. The brains were quickly removed and placed on ice. The hippocampus and cerebral cortices were dissected free, one quarter of the hippocampus and cerebral cortices was snap frozen in liquid nitrogen (Cryoproducts, Ireland) and stored at -80°C for later analysis of messenger ribonucleic acid (mRNA). The remaining hippocampus and cerebral cortices were sliced bi-directionally to a thickness of 350μm using a McIlwain tissue chopper and rinsed in Krebs buffer (composition of Krebs buffer in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $Mg_2SO_4.7H_2O$ 1.18, $NaHCO_3$ 16, Glucose 10, pH 7.5) containing $CaCl_2$ (2mM, Sigma Aldrich, UK). The slices were allowed to settle and were rinsed twice more in Krebs buffer containing $CaCl_2$ and finally rinsed in Krebs buffer containing $CaCl_2$ and 10% dimethylsulphoxide (DMSO, Sigma Aldrich, UK) and stored in this solution at -80°C until required for further analysis.

2.4.2. Protein quantification

In all cases, hippocampal slices were thawed on ice and rinsed thee times in ice-cold Krebs buffer containing $CaCl_2$. The slices were allowed to settle and then homogenised in Krebs buffer containing $CaCl_2$ using a polytron homogeniser (Kinematica, Germany) at maximum speed for 10 seconds. The protein concentration was assessed in the hippocampal homogenates using the bicinchonic acid (BCA) protein assay kit (Pierce, USA). Briefly standards (0-2000μg/ml) were prepared with bovine serum albumin (BSA) standard (2000μg/ml) diluted in Krebs buffer containing $CaCl_2$. Triplicate standards and samples (25μl) were pipetted into a 96-well plate to which 200μl of diluted working reagent was added; the plate was covered and incubated at 37°C for 30 minutes. The absorbance was read at 570nm in a 96-well plate reader (Labsystems Multiskan RC, UK). A standard curve was made by plotting the standards against their absorbance. Protein concentrations were calculated from the standard curve

and protein concentrations in samples were equalised by dilution in ice-cold Krebs buffer containing $CaCl_2$ to a concentration of 1mg/ml-1.5mg/ml.

2.5. Preparation of cultured cortical mixed glia, isolated microglia and isolated astrocytes

2.5.1. Preparation of sterile coverslips

Glass coverslips (10mm diameter) were sterilized in 70% ethanol for 1 hour and dried overnight under a sterile UV light in a fume hood. The following day the coverslips were incubated at 37°C in sterile poly-L-lysine (1mg/ml; diluted in sterile phosphate-buffered saline (PBS, composition in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1 and KH_2PO_4 1.5; pH 7.5) for 1 hour. After incubation, the coverslips were dried in a sterile fume hood and placed in 24-well plates

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

Primary cortical glial cells were prepared from one-day old wildtype and SIGIRR$^{-/-}$ mice (Bioresources Unit, Trinity College, Dublin 2, Ireland). Neonates were decapitated using a sterile scissors and brains removed, the cerebral cortices were dissected free and meninges removed. The cortical tissue was chopped bi-directionally using a sterile scalpel and placed into 1.5ml falcon tubes containing warm filter-sterilized culture media Dulbecco's Modified Eagle Medium (DMEM) (2ml; Sigma Aldrich, UK) supplemented with penicillin (100µl/ml; Gibco, UK), streptomycin (100µl/ml; Gibco, UK) and fetal bovine serum (FBS; 10% w/v; Gibco, UK). The tissue was incubated for 5 minutes in 5% CO_2 at 37°C, removed from the incubator and triturated using a sterile Pasteur pipette and filtered though a 50µl sterile nylon mesh filter (BD Biosciences, USA) into 50ml falcon tubes. The tubes were centrifuged at 2000*g* for 3 minutes at 20°C, the resulting supernatant was removed and the pellet reconstituted in 1ml warm filter-sterilized culture media and triturated. Resuspended glia were counted and equalized to a cell density of 1.5×10^5 cells/ml. Equal numbers of cells were plated onto the centre of each of the poly-L-lysine-coated coverslips and incubated for 2 hours in 5% CO_2 at 37°C to allow the cells to adhere. After 2 hours the cells were flooded with 400µl warm filter-sterilized culture media and incubated for 3 days

in 5% CO_2 at 37°C. Culture media was replaced with fresh culture media every 3 days for 12-14 days until cells were ready for treatments.

2.5.3. Preparation of cortical mixed glia from Wistar rats

Primary cortical glial cells were prepared from one-day old Wistar rats (BioResources Unit, Trinity College, Dublin 2, Ireland). Neonates were decapitated using a sterile scissors and brains removed, the cerebral cortices were dissected free and meninges removed. The cortical tissue was chopped bi-directionally using a sterile scalpel and placed into 15ml falcon tubes containing warm filter-sterilized culture media Dulbecco's Modified Eagle Medium (DMEM) (2ml; Sigma Aldrich, UK) supplemented with penicillin (100µl/ml; Gibco, UK), streptomycin (100µl/ml; Gibco, UK) and FBS (10% w/v; Gibco, UK). The tissue was incubated for 5 minutes in 5% CO_2 at 37°C, then removed from the incubator and triturated using a sterile Pasteur pipette and filtered though a 50µl sterile nylon mesh filter (BD Biosciences, USA) into 50ml falcon tubes. The tubes were centrifuged at 2000*g* for 3 minutes at 20°C, the resulting supernatant was removed and the pellet reconstituted in 1ml warm filter-sterilized culture media and triturated. Resuspended glia were counted and equalized to a cell density of 1.5×10^5 cells/ml. Equal numbers of cells were plated onto the centre of each of the poly-L-lysine-coated coverslips and incubated for 2 hours in 5% CO_2 at 37°C to allow the cells to adhere. After 2 hours the cells were flooded with 400µl warm filter-sterilized culture media and incubated for 3 days in 5% CO_2 at 37°C. Culture media was replaced with fresh culture media every 3 days for 12-14 days until cells were ready for treatments.

2.5.4. Preparation of isolated cortical microglia and astrocytes from Wistar rats

Primary cortical microglia and astrocytes were isolated from one-day old Wistar rats (Bio Resources Unit, Trinity College, Dublin 2, Ireland) using the same method for primary cortical glial cells as described above, except resuspended glia from each neonate were plated into two separate T25 flasks using a plastic Pasteur pipette and the glia were left in the incubator for 2 hours to allow the cells to adhere and then flooded with 8ml of culture media. The following day the media was taken off and replaced with culture media 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). Culture media was replaced with fresh culture media supplemented with M-CSF and GM-CSF every 3 days for 12 days until ready for separation into pure microglia and astrocytes. On day 13 the flasks (neck and cap) were wrapped with parafilm to make them air-tight and placed on an orbital shaker and shaken for 2 hours at 110rpm at room temperature. The microglia were isolated first; the flasks were taken back to the hood and each one was tapped about 10 times and the contents from all the flasks poured into a new 50ml falcon tube, which was centrifuged at 2000rpm for 5 minutes at 20°C. The supernatant was removed and the resulting pellet was resuspended in 1ml of standard culture media. The microglial cells were counted and equalized to a cell density of $1x10^5$cells/ml. The microglial cells were plated onto the centre of poly-L-lysine-coated coverslips and incubated for 2 hours in 5% CO_2 at 37°C to allow the cells to adhere, then flooded with 400µl of culture media. The astrocytes were incubated with 1ml Trypsin-EDTA for 15 minutes at 37°C and tapped about 10 times and the cell suspension from all the flasks was poured into a new 50ml falcon tube, this was spun at 2000rpm for 5 minutes at 20°C. The supernatant was removed and the resulting pellet was resuspended in 1ml standard culture media. The cells were counted and equalized to a cell density of $1x10^5$cells/ml. The cells were plated onto the centre of poly-L-lysine-coated coverslips and incubated for 2 hours in 5% CO_2 at 37°C to allow the cells to adhere, then flooded with 400µl of culture media.

2.5.5. Treatment of cortical mixed glia from wildtype and SIGIRR$^{-/-}$ mice

Confluent mixed glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice were pre-treated with IL-1F5 (3µg/ml; diluted in the well) or culture media for 2 hours. After 2 hours, mixed glial cells were treated with LPS (100ng/ml; Alexis Biochemicals, UK) or culture media and incubated for a further 24 hours. Following the indicated treatments, supernatants were removed into fresh tubes and stored at -80°C until required. Cells were harvested by washing once in PBS, lysed by addition of ice-cold lysis buffer for 15 minutes, scraped off and added to fresh tubes. Cells in lysis buffer were stored at -80°C until required for further analysis

2.5.6. Treatment of cortical mixed glia, isolated microglia and astrocytes from male Wistar rats

The effects of IL-1F5 on LPS-induced signalling were examined in mixed glial cells, isolated microglia and isolated astrocytes prepared from one-day old male Wistar rats. When these cells were confluent they were pre-treated with IL-1F5 (3μg/ml) or culture media for 2 hours. After 2 hours, cells were treated with LPS (100ng/ml; Alexis Biochemicals, UK) or culture media and incubated for a further 24 hours. Following the indicated treatments, supernatants were removed into fresh tubes and stored at -80°C until required for cytokine analysis and, in some cases, cells were fixed for confocal microscopy. Briefly, cells were washed in PBS, fixed by addition of ice-cold 70% ethanol for 15 minutes at -20°C, washed in PBS and kept in the fridge at 4°C until required for immunostaining. In other cases, cells were used for FACs analysis.

2.6. Analysis of mRNA by quantitative polymerase chain reaction (Q-PCR)

2.6.1. RNA extraction

Messenger RNA (mRNA) was extracted from snap frozen hippocampal tissue obtained from young and aged wildtype and SIGIRR$^{-/-}$ mice treated with LPS or $A\beta_{(1-42)}$ and from young IL-1F5-treated rats. In all cases, samples were thawed on ice and cell lysis mastermix (353.5μl; Nucleospin RNA II, Macherey-Nagel) was added to each Eppendorf tube. The samples and cell lysis mastermix were homogenised for extraction of RNA. To remove any insoluble tissue and reduce viscosity, the lysate was added to a NucleoSpin Filter placed in an Eppendorf collecting tube and centrifuged at 11,000*g* for 1 minute. The NucleoSpin Filter column was discarded and ethanol (350μl; 70%, Sigma, UK) was added to the lysate, the ethanol and lysate were mixed and loaded onto NucleoSpin RNA II columns (the addition of ethanol improves binding of the RNA to the column). The new column and lysate were added to new collecting tubes and these were centrifuged at 8,000*g* for 30 seconds. The column was removed and added to a new collecting tube, the silica membrane of the column was desalted (to allow the subsequent RNAase-free DNases to digest more effectively) by adding membrane-desalting buffer (350μl) and centrifuged at 11,000*g* for 1 minute to dry the membrane. DNA was digested by adding DNase

reaction mixture (95μl) to the column and incubated at room temperature for 15 minutes. The silica membrane of the column was washed with RA2 buffer (200μl) to inactivate the DNases, centrifuged at 11,000*g* for 30 seconds to dry and added to a new collecting tube. The silica membrane was washed with RA3 buffer (600μl), centrifuged at 11,000*g* for 30 seconds to dry, and added to a new collecting tube. The silica membrane was washed with RA3 buffer (250μl), centrifuged at 11,000*g* for 2 minutes to completely dry the membrane and placed into labelled nuclease-free collecting tubes 1.5ml Eppendorf tubes. RNA was eluted by adding RNase free H_20 (60μl) and centrifuged at 11,000*g*, for 1 minute and RNA concentration was quantified using a nanoDrop-spectrophotometer (ND-1000 V3.5, Nanodrop Technologies, Inc., USA).

2.6.2. Reverse transcription for cDNA synthesis

Total mRNA (3μg) was reverse transcribed into complementary DNA (cDNA) using a high-capacity cDNA archive kit (Applied Biosystems, Darmstadt, Germany). RNA (3μg) was added to fresh tubes containing the appropriate volume of nuclease-free H_2O to make a final volume of (25μl). A 2x mastermix was prepared containing the appropriate volumes of 10x RT buffer, 25x dNTPs, 10x random primer multiscribe reverse transcriptase (500U/μl). The mastermix (25μl) was added to the RNA and nuclease-free H_2O. Tubes were incubated for 10 minutes at 25°C followed by 2 hours at 37°C on a thermocycler (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences, Ireland).

2.6.3. cDNA amplification by Q-PCR

ICAM, CD40, IL-1β, IL-6 and TNF-α mRNA expression was assessed in hippocampal tissue obtained from wildtype and SIGIRR$^{-/-}$ mice. MHC II, CD40, CD11b, CD200, IL-6, IL-1ra and IL-4 mRNA expression was assessed in hippocampal tissue obtained from IL-1F5-treated rats. For mouse cDNA, a PCR mastermix was made up, containing Taqman Universal PCR Mastermix (Applied Biosystems, Darmstadt, Germany), mouse β-actin RNA as the endogenous control (Applied Biosystems, Darmstadt, Germany) and specific primers for each target gene probed (TaqMan® Gene Expression Assays, Applied Biosystems, Darmstadt, Germany; see Table 2.1 for primer assay numbers). For rat cDNA, a PCR mastermix was made up, containing Taqman Universal PCR Mastermix (Applied Biosystems,

Darmstadt, Germany), rat β-actin RNA as the endogenous control (Applied Biosystems, Darmstadt, Germany) and specific primers for each target gene probed (TaqMan® Gene Expression Assays, Applied Biosystems, Darmstadt, Germany; see Table 2.1 for primer assay numbers). In all cases, cDNA was diluted at a ratio of 1:5 (2μl cDNA and 8μl RNA-free H_20) and added in triplicate to each well of a 96-well plate. Mastermix for each target gene (15μl) was added to each well giving a total reaction volume of (25μl/well) and concentration of cDNA as (200pg/well). Plates were centrifuged at 2000*g* for 1 minute and real-time PCR was performed. The PCR consisted of forty cycles with the following conditions: 2 minutes at 50°C, 10 minutes at 95°C and for each cycle 15 seconds at 95 °C for denaturation and 1 minute at 60°C for transcription and to ensure complete extension of the PCR product (7300 real-time PCR system, Applied Biosystems, USA).

2.6.4. 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). These values were then normalized to an endogenous control 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).

Mouse primers	Primer Assay Numbers
CD40	Mm0041895_m1
ICAM	Mm00516023_m1
IL-1β	Mm00434228_m1
IL-6	Mm00446191_m1
TNF-α	Mm0043258_m1
Actin	Mm00607939_s1
Rat Primers	**Primer Assay Numbers**
MHC II	Rn01768597_m1
CD11b	Rn00709342_m1
CD40	Rn00584362_m1
CD200	Rn00580478_m1
IL-4	Rn01456866_m1
IL-6	Rn00561420_m1
IL-1ra	Rn00573488_m1
Actin	Rn00667869_m1

Table 2.1. Mouse and rat PCR primer assay numbers

2.7. Analysis of mRNA by reverse transcription PCR

2.7.1. RNA extraction

mRNA was extracted from snap frozen hippocampal tissue obtained from IL-1F5- and LPS- treated rats. Samples were thawed on ice and Tri-Reagent (600µl; Sigma, UK) was added to each eppendorf tube. The samples were homogenised using a polytron homogeniser (Kinematica, Germany) at maximum speed for 10 seconds. The samples of homogenate were placed into fresh autoclaved tubes and allowed to settle. To remove any insoluble tissue, the tubes were centrifuged at 12,000*g* for 10 minutes at 4°C. After centrifuging, a pellet was produced, the supernatants were removed and placed into new tubes and these were allowed to settle at room temperature for 5 minutes. Phase separation occurred following addition of chloroform (120µl; Sigma, USA) for 15 minutes at room temperature. The samples were centrifuged at 12,000*g* for 15 minutes at 4°C and this resulted in the formation of thee distinct layers: a lower pink, phenol-chloroform phase, the interphase which contains DNA and the upper aqueous layer which contains the RNA. The aqueous layer was carefully transferred into freshly autoclaved tubes containing isopropanol (300µl; Sigma, UK), samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000*g* for 10 minutes at 4°C. After centrifuging, the supernatant was removed and the pellet containing RNA was washed with ethanol (1ml; 75%, Sigma, UK) and mixed. Samples were centrifuged at 12 000*g* for 5 minutes at 4°C. After centrifuging, the ethanol was removed and the pellet was left to dry in a sterile hood for approximately 2 hours until all the ethanol had evapourated. The pellet was resuspended in deoxy-ribonuclease ribo-nuclease (DNase-RNase)-free H_2O (25µl; Invitrogen, UK). RNA samples were stored at -80°C until required for analysis.

2.7.2. Analysis of isolated RNA by gel electrophoresis

mRNA samples were run on an agarose gel (1% (w/v), Invitrogen, UK) to check the purity and integrity of the isolated RNA. The agarose gel was made up using 1.3g of agarose dissolved in 100ml 1X Tris borate EDTA (TBE) buffer (0.08M Tris; 0.04M boric acid; 1mM EDTA; pH 8.3) and this solution was boiled. Ethidium bromide (EtBr; Sigma, UK) was added and the gel was set in a

horizontal gel system. RNA samples (3μl) were mixed with DNase-RNase-free H_20 (2μl; Invitrogen, UK) and 6X gel loading dye (1μl; Promega, USA) and DNA ladder (10μl; Promega, USA) was mixed with 6X gel loading buffer (2μl; Promega, USA). RNA samples (5μl) and a DNA ladder (10μl; Promega, USA) were loaded onto the gel and separated by application of 90V for 60 minutes. Migration of the bromophenol blue dye towards the bottom of the gel indicated separation of samples. Following gel electrophoresis, the gel was visualized under a UV light, photographed using a UV transilluminator (Labworks, Ultra Violet Products (UVP) BioImaging Systems, UK) and quantified using densitometric analysis.

2.7.3. Reverse transcription

mRNA samples were thawed on ice and mRNA (8μl) was added to new tubes, DNase-RNase-free H_2O (2μl; Invitrogen, UK), Oligo-dT Primer (1μl; Invitrogen, UK) and nucleotide mix (dNTP mix; 1μl; containing 10mM each of dATP, dTTP, dCTP and dGTP; Promega, UK) were added to each tube containing the RNA. The tubes were mixed and placed in the thermocycler; the samples were incubated at 65°C for 5 minutes, removed and quickly placed on ice. Reaction buffer (4μl; 5X; Invitrogen, UK), 0.1M dithiotheitol (DTT; 2μl; Invitrogen, UK) and RNase Inhibitor (1μl; Promega, UK) were added to the tubes, these were mixed and placed in the thermocycler. The samples were incubated at 42°C for 2 minutes, removed and quickly placed on ice. Superscript Reverse Transcription enzyme (1μl; Invitrogen, UK) was added to each tube; the tubes were mixed and placed in the thermocycler. The samples were incubated at 42°C for 50 minutes for cDNA synthesis followed by incubation at 70°C for 15 minutes to stop the reverse transcription. The tubes containing the cDNA were removed from the thermocycler and placed in the -20°C freezer until required for further analysis.

2.7.4. Polymerase chain reaction for Interleukin-4

IL-4 mRNA expression was assessed by rat IL-4 and 18S gene Dual-PCR kit (Maxim Biotechnology Ltd., USA). cDNA (2μl) was added to fresh tubes and 17.5μl of mastermix provided with the kit (Maxim Biotechnology Ltd., USA) was added to each tube along with molecular-grade H_2O (5μl; Maxim Biotechnology

Ltd.,USA) and Jump-Start Taq Polymerase (0.5μl; Sigma, UK) the tubes were placed in the thermocycler. The amplification process started with an initial denaturing step of 95°C for 3 minutes, followed by 44 cycles consisting of a denaturing step of 95°C for 1 minute, an annealing step of 60°C for 1 minute (see Table 2.2 for optimal annealing temperatures) and an extension step of 72°C for 2 minutes. A final extension step at 72°C for 10 minutes was applied to ensure complete extension of PCR products. Equal volumes of PCR product from each sample and 100 base pair ladder (Promega, USA) were mixed with loading buffer (Promega, USA), loaded onto a 2% (w/v) agarose gel containing ethidium bromide and separated by application of 90V for 120 minutes. These primers generated IL-4 PCR products of 352bp (see table 2.2 for primer sequence) and visualized under an ultra violet (UV) light and photographed using a UV transilluminator (Labworks, Ultra Violet Products UVP, BioImaging Systems, UK).

Target gene	**Primer sequence**	**Annealing Temp (°C)**	**Size (bp)**
IL-4	For 5'-TCCATGCACCGAGATGTTTGTACC-3' Rev 5'- CGTAGGATGCTCCCTTTATGAACG-3'	60	352

Table 2.2. Rat IL-4 PCR primer sequence

2.7.5. Densitometry

RNA expression was quantified by densitometric analysis using two software packages, Grab It (Grab It Annotating Grabber 2.04.7, Synoptic; UVP Ltd., UK) and Gelworks (Gelworks ID, Version 2.51; UVP Ltd., UK) for photography and densitometry respectively. Gelworks provides a single value (in arbitrary units) representing the density of each blot.

2.8. Analysis of cytokine concentrations

2.8.1. Preparation of samples

The concentrations of IL-1β, IL-6, TNF-α and IL-4 were assessed by ELISA in supernatants from mixed glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice and from hippocampal homogenates prepared from wildtype and SIGIRR$^{-/-}$ mice. Hippocampal slices were thawed on ice and washed 3 times with ice-cold Krebs buffer containing $CaCl_2$, the slices were allowed to settle and homogenised in 400μl ice-cold Krebs buffer containing $CaCl_2$ using a polytron homogeniser (Kinematica, Germany) at maximum speed for 10 seconds. Protein concentrations were equalized to 1.5mg/ml. The concentrations of IL-1β, IL-6, TNF-α and IL-10 were assessed by ELISA in supernatants prepared from mixed glial cells, isolated astrocytes and isolated microglia from one-day old rats. Concentrations of IL-1β, IL-6, TNF-α and IL-10 were also measured in hippocampal homogenates prepared from rats. The hippocampal slices were thawed on ice rinsed thee times and homogenised in 600μl ice-cold Krebs buffer containing $CaCl_2$ using a polytron homogeniser (Kinematica, Germany) at maximum speed for 10 seconds. Protein concentrations in homogenates were equalized to 1mg/ml.

2.8.2. General ELISA protocol

In all cases 96-well plates (NUNC, Denmark) were incubated overnight at room temperature with capture antibody (50μl; see Table 2.3 and 2.4 for specific details). Plates were washed thee times with wash buffer (PBS containing 0.05% Tween-20) and incubated at room temperature for 1 hour in blocking buffer (300μl; see Table 2.3 and 2.4 for specific details). Plates were washed thee times

with wash buffer and incubated at room temperature for 1 hour with samples and standards (50μl in triplicate; see Table 2.3 and 2.4 for specific details). Plates were washed thee times with wash buffer and incubated at room temperature with detection antibody (50μl; see Table 2.3 and 2.4 for specific details). Plates were washed thee times with wash buffer and incubated in the dark at room temperature for 20 minutes with horseradish peroxidase-conjugated streptavidin (strep-HP) (100μl; 1:200 dilution in 1%BSA/PBS in the case of IL-1β and 10%FBS/PBS in the case of IL-6, TNF-α). Plates were washed thee times with wash buffer and incubated with substrate solution (100μl; 1:1 H_20_2: tetramethylbenzidine; R&D Systems, USA) in the dark at room temperature for 20-30 minutes or until colour developed. The reaction was stopped using a stop solution (50μl: 1M H_2SO_4) and absorbance was read at 450nm using a 96-well plate reader (Labsystem Multiskan RC, UK). A standard curve was made up by plotting the standards with their absorbance and concentrations were corrected for protein concentration and expressed as pg/mg protein in the case of tissue or pg/ml in the case of supernatants (GraphPad Prism v4.0 Macintosh; GraphPad Software, USA).

Cytokine	**Supplier**	**Block**	**Capture antibody**	**Standard antibody**	**Detection antibody**
IL-1β	R&D Systems, USA	1% BSA in PBS	Monoclonal anti-mouse IL-1β (4μg/ml;in PBS)	Mouse recombinant IL-1β standards (0-1000pg/ml; 1%BSA in PBS)	Biotinylated anti-mouse IL-1β (100ng/ml; 1%BSA and 2%normal goat serum in PBS)
IL-6	BD Biosciences, USA	3% BSA in PBS	Monoclonal anti-mouse IL-6 (1μg/ml; in PBS)	Mouse recombinant IL-6 standards (0-2000pg/ml; 3%BSA in PBS)	Biotinylated anti-mouse IL-6 (2μg/ml; in PBS)
TNF-α	BD Biosciences, USA	1% BSA and 5% sucrose in PBS	Monoclonal anti-mouse TNF-α (1μg/ml; in PBS)	Mouse recombinant TNF-α standards (0-4000pg/ml; 1%BSA in PBS)	Biotinylated anti-mouse TNF-α (2μg/ml; 1%BSA in PBS)
IL-4	BD Biosciences, USA	5% Milk in PBS	Monoclonal anti-mouse IL-4 (1μg/ml; in PBS)	Mouse recombinant IL-4 standards (0-2000pg/ml; in PBS)	Biotinylated anti-mouse IL-4 (1μg/ml; in PBS)

Table 2.3. Mouse cytokine analysis protocol

Cytokine	Supplier	Block	Capture antibody	Standard antibody	Detection antibody
IL-1β	R&D Systems, USA	1% BSA in PBS	Monoclonal anti-rat IL-1β (1μg/ml; in PBS)	Rat recombinant IL-1β standards (0-1000pg/ml; 1%BSA in PBS)	Biotinylated anti-rat IL-1β (350ng/ml; 1%BSA and 2% normal goat serum in PBS)
IL-6	BD Biosciences, USA	10% FBS in PBS	Monoclonal anti-rat IL-6 (4μg/ml; 0.1M Na_2CO_3 in PBS)	Rat recombinant IL-6 standards (0-4000pg/ml; 10%FBS in PBS)	Biotinylated anti-rat IL-6 (4μg/ml; 0.1M Na_2CO_3 in PBS)
TNF-α	BD Biosciences, USA	10% FBS in PBS	Monoclonal anti-rat TNF-α (4μg/ml; 0.1M Na_2CO_3 in PBS)	Rat recombinant TNF-α standards (0-4000pg/ml; 10%FBS in PBS)	Biotinylated anti-rat TNF-α (4μg/ml; 0.1M Na_2CO_3 in PBS)
IL-10	Biosource, USA	5% BSA and 5% FBS in PBS	Monoclonal anti-rat IL-10 (1.25μg/ml ;0.1M Na_2CO_3 in PBS)	Rat recombinant IL-10 standards (0-1000pg/ml; 5%BSA and 10%FBS in PBS)	Biotinylated anti-rat IL-10 (0.125μg/ml; 5% BSA and 5% FBS in PBS)

Table 2.4. Rat cytokine analysis protocols

2.9. Analysis of protein expression by western immunoblotting

2.9.1. Preparation of hippocampal tissue for gel electrophoresis

Phosphorylated forms of JNK (p-JNK) and unphosphorylated/total forms of JNK (t-JNK) were assessed by western immunoblotting in hippocampal lysates (100μl; 1.5mg/ml) prepared from wildtype and SIGIRR$^{-/-}$ mice. Phosphorylated forms of JNK (p-JNK), p38 (p-p38), ERK (p-ERK) and STAT6 (p-STAT6) and total forms of JNK (t-JNK), p-38 (total-p-38), ERK (t-ERK) and β-actin were assessed in hippocampal lysates (200μl; 1mg/ml) obtained from IL-1F5 and LPS treated rats. In all cases, hippocampal slices were thawed on ice and rinsed 3 times in ice-cold lysis buffer (composition in mM: Tris-HCl 10, NaCl 50, $Na_4P_20_7.H_2O$ 10, NaF 50, 1% Igepal, Na_3VO_4 1, PMSF 1, Protease Inhibitor cocktail 1) and homogenised in 400μl of lysis buffer using a polytron homogenizer (Kinematics, Germany). The protein concentration was assessed in the hippocampal lysates using the BCA protein assay (Pierce, USA) and samples were equalized to a protein concentration of 1.5mg/ml or 1mg/ml. Samples (100μl; 1.5mg/ml) and (200μl; 1mg/ml) were reconstituted in NuPage lithium dodecyl sulfate sample buffer (Invitrogen, UK) containing NuPage reducing agent (Invitrogen, UK) and the samples were placed in the -20°C freezer until required for further analysis.

2.9.2. Gel electrophoresis

NuPage Bis-Tris pre-cast gels with a sodium dodecylsulphate (SDS) concentration of 10% or 12% were used in these experiments (Invitrogen, UK). They were mounted in an electrophoretic unit (Xcell II Surelock Mini Cell System, Invitrogen, UK) and the unit was filled with NuPage electrode running buffer (Invitrogen, UK). Aliquots of samples (100μl; 1.5mg/ml) and (200μl; 1mg/ml) were heated to 72°C for 10 minutes to denature the protein, and samples (10-15μl) were loaded onto gels along with a pre-stained molecular weight marker (10μl; Biorad Dual Colour standards, USA). A current of 130V was passed across the unit to separate the proteins for 70 minutes. Following protein separation, the gel was placed onto a pre-soaked nitrocellulose membrane (Whatman,UK). Two pieces of filter paper (Whatman, UK) and two blotting pads (Invitrogen, UK), which were soaked in transfer buffer (Invitrogen, UK), were placed either side of the gel and membrane forming a sandwich. The sandwich was placed in the blotting unit which

was filled with transfer buffer (Invitrogen, UK); this was placed into the transferring unit which was filled with distilled water. A constant current of 30V was applied to the transferring unit for 75 minutes. Nitrocellulose membranes were immunoblotted with the appropriate antibody.

2.9.3. General protocol for western immunoblotting

The following describes a general protocol used to identify proteins of interest, which was carried out directly following transfer of proteins onto the nitrocellulose membrane. The specific details for every protein assessed by western immunoblotting are presented in Table 2.5. The nitrocellulose membranes were incubated overnight at 4°C with blocking buffer containing 5% Marvel in Tris-buffered saline (TBS) with 0.05% Tween (TBS-T) to block non-specific antibody binding. The nitrocellulose membranes were incubated for 2 hours at room temperature with a primary antibody raised against the appropriate protein. The primary antibodies were washed off with TBS-T (3x5 minutes) and incubated for 1 hour at room temperature with a secondary antibody conjugated to horseradish peroxidase (HP; Sigma-Aldrich, UK). The secondary antibodies were washed off (4x15 minutes) with TBS-T and the nitrocellulose membranes were incubated with a chemiluminescent detection solution (Supersignal (S/S) Ultra, Pierce, USA) or (Enhanced Chemiluminesence (ECL) Amersham, UK) to visualize protein complexes. The nitrocellulose membranes were then exposed to 5x7 inch photographic film (Pierce, USA) and developed using an AGFA film processor (Agfa-Gevart Group, Dublin, Ireland). The nitrocellulose membranes were stripped with antibody stripping solution (1:10 dilution in dH_2O; Reblot Plus Strong antibody stripping solution; Chemicon, California, USA) and re-probed for another target protein.

2.9.4. Densitometry

Protein bands exposed to photographic film were quantified by densitometric analysis using the GelDoc-It imaging system and Lab works image acquisition and analysis software (UVP Bioimaging systems, Cambridge, UK). In all cases ratios of phosphorylated target protein/target protein or phosphorylated target protein/total β-actin were expressed using arbitrary units.

Protein Target (source)	Protein Band size (kDa)	Block % in TBS-T	Antibody dilution % BSA/Milk (w/v) in TBS-T	Developing details
Total JNK (mouse)	54	5% Milk o/n @ 4°C	1° 1:200 1% BSA 2 hours @ RT 2° anti-mouse IgG 1:400 1% BSA 1 hour @ RT	ECL 5 min Exposure time: 5 min
Phosphorylated JNK (mouse)	54 46	5% Milk o/n @ 4°C	1° 1:200 1% BSA 2 hours @ RT 2° anti-mouse IgG 1:400 1% BSA 1 hour @ RT	ECL 5 min Exposure time: 15 min
Total p38 (mouse)	38	5% Milk o/n @ 4°C	1° 1:200 1% BSA 2 hours @ RT 2° anti-mouse IgG 1:1000 1% BSA 1 hour @ RT	ECL 5 min Exposure time: 10 min
Phosphorylated p38 (mouse)	38	5% Milk o/n @ 4°C	1° 1:200 1% BSA 2 hours @ RT 2° anti-mouse IgG 1:1000 1% BSA 1 hour @ RT	ECL 5 min Exposure time: 15 min
Total ERK (mouse)	42	5% Milk o/n @ 4°C	1° 1:1000 1% BSA 2 hours @ RT 2° anti-mouse IgG 1:1000 1% BSA 1 hour @ RT	S/S 5 min Exposure time: 2 s
Phosphorylated ERK (mouse)	44 42	5% Milk o/n @ 4°C	1° 1:3000 1% BSA 2 hours @ RT 2° anti-mouse IgG 1:1000 1% BSA 1 hour @ RT	ECL 5 min Exposure time: 5 min
Actin (mouse)	42	5% Milk o/n @ 4°C	1° 1:10000 3.5% Milk 2 hours @ RT 2° anti-mouse IgG 1:1000 3.5% Milk 1 hour @ RT	S/S 5 min Exposure time: 2 s
Phosphorylated STAT-6 (mouse)	110	5% Milk o/n @ 4°C	1° 1:200 1% BSA 2 hours @ RT 2° anti-mouse IgG 1:2000 1% BSA 1 hour @ RT	S/S 5 min Exposure time: 3 min
Abreviations: o/n = overnight, RT = room temperature, S/S = supersignal **Supplier:** Santa Cruz Biotechnology, USA				

Table 2.5. Western immunoblotting protocol

2.10. Immunofluorescent staining

2.10.1. Preparation of samples for immunofluorescent staining

Mixed glial cells were double-immunostained for SIGIRR and glial acidic fibrillary protein (GFAP) to determine the expression of SIGIRR on astrocytes. Mixed glial cells and isolated microglia were double-immunostained for SIGIRR and CD11b to determine the expression of SIGIRR on microglial cells. Mixed glial cells were double-immunostained with SIGIRR and IL-1F5 to determine co-localisation of SIGIRR with IL-1F5. The following protocol describes the general method used to identify the expression of SIGIRR on the various cell types on the CNS and the method used to determine the interaction between SIGIRR expressed on mixed glial cells and exogenous IL-1F5. The specific details for every protein assessed by immunofluorescent staining are presented in Table 2.6

2.10.2. General protocol for double immunofluorescent staining

Mixed glial cells and isolated microglia were fixed on glass coverslips with 70% ethanol for 15 minutes at -20°C and washed in PBS. Following fixation, cells were immunostained in the wells of 24-well plates. Cells were washed three times with PBS. Non-specific interactions were blocked using 10% blocking buffer (10% animal serum in PHEM buffer (composition in mM: Pipes 60, Hepes 25, EDTA 10, $MgCl_2$ 2) for 2 hours at room temperature on a rock'n'roller. Cells were incubated with primary antibody raised against the protein of interest (300µl/well; diluted in 5% blocking buffer) for 2 hours at room temperature. Negative controls were incubated with IgG antibody that the primary was raised in (300µl/well; diluted in 5% blocking buffer). The primary antibody was washed off with PHEM buffer (3x5 minutes) and cells were incubated with an appropriate secondary antibody conjugated to either Alexa Fluor®488 or Alexa Fluor®633 (300µl/well; diluted in 5% blocking buffer; Invitrogen, UK). The secondary antibody was washed off with dH_20 every 10 minutes for 1 hour to remove as much of the fluorescent background as possible and in the dark to avoid bleaching the fluorescent label. Non-specific interactions were blocked using 10% blocking buffer (10% animal serum in PHEM buffer) overnight at 4°C. The next day, cells were incubated with primary antibody raised against the protein of interest (300µl/well; diluted in 5% blocking buffer) for 2 hours at room temperature. Negative controls were incubated with IgG antibody

that the primary was raised in (300μl/well; diluted in 5% blocking buffer). The primary antibody was washed off with PHEM buffer (3x5 minutes) and cells were incubated with an appropriate secondary antibody conjugated to either Alexa Fluor®488 or Alexa Fluor®633 (300μl/well; diluted in 5% blocking buffer, Invitrogen, UK). The secondary antibody was washed off with dH_20 every 10 minutes for 1 hour. Glass slides were labelled and coverslips containing cells were mounted onto glass slides using Vectashield® fluorescent mounting media (Vector Laboratories, Peterborough, UK) and secured with a thin line of clear nail polish and stored in the dark at 4°C until required for analysis.

2.10.3. Confocal microscopy

The incorporated fluorophores were viewed using a confocal microscope (Zeiss, LSM-510-META, Karl Zeiss, UK) using appropriate excitation wavelength and filter sets. For Alexa Fluor®488™ the peak excitation/emission wavelengths were 495nm/520nm and the beam splitters were UV488/543/633nm and band pass (BP) 505-530. For Alexa Fluor®633™ the peak excitation/emission wavelengths were 632nm/647nm and the beam splitters were UV488/543/633nm and BP 575-640nm. Images were acquired and optimised using LSM 510 computer program at 40X and 60X magnifications.

Protein Target (source)	Supplier	Stock IgG Conc.	Block % in PHEM buffer	Antibody dilutions % block in PHEM buffer
SIGIRR (goat)	R&D Systems UK	100µg/ml	10% rabbit serum 2 h @ RT	1° 1:4000 5% rabbit serum 2 h @ RT 2° 1:4000 anti-goat-Alexa Fluor®633 5% rabbit serum 1 h @ RT
IL-1F5 (goat)	Santa Cruz Biotech USA	200ug/ml	10% rabbit serum 2 h @ RT	1° 1:500 5% rabbit serum 2 h @ RT 2° 1:4000 anti-goat Alexa Fluor®633 5% rabbit serum 1 h @ RT
GFAP (rabbit)	Dako Diagnostics Ireland	500µg/ml	10% goat serum 2 h @ RT	1° 1:500 5% goat serum 2 h @ RT 2° 1:4000 anti-rabbit- Alexa Fluor®488 5% goat serum 1 h @ RT
CD11b (mouse)	Serotec USA	1mg/ml	10% goat serum 2 h @ RT	1° 1:50 5% goat serum 2 h @ RT 2° 1:4000 anti-mouse- Alexa Fluor®488 5% goat serum 1 h @ RT
Abreviations: h = hour, RT = room temperature				

Table 2.6. Immunofluorescent staining protocols

2.11. Fluorescent immunostaining of mixed glial cells with CD11b and CD86 for fluorescent activated cell sorter (FACS) analysis

Mixed glial cells were prepared as described previously, plated onto 6-well plates and grown for 12 days. Cells were pre-treated with IL-1F5 (3μg/ml) for 2 hours and treated with LPS (100ng/ml) for 24 hours. Supernatants were removed from cells and added to labelled tubes. Cells were washed twice with PBS and 400μl of Trypsin-EDTA was added to each well. The plates were placed in the incubator for 5 minutes and tapped firmly to remove all cells. Media (1ml) was added to each well to stop the action of the Trypsin-EDTA and cells were harvested into labelled FACs tubes. Cells were centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatants were removed and the pellets were resuspended in ice-cold FACS buffer (2ml; PBS (500ml), FBS (10ml) and Sodium Azide (0.5g)). The cells were washed a further twice in FACS buffer and blocked for 30 minutes at room temperature in the dark in blocking buffer (1ml; PBS (40ml) and FBS (40ml)). The antibodies were made up in the dark, anti-rat CD11b conjugated to allophyscocyanin (APC) (1:100 dilution in FACS buffer; Alexa) for anti-rat CD86 conjugated to fluorescein (FITC) (1:50 dilution in FACS buffer, Acris). Beads were added to labelled FACS tubes (1 drop of anti-mouse IgG and 1 drop of negative control (BD Comp beads). The FACS tubes were centrifuged at 1200 rpm for 5 minutes at 4°C, the supernatants were removed and the pellets were washed 3 times with FACS buffer. The supernatants were removed and 10μl of antibody was added to the tubes which contained the beads and incubated in the dark for 30 minutes at 4°C. The cells and beads were washed 3 times with FACS buffer and, after the final wash, they were resuspended in a total volume of 300μl FACS buffer and read on the FACS machine.

2.12. Determination of binding of IL-1F5 with SIGIRR

The binding of IL-1F5 to SIGIRR was determined by ELISA. 96-well plates (NUNC, Denmark) were incubated for 2 hours at 37°C with capture antibodies to SIGIRR (50μl; 1μg/ml; goat anti-human SIGIRR antibody diluted in PBS; pH 7.3; R&D Systems, USA). Plates were washed 3 times with wash buffer (PBS containing 0.05% Tween-20) and incubated for 1 hour at 37°C with recombinant SIGIRR

containing the extracellular Ig domain (50μl; 1μg/ml; recombinant SIGIRR antibody diluted in PBS; pH 7.3; R&D Systems, USA). Plates were washed 3 times with wash buffer and incubated at 37°C for 1 hour in assay diluent (300μl; PBS containing 1% BSA, pH 7.3). Different concentrations of IL-1F5 (3μg/ml, 10μg/ml, 30μg/ml, 50μg/ml, 100μg/ml, 150μg/ml, 300μg/ml) were made up by making serial dilutions of recombinant human IL-1F5 (Alexa Biochemicals), these samples (50μl) were added to the appropriate wells and incubated at room temperature for 2 hours. Plates were washed 3 times with wash buffer and incubated at room temperature for 2 hours with detection antibody for IL-1F5 (50μl; 1μg/ml; mouse monoclonal anti-IL-1F5 antibody in PBS; R&D System, USA). Plates were washed 3 times with wash buffer and incubated at room temperature for 1 hour with biotinylated anti-mouse IgG antibody (50μl, 50ng/ml in 0.1% BSA in TBS; R&D Systems). Plates were washed 3 times with wash buffer and incubated at room temperature for 20 minutes with diluted horseradish peroxidase-conjugated strep-HRP (50μl, 1:200 dilution in assay diluent). Plates were washed 3 times with wash buffer and incubated with substrate solution (50μl; 1:1 dilution H_20_2: tetramethylbenzidine; R&D Systems, USA) in the dark at room temperature for 20-30 minutes or until colour developed. The reaction was stopped using a stop solution (1M H_2SO_4) and absorbance was read at 450nm using a 96-well plate reader (Labsystem Multiskan RC, UK).

2.13. Statistical analysis

Data are expressed as means ± standard error of mean (SEM). A one-tailed or two-tailed Student's t-test for unpaired means, a one-way analysis of variance (ANOVA) or two-way ANOVA was performed, where appropriate, to determine whether significant differences existed between conditions. When this analysis indicated significance (at the 0.05 level), a post-hoc student Newmann-Keuls test was used to determine which conditions were significantly different from each other (GraphPad Prism, USA).

Chapter 3

An investigation into the effects of LPS treatment on SIGIRR$^{-/-}$ mice

3.1. Introduction

Thomassen and colleagues (1999) were the first to identify a unique member of the IL-1R superfamily called SIGIRR, which is structurally and functionally distinct from the other members of the family. They reported that SIGIRR has only a single Ig domain in its extracellular region and is unable to activate IL-1-like signalling. Furthermore, several studies have highlighted the important negative regulatory role of SIGIRR on LPS- and IL-1β-induced inflammatory changes (Wald *et al.*, 2003, Garlanda *et al.*, 2004, Lech *et al.*, 2007). Moreover this attenuatory capacity of SIGIRR has been extended to microbial-induced inflammation (Garlanda *et al.*, 2004; Huang *et al.*, 2006; Xiao *et al.*, 2007). SIGIRR is highly expressed in peripheral organs such as the kidney, spleen, liver and gastrointestinal tract (Polentarutti *et al.*, 2003; Wald *et al.*, 2003; Garlanda *et al.*, 2004) and consistent with this expression profile SIGIRR-deficient kidney and intestinal epithelial cells and splenocytes exhibit exaggerated responsiveness to LPS- and IL-1β-induced inflammatory changes (Wald *et al.*, 2003; Lech *et al.*, 2007). Taken together these previous observations suggest a role for SIGIRR as a negative regulator of inappropriate inflammation in peripheral organs. Several studies have also shown that SIGIRR is expressed in the brain (Polentarutti *et al.*, 2003; Andre *et al.*, 2005; Dimcheff *et al.*, 2006; Costelloe *et al.*, 2008). Therefore, the first aim of this study was to determine if the negative regulatory capacity of SIGIRR on LPS-induced inflammatory changes could be extended to the brain.

There is accumulating evidence to support the idea that accompanying the deficits in memory performance with ageing and AD is an increase in inflammation in the brain (Hauss-Wegrzyniak *et al.*, 2002; Blasko *et al.*, 2004). The hippocampus is the area of the brain associated with learning, memory and specific forms of behaviour (Squire and Cave, 1991; Jarrard, 1993; Crusio *et al.*, 2001). Evidence suggests that the hippocampus is heavily affected by inflammatory changes in ageing and AD, including activation of microglial cells and increased production of pro-inflammatory cytokines (Griffin *et al.*, 2006). The second aim of this study was to determine if the exacerbated inflammation induced in the hippocampus of LPS-

treated SIGIRR$^{-/-}$ mice imparted functional consequences in the hippocampus, and in particular, if it induced heightened sickness behaviour in SIGIRR$^{-/-}$ mice.

3.2. Methods

Wildtype and SIGIRR$^{-/-}$ mice (2-4 months; weighing 20g-40g) were injected ip with sterile saline (200μl) or LPS (200μl; 50μg/mouse diluted in sterile saline). Following treatments, animals were assessed in the 'hole-board test' (see section 2.3.1 for specific details), killed by cervical dislocation three hours post-injections and tissue taken for analysis (see section 2.4.1 for specific details). Analysis of hippocampal tissue for microglial markers, cytokine and chemokine mRNA were assessed by PCR, cytokine concentrations were assessed by ELISA and MAPK by western immunoblotting (see sections 2.6. 2.8 and 2.9 for specific details). Data are expressed as means ± standard error of the mean and a two-way ANOVA was performed to determine whether significant differences existed between conditions. Post hoc Student Newmann-Keuls test was used to determine where the significance lied (see section 2.13 for specific details).

3.3. Results

Effect of LPS on CD40 and ICAM mRNA expression in SIGIRR$^{-/-}$ mice

In this study, we set out to determine if an LPS challenge elicited a greater effect on microglial activation in SIGIRR$^{-/-}$ compared with wildtype mice. Fully activated microglial cells are typified by expression of a variety of cell surface receptors. CD40 is one such receptor expressed on microglial cells and activation of the CD40 pathway can lead to the induction of pro-inflammatory cytokines, chemokines as well as other co-stimulatory molecules (Stout and Suttles, 1996). ICAM is a cellular adhesion molecule that facilitates the migration of blood-borne lymphocytes across the BBB into the CNS (Kleine and Benes, 2006). Importantly ICAM is upregulated in response to inflammatory stimuli such as LPS and IFN-γ (Wahl *et al.*, 1996). Consequently, CD40 and ICAM mRNA expression were

measured in snap frozen hippocampal tissue prepared from LPS-treated wildtype and SIGIRR$^{-/-}$mice.

Figure 3.1. shows that CD40 mRNA expression was significantly increased in tissue prepared from LPS-treated wildtype mice (16.35 ± 3.48 RQ; n=6) compared with tissue obtained from saline-treated control wildtype mice (0.91 ± 0.22 RQ; n=6; $^{**}p<0.01$; Student's *t* test). LPS-treated SIGIRR$^{-/-}$ mice (27.02 ± 9.51) compared with tissue obtained from saline-treated control SIGIRR$^{-/-}$ mice (0.11 ± 0.01 RQ; n=6; $^{**}p<0.01$; ANOVA). SIGIRR$^{-/-}$ mice showed enhanced responsiveness to LPS treatment compared with wildtype mice. Treatment with LPS induced a significant increase in CD40 mRNA expression in SIGIRR$^{-/-}$ mice (27.02 ± 9.51 RQ; n=6) compared with LPS-treated wildtype mice (16.35 ± 3.48 RQ; n=6; $^{+}p<0.05$; ANOVA).

Figure 3.2. shows that ICAM mRNA expression was significantly increased in tissue prepared from LPS-treated wildtype mice (66.55 ± 10.64 RQ; n=6) and SIGIRR$^{-/-}$mice (213.1 ± 30.46 RQ; n=6) compared with tissue obtained from saline-treated control wildtype mice (2.39 ± 0.65 RQ; n=6; $^{*}p<0.05$; ANOVA) and SIGIRR$^{-/-}$ mice (0.42 ± 0.06 RQ; n=6; $^{***}p<0.001$; ANOVA). SIGIRR$^{-/-}$ mice showed enhanced responsiveness to LPS treatment compared with wildtype mice. Treatment with LPS induced a significant increase in ICAM mRNA expression in SIGIRR$^{-/-}$ mice (213.1 ± 30.46 RQ; n=6) compared with LPS-treated wildtype mice (66.55 ± 10.64 RQ; n=6; $^{+++}p<0.001$; ANOVA).

Effect of LPS on MCP-1 and IP-10 mRNA expression in SIGIRR$^{-/-}$ mice

Studies by Wald and colleagues (2003) have shown that SIGIRR$^{-/-}$ mice exhibited a more exaggerated response to endotoxin challenge, including increased induction of chemokines in the lung and colon, compared with wildtype mice. In order to determine if peripheral LPS challenge to SIGIRR$^{-/-}$ mice leads to increased induction of chemokines in the CNS, MCP-1 and IP-10 mRNA expression were measured in snap-frozen hippocampal tissue from LPS-treated wildtype and SIGIRR$^{-/-}$ mice.

Figure 3.3. shows that mean MCP-1 mRNA expression was significantly increased in tissue prepared from LPS-treated wildtype (1.38 ± 0.28 RQ; n=6) and SIGIRR$^{-/-}$ mice (1.93 ± 0.11 RQ; n=6) compared with saline-treated control wildtype (0.55 ± 0.15 RQ; n=6; *p<0.05; ANOVA) and SIGIRR$^{-/-}$ mice (0.65 ± 0.13 RQ; n=6; ***p<0.001; ANOVA). SIGIRR$^{-/-}$ mice showed enhanced responsiveness to LPS treatment compared with wildtype mice; treatment with LPS induced a significant increase in MCP-1 mRNA expression in SIGIRR$^{-/-}$ mice (1.93 ± 0.11 RQ; n=6) compared with LPS-treated wildtype mice (1.38 ± 0.28 RQ; n=6; $^{+}$p<0.05; ANOVA).

Figure 3.4. shows that mean IP-10 mRNA expression was significantly increased in tissue prepared from LPS-treated wildtype (1625 ± 431.3 RQ; n=6) and SIGIRR$^{-/-}$ mice (1778 ± 178.0 RQ; n=6) compared with tissue obtained from saline-treated control wildtype (1.31 ± 0.17 RQ; n=6;***p<0.001; ANOVA) and SIGIRR$^{-/-}$ mice (5.51 ± 3.96 RQ; n=6; ***p<0.001; ANOVA). The effects of LPS-challenge on IP-10 mRNA expression was similar in SIGIRR$^{-/-}$ mice and wildtype mice.

Effect of LPS on IL-1β, IL-6 and TNF-α mRNA expression in SIGIRR$^{-/-}$ mice

Recent studies have shown that SIGIRR tempers cellular activation by IL-1β and LPS and may act as a 'brake' on the TLR system (O'Neill, 2003). To determine if the negative regulatory activity of SIGIRR extends to the brain, the effect of LPS administration on IL-1β, IL-6 and TNF-α mRNA expression was assessed in snap frozen hippocampal tissue prepared from LPS-treated wildtype and SIGIRR$^{-/-}$ mice. Figure 3.5. shows that mean IL-1β mRNA expression was significantly increased in hippocampal tissue prepared from LPS-treated wildtype (2.61 ± 0.79 RQ; n=6) and SIGIRR$^{-/-}$ mice (8.70 ± 3.35 RQ; n=6) compared with saline-treated wildtype (0.43 ± 0.19 RQ; n=6; *p<0.05; Student's *t* test) and SIGIRR$^{-/-}$ mice (0.05 ± 0.01 RQ; n=6; *p<0.05; ANOVA). LPS induced a significant increase in IL-1β mRNA expression in SIGIRR$^{-/-}$ mice (8.70 ± 3.35 RQ; n=6) compared with LPS-treated wildtype mice (2.61 ± 0.79 RQ; n=6; $^{+}$p<0.05; ANOVA).

Figure 3.6. shows that mean IL-6 mRNA expression was significantly increased in tissue from LPS-treated wildtype mice (452.4 ± 216.2 RQ; n=6) and SIGIRR$^{-/-}$ mice (1438 ± 420.6 RQ; n=6) compared with saline-treated wildtype mice

(1.83 ± 0.37 RQ; n=6; *$p<0.05$; Student's *t* test) and SIGIRR$^{-/-}$ mice (15.53 ± 14.91 RQ; n=6; ***$p<0.001$; ANOVA). LPS induced a significant increase in IL-6 mRNA expression in SIGIRR$^{-/-}$ mice (1438 ± 420.6 RQ; n=6) compared with LPS-treated wildtype mice (452.4 ± 216.2 RQ; n=6; $^{++}p<0.01$; ANOVA).

Figure 3.7. shows that mean TNF-α mRNA expression was significantly increased in tissue from LPS-treated wildtype (8.49.5 ± 423.9 RQ; n=6) and SIGIRR$^{-/-}$ mice (2053 ± 641.7 RQ; n=6) compared with saline-treated wildtype (1.64 ± 0.56 RQ; n=6; *$p<0.05$; Student's *t* test) and SIGIRR$^{-/-}$ mice (0.54 ± 0.14 RQ; n=6;**$p<0.01$; ANOVA). LPS induced a significant increase in TNF-α mRNA expression in SIGIRR$^{-/-}$ mice (2053 ± 641.7 RQ; n=6) compared with LPS-treated wildtype mice (849.5 ± 423.9 RQ; n=6; $^{+}p<0.05$; ANOVA).

Effect of LPS on IL-1β, IL-6 and TNF-α concentrations in SIGIRR$^{-/-}$ mice

To determine if SIGIRR negatively regulates the concentration of pro-inflammatory cytokines in the brain, the effect of LPS administration on IL-1β, IL-6 and TNF-α concentrations were assessed in hippocampal homogenates prepared from LPS-treated wildtype and SIGIRR$^{-/-}$ mice. Figure 3.8. shows IL-1β protein concentration was significantly increased in tissue prepared from LPS-treated wildtype mice (11.08 pg/mg ± 1.14; n=6) and SIGIRR$^{-/-}$ mice (17.34 pg/mg ± 2.86; n=6) compared with saline-treated control wildtype mice (5.79 pg/mg ± 1.62; n=6; *$p<0.05$; ANOVA) and SIGIRR$^{-/-}$ mice (8.10 pg/mg ± 2.31; n=6; *$p<0.05$; ANOVA). SIGIRR$^{-/-}$ mice showed enhanced responsiveness to LPS treatment compared with wildtype mice; treatment with LPS induced a significant increase in IL-1β protein concentration in SIGIRR$^{-/-}$ mice (17.34 pg/mg ± 2.86; n=6) compared with LPS-treated wildtype mice (11.08 pg/mg ± 1.14; n=6; $^{+}p<0.05$; ANOVA).

Figure 3.9. illustrates that mean IL-6 concentration was significantly increased in tissue prepared from LPS-treated wildtype mice (39.13 pg/mg ± 2.28; n=6) and SIGIRR$^{-/-}$mice (54.07 pg/mg ± 5.09; n=6) compared with saline-treated control wildtype mice (28.60 pg/mg ± 1.31; n=6; *$p<0.05$; ANOVA) and SIGIRR$^{-/-}$ mice (30.44 pg/mg ± 2.27; n=6;***$p<0.001$; ANOVA). SIGIRR$^{-/-}$ mice showed enhanced responsiveness to LPS treatment compared with wildtype mice; treatment

with LPS induced a significant increase in IL-6 protein concentration in SIGIRR$^{-/-}$ mice (54.07 pg/mg ± 5.09; n=6) compared with LPS-treated wildtype mice (39.13 pg/mg ± 2.28; n=6; $^{++}p<0.01$; ANOVA).

Figure 3.10. illustrates that mean TNF-α concentration was similar in all treatment groups, values were (16.49 pg/mg ± 2.73; n=6) and (16.24 pg/mg ± 2.04; n=6) in tissue prepared from control- and LPS-treated wildtype mice and (21.99 pg/mg ± 3.47; n=6) and (59.99 pg/mg ± 25.46; n=6) in tissue prepared from control- and LPS-treated SIGIRR$^{-/-}$mice.

SIGIRR$^{-/-}$ mice show enhanced JNK activation in response to LPS

JNK is a stress activated protein kinases which has been shown to be activated downstream of IL-1β (Loscher *et al.*, 2000), in order to determine if SIGIRR has the capacity to negatively regulate pathways downstream from IL-1β, the effect of LPS administration on JNK phosphorylation was assessed in hippocampal homogenates prepared from LPS-treated wildtype and SIGIRR$^{-/-}$ mice. Analysis of the mean data obtained from densitometric assessment indicated that JNK phosphorylation (expressed as a ratio of phosphorylated JNK to total JNK) was unchanged in hippocampal tissue prepared from LPS-treated wildtype mice compared with saline-treated wildtype mice. Figure 3.11. (A) shows two sample immunoblots indicating that LPS induced an increase in phosphorylated JNK (p-JNK) but not total JNK (t-JNK) tissue prepared from LPS-treated SIGIRR$^{-/-}$ mice compared with control-treated SIGIRR$^{-/-}$ mice. Figure 3.11. (B) shows that JNK phosphorylation was significantly increased in tissue prepared from LPS-treated SIGIRR$^{-/-}$ mice. (1.06 ± 0.14 arbitrary units; n=6) compared with saline-treated SIGIRR$^{-/-}$ mice (0.73 ± 0.05 arbitrary units; n=6; $^{*}p<0.05$; ANOVA). SIGIRR$^{-/-}$ mice showed enhanced sensitivity in response to LPS treatment compared with wildtype mice; treatment with LPS induced a significant increase in p-JNK in SIGIRR$^{-/-}$ mice (1.06 ± 0.14 arbitrary units; n=6) compared with LPS-treated wildtype mice (0.60 ± 0.06 arbitrary units; n=6; $^{++}p<0.01$; ANOVA).

Effect of LPS on head dipping behaviour in SIGIRR$^{-/-}$ mice in the hole-board test

Various reports have shown that an LPS-challenge to SIGIRR-deficient mice causes enhanced inflammatory responses (Garlanda *et al.*, 2004) and reduced survival rates (Wald *et al.*, 2003). One of the aims of this study was to establish if an LPS-challenge could induce exacerbated sickness behaviour in SIGIRR$^{-/-}$ mice, to this end the effect of LPS to alter the exploratory behaviour of SIGIRR$^{-/-}$ mice in a hole-board test was examined. Figure 3.12. shows that control-treated SIGIRR$^{-/-}$ mice exhibited a reduction in head dipping (10.83 counts ± 1.08; n=6) compared with control-treated wildtype mice (18.20 counts ± 0.97; n=6; $^{\$\$}p<0.01$; ANOVA). Treatment with LPS significantly decreased head dipping in wildtype (12.83 counts ± 1.54; n=6) and SIGIRR$^{-/-}$ mice (3.83 counts ± 1.42; n=6) compared with control-treated wildtype (18.20 counts ± 0.97; n=6, *p<0.05; ANOVA) and SIGIRR$^{-/-}$ mice (10.83 counts ± 1.08; n=6;**p<0.01; ANOVA). Although LPS decreased head dipping in wildtype and SIGIRR$^{-/-}$ mice this LPS-effect was exacerbated in SIGIRR$^{-/-}$ mice, such that there was a significant attenuation in head dipping in LPS-treated SIGIRR$^{-/-}$ mice (3.83 counts ± 1.42; n=6) compared with LPS-treated wildtype mice (12.83 counts ± 1.54; n=6; $^{+++}p<0.001$; ANOVA).

Effect of LPS on rearing behaviour in SIGIRR$^{-/-}$ mice in the hole-board test

Another determinant of exploratory behaviour is 'rearing' which was examined in LPS-treated wildtype and SIGIRR$^{-/-}$ mice. Figure 3.13. shows that treatment with LPS had no effect on rearing behaviour in LPS-treated wildtype mice (7.00 counts ± 1.55; n=6) compared with control-treated wildtype mice (9.20 counts ± 1.39; n=6). Conversely, treatment with LPS significantly decreased rearing behaviour in SIGIRR$^{-/-}$ mice (6.67 counts ± 1.31; n=6) compared with control-treated SIGIRR$^{-/-}$ mice (12.40 counts ± 0.68; n=6; **p<0.01; Student's *t* test).

Effect of LPS on the frequency of entries of SIGIRR$^{-/-}$ mice into the central zone of the hole-board test

The hole-board arena comprises an outer zone surrounding a central zone and anxiety behaviour is typified by avoidance of the central zone. In this study, the

frequency of entries into the central zone was assessed in control- and LPS-treated wildtype and SIGIRR$^{-/-}$ mice. Figure 3.14. shows that the frequency of entries into the central zone was reduced in control-treated SIGIRR$^{-/-}$ (10.67 counts ± 0.99; n=6) compared with control-treated wildtype mice (15.80 counts ± 1.24; n=6; $^{\$\$}p<0.01$; ANOVA). The frequency of entries into the central zone was significantly reduced in LPS-treated wildtype (9.83 counts ± 0.91; n=6) and SIGIRR$^{-/-}$ mice (2.60 counts ± 1.03; n=6) compared with control-treated wildtype (15.80 counts ± 1.24; n=6; $^{**}p<0.01$; ANOVA) and SIGIRR$^{-/-}$ mice (10.67 counts ± 0.99; n=6; $^{***}p<0.001$; ANOVA). Although LPS decreased head dipping in wildtype and SIGIRR$^{-/-}$ mice this LPS-effect was exacerbated in SIGIRR$^{-/-}$ mice, such that there was a significant difference between counts in LPS-treated SIGIRR$^{-/-}$ mice (2.60 counts ± 1.03; n=6) compared with LPS-treated wildtype mice (9.83 counts ± 0.91; n=6; $^{+++}p<0.001$; ANOVA).

Effect of LPS on the time SIGIRR$^{-/-}$ mice spend in each zone of the hole-board test

Since the hole-board test is divided into an outer and a central zone, the time spent in each zone can be calculated and expressed as a percentage of the total time spent in the arena. Figure 3.15. shows that LPS treatment had no effect on the time wildtype and SIGIRR$^{-/-}$ mice spend in the outer zone of the hole-board arena. Conversely control-treated SIGIRR$^{-/-}$ mice spend more time in the outer zone of a hole-board arena (81.45 % of entire duration ± 2.03; n=6) compared with control-treated wildtype mice (63.13 % of entire duration ± 4.59; n=6; $^{\$\$}p<0.01$; Student's *t* test) which is indicative of anxiety behaviour. Correspondingly, Figure 3.16. shows that control-treated SIGIRR$^{-/-}$ mice spend less time in the central zone of the hole-board arena (18.55 % of entire duration ± 2.03; n=6) than control-treated wildtype mice (36.87 % of entire duration ± 4.59; n=6; $^{\$\$}p<0.01$; Student's *t* test). LPS treatment had no effect on the time wildtype and SIGIRR$^{-/-}$ mice spend in the central zone of the hole-board arena.

Effect of LPS on the distance SIGIRR$^{-/-}$ mice stay from the border of the arena

Another measure of anxiety behaviour is the relative time spent in the area proximal to the border of a hole-board arena. Figure 3.17. shows that LPS treatment had no effect on the mean distance wildtype or SIGIRR$^{-/-}$ mice stayed from the border of the arena. Control-treated SIGIRR$^{-/-}$ mice stayed closer to the border of an hole-board arena (10.06 cm ± 0.25; n=6) compared with control-treated wildtype mice (15.18 cm ± 1.01; n=6; $^{\$\$}p<0.01$; Student's *t* test).

Effect of LPS on the total distance travelled by SIGIRR$^{-/-}$ mice

Figure 3.18. shows that LPS treatment reduced the total distance travelled by wildtype and SIGIRR$^{-/-}$ mice (1128 cm ± 123.7; n=6 and 827.6 cm ± 223.9; n=6 respectively) compared with control-treated wildtype (1831 cm ± 195; n=6; **$p<0.01$; ANOVA) and control-treated SIGIRR$^{-/-}$ mice (2049 cm ± 114.4; n=6; *$p<0.05$; ANOVA).

Effect of LPS on the maximum distance travelled by SIGIRR$^{-/-}$ mice at one time

Figure 3.19. shows that LPS treatment reduced the maximum distance travelled at one time by wildtype and SIGIRR$^{-/-}$ mice (9.21 cm ± 0.78; n=6 and 9.02 cm ± 1.08; n=6 respectively) compared with control-treated wildtype (13.18 cm ± 0.63; n=6; **$p<0.01$; ANOVA) and control-treated SIGIRR$^{-/-}$ mice (12.53 cm ± 0.69; n=6; *$p<0.05$; ANOVA).

Effect of LPS on the mean velocity travelled by SIGIRR$^{-/-}$ mice

Figure 3.20. shows that LPS treatment reduced the mean velocity travelled by wildtype and SIGIRR$^{-/-}$ mice (10.97 cm/s ± 1.28; n=6 and 8.28 cm/s ± 2.24; n=6 respectively) compared with control-treated wildtype (18.31 cm/s ± 1.95; n=6; **$p<0.01$; ANOVA) and control-treated SIGIRR$^{-/-}$ mice (20.51 cm/s ± 1.15; n=6; ***$p<0.001$; ANOVA).

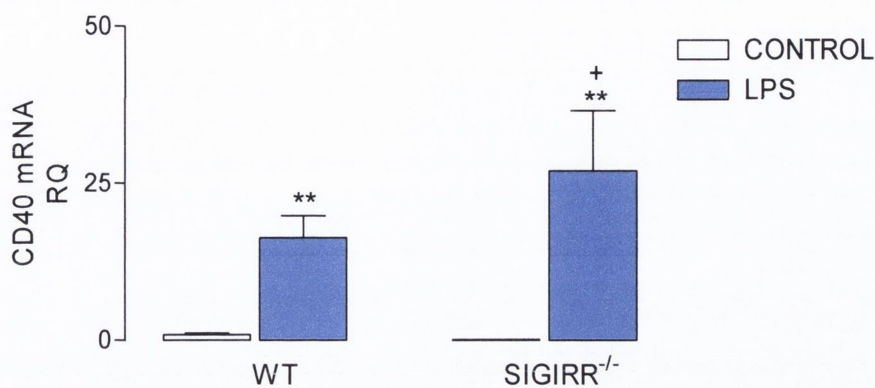


Figure 3.1. The effect of LPS on hippocampal CD40 mRNA expression is augmented in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and CD40 mRNA expression determined by QPCR. Mean CD40 mRNA expression was increased in hippocampal tissue prepared from LPS-treated wildtype compared with control mice (**$p<0.01$; Student's *t* test) and in LPS-treated SIGIRR$^{-/-}$ mice compared with control-treated wildtype and SIGIRR$^{-/-}$ mice (**$p<0.01$; ANOVA). Treatment with LPS induced a significantly greater effect in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{+}p<0.05$, ANOVA). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of CD40 mRNA to an endogenous control and are means ± SEM (n=6 in each group).

2-way ANOVA: LPS $_{effect}$ $F(1, 16) = 19.79$; $p=0.0004$, Strain $_{effect}$ $F(1, 16) = 2.00$; $p=0.1763$, Interaction $_{effect}$ $F(1, 16) = 2.44$; $p=0.1376$

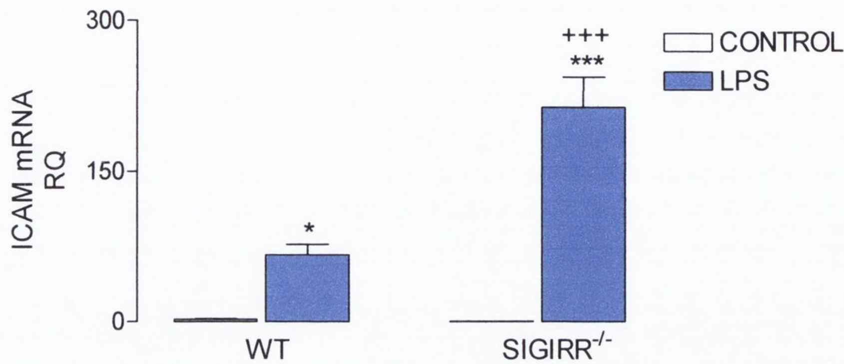


Figure 3.2. The effect of LPS on hippocampal ICAM mRNA expression is exacerbated in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200μl) or LPS (50μg/mouse; 200μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and ICAM mRNA expression determined by QPCR. Mean ICAM mRNA was increased in hippocampal tissue prepared from LPS-treated wildtype and treated SIGIRR$^{-/-}$ mice compared with hippocampal tissue obtained from saline-treated wildtype mice (*$p<0.05$; ANOVA) and SIGIRR$^{-/-}$ mice (***$p<0.001$; ANOVA). Treatment with LPS induced a significantly greater effect in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{+++}p<0.001$, ANOVA). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of ICAM mRNA to an endogenous control and are means ± SEM (n=6 in each group).

2-way ANOVA: LPS $_{effect}$ $F(1, 17) = 75.75$; $p<0.0001$, Strain $_{effect}$ $F(1, 17) = 20.66$; $p=0.0003$, Interaction $_{effect}$ $F(1, 17) = 21.80$; $p=0.0002$

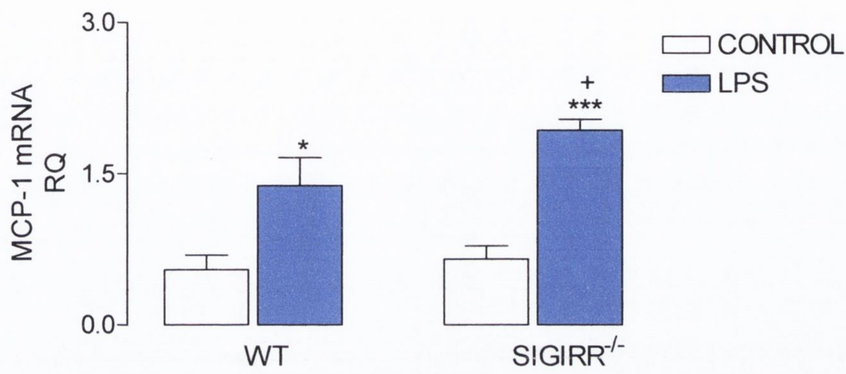


Figure 3.3. The effect of LPS on hippocampal MCP-1 mRNA expression is exacerbated in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 3 hours, mice were killed by decapitation; hippocampi dissected free, homogenized and MCP-1 mRNA expression determined by QPCR. Mean MCP-1 mRNA expression was significantly increased in hippocampal tissue prepared from LPS-treated wildtype and treated SIGIRR$^{-/-}$ mice compared with hippocampal tissue obtained from control-treated wildtype (*p<0.05, ANOVA) and SIGIRR$^{-/-}$ mice (***p<0.001, ANOVA). Treatment with LPS induced a significantly greater effect in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{+}$p<0.05, ANOVA). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of MCP-1 mRNA to an endogenous control and are means ± SEM (n=6 in each group).

2-way ANOVA: LPS $_{effect}$ F (1, 18) = 38.14; p<0.0001, Strain $_{effect}$ F (1, 18) = 3.64; p=0.0727, Interaction $_{effect}$ F (1, 18) = 1.69; p=0.2106

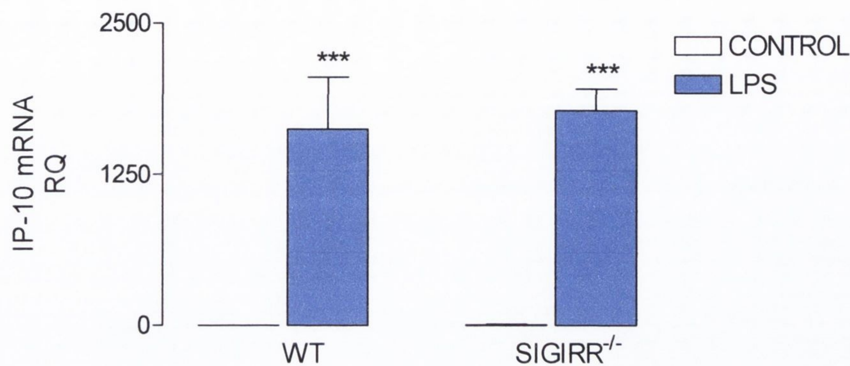


Figure 3.4. Effect of LPS on hippocampal IP-10 mRNA expression in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 3 hours, mice were killed by decapitation; hippocampi dissected free, homogenized and IP-10 mRNA expression determined by QPCR. Mean IP-10 mRNA expression was significantly increased in hippocampal tissue prepared from LPS-treated wildtype and SIGIRR$^{-/-}$ mice compared with hippocampal tissue obtained from control-treated wildtype mice (***p<0.001, ANOVA) and SIGIRR$^{-/-}$ mice (***p<0.001, ANOVA). IP-10 mRNA expression was similar in LPS-treated wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as relative quantities (RQ) obtained from calculating the ratio of IP-10 mRNA to an endogenous control and are means ± SEM (n=6 in each group).

2-way ANOVA: LPS $_{effect}$ F (1, 19) = 47.94; p<0.0001, Strain $_{effect}$ F (1, 19) = 0.10; p=0.7515, Interaction $_{effect}$ F (1, 19) = 0.09; p=0.7643

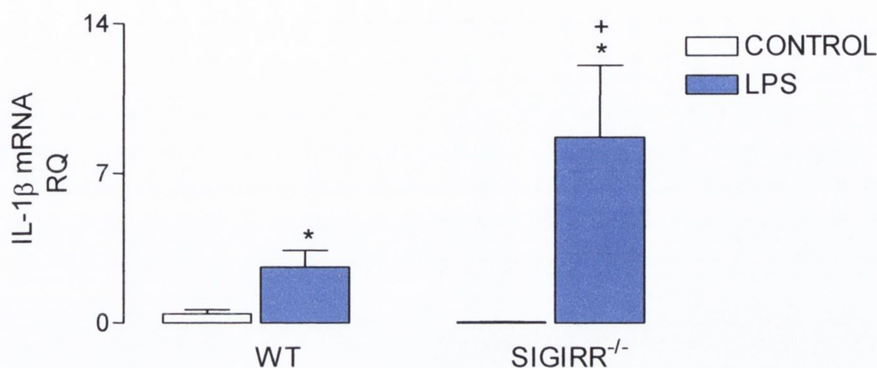


Figure 3.5. The effect of LPS on hippocampal IL-1β mRNA expression is exacerbated in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and IL-1β mRNA expression determined by QPCR. Mean IL-1β mRNA was significantly increased in hippocampal tissue prepared from LPS-treated wildtype mice compared with control-treated wildtype mice (*$p<0.05$; Student's *t* test). Mean IL-1β mRNA was significantly increased in hippocampal tissue prepared from LPS-treated SIGIRR$^{-/-}$ mice compared with control-treated SIGIRR$^{-/-}$ mice (*$p<0.05$; ANOVA) and this LPS-induced increase in IL-1β mRNA was significantly higher in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{+}p<0.05$; ANOVA). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of IL-1β mRNA to an endogenous control and are means ± SEM (n=6 in each group).

2-way ANOVA: LPS $_{effect}$ $F_{(1, 17)} = 7.16$; $p=0.0160$, Strain $_{effect}$ $F_{(1, 17)} = 2.00$; $p=0.1756$, Interaction $_{effect}$ $F_{(1, 17)} = 2.56$; $p=0.1283$

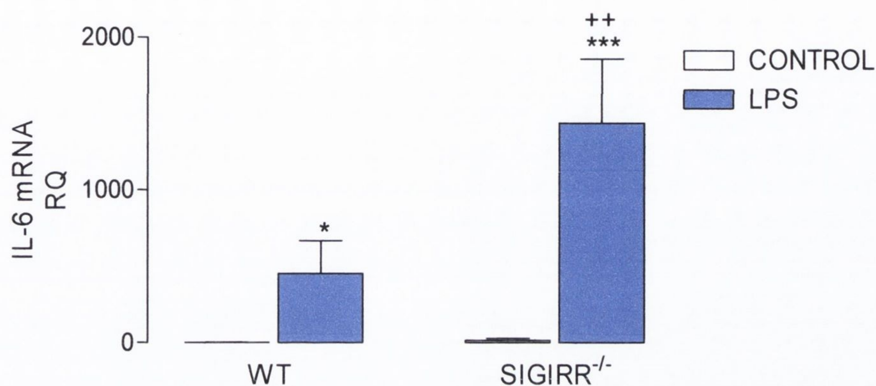


Figure 3.6. The effect of LPS on hippocampal IL-6 mRNA expression is exacerbated in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 3 hours, mice were killed by decapitation; hippocampi dissected free, homogenized and IL-6 mRNA expression determined by QPCR. Mean IL-6 mRNA expression was significantly increased in hippocampal tissue prepared from LPS-treated wildtype mice compared with control-treated wildtype mice (*$p<0.05$; Student's *t* test). Mean IL-6 mRNA was significantly increased in hippocampal tissue prepared from LPS-treated SIGIRR$^{-/-}$ mice compared with controls (***$p<0.001$; ANOVA) and the LPS-induced increase in IL-6 mRNA was significantly higher in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{++}p<0.01$; ANOVA). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of IL-6 mRNA to an endogenous control and are means ± SEM (n=6 in each group).

2-way ANOVA: LPS $_{effect}$ $F(1, 39) = 14.76$; $p=0.0004$, Strain $_{effect}$ $F(1, 39) = 4.20$; $p=0.0472$, Interaction $_{effect}$ $F(1, 39) = 3.97$; $p=0.0533$

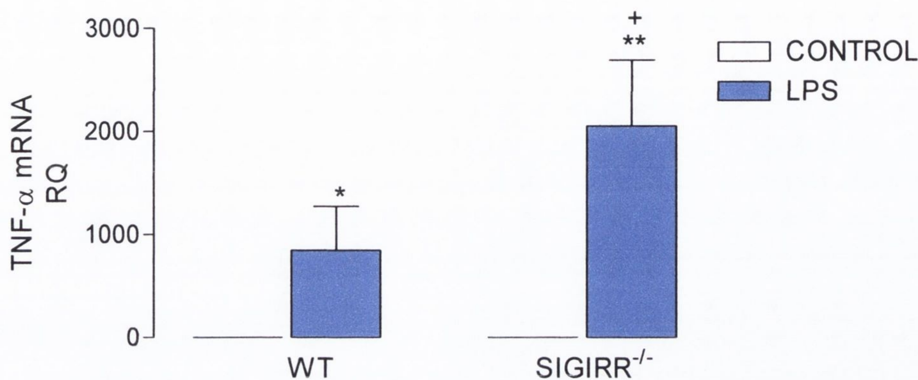


Figure 3.7. The effect of LPS on hippocampal TNF-α mRNA expression is exacerbated in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200μl) or LPS (50μg/mouse; 200μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and TNF-α mRNA expression determined by QPCR. Mean TNF-α mRNA was significantly increased in hippocampal tissue prepared from LPS-treated wildtype mice compared with control-treated wildtype mice (*$p<0.05$; Student's *t* test). Mean TNF-α mRNA was significantly increased in hippocampal tissue prepared from LPS-treated SIGIRR$^{-/-}$ mice compared with controls (**$p<0.01$; ANOVA) and this LPS-induced increase in TNF-α mRNA was significantly higher in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{+}p<0.05$; ANOVA). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of TNF-α mRNA to an endogenous control and are means ± SEM (n=6 in each group).

2-way ANOVA: LPS $_{effect}$ $F(1, 18) = 12.95$; $p=0.0021$, Strain $_{effect}$ $F(1, 18) = 2.23$; $p=0.1531$, Interaction $_{effect}$ $F(1, 18) = 2.23$; $p=0.1524$

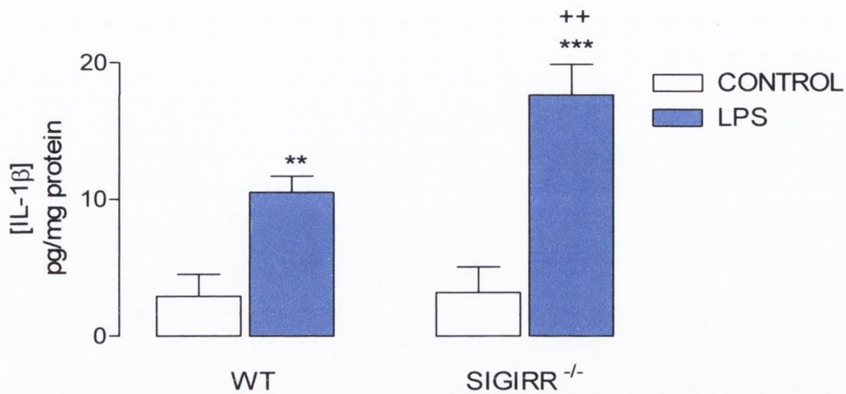


Figure 3.8. The effect of LPS on hippocampal IL-1β concentration is exacerbated in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200μl) or LPS (50μg/mouse; 200μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and IL-1β concentration determined by ELISA. Mean IL-1β concentration was significantly increased in hippocampal homogenates prepared from LPS-treated wildtype and SIGIRR$^{-/-}$ mice compared with hippocampal homogenates obtained from control-treated wildtype (**$p<0.01$, ANOVA) and SIGIRR$^{-/-}$ mice (***$p<0.001$, ANOVA). Treatment with LPS induced a significant increase in IL-1β concentration in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{++}p<0.01$, ANOVA). Values are expressed as pg IL-1β/mg protein and presented as means ± SEM of 6 observations per group.

2-way ANOVA: LPS $_{effect}$ $F (1, 48) = 40.41$; $p<0.0001$, Strain $_{effect}$ $F (1, 48) = 4.54$; $p=0.0384$, Interaction $_{effect}$ $F (1, 48) = 3.88$; $p=0.0546$

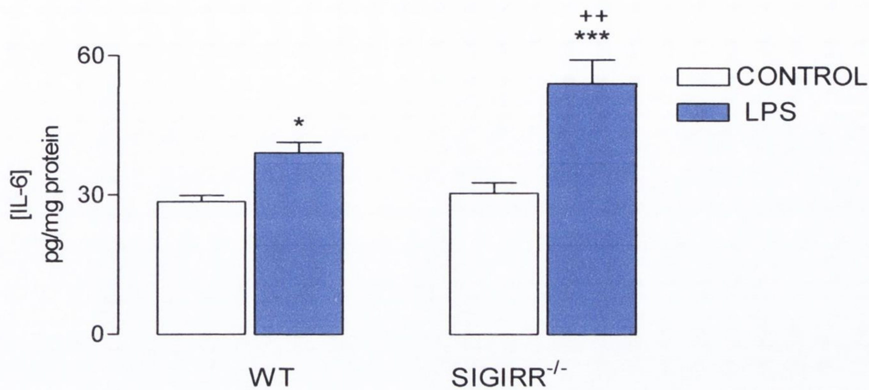


Figure 3.9. The effect of LPS on hippocampal IL-6 concentration is exacerbated in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200μl) or LPS (50μg/mouse; 200μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and IL-6 concentration determined by ELISA. Mean IL-6 concentration was significantly increased in hippocampal homogenates prepared from LPS-treated wildtype and SIGIRR$^{-/-}$ mice compared with hippocampal homogenates obtained from control-treated wildtype (*p<0.05, ANOVA) and SIGIRR$^{-/-}$ mice (***p<0.001, ANOVA). Treatment with LPS induced a significant increase in IL-6 concentration in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{++}$p<0.01, ANOVA). Values are expressed as pg IL-6/mg protein and presented as means ± SEM of 6 observations per group.

2-way ANOVA: LPS $_{effect}$ F (1, 18) = 34.58; p<0.0001, Strain $_{effect}$ F (1, 18) = 8.35; p=0.0098, Interaction $_{effect}$ F (1, 18) = 5.08; p=0.0370

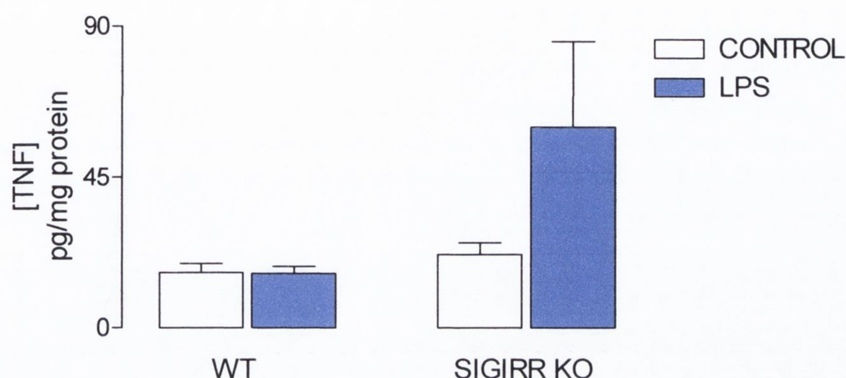


Figure 3.10. Effect of LPS on hippocampal TNF-α concentration in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200μl) or LPS (50μg/mouse; 200μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and TNF-α concentration determined by ELISA. Mean hippocampal TNF-α concentration was similar in control- and LPS-treated wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as pg TNF-α /mg protein and presented as means ± SEM of 6 observations per group.

2-way ANOVA: LPS $_{effect}$ F (1, 17) = 1.59; p=0.2249, Strain $_{effect}$ F (1, 17) = 2.70; p=0.1187, Interaction $_{effect}$ F (1, 17) = 1.63; p=0.2197

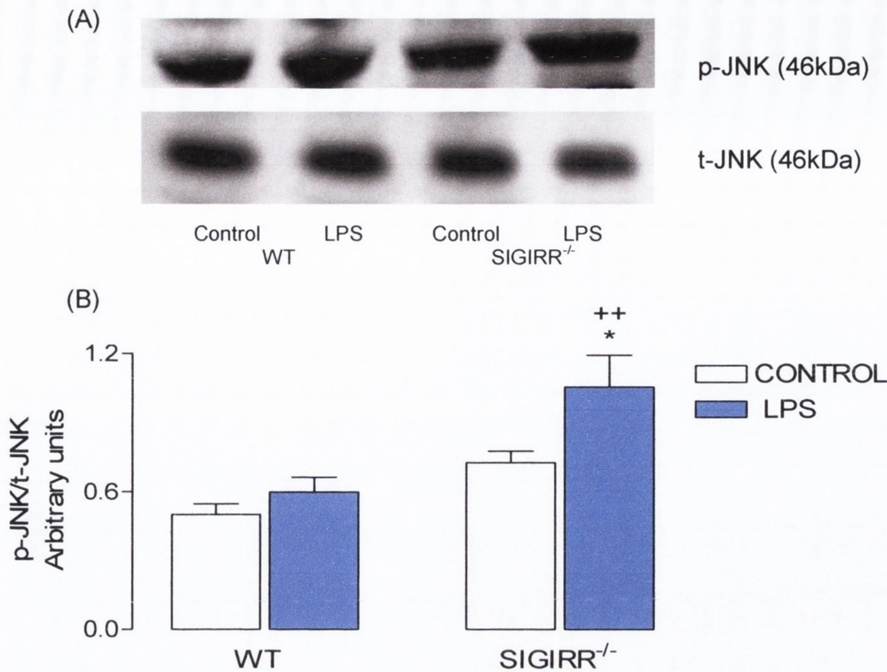


Figure 3.11. The effect of LPS on hippocampal JNK activation is exacerbated in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200μl) or LPS (50μg/mouse; 200μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and JNK phosphorylation determined by Western immunoblotting (expressed as a ratio of phosphorylated to total JNK in arbitrary units). (A) Sample immunoblots indicating density of phosphorylated JNK and total JNK (p-JNK and t-JNK) for hippocampal tissue prepared from control- and LPS-treated wildtype and SIGIRR$^{-/-}$ mice. (B) JNK phosphorylation was similar in tissue prepared from control- and LPS-treated wildtype mice. JNK phosphorylation was significantly increased in tissue prepared from LPS-treated SIGIRR$^{-/-}$ mice compared with control-treated SIGIRR$^{-/-}$ mice (*$p<0.05$, ANOVA). Treatment with LPS induced a significantly greater effect in SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{++}p<0.01$, ANOVA). Values are expressed in arbitrary units obtained from calculating the ratio of p-JNK to t-JNK provided by densitometric analysis and are means ± SEM (n=6 values in all groups).

2-way ANOVA: LPS $_{effect}$ $F_{(1, 17)} = 6.84$; p=0.0181, Strain $_{effect}$ $F_{(1, 17)} = 17.50$; p=0.0006, Interaction $_{effect}$ $F_{(1, 17)} = 2.01$; p=0.1742

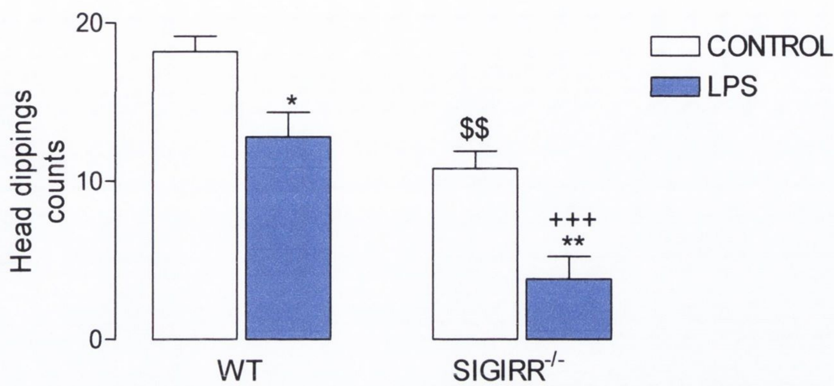


Figure 3.12. Effect of LPS on head dipping behaviour in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. Control-treated SIGIRR$^{-/-}$ mice showed reduced head dipping compared with control-treated wildtype mice ($^{\$\$}p<0.01$; ANOVA). Treatment with LPS significantly reduced head dipping in wildtype and SIGIRR$^{-/-}$ mice compared with control treated wildtype and SIGIRR$^{-/-}$ mice (*$p<0.05$, **$p<0.01$; ANOVA). This LPS-effect was enhanced in SIGIRR$^{-/-}$ such that there was a significantly greater LPS-induced change in SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p<0.001$; ANOVA). Values are expressed as counts and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ F (1, 19) = 22.40; p=0.0001, Strain $_{effect}$ F (1, 19) = 39.24; p<0.0001, Interaction $_{effect}$ F (1, 19) = 0.39; p=0.5393

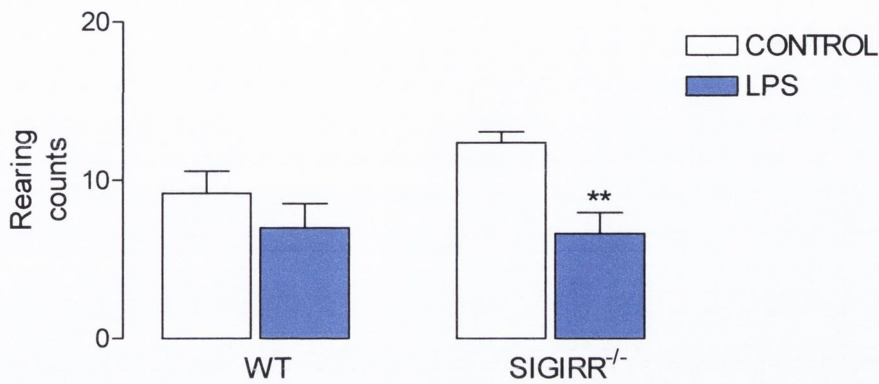


Figure 3.13. Effect of LPS on rearing behaviour in SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. Rearing was similar in control- and LPS-treated wildtype mice. Treatment with LPS significantly decreased rearing in SIGIRR$^{-/-}$ mice compared with control-treated SIGIRR$^{-/-}$ mice (**$p<0.01$; Student's *t* test). Values are expressed as counts and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ $F(1, 19) = 10.85$; $p=0.0038$, Strain $_{effect}$ $F(1, 19) = 1.79$; $p=0.1966$, Interaction $_{effect}$ $F(1, 19) = 2.55$; $p=0.1271$

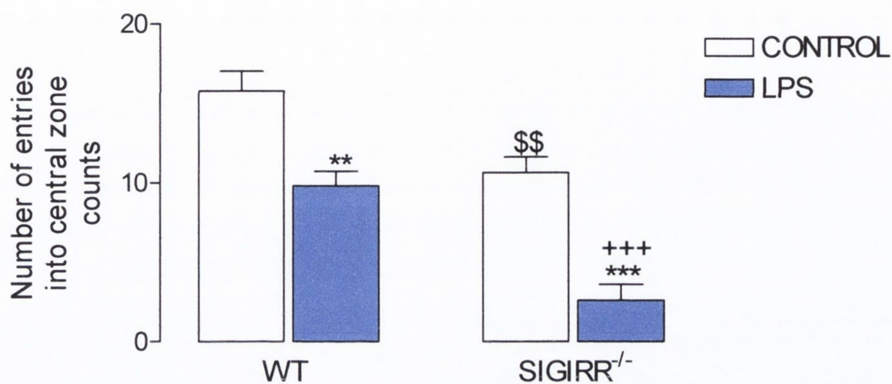


Figure 3.14. Effect of LPS on the number of entries of SIGIRR$^{-/-}$ mice into the central zone of the hole-board test

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200μl) or LPS (50μg/mouse; 200μl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. The frequency of entries into the central zone of a hole-board arena was decreased in control-treated SIGIRR$^{-/-}$ mice compared with control-treated wildtype mice ($^{\$\$}p<0.01$; ANOVA). Treatment with LPS significantly reduced the frequency of entry into the central zone in wildtype and SIGIRR$^{-/-}$ mice compared with control-treated wildtype and SIGIRR$^{-/-}$ mice (**$p<0.01$, ***$p<0.001$; ANOVA). This LPS-induced effect was enhanced in SIGIRR$^{-/-}$ mice such that there was a significant attenuation in LPS-treated SIGIRR$^{-/-}$ mice compared with LPS-treated wildtype mice ($^{+++}p<0.001$; ANOVA). Values are expressed as counts and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ $F_{(1, 18)} = 45.53$; $p<0.0001$, Strain $_{effect}$ $F_{(1, 18)} = 35.36$; $p<0.0001$, Interaction $_{effect}$ $F_{(1, 18)} = 1.02$; $p=0.3260$

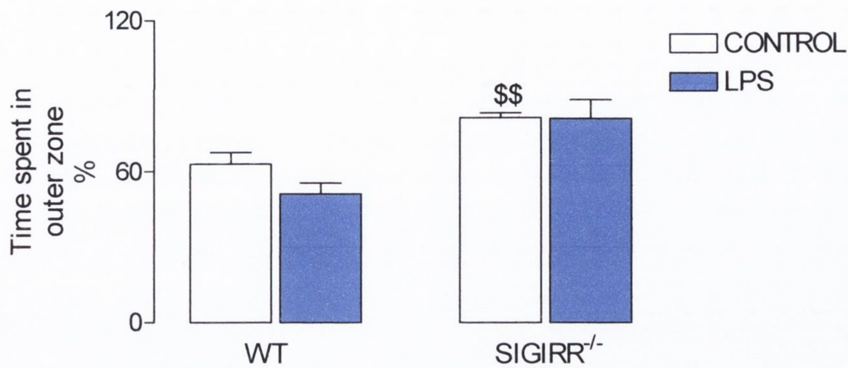


Figure 3.15. Effect of LPS on the time SIGIRR$^{-/-}$ mice spend in the outer zone

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. Control-treated SIGIRR$^{-/-}$ mice spend more time in the outer zone of a hole-board arena compared with control-treated wildtype mice ($^{\$\$}p<0.01$; Student's *t* test). LPS treatment had no effect on the amount of time wildtype and SIGIRR$^{-/-}$ mice spend in the outer zone compared with control-treated wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as percentages and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ F (1, 19) = 1.48; p=0.2384, Strain $_{effect}$ F (1, 19) = 23.21; p=0.0001, Interaction $_{effect}$ F (1, 19) = 1.37; p=0.2556

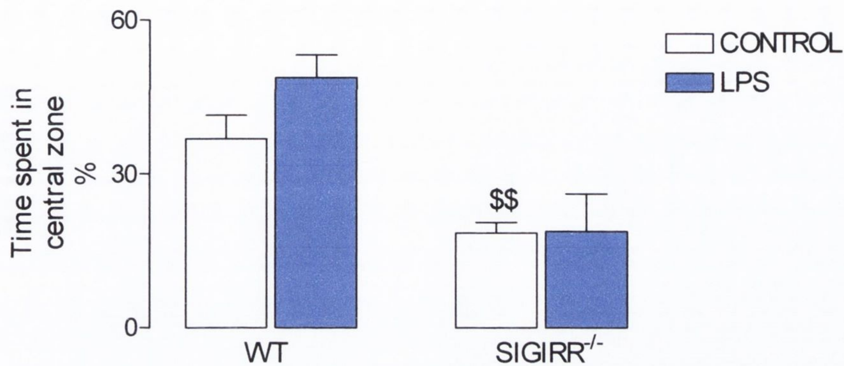


Figure 3.16. Effect of LPS on the time SIGIRR$^{-/-}$ mice spend in the central zone
Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. Control-treated SIGIRR$^{-/-}$ mice spend less time in the central zone of a hole-board arena compared with control-treated wildtype mice ($^{\$\$}p<0.01$; Student's *t* test). LPS treatment had no effect on the amount of time wildtype and SIGIRR$^{-/-}$ mice spend in the central zone compared with control-treated wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as percentages and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ $F(1, 18) = 0.69$; $p=0.4168$ Strain $_{effect}$ $F(1, 18) = 32.04$; $p<0.0001$, Interaction $_{effect}$ $F(1, 18) = 3.02$; $p=0.0991$

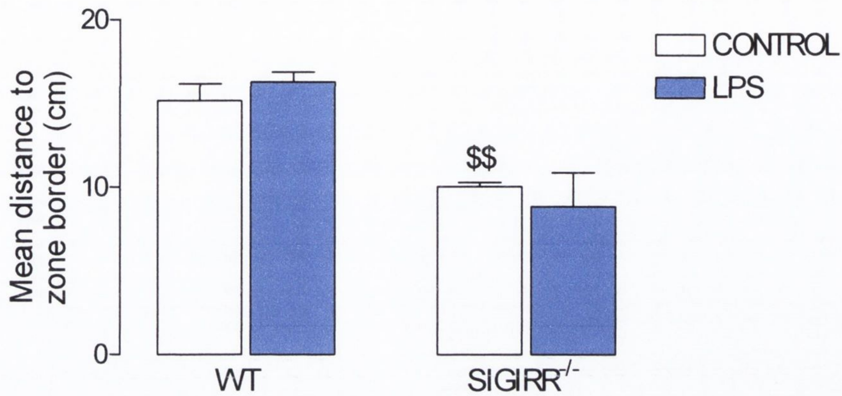


Figure 3.17. Effect of LPS on the distance SIGIRR$^{-/-}$ mice stay from the border of the arena

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. Control-treated SIGIRR$^{-/-}$ mice stayed closer to the border of a hole-board arena compared with control-treated wildtype mice, such that the mean distance to the arena border is reduced in control-treated SIGIRR$^{-/-}$ mice compared with control-treated wildtype mice ($^{\$\$}p<0.01$; Student's *t* test). LPS treatment had no effect on the distance wildtype or SIGIRR$^{-/-}$ mice spent to the border of the arena. Values are expressed as cm and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ $F(1, 19) = 0.00$; $p=0.9701$ Strain $_{effect}$ $F(1, 19) = 27.21$; $p<0.0001$, Interaction $_{effect}$ $F(1, 19) = 0.94$; $p=0.3436$

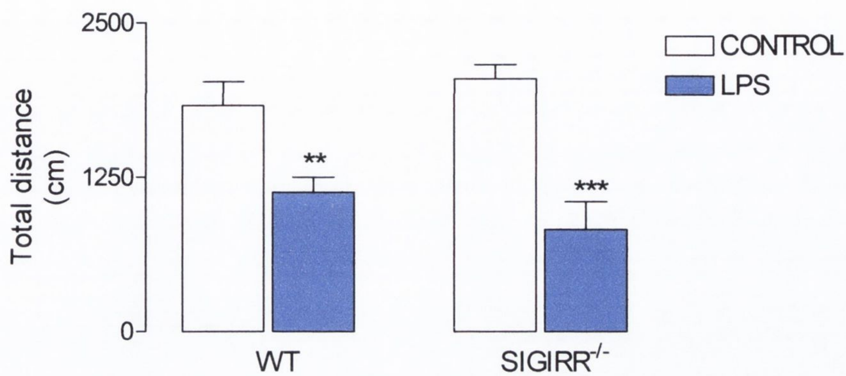


Figure 3.18. Effect of LPS on the total distance travelled by SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. LPS reduced the total distance travelled by wildtype and SIGIRR$^{-/-}$ mice such that the total distance travelled was reduced in LPS-treated wildtype and SIGIRR$^{-/-}$ mice compared with control-treated wildtype (**p<0.01; ANOVA) and control-treated SIGIRR$^{-/-}$ mice (***p<0.001; ANOVA). Values are expressed as cm and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ $F(1, 19) = 32.36$; $p<0.001$, Strain $_{effect}$ $F(1, 19) = 0.06$; $p=0.8113$, Interaction $_{effect}$ $F(1, 19) = 2.34$; $p=0.1425$

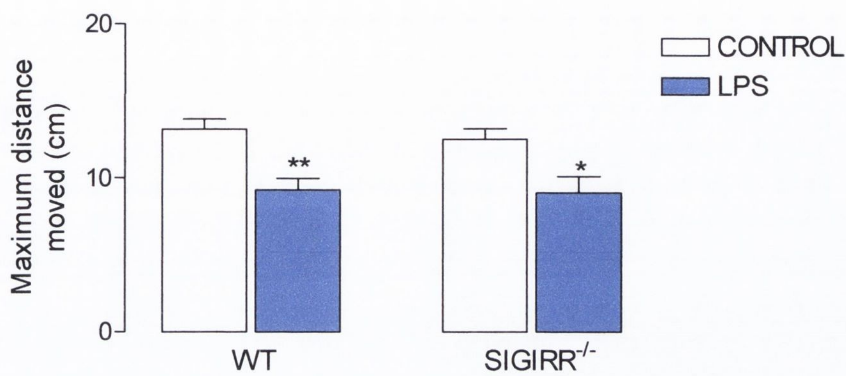


Figure 3.19. Effect of LPS on the maximum distance travelled by SIGIRR$^{-/-}$ mice at one time

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. LPS reduced the maximum distance travelled by wildtype and SIGIRR$^{-/-}$ mice such the distance travelled was reduced in LPS-treated wildtype and SIGIRR$^{-/-}$ mice compared with control-treated wildtype (**p<0.01; ANOVA) and control-treated SIGIRR$^{-/-}$ mice (*p<0.05; ANOVA). Values are expressed as cm and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ F (1, 19) = 20.25; p=0.0002, Strain $_{effect}$ F (1, 19) = 0.26; p=0.6175, Interaction $_{effect}$ F (1, 19) = 0.08; p=0.7803

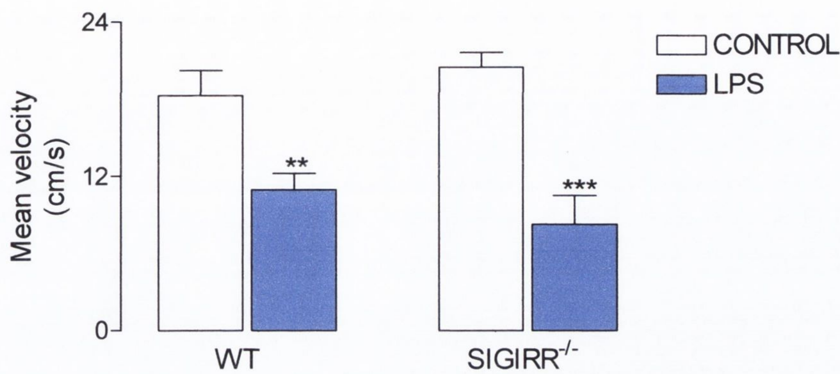


Figure 3.20. Effect of LPS on the mean velocity travelled by SIGIRR$^{-/-}$ mice

Wildtype and SIGIRR$^{-/-}$ mice were injected ip with sterile saline (200µl) or LPS (50µg/mouse; 200µl). After 30 minutes mice were assessed for sickness behaviour in the 'hole-board test' for 2 minutes. LPS reduced the mean velocity travelled by wildtype and SIGIRR$^{-/-}$ mice such the mean velocity was reduced in LPS-treated wildtype and SIGIRR$^{-/-}$ mice compared with control-treated wildtype (**p<0.01; ANOVA) and control-treated SIGIRR$^{-/-}$ mice (***p<0.001; ANOVA). Values are expressed as cm/s and are means ± SEM (n=6 observations in all groups).

2-way ANOVA: LPS $_{effect}$ F (1, 19) =32.83; p<0.0001, Strain $_{effect}$ F (1, 19) = 0.02; p=0.8891, Interaction $_{effect}$ F (1, 19) = 2.05; p=0.1685

3.4. Discussion

One of the central aims of this study was to determine if SIGIRR is an inflammatory watchdog in the CNS. The motivations for this study were based on two key facts; firstly that SIGIRR is expressed in the brain (Polentarutti *et al.*, 2003; Andre *et al.*, 2005) and secondly that SIGIRR is a negative regulator of TLR-mediated innate-immunity (Wald *et al.*, 2003; Garlanda *et al.*, 2004; Qin *et al.*, 2005) and microbial- and auto-immune-induced adaptive-immunity (Huang *et al.*, 2006; Lech *et al.*, 2008). The hypothesis advanced was that a deficiency in SIGIRR, combined with an LPS challenge, would lead to increased inflammation in the CNS.

The most significant findings of this study are that an LPS challenge induced exacerbated inflammation in the hippocampus of SIGIRR$^{-/-}$ mice. This inflammation is characterised by up-regulated microglial activation and enhanced production of chemokines and pro-inflammatory cytokines. Although this is the first study to reveal enhanced inflammation in the CNS of SIGIRR$^{-/-}$ mice in response to an LPS challenge, it is consistent with other studies which have shown that endotoxic shock, dextran-induced colitis, *P. aeruginosa* keratitis and systemic lupus erythematosus (SLE) can induce exaggerated inflammation in SIGIRR$^{-/-}$mice (Wald *et al.*, 2003; Garlanda *et al.*, 2004; Huang *et al.*, 2006; Lech *et al.*, 2008).

A single intraperitoneal injection of LPS significantly increased mean CD40 mRNA expression in hippocampus of wildtype and SIGIRR$^{-/-}$ mice however; this LPS-induced effect was exacerbated in SIGIRR$^{-/-}$ mice. These findings suggest that SIGIRR is involved in dampening the CD40 pathway in response to LPS. The mechanisms by which SIGIRR negatively regulates TLR-IL-1R-mediated signalling events has not been fully elucidated, however studies have shown that SIGIRR interacts with the TLR-IL-1R-downstream signalling components IRAK and TRAF-6 (Wald *et al.*, 2003; Qin *et al.*, 2005). In view of the fact that CD40 is a member of the TNF receptor superfamily which requires recruitment of TRAF-6 to initiate its inflammatory cascade (Arch *et al.*, 1998), these data suggest that perhaps SIGIRR dampens TLR-mediated CD40 activation through inhibition of TRAF-6.

Lymphocyte entry into the CNS is a critical step in the pathogenesis of disease and the mechanism of entry of lymphocytes into the CNS is through

interaction with cellular adhesion molecules expressed on endothelial cells. ICAM-1 is a cellular adhesion molecule that facilitates the migration of blood-borne lymphocytes across the BBB into the CNS (Kleine and Benes, 2006). ICAM-1 is also up-regulated in response to inflammatory stimuli such as LPS and IFN-γ (Wahl *et al.*, 1996) and importantly studies have shown that CD40-CD40L interactions can up-regulate ICAM-1 expression (Saito *et al.*, 2007). The data presented here show that the LPS-induced increase in ICAM-1 expression is exaggerated in SIGIRR$^{-/-}$ mice compared with wildtype control mice. These results provide a testable hypothesis that in response to an inflammatory stimulus such as LPS, SIGIRR negatively regulates CD40 and ICAM-1 mRNA expression and possibly limits the traffic of lymphocytes into the CNS.

There was heightened MCP-1 mRNA expression in the hippocampus of LPS-treated SIGIRR$^{-/-}$ mice compared with wildtype mice. This finding is concordant with that of Lech and colleagues (2007) who found increased MCP-1 and KC chemokine expression in response to LPS in renal myeloid cells. Likewise Garlanda and colleagues (2007) found increased MCP-1, MIP-1 and KC chemokine expression in DCs from SIGIRR$^{-/-}$ mice treated with bacillus Calmette-Guerin (BCG). Xiao and colleagues (2007) showed constitutive up-regulation of the chemokines MIP-2, KC and MCP-1 in the colon of SIGIRR$^{-/-}$ mice. Moreover, MIP-2 expression was increased in response to *P. aeruginosa* combined with an anti-SIGIRR antibody (Huang *et al.*, 2006). All of these results suggest that SIGIRR is significantly involved in dampening the expression of chemokines during an inflammatory response.

In this study, the expression of IP-10 mRNA was also examined in the hippocampus of SIGIRR$^{-/-}$ mice treated with LPS and it seems that IP-10 is the pariah of the chemokine system. Surprisingly, LPS-induced similar expression of IP-10 mRNA in the hippocampus of both wildtype and SIGIRR$^{-/-}$ mice suggesting that SIGIRR is not a negative regulator of this chemokine. On the other hand, another study showed that a deficiency in SIGIRR caused heightened activation of IP-10 mRNA expression in the lung, but not in the liver, in response to IL-1β and that treatment with TNF-α had no effect in either tissue (Wald *et al.*, 2003). This was a

surprising outcome considering that SIGIRR has been shown to dampen Thl cytokines such as IL-12 and IL-18, and especially IFN-γ (Huang *et al.*, 2006) and since IP-10, IFN-γ-inducible-protein 10, was originally named for its actions on IFN-γ, it might have been anticipated that down-regulation of IFN-γ would lead to subsequent down-regulation of IP-10. Perhaps this suggests that SIGIRR regulates IP-10 mRNA expression in certain cell types and under certain inflammatory conditions, and perhaps that IP-10 is regulated by other mechanisms.

There is a plethora of studies to suggest that SIGIRR acts differently to the other members of the IL-1R super-family and is incapable of inducing IL-1-like signalling (Thomassen *et al.*, 1999; Sims, 2002; Wald *et al.*, 2003; Polentarutti *et al.*, 2003). The first studies that pioneered the characterisation of SIGIRR showed that the intracellular domain of the receptor could not activate NFκB or AP-1 because it lacks two essential amino acids (Ser_{447} and Tyr_{536}) in its highly conserved TIR domain which are essential for IL-1β signalling (Thomassen *et al.*, 1999). Furthermore the intracellular domain extends that of the typical IL-1R family members by more than 73 amino acids at the C-terminus and this extra-long cytoplasmic region does not fold into a conformation that is sufficient to spontaneously bind to and activate the NFκB signalling machinery (Thomassen *et al.*, 1999). Indeed studies have shown that this extra long tail is reminiscent of the Toll receptor and plays a pivotal role in regulating Toll signalling (Norris *et al.*, 1995). Furthermore, there is also a growing body of evidence to suggest that not only does SIGIRR fail to initiate IL-1β signalling, but it is a potent negative regulator of IL-1β signalling. Over-expression of SIGIRR in Jurkat and HepG2 cells down-regulated IL-1β- and IL-18-mediated NFκB expression (Wald *et al.*, 2003). In the same way a deficiency in SIGIRR increased LPS-induced chemokine and cytokine expression (Wald *et al.*, 2003; Garlanda *et al.*, 2004; Lech *et al.*, 2007). Another significant finding of this study is that both the mRNA expression and concentration of the pro-inflammatory cytokines IL-1β and IL-6, are up-regulated in hippocampal tissue prepared from $SIGIRR^{-/-}$ mice. Conversely the expression of TNF-α mRNA only is increased in tissue prepared from $SIGIRR^{-/-}$ mice. This corroborates previous earlier findings that SIGIRR is a negative regulator of LPS-

induced signalling, however, this is the first study to show that the effects of SIGIRR are extended to the CNS.

The finding that IL-1β and IL-6 concentrations are up-regulated in response to LPS, whereas TNF-α concentrations are similar to wildtype mice, suggests that the regulatory effects of SIGIRR are not extended to TNF-α signalling. This finding is similar to previous reports; Wald and colleagues (2003) showed that there was increased expression of chemokines in the lung of SIGIRR$^{-/-}$ mice in response to IL-1β, but TNF-α had no effect. The lack of effects of TNF-α was attributed to the fact that TNF is not a member of the TLR/IL-1R family and therefore is not regulated by SIGIRR. Even though in this study SIGIRR$^{-/-}$ mice were treated with LPS and not TNF-α, this could be a possible explanation as to why TNF-α concentration is not up-regulated in SIGIRR$^{-/-}$ mice. The data presented here also show that SIGIRR exerts its negative regulatory capacity directly on LPS-induced downstream signalling components. JNK activation was significantly increased in SIGIRR$^{-/-}$ mice in response to LPS and other studies have reported this heightened JNK activation in response to LPS in SIGIRR$^{-/-}$ mice (Wald *et al.*, 2003; Qin *et al.*, 2005; Xiao *et al.*, 2007).

This study along with others definitively proves a role for SIGIRR in negatively regulating LPS-induced signalling. However the exact mechanisms by which SIGIRR mediates its anti-inflammatory actions has yet to be elucidated, some studies have suggested that SIGIRR interacts with IL-1RI and TLR4 signalling components such as IRAK and TRAF6 (Wald *et al.*, 2003; Qin *et al.*, 2005), either by sequestering these components or causing their dissociation. On the other hand, considering that SIGIRR is a member of the IL-1 super-family this suggests that it must bind a ligand to induce activation and downstream signalling. Yet other studies believe that the single Ig domain of SIGIRR is too short to fold around an IL-1-like ligand in the same manner as the other IL-1 receptors (Thomassen *et al.*, 1999). In contrast, O'Neill (2003) suggested that even with a single Ig domain SIGIRR would be able to bind to a ligand. Consistent with this idea, results from our previous studies have shown that a novel member of the IL-1 family, the anti-inflammatory cytokine IL-1F5, mediates its effects through interaction with SIGIRR (Costelloe *et al.*, 2008).

Taken together, this suggests that IL-1F5 and SIGIRR may be a novel immunomodulatory ligand-receptor complex in the CNS.

An important objective of this study was to determine if exacerbated inflammation in the hippocampus of LPS-treated SIGIRR$^{-/-}$ mice was accompanied by enhanced sickness behaviour. As a first step to assess this, the hole-board test which measures sickness behaviour in particular exploratory behaviour, anxiety-related behaviour and locomotory behaviour was employed here. The behavioural results from the hole-board test in this study pose some interesting questions. LPS treatment impaired locomotion to the same extent in wildtype and SIGIRR$^{-/-}$ mice. In particular LPS-treated wildtype and SIGIRR$^{-/-}$ mice travelled less distance and at a slower velocity than control-treated wildtype and SIGIRR$^{-/-}$ mice. Interestingly, LPS treatment did not have an additively adverse effect on locomotion in SIGIRR$^{-/-}$ mice. Considering SIGIRR's role in inflammation this finding is not that surprising and suggests that SIGIRR is not involved in regulating locomotion and that this LPS-induced effect is due to general sickness behaviour.

Another finding of this study was that control-treated SIGIRR$^{-/-}$ mice exhibited increased anxiety behaviour. SIGIRR$^{-/-}$ mice stayed closer to the border of the hole-board arena and spent less time in the central zone, and consequently more time in the outer zone, compared with control-treated wildtype mice. In light of the previous data showing an absence of inflammation in control-treated SIGIRR$^{-/-}$ mice, this is an intriguing result and suggests that a deficiency in the SIGIRR gene itself increases anxiety and therefore points to a role of SIGIRR outside of the immune system. The gene for SIGIRR is located on a different chromosome to other members of the IL-1R super-family, on chromosome 11 (Thomassen *et al.*, 1999). Chromosome 11 is heavily associated with bipolar disorder and schizophrenia (Klar, 2004), and this could be taken to mean that a deficiency in SIGIRR may play a role in these disorders, however further studies are required to fully understand the role, if any of SIGIRR in these disorders.

More pertinent to this study, was the findings that SIGIRR$^{-/-}$ mice treated with LPS exhibited reduced exploratory behaviour than LPS-treated wildtype mice. Head dipping and rearing behaviour were decreased in SIGIRR$^{-/-}$ mice and these mice

entered the central zone fewer times than LPS-treated wildtype mice. These behavioural results, along with the exacerbated inflammation in LPS-treated SIGIRR$^{-/-}$ mice, point to a role for SIGIRR in regulating inflammation in the hippocampus, as a deficiency in SIGIRR accompanied with LPS produced heightened inflammation and altered hippocampal-dependent behaviour. These results also suggest that there could be structural and functional damage of hippocampal neurons caused by the heightened LPS-induced inflammation in SIGIRR$^{-/-}$ mice leading to an impairment in hippocampal function, however further studies need to be carried out to assess the integrity of neurons and to assess neuronal damage. In support of this hypothesis, one study showed that in a model of SLE, a disease characterised by auto-immunity and inflammation there was neuronal damage in the CA3 region of the hippocampus which led to impaired hippocampal function and spatial memory in rodents (Ballok *et al.*, 2004). Considering a recent study was published on the role of SIGIRR in regulating autoimmunity in a mouse model of SLE (Lech *et al.*, 2008), this suggests that SIGIRR may play a role in regulating the inflammation associated with this disease.

Chapter 4

An investigation into the effects of age and Aβ treatment on SIGIRR$^{-/-}$ mice

4.1. Introduction

The role of SIGIRR in dampening innate immune reactions has been well characterised in this study and by others (Wald *et al.*, 2003; Huang *et al.*, 2006). More recently studies have focused on the role of SIGIRR in more chronic inflammatory states. Lech and colleagues (2007) characterised the role of SIGIRR in a mouse model of the auto-immune disease, systemic lupus erythematosus (SLE). Xiao and colleagues (2007) demonstrated that there was exacerbated tumour formation in aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice. One of the aims of this study was to build on the idea that SIGIRR is involved in dampening chronic inflammatory changes over an extended period of time and to determine if SIGIRR$^{-/-}$ mice had heightened inflammation characterised by microglial activation and increased pro-inflammatory cytokine production in response to age.

Previous observations by Wald and colleagues (2003), and the results presented in Chapter 3 highlight the negative regulatory role of SIGIRR on LPS- and IL-1β-mediated inflammatory changes. Additional studies have shown that SIGIRR also has the ability to attenuate inflammatory changes induced by other TLR ligands, such as bacterial CpG-DNA (Garlanda *et al.*, 2004; Qin *et al.*, 2005; Lech *et al.*, 2007) and Pam3Cys (Lech *et al.*, 2007). Therefore, it was considered that SIGIRR may act as a negative regulator of other initiators of inflammation, and since Aβ is a potent inducer of pro-inflammatory cytokines and activator of MAPKs in the brain (Minogue *et al.*, 2003), the aim of this study was to determine if SIGIRR could negatively regulate inflammation induced by Aβ.

SIGIRR is widely expressed in peripheral organs such as kidney, liver, spleen, and gastrointestinal tract (Thomassen *et al.*, 1999; Polentarutti *et al.*, 2003; Wald *et al.*, 2003; Garlanda *et al.*, 2004). However studies have shown that the expression profile of SIGIRR is limited to certain cell types and does not appear to be expressed on cells of the myeloid lineage (Polentarutti *et al.*, 2003; Wald *et al.*, 2003). SIGIRR expression was also found in the brain (Polentarutti *et al.*, 2003) and results from our previous studies revealed the expression of SIGIRR on mixed glial cells (Costelloe *et al.*, 2008). Consequently one of the aims of this study was to determine the exact cellular expression of SIGIRR in the brain.

4.2. Methods

Young and aged wildtype and SIGIRR$^{-/-}$ mice were injected icv with sterile saline or A$\beta_{(1-42)}$ (5μl; 1mg/ml), 3 hours post-injections, animals were killed by cervical dislocation and tissue taken for analysis (see section 2.4.1 for specific details). Analysis of hippocampal tissue for microglial markers, cytokine and chemokine mRNA was assessed by PCR and cytokine concentrations were assessed by ELISA (see sections 2.6. 2.8 and 2.9 for specific details).

Mixed glia cells and isolated microglia were prepared from one-day old male Wistar rats, cells were fixed and immunofluorescently stained to determine the expression of SIGIRR on mixed glial cells, microglia and astrocytes (see section 2.10 for specific details). Data are expressed as means ± standard error of the mean and a two-way ANOVA was performed to determine whether significant differences existed between conditions. Post hoc Student Newmann-Keuls test was used to determine where the significance lied (see section 2.13 for specific details).

4.3. Results

Age-related changes in hippocampal expression of CD40 mRNA is increased in SIGIRR$^{-/-}$ mice

The evidence from several studies suggests that inflammatory changes contribute to brain ageing and since the data in chapter 3 showed that SIGIRR$^{-/-}$ mice have an inflammatory phenotype, it was hypothesised that inflammatory changes in aged SIGIRR$^{-/-}$ mice would be more profound that aged wildtype mice. Figure 4.1. shows that CD40 mRNA expression was significantly increased in snap frozen hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice (747.0 ± 233.3 fold increase; n=6) compared with young control SIGIRR$^{-/-}$ mice (74.23 ± 9.10 fold increase; n=6; $^{**}p<0.01$; ANOVA). Hippocampal CD40 mRNA expression was significantly greater in aged SIGIRR$^{-/-}$ mice (747.0 ± 233.3 fold increase; n=6) compared with aged wildtype mice (82.55 ± 11.50 fold increase; n=6; $^{+}p<0.05$; ANOVA).

Age-related changes in hippocampal expression of pro-inflammatory cytokines are increased in SIGIRR$^{-/-}$ mice

The expression of IL-1β, IL-6 and TNF-α mRNA was assessed in snap frozen hippocampal tissue prepared from young and aged wildtype and SIGIRR$^{-/-}$ mice. Figure 4.2. shows that that mean IL-1β mRNA expression was significantly increased in snap frozen hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice (1758 ± 485.9 fold increase; n=6) compared with young SIGIRR$^{-/-}$ mice (74.62 ± 9.63 fold increase; n=6; *$p<0.05$; ANOVA). Aged SIGIRR$^{-/-}$ mice have increased hippocampal IL-1β mRNA expression (1758 ± 485.9 fold increase; n=6) compared with aged wildtype mice (317.4 ± 146.0 fold increase; n=6; $^{+}p<0.05$; ANOVA).

In parallel with the age-related increase in IL-1β mRNA expression in SIGIRR$^{-/-}$ mice, Figure 4.3. shows that mean IL-6 mRNA expression was significantly increased in snap frozen hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice (99.78 ± 19.78 fold increase; n=6) compared with young SIGIRR$^{-/-}$ mice (6.23 ± 5.86 fold increase; n=6; ***$p<0.001$; ANOVA). Aged SIGIRR$^{-/-}$ mice have increased hippocampal IL-6 mRNA expression (99.78 ± 19.78 fold increase; n=6) compared with aged wildtype mice (37.39 ± 5.64 fold increase; n=6; $^{++}p<0.01$; ANOVA).

Figure 4.4. shows that mean TNF-α mRNA was significantly increased in snap frozen hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice (215.0 ± 57.26 fold increase; n=6) compared with young SIGIRR$^{-/-}$ mice (57.60 ± 18.35 fold increase; n=6; *$p<0.05$; ANOVA). Aged SIGIRR$^{-/-}$ mice have increased hippocampal TNF-α mRNA expression (215.0 ± 57.26 fold increase; n=6) compared with aged wildtype mice (48.82 ± 13.52 fold increase; n=6; $^{+}p<0.05$; ANOVA).

Age-related changes in hippocampal concentrations of pro-inflammatory cytokines are increased in SIGIRR$^{-/-}$ mice

The concentrations of IL-1β, IL-6 and TNF-α were assessed in hippocampal homogenates prepared from young and aged wildtype and SIGIRR$^{-/-}$ mice. Figure 4.5. shows that IL-1β concentration was significantly increased in hippocampal homogenates prepared from aged wildtype mice (51.12 pg/mg ± 4.47; n=6) compared

with young wildtype mice (3.94 pg/mg ± 1.27; n=6; *p<0.05; ANOVA). IL-1β was also significantly increased in hippocampal homogenates prepared from aged SIGIRR$^{-/-}$ mice (61.90 pg/mg ± 4.34; n=6) compared with young SIGIRR$^{-/-}$ mice (6.53 pg/mg ± 2.75; n=6; *p<0.05; ANOVA). The age-related increase in hippocampal IL-1β in SIGIRR$^{-/-}$ mice (61.90 pg/mg ± 4.34; n=6) was significantly greater than that in aged wildtype mice (51.12 pg/mg ± 4.47; n=6; $^{+}$p<0.05; ANOVA).

Figure 4.6 shows IL-6 concentration was significantly increased in hippocampal homogenates prepared from aged SIGIRR$^{-/-}$ mice (711.3 pg/mg ± 277.3; n=6) compared with young SIGIRR$^{-/-}$ mice (30.44 pg/mg ± 2.27; n=6; *p<0.05; ANOVA). There was a significant increase in IL-6 concentration in aged SIGIRR$^{-/-}$ mice (711.3 pg/mg ± 277.3; n=6) compared with aged wildtype mice (169.5 pg/mg protein ± 59.24; n=6; $^{+}$p<0.05; ANOVA).

Figure 4.7 shows TNF-α concentration was significantly increased in hippocampal homogenates prepared from aged wildtype mice (108.4 pg/mg ± 18.38; n=6) and aged SIGIRR$^{-/-}$ mice (104.6 pg/mg ± 5.02; n=6) compared with young wildtype mice and SIGIRR$^{-/-}$ mice (20.05 pg/mg ± 11.96; n=6; *p<0.05; ANOVA and 31.59 pg/mg ± 11.04; n=6; *p<0.05; ANOVA respectively). There was no significant difference in TNF-α in hippocampal tissue prepared from aged wildtype and SIGIRR$^{-/-}$ mice.

Effect of Aβ on age-related changes in cytokine expression

Given that LPS-treated and aged SIGIRR$^{-/-}$ mice exhibited a heightened immune response compared with control-treated and young SIGIRR$^{-/-}$ mice respectively, it was considered that the combination of age and Aβ-treatment might further enhance this response. Figure 4.8. shows that mean IL-1β mRNA expression was similar in snap frozen hippocampal tissue obtained from aged Aβ-treated wildtype (135.6 ± 70.09 fold increase; n=6) and SIGIRR$^{-/-}$ mice (357.5 ± 257.5 fold increase; n=6) and in aged control-treated wildtype (54.34 ± 24.98 fold increase; n=6) and SIGIRR$^{-/-}$ mice (44.88 ± 12.40 fold increase; n=6).

Figure 4.9. shows that mean IL-6 mRNA expression was similar in snap frozen hippocampal tissue obtained from aged Aβ-treated wildtype (36.19 ± 8.05 fold

increase; n=6) and SIGIRR$^{-/-}$ mice (45.39 ± 14.56 fold increase; n=6) and in aged control-treated wildtype (10.34 ± 2.91 fold increase; n=6) and SIGIRR$^{-/-}$ mice (40.66 ± 20.17 fold increase; n=6).

Figure 4.10. shows that mean TNF-α mRNA expression was similar in snap frozen hippocampal tissue obtained from aged Aβ-treated wildtype (1266 ± 570.7 fold increase; n=6) and SIGIRR$^{-/-}$ mice (13090 ± 10366 fold increase; n=6) and in aged control-treated wildtype (62.42 ± 18.19 fold increase; n=6) and SIGIRR$^{-/-}$ mice (46.04 ± 11.87 fold increase; n=6).

Effect of Aβ on age-related changes in cytokine concentration

The concentrations of IL-1β, IL-6 and TNF-α were assessed in hippocampal homogenates prepared from young and aged Aβ-treated wildtype and SIGIRR$^{-/-}$ mice. Figure 4.11. shows that mean IL-1β concentration was similar in hippocampal homogenates obtained from aged Aβ-treated wildtype (59.35 pg/mg ± 8.92; n=6) and SIGIRR$^{-/-}$ mice (81.10 pg/mg ± 19.53; n=6) and in aged control-treated wildtype (51.12 pg/mg ± 4.47; n=6) and SIGIRR$^{-/-}$ mice (65.20 pg/mg ± 3.44; n=6). IL-1β concentration was significantly increased in hippocampal homogenates obtained from aged SIGIRR$^{-/-}$ mice (65.20 pg/mg ± 3.44; n=6) with aged wildtype mice (51.12 pg/mg ± 4.47; n=6 $^{\$}p<0.05$; Student's *t* test).

Figure 4.12. shows that mean IL-6 concentration was similar in hippocampal homogenates obtained from aged Aβ-treated wildtype (137.1 pg/mg ± 41.50; n=6) and SIGIRR$^{-/-}$ mice (34.07 pg/mg ± 21.65; n=6) and in aged control-treated wildtype (169.5 pg/mg ± 59.24; n=6) and SIGIRR$^{-/-}$ mice (711.3 pg/mg ± 277.3; n=6). IL-6 concentration was significantly increased in hippocampal homogenates obtained from aged SIGIRR$^{-/-}$mice (711.3 pg/mg ± 277.3; n=6) with aged wildtype mice (169.5 pg/mg ± 59.24; n=6; $^{\$}p<0.05$; ANOVA).

Figure 4.13. shows that mean TNF-α concentration was similar in hippocampal homogenates obtained from aged Aβ-treated wildtype (123 pg/mg ± 5.37; n=6) and SIGIRR$^{-/-}$ mice (117 pg/mg ± 7.67; n=6) and in aged control-treated wildtype (126.7 pg/mg ± 2.15; n=6) and SIGIRR$^{-/-}$ mice (104.6 pg/mg ± 5.02; n=6).

The expression of SIGIRR is cell-type specific

Studies on the expression profile of SIGIRR are often conflicting. Certain studies have shown that SIGIRR mRNA is highly expressed on epithelial cells in peripheral tissues like kidney, gut and liver (Polentarutti *et al.*, 2003) and only weakly expressed in the brain and muscle (Wald *et al.*, 2003). However, searches of expression sequence tag (EST) databases have showed that SIGIRR is expressed in the brain (Thomassen *et al.*, 1999). In order to determine the cell type on which SIGIRR is expressed in the CNS, mixed glial cells and microglial cells were prepared and immunostained for specific markers and for the expression of SIGIRR.

Mixed glial cells were double-immunostained for SIGIRR and the astrocytic marker GFAP and visualised by confocal microscopy to determine if SIGIRR is expressed on astrocytes. The micrographs which are representative of 4 separate experiments in Figure 4.14. show double-immunofluorescent staining with anti-SIGIRR and anti-GFAP. Figure 4.14. (A) shows low (40X) and higher (60X) magnification images of GFAP staining on mixed glial cells indicated by the green Alexa staining. Figure 4.14. (B) shows low (40X) and higher (60X) magnification images of SIGIRR staining on mixed glial cells indicated by the red Alexa staining. Figure 4.14. (C) shows an overlay image at the higher magnification, which shows co-localisation of GFAP with SIGIRR indicated by the yellow merged photo with white arrows indicating individual cells.

Figure 4.15. (A) shows a scatter plot depicting the relationship between GFAP (quadrant 1) on the x axis and SIGIRR (quadrant 2) on the y axis of the previous micrographs. The pixels are pseudocoloured blue so that their colour represents the frequency of the green-red pixel overlap in the original images and thus shows very high co-localisation. The blue stain on the surface of the astrocytes depicts areas of co-localisation between GFAP and SIGIRR (Figure 4.15. B).

Mixed glial cells were double-immunostained for SIGIRR and CD11b to determine if SIGIRR was expressed on microglia and visualised by confocal microscopy. The micrographs which are representative of 4 separate experiments in Figure 4.16. show double-immunofluorescent staining with anti-SIGIRR and anti-CD11b. Figure 4.16. (A) shows low (40X) and higher (60X) magnification images of

CD11b staining on mixed glial cells indicated by the green ALEXA staining, Figure 4.16. (B) shows low (40X) and higher (60X) magnification images of SIGIRR staining on mixed glial cells indicated by the red Alexa staining; this staining appears to be distinct from the staining of the CD11b. Figure 4.16. (C) shows an overlay image at the higher magnification which shows no apparent overlap between SIGIRR and CD11b with white arrows indicating individual cells.

Figure 4.17. (A) shows a scatter plot depicting the relationship between CD11b (quadrant 1) on the x axis and SIGIRR (quadrant 2) on the y axis. The graph shows minimal if any co-localisation between CD11b and SIGIRR as revealed by the blue pixels and there is no shift of these blue pixels into quadrant 4. Figure 4.17. (B) shows that there is no dye-overlap on the surface of the microglia and thus no areas of co-localisation between CD11b and SIGIRR.

To further validate the finding that SIGIRR is not expressed on microglial cells, isolated microglia were prepared, double-immunostained for SIGIRR and CD11b and visualised by confocal microscopy. The micrographs which are representative of 4 separate experiments in Figure 4.18. (A+B) show double-immunofluorescent staining with anti-SIGIRR (red) and anti-CD11b (green) at low (40X) and higher (60X) magnifications. The red staining is indistinguishable from the negative controls. Figure 4.18. (C) shows an overlay image at the higher magnification which shows no apparent overlap between SIGIRR and CD11b; white arrows indicating individual cells.

Figure 4.19. (A) shows a scatter plot depicting the relationship between CD11b (quadrant 1) on the x axis and SIGIRR (quadrant 2) on the y axis. The graph shows minimal if any co-localisation between CD11b and SIGIRR as revealed by the blue pixels and there is no shift of these blue pixels into quadrant 4. Figure 4.19. (B) shows that there is no dye-overlap on the surface of the microglia and thus no areas of co-localisation between CD11b and SIGIRR.

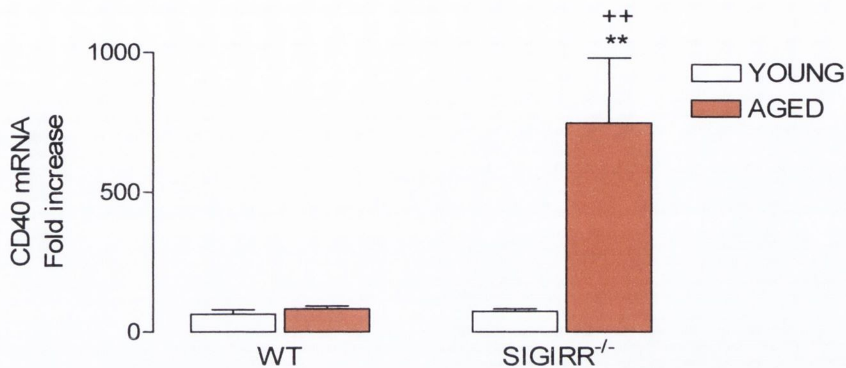


Figure 4.1. CD40 mRNA expression is increased in the hippocampus of aged SIGIRR$^{-/-}$ mice

Young (2-4 months) and aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were killed by decapitation, hippocampi dissected free, homogenized and CD40 mRNA expression determined by QPCR. Mean CD40 mRNA was increased in hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice compared with hippocampal tissue obtained from young control SIGIRR$^{-/-}$ mice (**$p<0.01$; ANOVA). CD40 mRNA expression was increased in hippocampus of aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{++}p<0.01$, ANOVA). Values are expressed as fold increases and are means ± SEM (n=6 in each group).

2-way ANOVA: Age $_{effect}$ F (1, 14) = 6.77; p=0.0209, Strain $_{effect}$ F (1, 14) = 6.45; p=0.0236, Interaction $_{effect}$ F (1, 14) = 6.10; p=0.0270

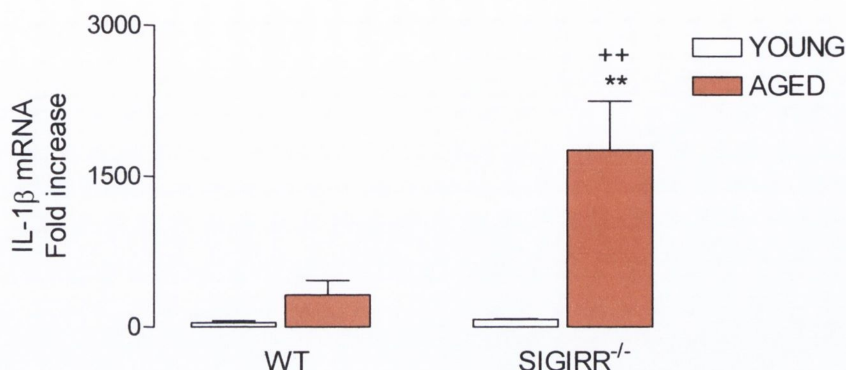


Figure 4.2. IL-1β mRNA expression is increased in the hippocampus of aged SIGIRR$^{-/-}$ mice

Young (2-4 months) and aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were killed by decapitation, hippocampi dissected free, homogenized and IL-1β mRNA expression determined by QPCR. Mean IL-1β mRNA expression was significantly increased in hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice compared with hippocampal tissue obtained from young control SIGIRR$^{-/-}$ mice (**$p<0.01$, ANOVA). IL-1β mRNA expression was increased in hippocampus of aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{++}p<0.01$, ANOVA). Values are expressed as fold increases and are means ± SEM (n=6 in each group).

2-way ANOVA: Age $_{effect}$ $F_{(1, 15)} = 9.04$; $p=0.0088$, Strain $_{effect}$ $F_{(1, 15)} = 5.10$; $p=0.0392$, Interaction $_{effect}$ $F_{(1, 15)} = 4.68$; $p=0.0470$

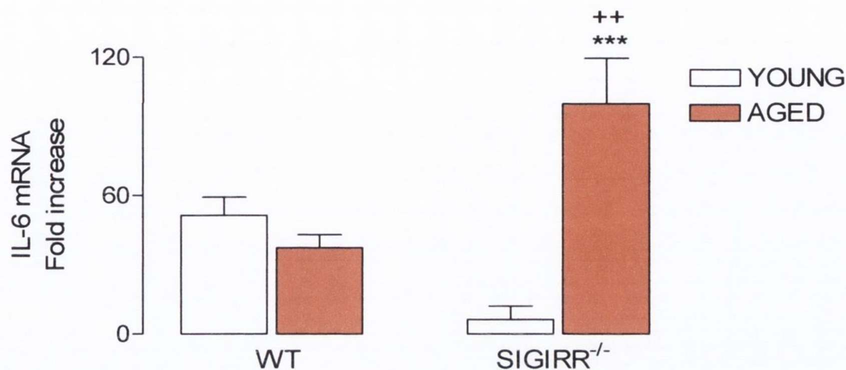


Figure 4.3. IL-6 mRNA expression is increased in the hippocampus of aged SIGIRR$^{-/-}$ mice

Young (2-4 months) and aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were killed by decapitation, hippocampi dissected free, homogenized and IL-6 mRNA expression determined by QPCR. Mean IL-6 mRNA expression was significantly increased in hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice compared with hippocampal tissue obtained from young control SIGIRR$^{-/-}$ mice (***p<0.001, ANOVA). IL-6 mRNA expression was increased in hippocampus of aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{++}$p<0.01, ANOVA). Values are expressed as fold increases and are means ± SEM (n=6 in each group).

2-way ANOVA: Age $_{effect}$ F (1, 36) = 13.32; p=0.0008, Strain $_{effect}$ F (1, 36) = 0.62; p=0. 4379, Interaction $_{effect}$ F (1, 36) = 24.51; p<0.0001

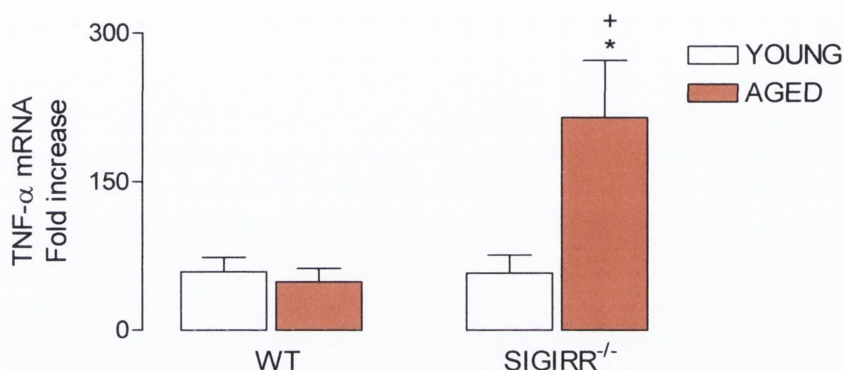


Figure 4.4. TNF-α mRNA expression is increased in the hippocampus of aged SIGIRR$^{-/-}$ mice

Young (2-4 months) and aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were killed by decapitation, hippocampi dissected free, homogenized and TNF-α mRNA expression determined by QPCR. Mean TNF-α mRNA expression was significantly increased in hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice compared with hippocampal tissue obtained from young control SIGIRR$^{-/-}$ mice (*p<0.05, ANOVA). TNF-α mRNA expression was increased in hippocampus of aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{+}$p<0.05, ANOVA). Values are expressed as fold increases and are means ± SEM (n=6 in each group).

2-way ANOVA: Age$_{effect}$ F (1, 14) = 4.37; p=0.0552, Strain$_{effect}$ F (1, 14) = 5.48; p=0.0345, Interaction$_{effect}$ F (1, 14) = 5.68; p=0.0319

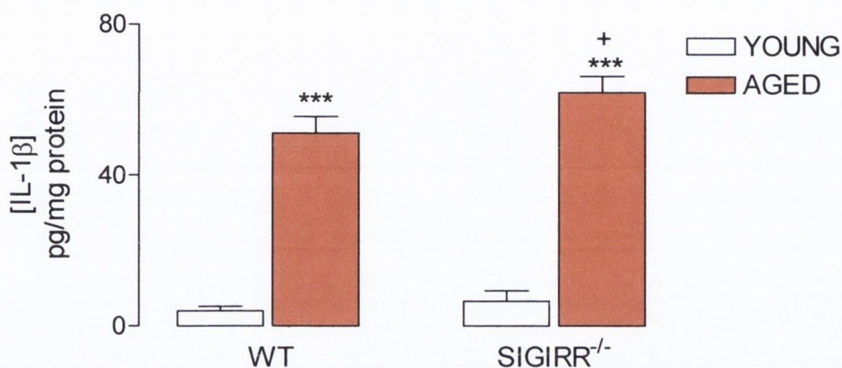


Figure 4.5. The age-related increase in hippocampal IL-1β concentration is greater in SIGIRR$^{-/-}$ mice

Young (2-4 months) and aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were killed by decapitation, hippocampi dissected free, homogenized and IL-1β concentration determined by ELISA. Mean IL-1β concentration was significantly increased in hippocampal homogenates prepared from aged wildtype and aged SIGIRR$^{-/-}$ mice compared with hippocampal homogenates obtained from young wildtype mice (***p<0.001, ANOVA) and young SIGIRR$^{-/-}$ mice (***p<0.001, ANOVA). IL-1β concentration was significantly greater in hippocampus of aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{+}$p<0.05, ANOVA). Values are expressed as pg IL-1β/mg protein and are means ± SEM (n=6 in each group).

2-way ANOVA: Age$_{effect}$ F (1, 33) = 286.36; p<0.0001, Strain$_{effect}$ F (1, 33) = 4.87; p=0.0344, Interaction$_{effect}$ F (1, 33) = 1.82; p=0.1862

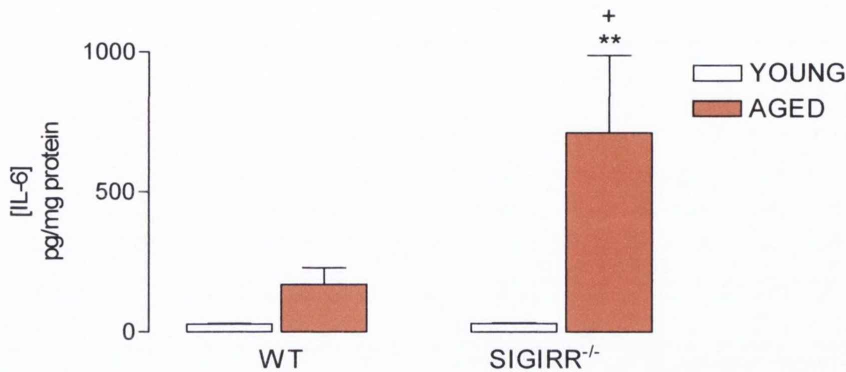


Figure 4.6. The age-related increase in hippocampal IL-6 concentration is greater in SIGIRR$^{-/-}$ mice

Young (2-4 months) and aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were killed by decapitation, hippocampi dissected free, homogenized and IL-6 concentration determined by ELISA. Mean IL-6 concentration was significantly increased in hippocampal homogenates prepared from aged SIGIRR$^{-/-}$ mice compared with hippocampal homogenates obtained from young SIGIRR$^{-/-}$ mice (**$p<0.001$, ANOVA). IL-6 concentration was significantly greater in hippocampus of aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{+}p<0.05$, ANOVA). Values are expressed as pg IL-6/mg protein and are means ± SEM (n=6 in each group).

2-way ANOVA: Age$_{effect}$ $F(1, 17) = 9.28$; $p=0.0073$, Strain$_{effect}$ $F(1, 17) = 4.06$; $p=0.0600$, Interaction$_{effect}$ $F(1, 17) = 4.00$; $p=0.0616$

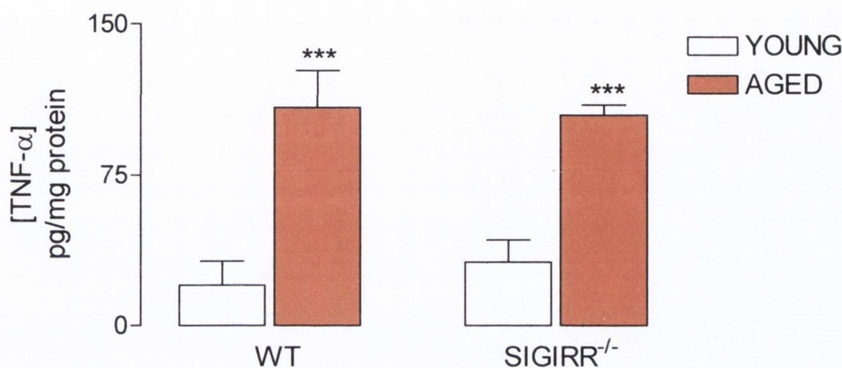


Figure 4.7. The age-related increase in TNF-α concentration was similar in hippocampus of wildtype and SIGIRR$^{-/-}$ mice

Young (2-4 months) and aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were killed by decapitation, hippocampi dissected free, homogenized and TNF-α concentration determined by ELISA. Mean TNF-α concentration was significantly increased in hippocampal homogenates prepared from aged wildtype and aged SIGIRR$^{-/-}$ mice compared with hippocampal homogenates obtained from young wildtype mice (***p<0.001, ANOVA) and young SIGIRR$^{-/-}$ mice (***p<0.001, ANOVA). Hippocampal TNF-α concentration was similar in aged wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as pg TNF-α/mg protein and are means ± SEM (n=6 in each group).

2-way ANOVA: Age$_{effect}$ F (1, 17) = 49.92; p<0.0001, Strain$_{effect}$ F (1, 17) = 0.12; p=0.7383, Interaction$_{effect}$ F (1, 17) = 0.45; p=0.5112

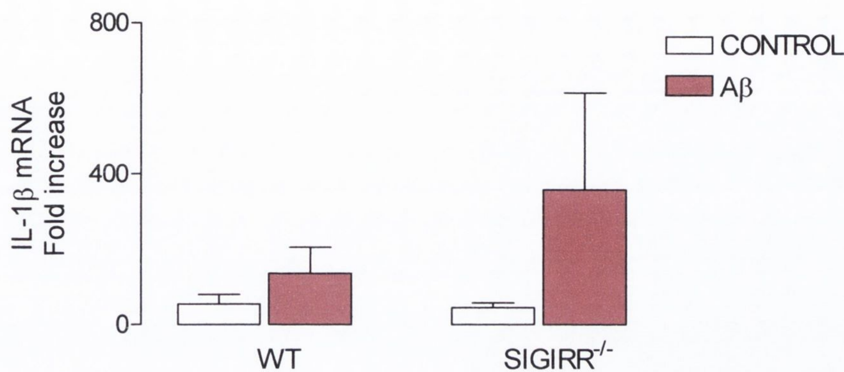


Figure 4.8. Aβ did not affect hippocampal IL-1β mRNA expression in wildtype and SIGIRR$^{-/-}$ mice

Aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were injected icv with sterile saline (5μl) or Aβ $_{(1-42)}$ (1mg/ml; 5μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and IL-1β mRNA expression determined by QPCR. Mean IL-1β mRNA expression was similar in snap frozen hippocampal tissue prepared from aged control- and Aβ-treated wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as fold increases and are means ± SEM (n=6 in each group).

2-way ANOVA: Aβ $_{effect}$ F (1, 17) = 1.48; p=0.2411, Strain $_{effect}$ F (1, 17) = 0.43; p=0.5212, Interaction $_{effect}$ F (1, 17) = 0.51; p=0.4853

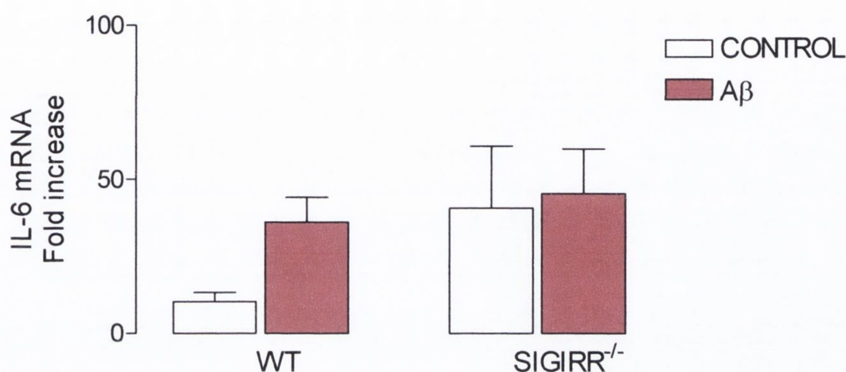


Figure 4.9. Aβ did not affect hippocampal IL-6 mRNA expression in wildtype and SIGIRR$^{-/-}$ mice

Aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were injected icv with sterile saline (5µl) or Aβ $_{(1\text{-}42)}$ (1mg/ml; 5µl). After 3 hours, mice were killed by decapitation; hippocampi dissected free, homogenized and IL-6 mRNA expression determined by QPCR. Mean IL-6 mRNA expression was similar in snap frozen hippocampal tissue prepared from aged control- and Aβ-treated wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as fold increases and are means ± SEM (n=6 in each group).

2-way ANOVA: Aβ $_{effect}$ $F(1, 14) = 1.09$; $p=0.3133$, Strain $_{effect}$ $F(1, 14) = 1.83$; $p=0.1977$, Interaction $_{effect}$ $F(1, 14) = 0.52$; $p=0.4819$

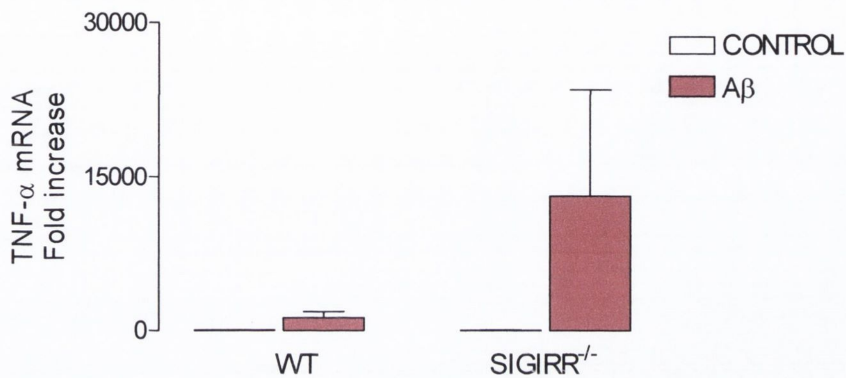


Figure 4.10. Aβ did not affect hippocampal TNF-α mRNA expression in wildtype and SIGIRR$^{-/-}$ mice

Aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were injected icv with sterile saline (5μl) or Aβ $_{(1\text{-}42)}$ (1mg/ml; 5μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and TNF-α mRNA expression determined by QPCR. Mean TNF-α mRNA expression was similar in snap frozen hippocampal tissue prepared from aged control- and Aβ-treated wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as fold increases and are means ± SEM (n=6 in each group).

2-way ANOVA: Aβ $_{effect}$ $F_{(1, 18)} = 1.79$; $p=0.1971$, Strain $_{effect}$ $F_{(1, 18)} = 0.78$; $p=0.3901$, Interaction $_{effect}$ $F_{(1, 18)} = 0.80$; $p=0.3822$

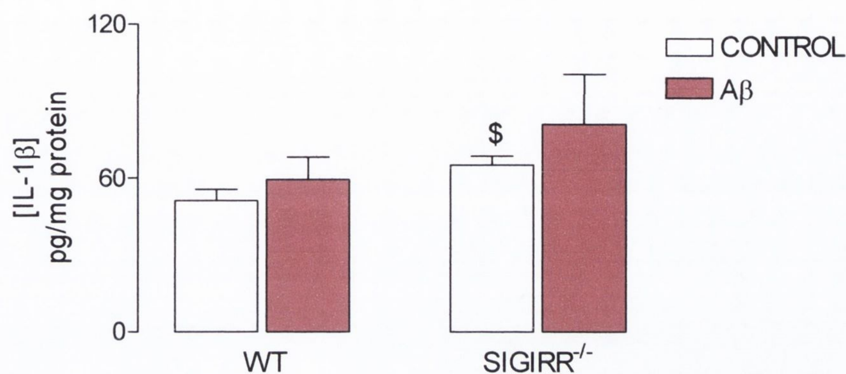


Figure 4.11. Aβ did not affect hippocampal IL-1β concentration in wildtype and SIGIRR$^{-/-}$ mice

Aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were injected icv with sterile saline (5µl) or Aβ $_{(1-42)}$ (1mg/ml; 5µl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and IL-1β concentration determined by ELISA. Mean IL-1β concentration was similar in snap frozen hippocampal tissue prepared from aged control- and Aβ-treated wildtype and SIGIRR$^{-/-}$ mice. Mean IL-1β concentration was significantly greater in hippocampus of aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{\$}p<0.05$, ANOVA). Values are expressed as pg IL-1β/mg protein and are means ± SEM (n=6 in each group).

2-way ANOVA: Aβ $_{effect}$ $F(1, 17) = 0.88$; $p=0.3607$, Strain $_{effect}$ $F(1, 17) = 1.95$; $p=0.1808$, Interaction $_{effect}$ $F(1, 17) = 0.09$; $p=0.7686$

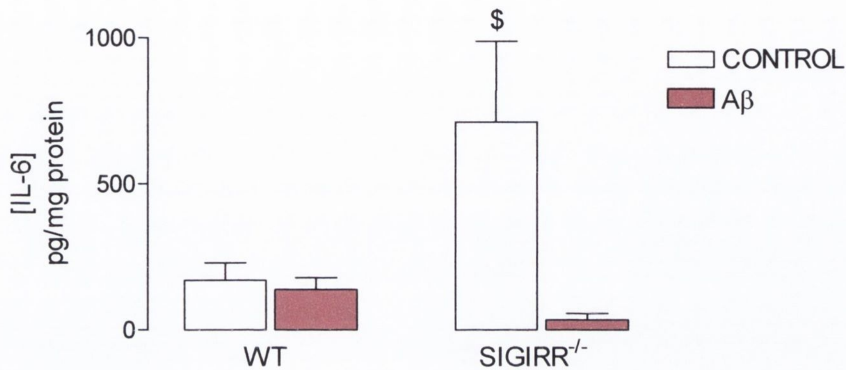


Figure 4.12. Aβ did not affect hippocampal IL-6 concentration in wildtype and SIGIRR$^{-/-}$ mice

Aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were injected icv with sterile saline (5μl) or Aβ $_{(1-42)}$ (1mg/ml; 5μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and IL-6 concentration determined by ELISA. Mean IL-6 concentration was similar in snap frozen hippocampal tissue prepared from aged control- and Aβ-treated wildtype and SIGIRR$^{-/-}$ mice. Mean IL-6 concentration was significantly greater in hippocampus of aged SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{\$}p<0.05$, ANOVA). Values are expressed as pg IL-6/mg protein and are means ± SEM (n=6 in each group).

2-way ANOVA: Aβ $_{effect}$ F (1, 16) = 6.01; p=0.0261; Strain $_{effect}$ F (1, 16) = 2.30; p=0.1490, Interaction $_{effect}$ F (1, 16) = 4.97; p=0.0405

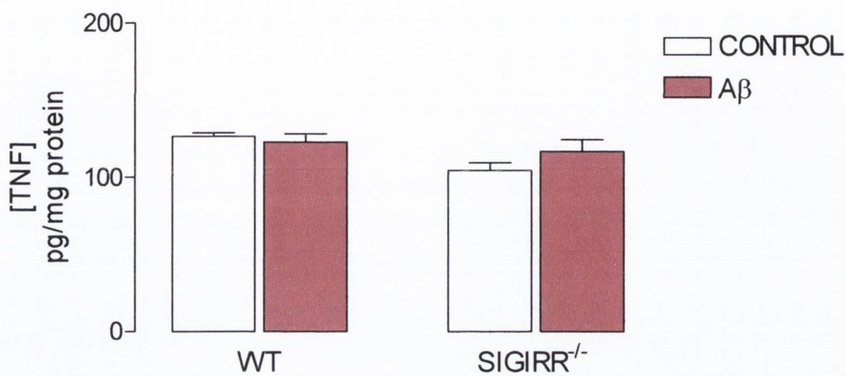


Figure 4.13. Aβ did not affect hippocampal TNF-α concentration in wildtype and SIGIRR$^{-/-}$ mice

Aged (10-12 months) wildtype and SIGIRR$^{-/-}$ mice were injected icv with sterile saline (5μl) or Aβ $_{(1-42)}$ (1mg/ml; 5μl). After 3 hours, mice were killed by decapitation, hippocampi dissected free, homogenized and TNF-α concentration determined by ELISA. Mean TNF-α concentration was similar in hippocampal homogenates obtained from aged control- and Aβ-treated wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as pg TNF-α/mg protein and are means ± SEM (n=6 in each group).

2-way ANOVA: Aβ $_{effect}$ F (1, 18) = 2.18; p=0.1570, Strain $_{effect}$ F (1, 18) = 0.32; p=0.5757, Interaction $_{effect}$ F (1, 18) = 0.57; p=0.4589

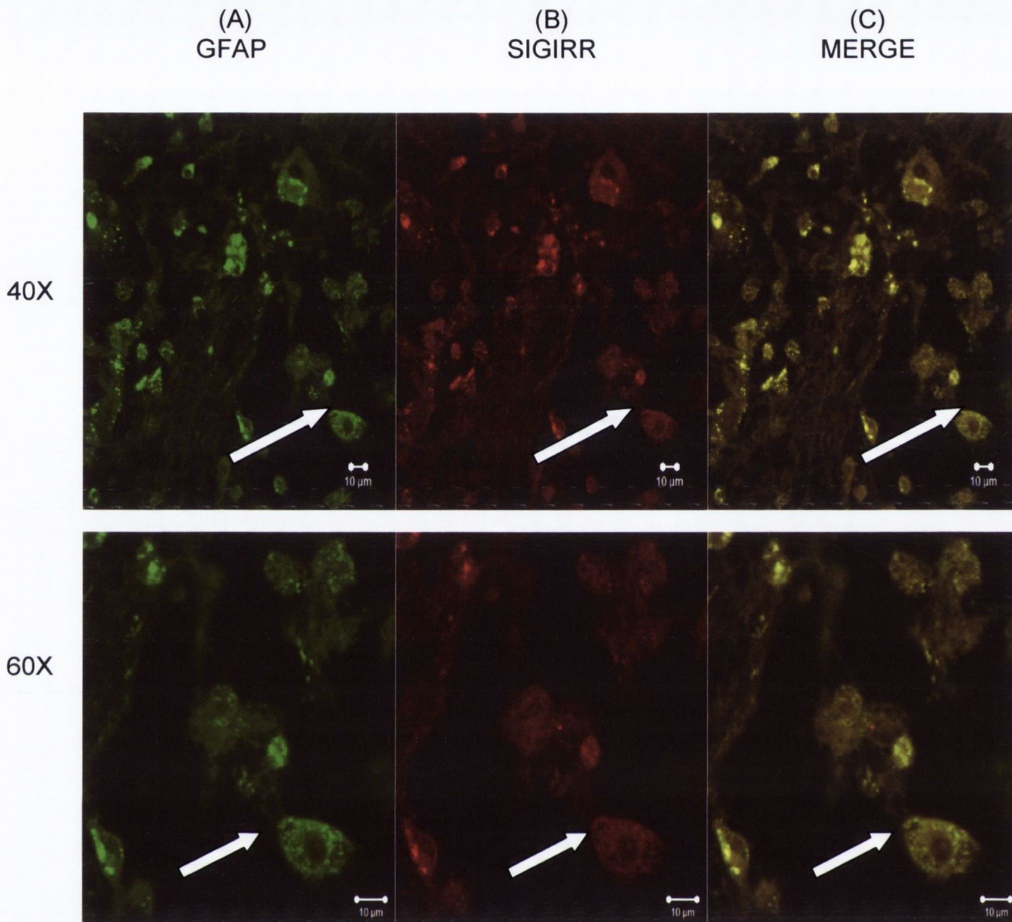


Figure 4.14. SIGIRR expression on astrocytes

Rat mixed glial cells were cultured with medium for 10-14 days, supernatants removed, cells fixed with ethanol, immunostained for GFAP and SIGIRR and visualised by confocal microscopy. (A) GFAP immunostaining with an ALEXA-labelled antibody (green stain) shows individual astrocyte cells in a mixed glial population. (B) SIGIRR immunostaining with an Alexa-labelled antibody (red stain) confirms SIGIRR expression on the cellular surface of mixed glial cells. (C) A yellow signal is obtained as a result of co-localisation of GFAP (astrocytes) with SIGIRR. White arrows indicate individual cells. (Magnifications 40X, 60X; scale bar 10µm).

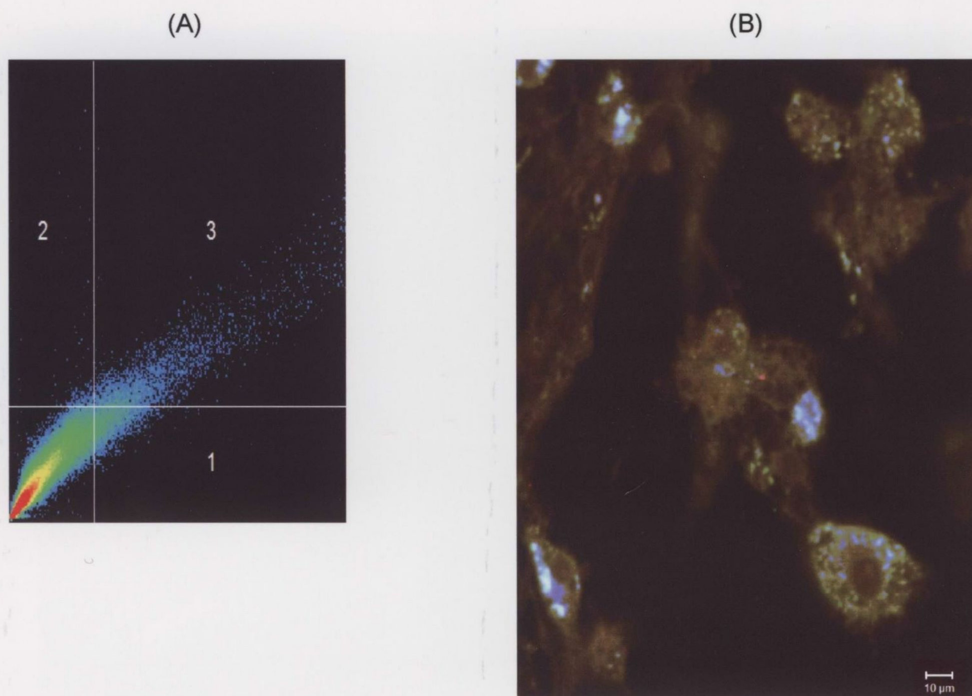


Figure 4.15. Co-localisation of SIGIRR and GFAP in a mixed glial population

Rat mixed glial cells were cultured with medium for 10-14 days, supernatants removed, cells fixed with ethanol, immunostained for GFAP and SIGIRR and visualised by confocal microscopy. (A) Scatter plot illustrating the relationship between GFAP (quadrant 1) and SIGIRR (quadrant 2). The graph shows high co-localisation between GFAP and SIGIRR. The frequency of GFAP (green stain) and SIGIRR (red stain) overlap produces the strong blue colour in this graph and the shift of these blue pixels into quadrant 4 demonstrates co-localisation. (B) The blue pixels on the surface of the astrocytes denote the areas of co-localisation between GFAP and SIGIRR. (Magnification 60X; scale bar 10µm).

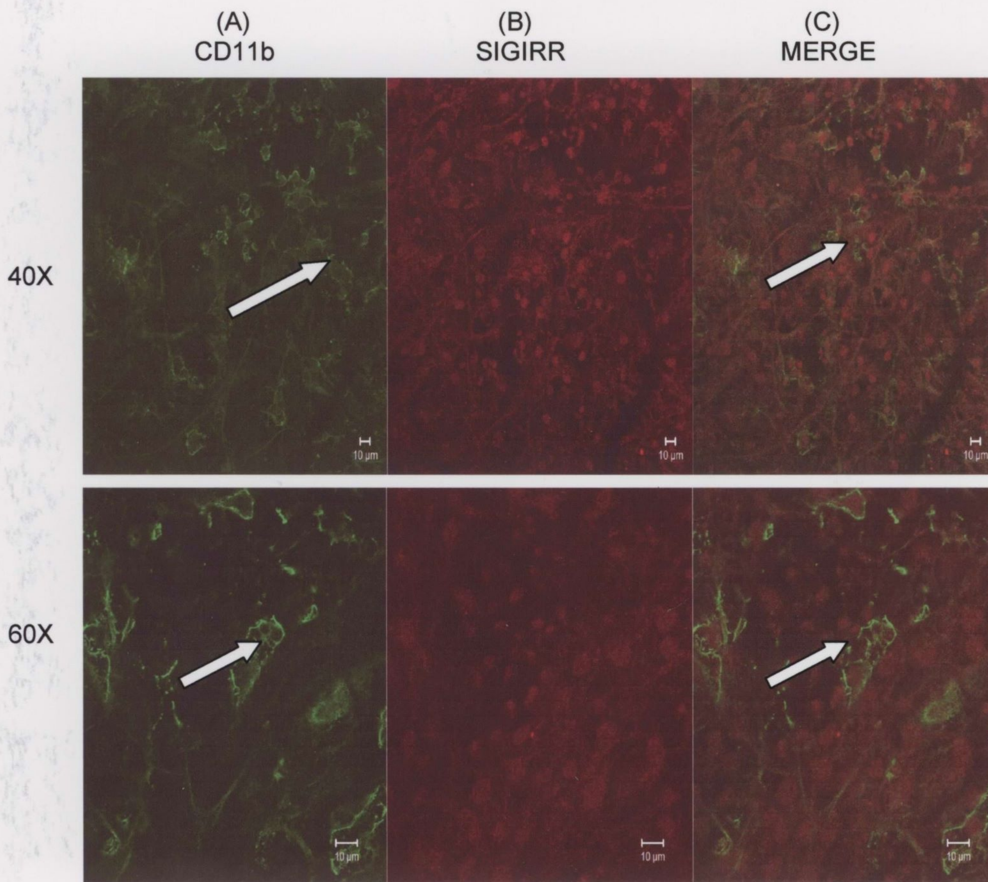


Figure 4.16. Lack of SIGIRR expression on microglia in a mixed glial population
Rat mixed glial cells were cultured with medium for 10-14 days, supernatants removed, cells fixed with ethanol, immunostained for CD11b and SIGIRR and visualised by confocal microscopy. (A) CD11b immunostaining with an ALEXA-labelled antibody (green stain) shows individual microglial cells in a mixed glial population. (B) SIGIRR immunostaining with an Alexa-labelled antibody (red stain) confirms SIGIRR expression on the cell surface of mixed glial cells. (C) In the merged micrograph there is no apparent overlap of signal between the CD11b and SIGIRR suggesting no co-localisation. White arrows indicate individual cells. (Magnifications 40X, 60X; scale bar 10μm).

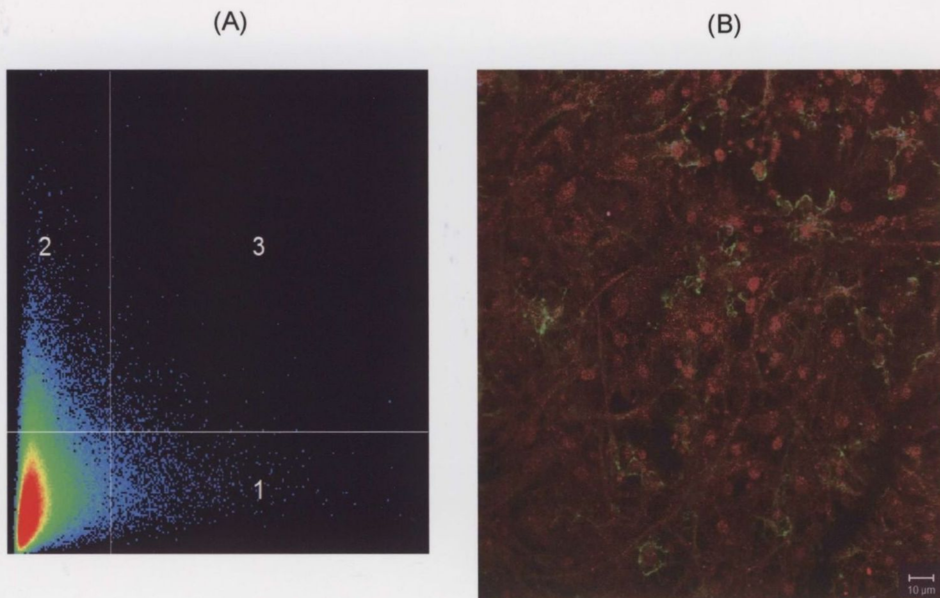


Figure 4.17. Lack of co-localisation of SIGIRR and CD11b in a mixed glial cell population

Rat mixed glial cells were cultured with medium for 10-14 days, supernatants removed, cells fixed with ethanol, immunostained for CD11b and SIGIRR and visualised by confocal microscopy. (A) Scatter plot illustrating the relationship between CD11b (quadrant 1) and SIGIRR (quadrant 2). The graph shows little co-localisation between CD11b and SIGIRR with minimal frequency overlap between CD11b and SIGIRR, denoted by the blue pixels and no shift of these blue pixels into quadrant 4. (B) There is no dye-overlap on the surface of the microglia and thus no areas of co-localisation between CD11b and SIGIRR. (Magnification 60X, scale bar 10μm).

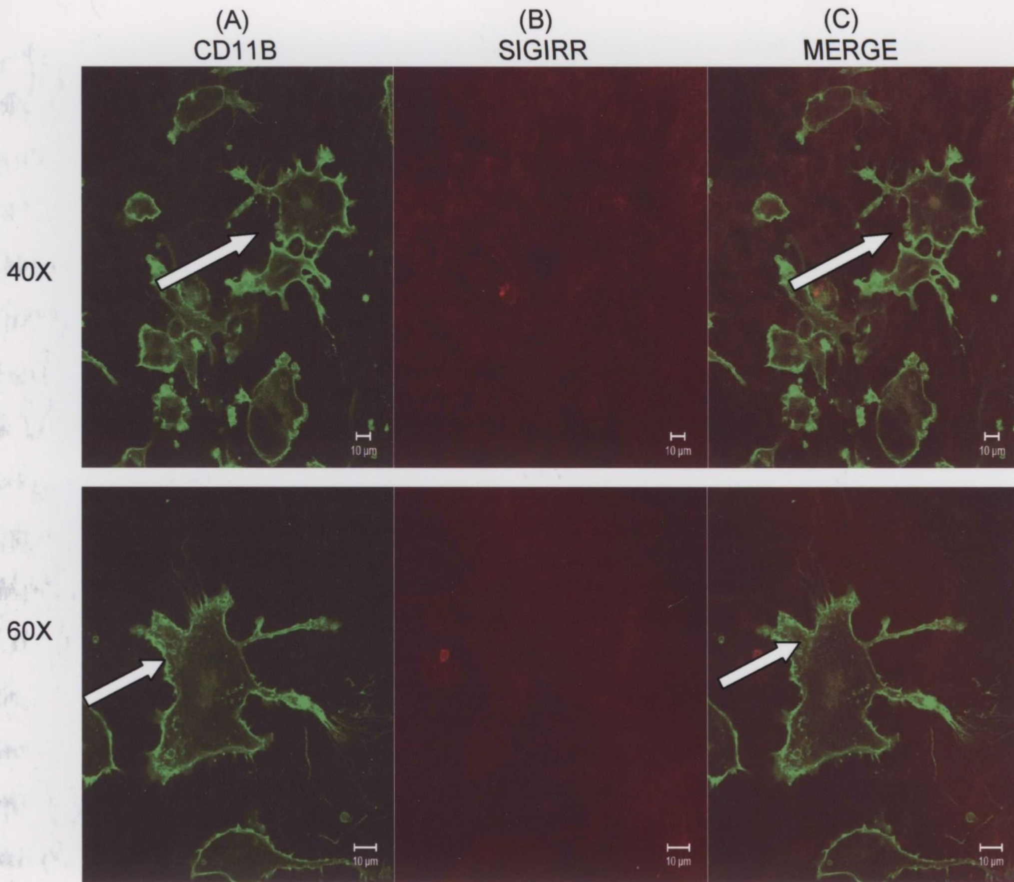


Figure 4.18. Lack of SIGIRR expression on isolated microglial cells:

Rat microglial cells were cultured with medium for 10-14 days, supernatants removed, cells fixed with ethanol, immunostained for CD11b and SIGIRR and visualised by confocal microscopy. (A) CD11b immunostaining with an ALEXA-labelled antibody (green stain) shows individual microglial cells in isolated microglia (B) SIGIRR immunostaining with an Alexa-labelled antibody (red stain) is undetectable on the cell surface of microglial cells. (C) In the merged micrograph there is no apparent overlap of signal between the CD11b and SIGIRR suggesting no co-localisation. White arrows indicate individual cells. (Magnifications 40X, 60X; scale bar 10μm).

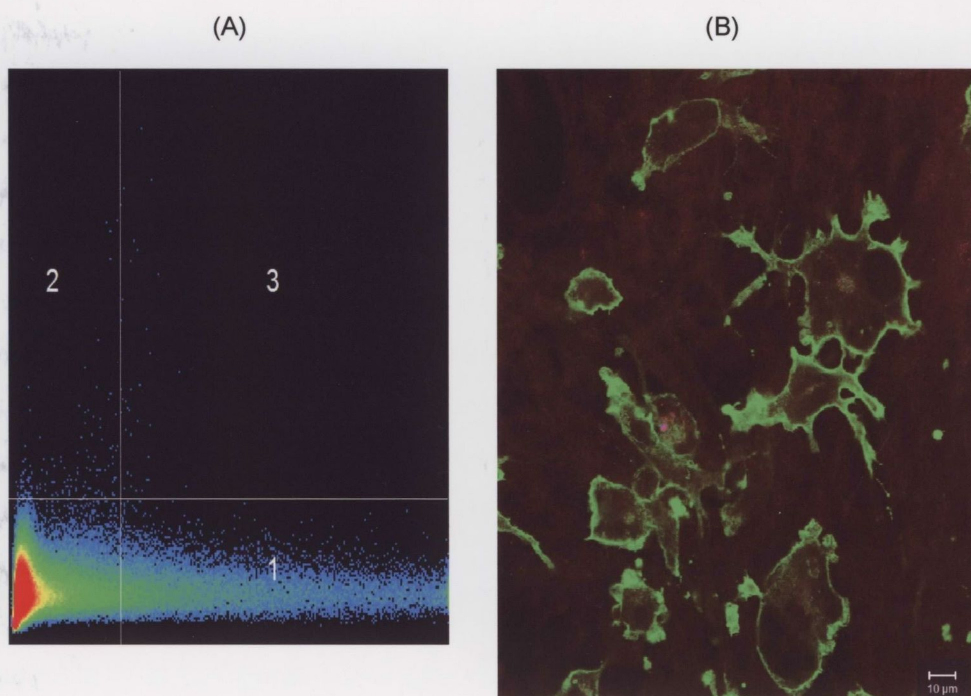


Figure 4.19. Lack of co-localisation of SIGIRR and CD11b on isolated microglial cells

Rat microglial cells were cultured with medium for 10-14 days, supernatants removed, cells fixed with ethanol, immunostained for CD11b and SIGIRR and visualised by confocal microscopy.(A) Scatter plot illustrating the relationship between CD11b (quadrant 1) and SIGIRR (quadrant 2). The graph shows little co-localisation between CD11b and SIGIRR with minimal frequency overlap between CD11b and SIGIRR denoted by the blue pixels and there is no shift of these blue pixels into quadrant 4. (B) There is no dye-overlap on the surface of the microglia and thus no areas of co-localisation between CD11b and SIGIRR. (Magnification 60X; scale bar 10µm).

4.4. Discussion

Evidence from several studies suggests that inflammatory changes contribute to brain ageing (Lynch and Lynch, 2002; Maher *et al.*, 2005; Griffin *et al.*, 2006). Since the data presented so far in this study showed that SIGIRR$^{-/-}$ mice have an inflammatory phenotype, one of the intentions of this study was to determine if inflammatory changes would be exacerbated in aged SIGIRR$^{-/-}$ mice. The data presented here show that, accompanying the LPS-induced increase in microglial activation and pro-inflammatory cytokine concentrations in SIGIRR$^{-/-}$ mice, is an age-associated up-regulation in the mRNA expression of CD40, IL-1β, IL-6 and TNF-α. Coupled with these changes in mRNA expression is an age-associated increase in IL-1β and IL-6 concentrations. The studies on the anti-inflammatory capacity of SIGIRR on IL-1β and LPS-induced signalling are exhaustive, however there is little known on the effects of SIGIRR in the aged animal. Indeed the only study on aged animals is the recent work of Xiao and colleagues (2007). They found increased cellular proliferation and survival of cells in the colonic epithelium of aged SIGIRR$^{-/-}$ mice resulting in enhanced colitis-associated tumourigenesis compared with aged wildtype mice. Taken together, the results presented here and those of Xiao and colleagues suggest that SIGIRR may play a role in regulating inflammation with age.

However, since studies have shown an age-related decrease in SIGIRR expression (Lech *et al.*, 2007), this begs the question of whether SIGIRR is more important in acute or chronic disease states. To test this idea further, aged wildtype and SIGIRR$^{-/-}$ mice were injected icv with Aβ $_{(1-42)}$ and hippocampal tissue was extracted and analysed for cytokine production. The data presented show that the mRNA expression and concentrations of IL-1β, IL-6 and TNF-α were all the same in all treatment groups. This suggests that SIGIRR may not be involved in down-regulating Aβ-induced inflammation and may not play a part in regulating chronic inflammatory diseases such as AD. It is worthy of note that in this study the SIGIRR$^{-/-}$ mice used were 10-12 months old when they received the injection with Aβ $_{(1-42)}$ and perhaps it would have been beneficial to look at the effects of an injection of

$A\beta_{(1\text{-}42)}$ in young rats first to determine if SIGIRR could dampen down Aβ-induced inflammation.

It is also worthy of note that the aged SIGIRR$^{-/-}$ mice used in this study had a very similar pattern of inflammation as SIGIRR$^{-/-}$ mice treated with LPS. The exacerbated mRNA expression of CD40, IL-1β, IL-6 and TNF-α was the same in aged SIGIRR$^{-/-}$ mice as LPS treated SIGIRR$^{-/-}$ mice. Similarly IL-1β and IL-6 concentrations were enhanced in hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice and LPS-treated SIGIRR$^{-/-}$ mice. In contrast there was an age-related increase in TNF-α concentration in hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice but this was not seen in LPS-treated SIGIRR$^{-/-}$ mice, however, this age-related increase in TNF-α concentration was also observed in wildtype mice and was not further increased in SIGIRR$^{-/-}$ mice. Combining the findings presented here and in Chapter 3 this corroborates the hypothesis that ageing is associated with inflammation and provides a role for SIGIRR in regulating inappropriate inflammation.

One of the primary aims of this study was to determine the cellular expression of SIGIRR in the CNS. The hypothesis was that SIGIRR would be expressed on the two main types of immune-modulating cells in the CNS, microglial cells and astrocytes, since it is a negative regulator of TLR/IL-1RI signalling (Wald *et al.*, 2003). The immunofluorescent staining results reveal that the expression profile of SIGIRR is cell-type specific; it is highly expressed on astrocytes but not expressed on microglial cells. The expression of SIGIRR on astrocytes was confined to the cellular surface on the outer membranes; this pattern is analogous to other studies which showed cellular surface and not cytoplasmic expression of SIGIRR on primary tubular epithelial cells in the kidney by flow cytometry (Lech *et al.*, 2007) and others that confirmed SIGIRR in the membrane fraction of COS-7 cells (Thomassen *et al.*, 1999). In mixed glial cells there was no co-localisation of the microglial marker CD11b and SIGIRR found, indicating that SIGIRR is not present on microglia. SIGIRR appeared to be expressed on a completely different cell and considering these were mixed glial cells, it would be reasonable to suggest that SIGIRR was being expressed on astrocytes however as these cells were not stained with GFAP, this cannot be irrefutably stated. In isolated microglial cells immunostained with

CD11b and SIGIRR, SIGIRR staining was completely absent, further corroborating the evidence that SIGIRR is not expressed on microglial cells.

The selective nature of expression of SIGIRR in the CNS is in agreement to that observed by others in the periphery. Wald and colleagues (2003) showed that SIGIRR is expressed ubiquitously in peripheral tissues; however RNA blot analysis revealed compartmentalisation of this expression. SIGIRR was highly expressed in epithelial cell lines, moderately expressed in splenocytes, fibroblast and endothelial cell lines but remarkably, not expressed in BMDMs (Wald *et al.*, 2003). Likewise, Polentarutti (2003) localised SIGIRR expression to epithelial cells and found little or no expression on monocytes and macrophages, although its expression was found in the mouse macrophage GG2EE cell line. Various pro- and anti-inflammatory stimuli also failed to induce SIGIRR expression in mononuclear phagocytes. Garlanda and colleagues (2004) reported that SIGIRR has a distinct pattern of expression that includes epithelial tissue and DCs. SIGIRR was expressed at low levels in monocytes and was almost undetectable in macrophages. Considering that microglial cells and macrophages share most phenotypical markers and exert similar effector functions (Stoll and Jander, 1999), this supports the finding from this study, that SIGIRR is not expressed on microglial cells. Furthermore SIGIRR is mainly expressed on epithelial cell types (Wald *et al.*, 2003) and astrocytes are derived from the neuroectoderm which is the primitive neuroepithelial cells of the neural tube (Morest & Silver, 2003)

In contrast to these observations a previous study showed by RT-PCR that SIGIRR is expressed on astrocytes, microglia and cortical neurons (Andre *et al.*, 2005). Similarly another study showed by RT-PCR that SIGIRR was strongly expressed in tubular epithelial cells and on resident intrarenal myeloid cells, which are thought to share a common ancestry with microglial cells (Lech *et al.*, 2007). A further study showed that isolated microglia treated with a virulent form of a murine leukaemia virus induced SIGIRR gene expression as measured by gene array analysis and RT-PCR but induced no increase in SIGIRR protein expression as measured by western immunoblotting (Dimcheff *et al.*, 2006). A possible explanation for the disparity between SIGIRR mRNA and SIGIRR protein expression in microglial cells could be due to the techniques employed. In this study using immunofluorescent

staining techniques SIGIRR expression was not detected in isolated microglial cells, whereas others detected SIGIRR expression by RT-PCR and gene array analysis (Andre *et al.*, 2005; Dimcheff *et al.*, 2006). It is possible that the detection method used in this study was not sensitive enough to identify SIGIRR expression, as optimum immunostaining requires preservation of the proteins tertiary structure in the region of the relevant epitopes for the antibody to bind (Pascal *et al.*, 2008) and if this structure was disrupted in any way then the protein would not be detected by this technique. Additionally, it must be acknowledged that immunostaining is only semi-quantitative and perhaps less sensitive than quantitative techniques such as flow cytometry. However, relying solely on RNA analysis such as RT-PCR or gene array analysis to predict the expression of SIGIRR protein is likely to be problematic as mRNA levels are an unreliable surrogate of protein concentrations; this is illustrated by one study which showed that changes in protein expression were only 20-40% attributable to changes in mRNA levels (Nie *et al.*, 2006). Therefore, the studies that predict the expression of SIGIRR protein on myeloid and microglial cells by RNA analysis should be considered in the light of these limitations. With this conflicting evidence, the expression of SIGIRR on myeloid cells and microglial cells requires further scrutiny and therefore additional integrated experimentation measuring transcriptomic and proteomic SIGIRR expression would be essential to categorically address this question.

It is important to highlight the fact that cells that do not appear to express SIGIRR exhibit different responses to inflammatory signals, and the effects of SIGIRR are therefore cell-type specific. Certainly, other studies have highlighted the cell-type specific functions of SIGIRR (Wald *et al.*, 2003; Lech *et al.*, 2007). SIGIRR-deficient cells from kidney and spleen exhibited increased NFκB activation and prolonged JNK activation in response to LPS and IL-1β; however LPS and IL-1β had no effects in SIGIRR-deficient BMDMs (Wald *et al.*, 2003). The authors attributed these cell-type-specific effects to the lack of SIGIRR expression on BMDMs. Similarly, another study showed that SIGIRR-deficient DCs had increased responsiveness to LPS in terms of IL-6 and IP-10, whereas BMDMs and peritoneal macrophages from SIGIRR-deficient mice showed normal responsiveness to LPS, as

expected on the basis of the low expression of SIGIRR on these cells (Garlanda *et al.*, 2004). Additionally, published results from this group showed that IL-1F5 could not impart its anti-inflammatory role in BMDMs (Costelloe *et al.*, 2008), where SIGIRR is not expressed (Wald *et al.*, 2003).

It could be concluded that the effects of SIGIRR are cell-type specific, because of differential expression of SIGIRR but another possible explanation for these cell-type specific functions of SIGIRR identified in this study and others, is that an alternative form of SIGIRR may be expressed. For example post-translational modifications, such as glycosylation processes, which can disrupt protein folding and thus the functions of immune mediators could explain different findings. Indeed, the first study to characterise SIGIRR revealed that it is highly glycosylated with five N-glycosylation sites in the extracellular domain (Thomassen *et al.*, 1999). Lech and colleagues (2007) showed that, in some cases, the degree of glycosylation could explain the differential functions of SIGIRR in different cell types. In particular, this study revealed that SIGIRR was glycosylated differently on the two different cells types in the kidney, SIGIRR in tubular epithelial cells is N- and O-glycosylated, whereas SIGIRR in intrarenal CD11b-positive myeloid cells is only N-glycosylated. It remains possible that SIGIRR in astrocytes is differentially glycosylated to SIGIRR in microglial cells and this shift in glycosylation affects the function of SIGIRR in these two cell types.

It seems counter-intuitive that the expression and thus functionality of SIGIRR should be restricted to astrocytes and not microglia in the CNS, considering that microglia are the main immune effector cells of the CNS, which rapidly respond to various kinds of CNS injury (Stoll and Jander, 1999). On the other hand, perhaps this restricted functionality is logical as, more recently, attention has shifted to the role of astrocytes in the innate immune response. Studies have shown that astrocytes have the capacity to express TLRs and that astrocytes share with microglia a role in the innate immune response (Owens, 2005).

Chapter 5

An investigation into the anti-inflammatory effects of IL-1F5 on three models of inflammation in rat hippocampus

5.1. Introduction

IL-1F5 is a newly characterised member of the IL-1 superfamily, which shares 44% amino acid sequence homology with IL-1ra suggesting it may have an antagonistic role (Dunn *et al.*, 2003). The exact biological role of IL-1F5 has remained elusive; it was considered that IL-1F5 might exert antagonistic properties similar to IL-1ra, as it was shown to be capable of antagonising IL-1F9-mediated NFκB activation in Jurkat cells transfected with IL-1Rrp2 (Debets *et al.*, 2001). In support of this, another study reported that skin abnormalities such as acanthosis, hyperkeratosis and inflammatory cell infiltrate in IL-1F6 transgenic mice were exacerbated by an IL-1F5 deficiency, further suggesting that IL-1F5 antagonises inflammation at IL-1Rrp2 (Blumberg *et al.*, 2007). However Towne and colleagues (2004) observed no antagonistic effect of IL-1F5 on IL-F6-, IL-F8- and IL-1F9-induced inflammatory signalling. Furthermore Barton and colleagues (2000) showed that IL-1F5 did not attenuate IL-6 production from fibroblasts and endothelial cells in response to LPS or reduce IFN-γ production in response to IL-18 in KG-1 cells. Results from our previous study revealed an anti-inflammatory role for IL-1F5 in rat hippocampus and in mixed glial cells. IL-1F5 attenuated the inflammatory effects of IL-1β and LPS on LTP, pro-inflammatory cytokine production and MAPK activation, however IL-1F5 could not exert its anti-inflammatory effects on LPS-induced signalling in peripheral spleen macrophages and DCs (Costelloe *et al.*, 2008). Taken together these findings suggest that IL-1F5 exerts differential effects in different cell types and under different pro-inflammatory stimuli. The aim of this study was twofold, firstly to examine if IL-1F5 was capable of antagonising LPS-induced pro-inflammatory cytokines production from mixed glial cells, isolated astrocytes and microglial cells in order to identify the cellular target for the effects of IL-1F5 in the brain. The second aim of this study was to investigate if IL-1F5 was capable of antagonising the effects of three models which induce inflammatory changes, in particular LPS- and Aβ-induced and age-associated pro-inflammatory cytokine production and downstream signalling in rat hippocampus.

5.2. Methods

Mixed glial cells, isolated astrocytes and isolated microglial were prepared from one-day old male Wistar rats, cultured for 10-14 days and treated with IL-1F5 (3µg/ml) in the presence and absence of LPS (100ng/ml) (see section 2.5.6 for specific details). Young male Wistar rats were anaesthetised and injected icv with sterile saline or IL-1F5 (5µl; 30ng/ml; diluted in sterile saline) following this treatment rats were injected icv with sterile saline or $A\beta_{(1\text{-}42)}$ (5µl; 1mg/ml) or injected ip with sterile saline or LPS (200µl; 100µg/kg) (see section 2.2.2 for specific details). Young and aged male Wistar rats were anaesthetised and injected icv with sterile saline or IL-1F5 (5µl; 30ng/ml; diluted in sterile saline). In all cases, rats were left for 3 hours post-injections and killed by cervical dislocation and tissue taken for analysis (see section 2.4 for specific details). Analysis of peripheral cytokine concentrations were assessed by ELISA, microglial markers by FACS and MAPK by western immunoblotting (see sections 2.8, 2.9 and 2.11 for specific details). Data are expressed as means ± standard error of the mean. A Student's two-way ANOVA was performed to determine whether significant differences existed between conditions. Post hoc Student Newmann-Keuls test was used to determine where the significance lied (see section 2.13 for specific details).

5.3. Results

Anti-inflammatory effects of IL-1F5 in vitro

It is known that glial cells are the primary cell source of pro-inflammatory cytokines in particular IL-1β, IL-6 and TNF-α in the CNS. It has also been shown that anti-inflammatory cytokines like IL-4 and IL-10 can modulate the production of these pro-inflammatory cytokines by glia (Szczepanik *et al.*, 2001). Recent evidence has indicated that IL-1F5 possesses anti-inflammatory properties (Debets *et al.*, 2001). The objectives of the following series of experiments were to establish whether IL-1F5 might modulate LPS-induced inflammatory changes in mixed glia, astrocytes and microglial cells.

IL-1F5 attenuates the LPS-induced increase in CD86 expression in mixed glia

Mixed glial cells were pre-treated with IL-1F5 and incubated in the presence of LPS, stained for CD86 and CD11b and analysed by flow cytometry. Figure 5.1. shows that CD86 and CD11b expression is markedly increased in LPS-treated samples compared with control-treated samples. Pre-treatment with IL-1F5 reduced the LPS-stimulated increases in CD86 and CD11b expression. Plots are representatives of 3 separate cultures.

The mean data from the 3 experiments are shown in Figure 5.2. treatment with LPS induced higher percentages of CD86 and CD11b expression (1.145 % ± 0.29; n=6) compared with control-treated cells (0.09 % ± 0.06; n=3; $^{*}p<0.05$; ANOVA). Pre-treatment with IL-1F5 significantly attenuated the LPS-induced increase in CD86 and CD11b expression (0.32 % ± 0.14; n=3; $^{+}p<0.05$; ANOVA). Data are expressed as means ± SEM of 3 separate cultures.

IL-1F5 attenuates the LPS-induced increase in IL-1β concentration in mixed glial cells

To determine if IL-1F5 might modulate LPS-induced cytokine production. Primary cultures of mixed glia were pre-treated with IL-1F5 and incubated in the presence of LPS. Figure 5.3. shows that mean IL-1β concentration was significantly increased in supernatants prepared from LPS-treated mixed glia (280.2 pg/ml ± 16.32; n=12) compared with control-treated mixed glia (50.64 pg/ml ± 10.89; n=12; $^{**}p<0.01$; ANOVA). Pre-treatment with IL-1F5 significantly attenuated the LPS-induced increase in IL-1β concentration (148.1 pg/ml ± 19.61; n=12; $^{++}p<0.01$; ANOVA).

Effect of LPS and IL-1F5 on IL-1β concentration in isolated astrocytes and microglial cells

In an effort to identify the target cells for the actions of IL-1F5 in mixed glial cultures, the effects of IL-1F5 was assessed on LPS-induced IL-1β concentration in astrocytes and microglial cells. Figure 5.4. illustrates that mean IL-1β concentration was significantly increased in supernatants from LPS-treated astrocytes (73.86 pg/ml

± 10.48; n=5) compared with control-treated astrocytes (12.43 pg/ml ± 3.20; n=5; ***$p<0.001$; ANOVA). Pre-treatment of astrocytes with IL-1F5 significantly decreased the LPS-induced increase in IL-1β protein expression (56.42 pg/ml ± 3.56; n=5; $^{+}p<0.05$; ANOVA).

Figure 5.5. illustrates that mean IL-1β concentration was significantly increased in LPS-treated microglial cells (130.6 pg/ml ± 32.34; n=6) compared with control-treated microglia (2.78 pg/ml ± 2.02; n=6; *$p<0.05$; Student's *t* test). Pre-treatment of microglial cells with IL-1F5 failed to attenuate the LPS-induced increase in IL-1β concentration. Conversely pre-treatment of microglia cells with IL-1F5 induced a significant increase in IL-1β concentration (776.7 pg/ml ± 66.59; n=6) compared with control-treated microglial cells (2.78 pg/ml ± 2.02; n=6; $^{\$\$\$}p<0.001$; ANOVA).

IL-1F5 attenuates the LPS-induced increase in IL-6 concentration in mixed glial cells

To further establish a role for IL-1F5 as a modulator of LPS-induced signalling, primary cultures of mixed glia were pre-treated with IL-1F5 and incubated in the presence of LPS. Figure 5.6. shows that mean IL-6 concentration was significantly increased in supernatants prepared from LPS-treated cultured mixed glia (675.2 pg/ml ± 23.80; n=6) compared with control-treated mixed glia (189.2 pg/ml ± 6.105; n=6; ***$p<0.001$; ANOVA). Pre-treatment with IL-1F5 significantly attenuated the LPS-induced increase in IL-6 concentration (597.4 pg/ml ± 33.0; n=6; $^{+}p<0.05$; ANOVA).

Effect of LPS and IL-1F5 on IL-16 concentration in isolated astrocytes and microglial cells

In an effort to identify the target cells for the actions of IL-1F5 in mixed glial cultures, the effect of IL-1F5 was assessed on LPS-induced IL-6 concentration in astrocytes and microglial cells. Figure 5.7. illustrates that mean IL-6 concentration was significantly increased in supernatants prepared from LPS-treated astrocytes (1574 pg/ml ± 156.1; n=5) compared with control-treated astrocytes (0 pg/ml; n=5; ***$p<0.001$; ANOVA). Pre-treatment of astrocytes with IL-1F5 significantly

decreased the LPS-induced increase in IL-1β protein expression (1227 pg/ml ± 97.98; n=5; $^{+}p<0.05$; ANOVA).

Figure 5.8. illustrates that mean IL-6 concentration was significantly increased in supernatants prepared from LPS-treated microglial cells (4.35 ng/ml ± 0.22; n=6) compared with control-treated microglia (0.45 ng/ml ± 0.18; n=6; $^{***}p<0.001$; ANOVA). Pre-treatment of microglial cells with IL-1F5 failed to attenuate the LPS-induced increase in IL-6 concentration. Conversely pre-treatment of microglia cells with IL-1F5 induced a significant increase in IL-6 concentration (8.47 ng/ml ± 0.30; n=6) compared with control-treated microglial cells (0.45 ng/ml ± 0.18; n=6; $^{\$\$\$}p<0.001$; ANOVA).

IL-1F5 attenuates the LPS-induced increase in TNF-α concentration in mixed glial cells

To further establish a role for IL-1F5 as a modulator of LPS-induced signalling, primary cultures of mixed glia were pre-treated with IL-1F5 and incubated in the presence of LPS. Figure 5.9. illustrates that mean TNF-α concentration was significantly increased in supernatants prepared from LPS-treated mixed glia (1512 pg/ml ± 108; n=5) compared with control-treated mixed glia (29.51 pg/ml ± 24.40; n=6; $^{***}p<0.001$; ANOVA). Pre-treatment of mixed glia with IL-1F5 significantly decreased the LPS-induced increase in TNF-α concentration (1067 pg/ml ± 164.7; n=6; $^{++}p<0.01$; ANOVA).

Effect of LPS and IL-1F5 on TNF-α concentration in isolated astrocytes and microglial cells

In an effort to identify the target cells for the actions of IL-1F5 in mixed glial cultures, the effect of IL-1F5 was assessed on LPS-induced TNF-α concentration in astrocytes and microglial cells. Figure 5.10. illustrates that mean TNF-α concentration was significantly increased in supernatants prepared from LPS-treated astrocytes (228.7 pg/ml ± 14.78; n=5) compared with control-treated astrocytes (4.29 pg/ml ± 4.29; n=5; $^{***}p<0.001$; ANOVA). Pre-treatment of astrocytes with IL-1F5

significantly decreased the LPS-induced increase in TNF-α concentration (188.8 pg/ml ± 15.25; n=5; $^{+}p<0.05$; ANOVA).

Figure 5.11. illustrates that mean TNF-α concentration was significantly increased in supernatants prepared from LPS-treated microglial cells (1198 pg/ml ± 25.80; n=6) compared with control-treated microglia (4.75 pg/ml ± 4.84; n=6; $^{***}p<0.001$; ANOVA). Pre-treatment of microglial cells with IL-1F5 failed to attenuate the LPS-induced increase in TNF-α concentration. Conversely pre-treatment of microglia cells with IL-1F5 induced a significant increase in TNF-α concentration (1525 pg/ml ± 56.42; n=6) compared with control-treated microglial cells (4.75 pg/ml ± 4.84; n=6; $^{\$\$\$}p<0.001$; ANOVA).

Anti-inflammatory effects of IL-1F5 in vivo

The data presented here indicate that IL-1F5 appears to exert an anti-inflammatory effect *in vitro* and in particular in astrocytes rather than microglial cells. To assess whether IL-1F5 exerted a similar anti-inflammatory effect *in vivo*, we have assessed the modulatory effects of IL-1F5 in LPS-treated, Aβ-treated and aged rats, 3 models in which inflammatory changes have been described.

IL-1F5 modulates the LPS-induced increase in hippocampal cytokine production

The concentration of the pro-inflammatory cytokines IL-1β and IL-6 were assessed in hippocampal homogenates prepared from rats treated with LPS and IL-1F5. Figure 5.12. shows that mean IL-1β concentration was significantly increased in tissue prepared from LPS-treated rats (96.89 pg/mg ± 9.94; n=6) compared with saline-treated control rats (21.73 pg/mg ± 9.03; n=6; $^{*}p<0.05$; ANOVA). The data show that mean IL-1β concentration was decreased significantly in the hippocampus of LPS-treated rats which received IL-1F5 and mean IL-1β concentration was similar to that in tissue prepared from saline-treated control rats (40.61 pg/mg ± 13.51; n=6; $^{+}p<0.05$; ANOVA).

Figure 5.13. illustrates that IL-6 concentration was significantly increased in hippocampal homogenates prepared from LPS-treated rats (190.3 pg/mg ± 36.09; n=6) compared with hippocampal homogenates prepared from saline-treated control rats (100.4 pg/mg ± 21.41; n=6; $^{*}p<0.05$; ANOVA). Mean IL-6 concentration was

significantly reduced in the hippocampus following treatment with LPS in combination with IL-1F5 compared with LPS alone (115.7 pg/mg ± 8.86; n=6; $^{+}p<0.05$; ANOVA).

IL-1F5 attenuates LPS-induced changes in signalling in the hippocampus

Signalling by IL-1β is initiated by binding to its receptor, IL-1RI. Among the downstream effects of IL-1β signalling is activation of the stress-activated protein kinases (SAPKs) c-Jun-NH_2-terminal kinase (JNK) and p38. In the hippocampus, activation of JNK and p38 is coupled with elevated IL-1β concentrations in aged rats (O'Donnell *et al.*, 2000), similarly studies have shown that IL-6 can activate p38 (Zauberman *et al.*, 1999). In this study, activation of JNK was assessed by gel electrophoresis and western immunoblotting using antibodies which specifically identifies the phosphorylated and total forms of JNK and activation is expressed as a ratio of pJNK to t-JNK. Figure 5.14. (A) shows two sample immunoblots illustrating expression of p-JNK and t-JNK in hippocampal samples prepared from control-, LPS-, IL-1F5- and LPS and IL-1F5-treated rats. Figure 5.14. (B) represents the mean data obtained from densitometric analysis and shows a significant increase in JNK activity, expressed as a ratio of p-JNK to t-JNK in hippocampal lysates prepared from LPS-treated rats (3.36 ± 0.33 arbitrary units; n=6), compared with saline-treated controls (2.15 ± 0.24 arbitrary units; n=6; $^{*}p<0.05$; ANOVA). The sample immunoblots in Figure 5.14. (A) and the histogram in Figure 5.14. (B) show that JNK activation was significantly decreased in tissue prepared from rats treated with LPS and IL-1F5 compared with LPS alone (1.68 ± 0.24 arbitrary units; n=6; $^{+}p<0.05$; ANOVA).

Data from several studies have shown that, concomitant with the increase in JNK activation, there is a parallel increase in p38 activity following treatment with LPS (Kelly *et al.*, 2003). Here, the expression of the activated form of p38 was assessed by western immunoblotting using antibodies which specifically identifies p38 phosphorylation and activation is expressed as a ratio of phosphorylated p38 to total p38. Figure 5.15. (A) shows two sample immunoblots illustrating expression of p-p38 and t-p38 in hippocampal samples prepared from control-, LPS-, IL-1F5- and

LPS and IL-1F5-treated rats. Figure 5.15. (B) represents the mean data obtained from densitometric analysis showing a significant increase in p38 activity, expressed as a ratio of phosphorylated p38 to total p38 expression in hippocampal lysates prepared from LPS-treated rats (6.09 ± 0.78 arbitrary units; n=6), compared with saline-treated controls (3.64 ± 0.28 arbitrary units; n=6; *p<0.05; ANOVA). The histogram in Figure 5.15. (B) shows that p38 activation was decreased in tissue prepared from rats treated with LPS and IL-1F5 compared with LPS alone (4.49 ± 0.60 arbitrary units; n=6).

IL-1F5 modulates Aβ-induced cytokine production in hippocampus

Figure 5.16. shows that IL-1β concentration was significantly increased in hippocampal tissue prepared from Aβ-treated rats (164.9 pg/mg ± 29.24; n=6) compared with tissue prepared from saline-treated control rats (43.03 pg/mg ± 8.24; n=6; **p<0.01; ANOVA). Mean IL-1β concentration was decreased significantly in the hippocampus of Aβ-treated rats which received IL-1F5 (82.08 ± 21.61 pg/mg; n=6;$^{+}$p<0.05; ANOVA) compared with Aβ-treated rats.

Figure 5.17. shows that TNF-α concentration was significantly increased in hippocampal homogenates prepared from Aβ-treated rats (575.7 pg/mg ± 22.29; n=6) compared with tissue prepared from saline-treated control rats (315 pg/mg ± 21.65; n=6; **p<0.01; ANOVA). Mean TNF-α concentration was decreased significantly in the hippocampus of Aβ-treated rats which received IL-1F5 (399.3 pg/mg ± 29.36; n=6; $^{+}$p<0.05; ANOVA) compared with Aβ-treated rats (575.7 pg/mg ± 22.29; n=6).

Figure 5.18. shows that IL-6 concentration was significantly increased in hippocampal homogenates prepared from Aβ-treated rats (1121 pg/mg ± 42.32; n=6) compared with tissue prepared from saline-treated control rats (699.9 pg/mg ± 37.39; n=6; **p<0.01; ANOVA). Mean IL-6 concentration was significantly decreased in the hippocampus of Aβ-treated rats which received IL-1F5 (891.8 pg/mg ± 46.22; n=6; $^{++}$p<0.01; ANOVA)compared with Aβ-treated rats.

IL-1F5 attenuated age-related changes in hippocampal cytokine production

The concentration of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were assessed in hippocampal homogenates prepared from control-treated aged rats and aged rats treated with IL-1F5. Figure 5.19. shows that IL-1β concentration was significantly increased in hippocampal homogenates prepared from aged (149.4 pg/mg ± 6.09; n=6) compared with young (129.1 pg/mg ± 5.30; n=6; *$p<0.05$; ANOVA) rats. Mean IL-1β concentration was significantly decreased in hippocampal tissue of aged rats which received IL-1F5 (124.4 pg/mg ± 7.46; n=6; *$p<0.05$; ANOVA) compared with control-treated aged rats; in this case mean IL-1β concentration was similar to that in tissue prepared from young control rats (129.1 pg/mg ± 5.302; n=6).

Similar to the age-associated increase in IL-1β, Figure 5.20. shows that TNF-α concentration was significantly increased in hippocampal homogenates prepared from aged rats (1612 pg/mg ± 79.09; n=6) compared with tissue prepared from young rats (157.3 pg/mg ± 10.81; n=6; ***$p<0.001$; ANOVA). Mean TNF-α concentration was decreased significantly in the hippocampus of aged rats which received IL-1F5 (1386 pg/mg ± 66.81; n=6; $^{++}p<0.01$; ANOVA) compared with control-treated aged rats.

In contrast to the age-associated increases in IL-1β and TNF-α, Figure 5.21. shows that there was no age-associated increase in IL-6 concentration in hippocampal tissue prepared from aged (803.7 pg/mg ± 82.13; n=6) compared with young (746.3 pg/mg ± 108.5; n=6) rats. Mean IL-6 concentrations were also similar in IL-1F5-treated young (661.9 pg/mg ± 85.31; n=6) and aged (765.6 pg/mg ± 61.95; n=6) rats.

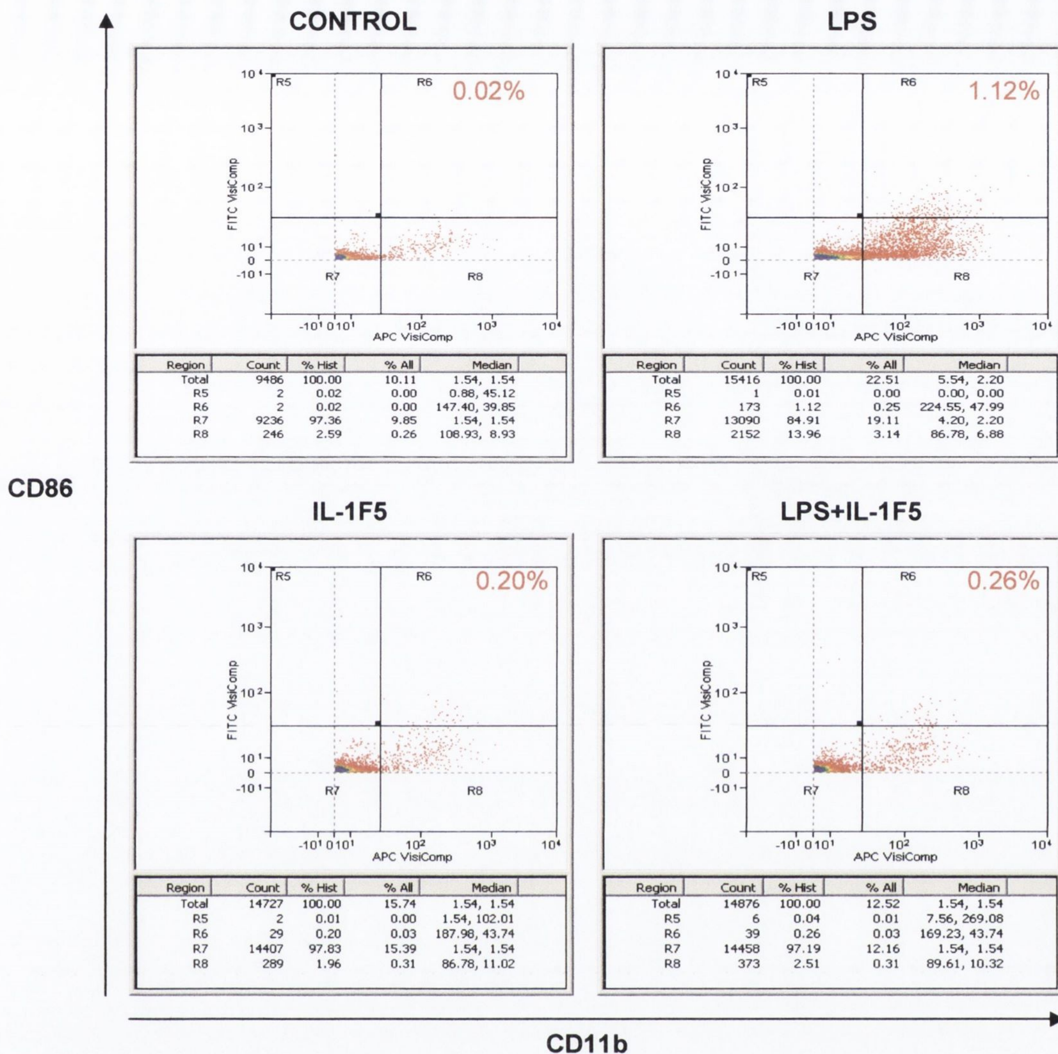


Figure 5.1. IL-1F5 attenuates the LPS-induced increase in CD86 expression

Rat mixed glial cells were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours CD86 and CD11b expression was determined in cells by flow cytometry. LPS increased the percentages of CD86 and CD11b positive cells and pre-treatment with IL-1F5 attenuated the LPS-induced increase in CD86 and CD11b in mixed glial cells. Plots are representative of 3 separate cultures.

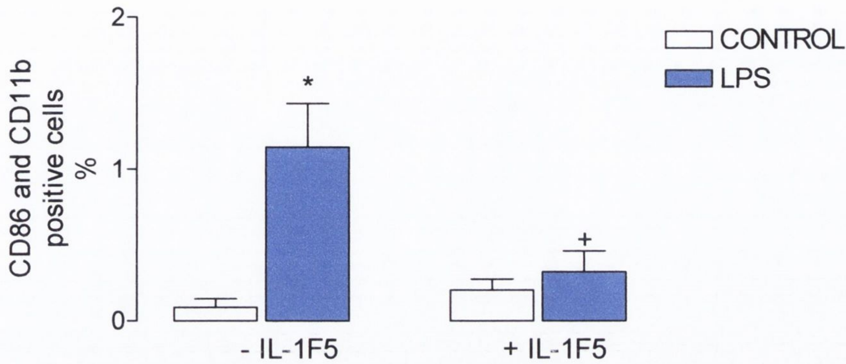


Figure 5.2. IL-1F5 attenuates the LPS-induced increase in CD86 and CD11b positive cells

Rat mixed glial cells were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours CD86 and CD11b expression was determined in cells by flow cytometry. The data show that LPS increases the percentages of CD86 and CD11b positive cells (*$p<0.05$; ANOVA). Pre-treatment with IL-1F5 attenuated the LPS-induced increases in CD86 and CD11b positive cells ($^{+}p<0.05$; ANOVA). Values are expressed as percentages and are means ± SEM of 3 separate cultures.

2-way ANOVA: LPS $_{effect}$ $F_{(1, 11)} = 4.94$; $p=0.0482$, IL-1F5 $_{effect}$ $F_{(1, 11)} = 1.79$; $p=0.2075$, Interaction $_{effect}$ $F_{(1, 11)} = 3.13$; $p=0.1047$

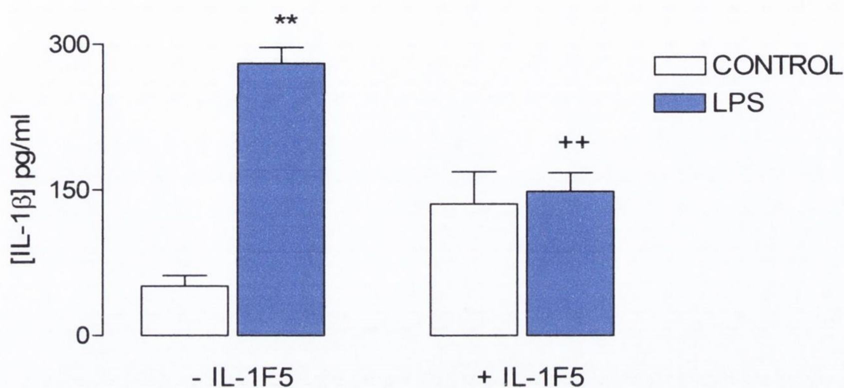


Figure 5.3. IL-1F5 attenuates the LPS-induced increase in IL-1β in mixed glia

Rat mixed glial cells were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours IL-1β concentration was determined in supernatants by ELISA. Mean IL-1β concentration was significantly increased in supernatants prepared from LPS-treated mixed glia compared with controls (**$p<0.01$; ANOVA). Pre-treatment with IL-1F5 significantly decreased the LPS-induced increase in IL-1β ($^{++}p<0.01$; ANOVA). Values are expressed as pg IL-1β/ml and are means ± SEM of 12 cultures.

2-way ANOVA: LPS $_{effect}$ $F(1, 40) = 31.18$; $p<0.0001$, IL-1F5 $_{effect}$ $F(1, 40) = 1.19$; $p=0.2819$, Interaction $_{effect}$ $F(1, 40) = 24.96$; $p<0.0001$

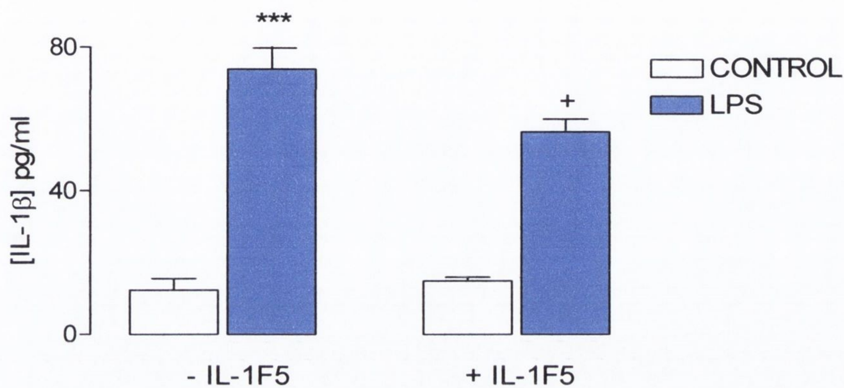


Figure 5.4. IL-1F5 attenuates the LPS-induced increase in IL-1β in astrocytes

Rat astrocytes were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours IL-1β concentration was determined in supernatants by ELISA. Mean IL-1β concentration was significantly increased in supernatants prepared from LPS-treated cultured astrocytes compared with controls (***$p<0.001$; ANOVA). Pre-treatment with IL-1F5 significantly decreased the LPS-induced change ($^{+}p<0.05$; ANOVA). Values are expressed as pg IL-1β/ml and are means ± SEM of 5 cultures.

2-way ANOVA: LPS $_{effect}$ $F(1, 15) = 103.92$; $p<0.0001$, IL-1F5 $_{effect}$ $F(1, 15) = 2.18$; $p=0.1605$, Interaction $_{effect}$ $F(1, 15) = 3.92$; $p=0.0665$

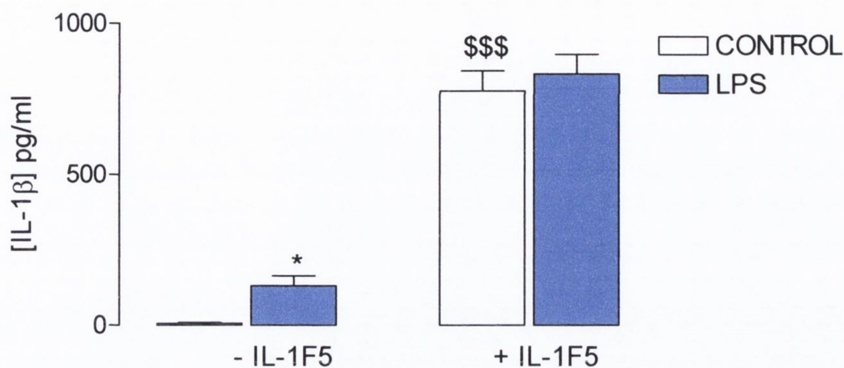


Figure 5.5. Effect of IL-1F5 on LPS-induced increase in IL-1β in microglial cells
Rat microglial cells were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours IL-1β concentration was determined in supernatants by ELISA. Mean IL-1β concentration was significantly increased in supernatants prepared from LPS-treated microglial cells compared with controls (*$p<0.05$; Student's *t* test). Pre-treatment of microglial cells with IL-1F5 failed to attenuate the LPS-induced increase in IL-1β concentration and treatment with IL-1F5 induced a significant increase in IL-1β concentration compared with controls ($^{\$\$\$}p<0.001$; ANOVA). Values are expressed as pg IL-1β/ml and are means ± SEM of 6 cultures.

2-way ANOVA: LPS $_{effect}$ $F(1,20) = 3.47$; $p=0.0771$, IL-1F5 $_{effect}$ $F(1,20) = 223.70$; $p<0.0001$, Interaction $_{effect}$ $F(1,20) = 0.53$; $p=0.4760$

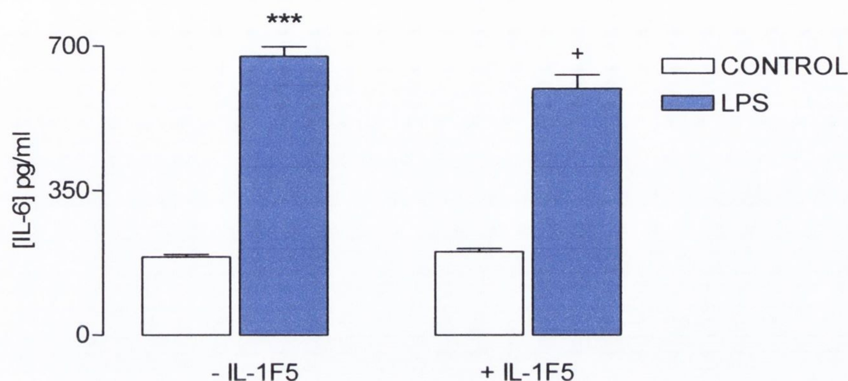


Figure 5.6. IL-1F5 attenuates the LPS-induced increase in IL-6 in mixed glia

Rat mixed glial cells were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3µg/ml), after 24 hours IL-6 concentration was determined in supernatants by ELISA. Mean IL-6 concentration was significantly increased in supernatants prepared from LPS-treated mixed glia compared with controls (***p<0.001; ANOVA). Pre-treatment with IL-1F5 significantly attenuates the LPS-induced increase in IL-6 concentration in mixed glia (^{+}p<0.05; ANOVA). Values are expressed as pg IL-6/ml and are means ± SEM of 12 cultures.

2-way ANOVA: LPS $_{effect}$ F (1, 19) = 450.21; p<0.0001, IL-1F5 $_{effect}$ F (1, 19) = 2.42; p=0.1361, Interaction $_{effect}$ F (1, 19) = 4.79; p=0.0414

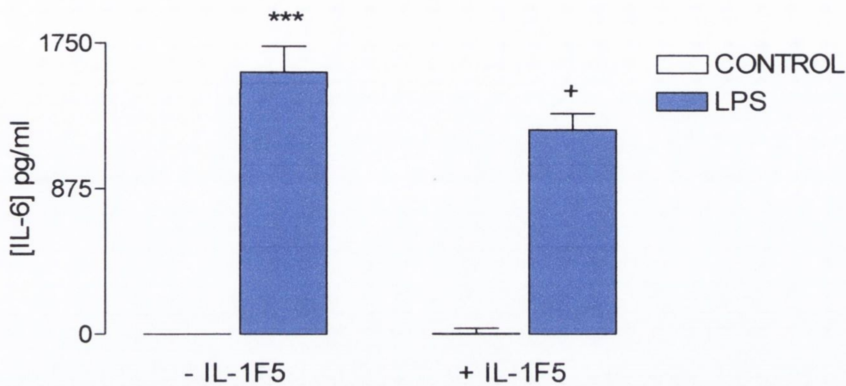


Figure 5.7. IL-1F5 decreases the LPS-induced increase in IL-6 in astrocytes

Rat astrocytes were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours IL-1β concentration was determined in supernatants by ELISA. Mean IL-6 concentration was significantly increased in supernatants prepared from LPS-treated cultured astrocytes compared with controls (***p<0.001; ANOVA). Pre-treatment with IL-1F5 significantly attenuates the LPS-induced increase in IL-6 concentration in cultured astrocytes (^{+}p<0.05; ANOVA). Values are expressed as pg IL-6/ml and are means ± SEM of 5 cultures.

2-way ANOVA: LPS $_{effect}$ F (1, 17) = 247.40; p<0.0001, IL-1F5 $_{effect}$ F (1, 17) = 3.25; p=0.0891, Interaction $_{effect}$ F (1, 17) = 4.55; p=0.0479

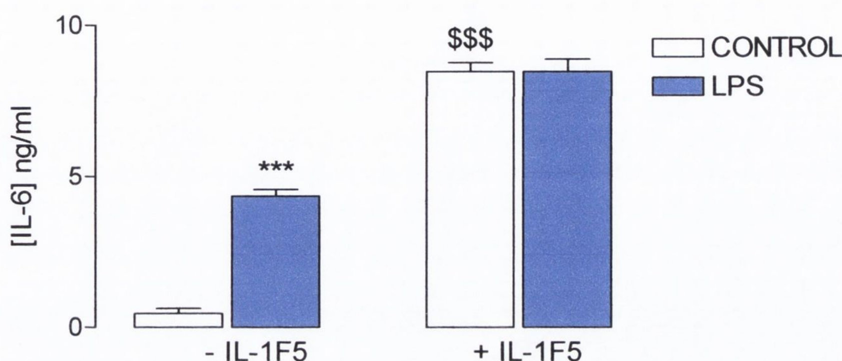


Figure 5.8. Effect of IL-1F5 on LPS-induced increase in IL-6 in microglial cells
Rat microglial cells were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours IL-6 concentration was determined in supernatants by ELISA. Mean IL-6 concentration was significantly increased in supernatants prepared from LPS-treated microglial cells compared with controls (***p<0.001; ANOVA). Pre-treatment of microglial cells with IL-1F5 failed to attenuate the LPS-induced increase in IL-6 concentration and treatment with IL-1F5 induced a significant increase in IL-6 concentration compared with controls ($^{\$\$\$}$p<0.001; ANOVA). Values are expressed as ng IL-6/ml and are means ± SEM of 6 cultures.

2-way ANOVA: LPS $_{effect}$ F (1, 20) = 45.68; p<0.0001, IL-1F5 $_{effect}$ F (1, 20) = 446.16; p<0.0001, Interaction $_{effect}$ F (1, 20) = 45.55; p<0.0001

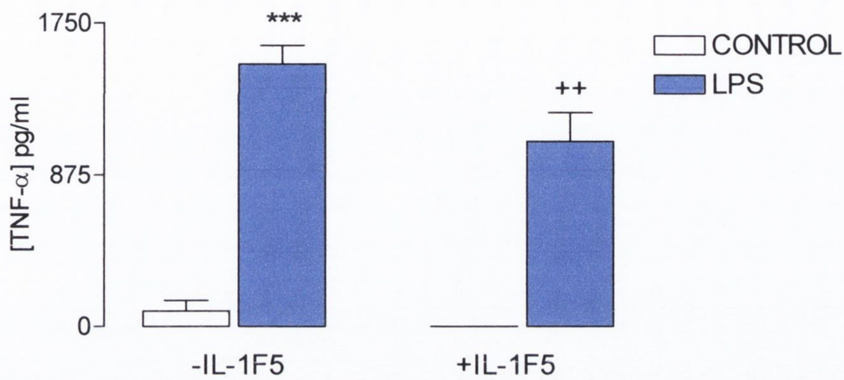


Figure 5.9. IL-1F5 attenuates the LPS-induced increase in TNF-α in mixed glial cells

Rat mixed glial cells were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3µg/ml), after 24 hours TNF-α concentration was determined in supernatants by ELISA. Mean TNF-α concentration was significantly increased in supernatants prepared from LPS-treated mixed glial cells compared with controls (****p*<0.001; ANOVA). Pre-treatment with IL-1F5 attenuated the LPS-induced increase in IL-6 concentration in mixed glial cells ($^{++}p<0.01$; ANOVA). Values are expressed as pg TNF-α/ml and are means ± SEM of 12 cultures.

2-way ANOVA: LPS $_{effect}$ F (1, 19) = 193.89; p<0.0001, IL-1F5 $_{effect}$ F (1, 19) = 6.72; p=0.0179, Interaction $_{effect}$ F (1, 19) = 5.15; p=0.0350

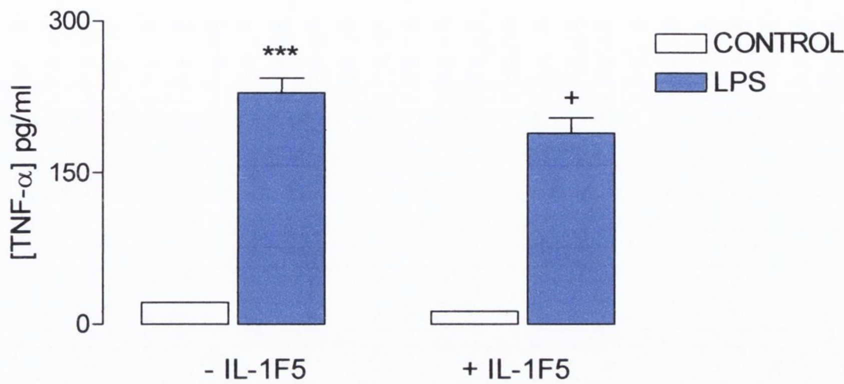


Figure 5.10. IL-1F5 attenuates the LPS-induced increase in TNF-α in astrocytes

Rat astrocytes were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours TNF-α concentration was determined in supernatants by ELISA. Mean TNF-α concentration was significantly increased in supernatants prepared from LPS-treated astrocytes compared with controls (***$p<0.001$; ANOVA). Pre-treatment with IL-1F5 significantly attenuated the LPS-induced increase in TNF-α concentration in astrocytes ($^{+}p<0.05$; ANOVA). Values are expressed as pg TNF-α/ml and are means ± SEM of 5 cultures.

2-way ANOVA: LPS $_{effect}$ F (1, 16) = 354.20; p<0.0001, IL-1F5 $_{effect}$ F (1, 16) = 3.65; p=0.0742, Interaction $_{effect}$ F (1, 16) = 3.06; p=0.0922

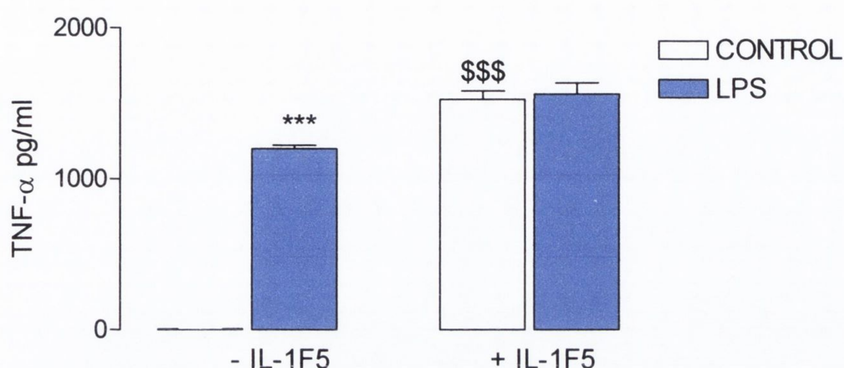


Figure 5.11. Effect of IL-1F5 on LPS-induced increase in TNF-α in microglial cells

Rat microglial cells were cultured with LPS (100ng/ml), following 2 hour pre-treatment with IL-1F5 (3μg/ml), after 24 hours TNF-α concentration was determined in supernatants by ELISA. Mean TNF-α concentration was significantly increased in supernatants prepared from LPS-treated microglial cells compared with controls (****p*<0.001; ANOVA). Pre-treatment of microglial cells with IL-1F5 failed to attenuate the LPS-induced increase in TNF-α concentration and treatment with IL-1F5 induced a significant increase in TNF-α concentration compared with controls ($^{\$\$\$}$p<0.001; ANOVA).Values are expressed as pg TNF-α/ml and are means ± SEM of 6 cultures.

2-way ANOVA: LPS $_{effect}$ F (1, 20) = 159.37; p<0.0001, IL-1F5 $_{effect}$ F (1, 20) = 374.50; p<0.0001, Interaction $_{effect}$ F (1, 20) = 140.99; p<0.0001

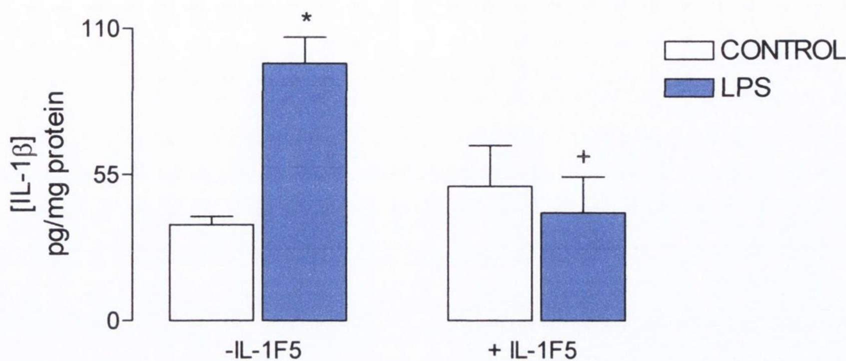


Figure 5.12. IL-1F5 attenuates the LPS-induced increase in hippocampal IL-1β
Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl) and 5 minutes later were injected ip with LPS (100μg/kg). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-1β concentration determined by ELISA. Mean IL-1β concentration was significantly increased in the hippocampus of LPS-treated rats compared with control-treated rats (*$p<0.05$; ANOVA). Mean IL-1β concentration was significantly decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS alone ($^{+}p<0.05$; ANOVA). Values are expressed as pg IL-1β/mg protein and are means ± SEM of 6 values in all groups.

2-way ANOVA: LPS $_{effect}$ $F(1, 13) = 5.85$; $p=0.0310$, IL-1F5 $_{effect}$ $F(1, 13) = 0.95$; $p=0.3470$, Interaction $_{effect}$ $F(1, 13) = 2.97$; $p=0.1083$

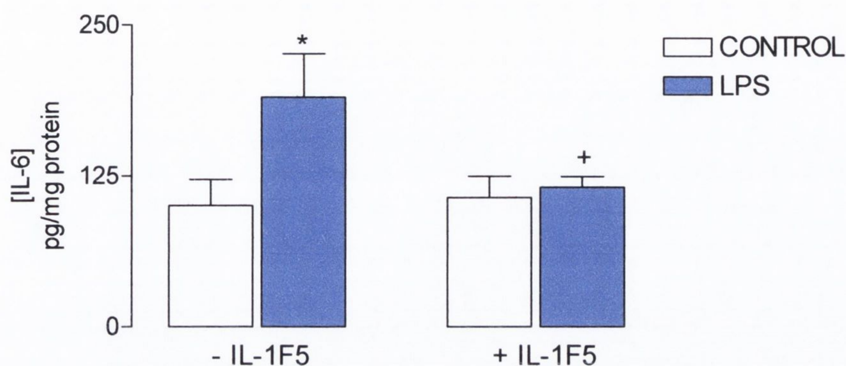


Figure 5.13. IL-1F5 attenuates the LPS-induced increase in hippocampal IL-6

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl) and 5 minutes later were injected ip with LPS (100μg/kg). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-6 concentration determined by ELISA. Mean IL-6 concentration was significantly increased in the hippocampus of LPS-treated rats compared with control-treated rats (*$p<0.05$; ANOVA). Mean IL-6 concentration was significantly decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS alone ($^{+}p<0.05$; ANOVA). Values are expressed as pg IL-6/mg protein and are means ± SEM of 6 values in all groups.

2-way ANOVA: LPS_{effect} $F(1, 13) = 5.10$; $p=0.0359$, IL-1F5 $_{effect}$ $F(1, 13) = 2.42$; $p=0.1360$, Interaction $_{effect}$ $F(1, 13) = 3.48$; $p=0.0778$

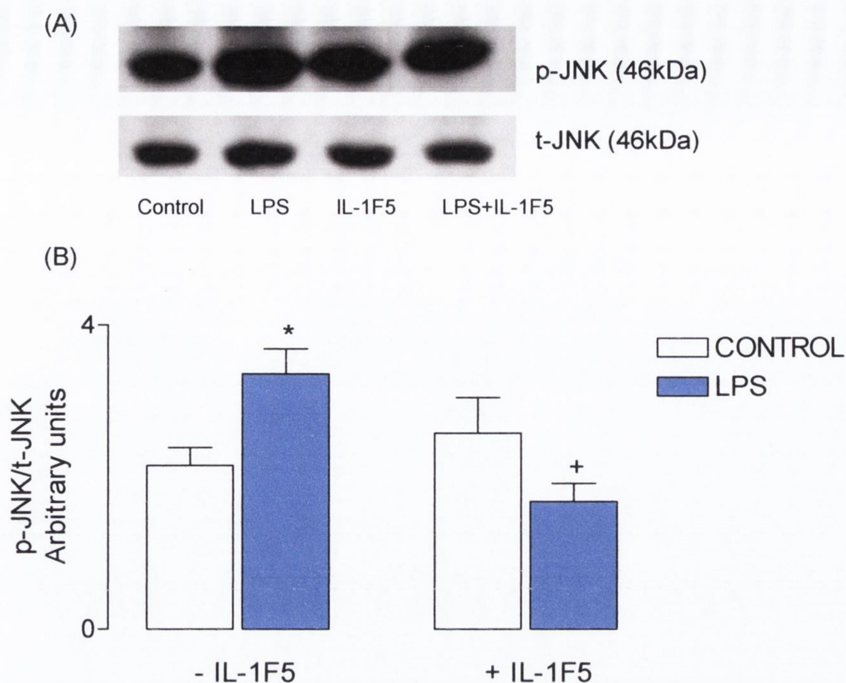


Figure 5.14. IL-1F5 attenuates the LPS-induced increase in JNK phosphorylation

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5µl) or IL-1F5 (30ng/ml; 5µl) and 5 minutes later were injected ip with LPS (100µg/kg). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and JNK phosphorylation determined by Western immunoblotting (expressed as a ratio of phosphorylated to total JNK in arbitrary units). (A) Sample immunoblots indicating density of phosphorylated JNK and total JNK (p-JNK and t-JNK) in tissue prepared from control-, LPS-, IL-1F5- and LPS and IL-1F5-treated rats. (B) JNK phosphorylation was significantly increased in hippocampus of LPS-treated rats compared with control-treated rats ($^*p<0.05$; ANOVA). Mean JNK phosphorylation was significantly decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS alone ($^+p<0.05$; ANOVA). Values are expressed in arbitrary units obtained from densitometric analysis and are means ± SEM of 6 values in all groups.

2-way ANOVA: LPS $_{effect}$ F(1,18) = 0.20; p=0.6615, IL-1F5 $_{effect}$ F(1,18) = 3.34; p=0.0840, Interaction $_{effect}$ F(1,18) = 9.49; p=0.0065

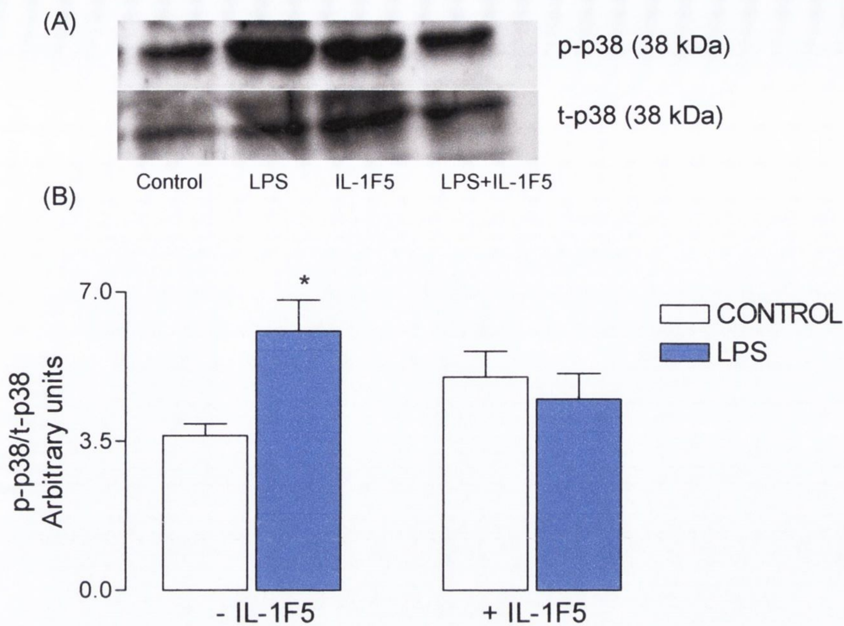


Figure 5.15. IL-1F5 attenuates the LPS-induced increase in p38 phosphorylation
Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5µl) or IL-1F5 (30ng/ml; 5µl) and 5 minutes later were injected ip with LPS (100µg/kg). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and p38 phosphorylation determined by Western immunoblotting (expressed as a ratio of phosphorylated to total p38 in arbitrary units). (A) Sample immunoblots indicating density of phosphorylated p38 and total p38 (p-p38 and t-p38) in tissue prepared from control-, LPS-, IL-1F5- and LPS and IL-1F5-treated rats. (B) p38 phosphorylation was significantly increased in hippocampus of LPS-treated rats compared with control-treated rats (*$p<0.05$; ANOVA). Mean p38 phosphorylation was decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS. Values are expressed in arbitrary units obtained from densitometric analysis and are means ± SEM of 6 values in all groups.

2-way ANOVA: LPS $_{effect}$ $F(1, 19) = 2.87$; $p=0.1063$, IL-1F5 $_{effect}$ $F(1, 19) = 0.04$; $p=0.8422$, Interaction $_{effect}$ $F(1, 19) = 6.84$; $p=0.0170$

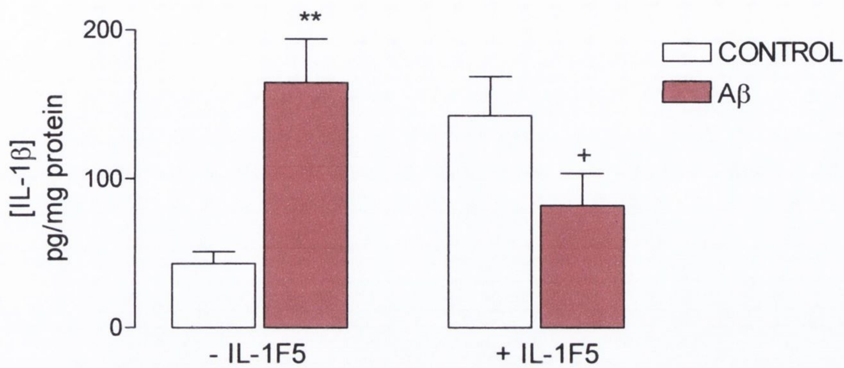


Figure 5.16. IL-1F5 attenuates the Aβ-induced increase in hippocampal IL-1β
Young (2-3 months) male Wistar rats were injected i.c.v. with IL-1F5 (30ng/ml; 5μl) or Aβ $_{(1-42)}$ (1mg/ml; 5μl) or both. After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-1β concentration determined by ELISA. Mean IL-1β concentration was significantly increased in the hippocampus of Aβ-treated rats compared with control-treated rats (**$p<0.01$; ANOVA). Mean IL-1β concentration was significantly decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS alone ($^{+}p<0.05$; ANOVA). Values are expressed as pg IL-1β/mg protein and are means ± SEM of 6 values in all groups.

2-way ANOVA: Aβ $_{effect}$ $F(1, 19) = 1.89$; $p=0.1854$, IL-1F5 $_{effect}$ $F(1, 19) = 0.14$; $p=0.7139$, Interaction $_{effect}$ $F(1, 19) = 16.63$; $p=0.0006$

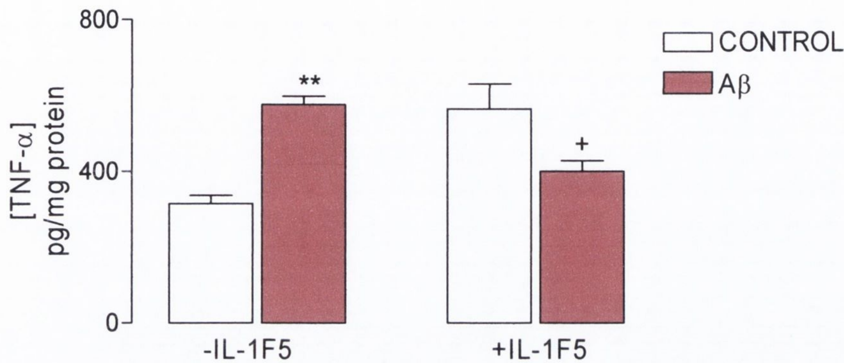


Figure 5.17. IL-1F5 attenuates the Aβ-induced increase in hippocampal TNF-α

Young (2-3 months) male Wistar rats were injected i.c.v. with IL-1F5 (30ng/ml; 5μl) or Aβ $_{(1-42)}$ (1mg/ml; 5μl) or both. After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and TNF-α concentration determined by ELISA. Mean TNF-α concentration was significantly increased in the hippocampus of Aβ-treated rats compared with control-treated rats (**p<0.01; ANOVA). Mean TNF-α concentration was significantly decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS alone ($^{+}$p<0.05; ANOVA). Values are expressed as pg TNF-α/mg protein and are means ± SEM of 6 values in all groups.

2-way ANOVA: Aβ $_{effect}$ F (1, 18) = 1.23; p=0.2827, IL-1F5 $_{effect}$ F (1, 18) = 0.74; p=0.4025, Interaction $_{effect}$ F (1, 18) = 24.69; p<0.0001

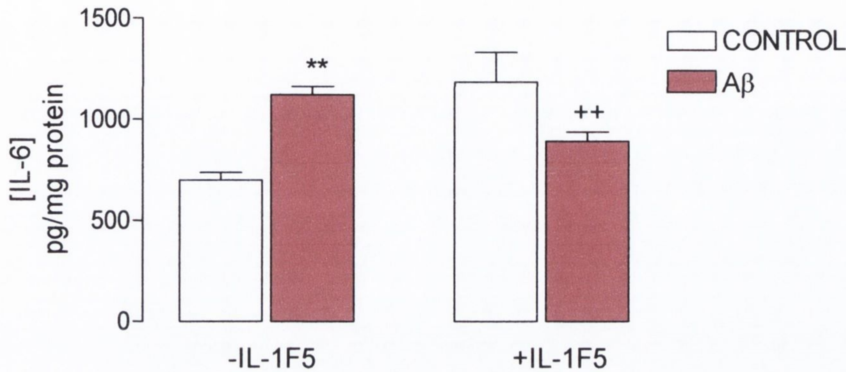


Figure 5.18. IL-1F5 attenuates the Aβ-induced increase in hippocampal IL-6

Young (2-3 months) male Wistar rats were injected i.c.v. with IL-1F5 (30ng/ml; 5μl) or Aβ $_{(1-42)}$ (1mg/ml; 5μl) or both. After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-6 concentration determined by ELISA. Mean IL-6 concentration was significantly increased in the hippocampus of Aβ-treated rats compared with control-treated rats (**$p<0.01$; ANOVA). Mean IL-6 concentration was significantly decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS alone ($^{++}p<0.01$; ANOVA). Values are expressed as pg IL-6/mg protein and are means ± SEM of 6 values in all groups.

2-way ANOVA: Aβ $_{effect}$ $F(1, 19) = 0.57$; $p=0.4587$, IL-1F5 $_{effect}$ $F(1, 19) = 2.30$; $p=0.1462$, Interaction $_{effect}$ $F(1, 19) = 17.94$; $p=0.0004$

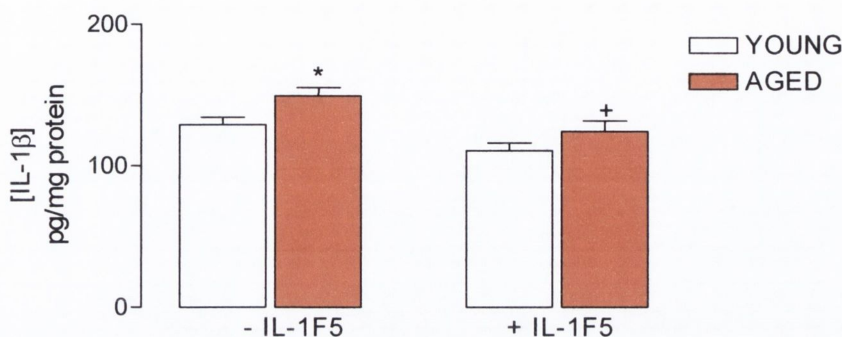


Figure 5.19. IL-1F5 attenuates the age-associated increase in hippocampal IL-1β
Young (2-3 months) and aged (22-24 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-1β concentration determined by ELISA. Mean IL-1β concentration was significantly increased in the hippocampus of aged rats compared with young rats (*$p<0.05$; ANOVA). Mean IL-1β concentration was significantly decreased in hippocampal tissue prepared from aged rats which received IL-1F5 compared with control-treated aged rats ($^{+}p<0.05$; ANOVA). Values are expressed as pg IL-1β/mg protein and are means ± SEM of 6 values in all groups.

2-way ANOVA: Age$_{effect}$ F (1, 20) = 7.68; p=0.0118, IL-1F5$_{effect}$ F (1, 20) = 12.59; p=0.0020, Interaction$_{effect}$ F (1, 20) = 0.31; p=0.5812

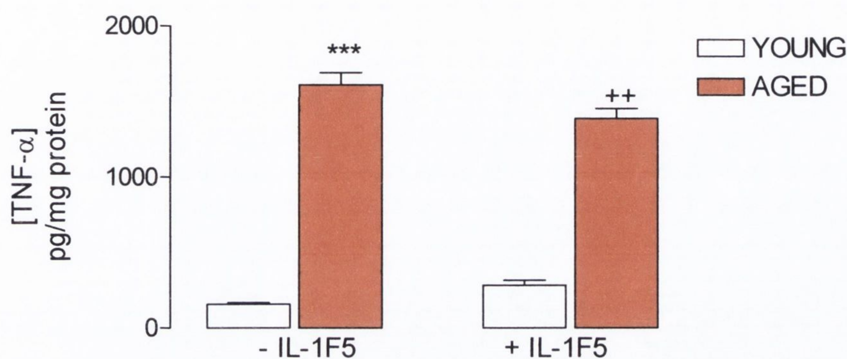


Figure 5.20. IL-1F5 attenuates the age-associated increase in hippocampal TNF-α

Young (2-3 months) and aged (22-24 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and TNF-α concentration determined by ELISA. Mean TNF-α concentration was significantly increased in the hippocampus of aged rats compared with young rats (***$p<0.001$; ANOVA). Mean TNF-α concentration was significantly decreased in hippocampal tissue prepared from aged rats which received IL-1F5 compared with control-treated aged rats ($^{++}p<0.01$; ANOVA). Values are expressed as pg TNF-α/mg protein and are means ± SEM of 6 values in all groups.

2-way ANOVA: Age $_{effect}$ F (1, 18) = 641.46; p<0.0001, IL-1F5 $_{effect}$ F (1, 18) = 1.01; p=0.3281, Interaction $_{effect}$ F (1, 18) = 12.08; p=0.0027

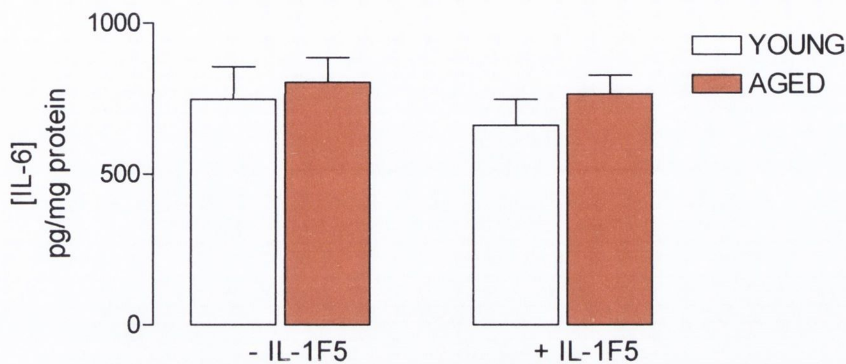


Figure 5.21. Effect of age and IL-1F5 on hippocampal IL-6 concentration

Young (2-3 months) and aged (22-24 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-6 concentration determined by ELISA. Mean IL-6 concentration was similar in the hippocampus of aged rats compared with young rats. Pre-treatment with IL-1F5 had no effect on IL-6 concentration in hippocampal homogenates obtained from young or aged rats. Values are expressed as pg IL-6/mg protein and are means ± SEM of 6 values in all groups.

2-way ANOVA: Age $_{effect}$ F (1, 18) = 0.82; p=0.3774, IL-1F5 $_{effect}$ F (1, 18) = 0.47; p=0.4999, Interaction $_{effect}$ F (1, 18) = 0.07; p=0.7978

5.4. Discussion

It is well established that glial cells are the primary source of pro-inflammatory cytokines in the CNS (Szczepanik *et al.*, 2001) and in particular are the primary source of IL-1β (Van Dam *et al.*, 1995). It is also well known that anti-inflammatory cytokines can modulate the production of these pro-inflammatory cytokines (Lyons *et al.*, 2007). IL-1ra is an anti-inflammatory cytokine and also an endogenous antagonist of IL-1β signalling. The similarity between IL-1F5 with IL-1ra has led to the idea that IL-1F5 might have the capacity to act like IL-1ra, as an antagonist of the IL-1 super-family. In support of this, recent evidence has revealed that IL-1F5 antagonised the production of NFκB by IL-1F9 in Jurkat cells (Debets *et al.*, 2001) and results from our previous studies have shown that IL-1F5 restores the IL-1β- and LPS-induced deficit in LTP and attenuates IL-1β- and LPS-induced pro-inflammatory cytokine production *in vitro* and *in vivo* (Costelloe *et al.*, 2008). The main objectives of this study were to establish whether IL-1F5 might antagonise LPS-induced pro-inflammatory cytokine production from mixed glial cells and to identify the cellular target for these effects.

The evidence presented here demonstrates for the first time that IL-1F5 antagonises LPS-induced IL-1β, IL-6 and TNF-α concentration in mixed glial cells. The data show that LPS induced a robust increase in IL-1β concentration in mixed glial cells similar to previous reports (Giulian *et al.*, 1986). Pre-treatment with IL-1F5 markedly attenuated this LPS-induced effect. There is a plethora of evidence to suggest that, in parallel with an increase in IL-1β concentration from mixed glial cells in response to LPS, there is an increase in IL-6 and TNF-α concentration (Frei *et al.*, 1989; Suzumura *et al.*, 1996; Sherwin and Fern, 2005; Suzumura *et al.*, 2006); the findings from the present study support these data. The data show a concomitant increase in the concentration of the pro-inflammatory cytokines IL-6 and TNF-α from mixed glial cells treated with LPS. Importantly, the results from this study also reveal that pre-treatment with IL-1F5 antagonised this LPS-induced effect, however not to the same extent as IL-1β. These results are in contrast to a study by Barton and colleagues (2000), which showed that IL-1F5 was unable to decrease IL-1β-induced IL-6 production from fibroblasts and endothelial cells. Likewise, in previous

investigations we observed that IL-1F5 decreases LPS-induced IL-1β in mixed glial cells but exhibits no anti-inflammatory effects on LPS-induced IL-1β in macrophages and DCs (Costelloe *et al.*, 2008).

This suggests that the anti-inflammatory effects of IL-1F5 are only evident in certain cell types and it was therefore important to identify the cellular target of actions of IL-1F5 in the CNS. To this end, isolated astrocytes and microglia were prepared and incubated with LPS in the presence of IL-1F5 and analysed for cytokine concentrations. The data show that, similar to the effects of LPS on mixed glial cells found in this study and elsewhere, LPS induced a strong increase in the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α from both astrocytes and microglial cells. This finding supports previous reports that have demonstrated that both microglia and astrocytes are capable of producing pro-inflammatory cytokines (Acarin *et al.*, 2000). The results from this study also revealed, that the capacity of IL-1F5 to attenuate LPS-induced pro-inflammatory cytokines in mixed glial cells were mirrored in astrocytes. IL-1F5 reduced the production of IL-1β, IL-6 and TNF-α concentration from LPS-treated astroctyes to the same extent as in mixed glial cells.

However, the anti-inflammatory effects of IL-1F5 on LPS-induced pro-inflammatory cytokines were abolished in isolated microglial cells. LPS induced an increase in the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. However, surprisingly, treatment of isolated microglial cells with IL-1F5 alone produced a marked increase in IL-1β, IL-6 and TNF-α concentration. The reasons for this robust effect of IL-1F5 on pro-inflammatory cytokines are unclear, other studies are in sharp contrast and have shown that IL-1F5 alone is incapable of inducing IL-1-like signalling, IL-1F5 failed to induce IL-6 production from fibroblasts and endothelial cells and IFN-γ from myelomonocytes (Barton *et al.*, 2000). Similarly, in two separate studies IL-1F5 did not activate NFκB in Jurkat T cells (Debets *et al.*, 2001; Towne *et al.*, 2004). Some studies suggest that the microglial isolation process itself changes the morphology and functionality of these cells and perhaps this is a reason for the heightened inflammation, however this does not account for the heightened inflammatory effects induced by IL-1F5 which was not observed before. Another

possibility is that IL-1F5 is exerting pro-inflammatory effects at another as yet uncharacterised receptor and this merits further investigations.

Nevertheless, a certainty from this study is that the anti-inflammatory effects of IL-1F5 are targeted and confined to astrocytes but not effectual in microglial cells. The conclusion from these findings is that IL-1F5 has a completely different mode of action in astrocytes and mixed glial cells which comprises about 75% astrocytes, compared with microglial cells. Since IL-1F5 is a member of the IL-1 family, it must therefore bind to a receptor to induce its response (Dinarello, 1998). However, the receptor for IL-1F5 has not yet been identified; studies have shown that IL-1F5 is unable to bind to the classical IL-1 receptors IL-1RI, AcP, IL-18R and AcPL (Born *et al.*, 2000; Smith *et al.*, 2000; Dunn *et al.*, 2001). Similarly evidence from sequence and structural analysis suggests that IL-1F5 is more similar to IL-1β in the loop regions and might therefore convey agonistic properties (Dunn *et al.*, 2003). Taken together these studies formed the basis of the rationale that IL-1F5 might be a ligand for an otherwise orphan receptor. It was considered that due to its known anti-inflammatory properties, SIGIRR might be a possible receptor for IL-1F5, leading to an anti-inflammatory signal which could indirectly attenuate pro-inflammatory signalling. In support of this, results from our previous studies have shown that the anti-inflammatory effects of IL-1F5 on LPS-induced signalling are eliminated in the presence of an antibody to SIGIRR and in SIGIRR$^{-/-}$ mice suggesting that the anti-inflammatory role of IL-1F5 is mediated through its interaction with SIGIRR.

The results from this study showed the expression of SIGIRR on astrocytes, but not on microglial cells. This proves that the differential effects of IL-1F5 in astrocytes and microglia were due to the presence and absence of SIGIRR. In support of this, studies have shown that SIGIRR is not expressed on monocytes and macrophages (Wald *et al.*, 2003; Garlanda *et al.*, 2004) and results from our previous studies have demonstrated that IL-1F5 fails to attenuate LPS-induced IL-1β in these cells (Costelloe *et al.*, 2008). The findings presented here further back up the hypothesis that IL-1F5 requires the receptor SIGIRR to induce its anti-inflammatory actions.

To assess whether IL-1F5 exerted a similar an anti-inflammatory role *in vivo*, the modulatory effects of IL-1F5 were examined in hippocampal tissue prepared from LPS-, Aβ-treated and aged rats. The data show that the potent inflammatory effects of LPS *in vitro* were mimicked in rat hippocampus. Systemic administration of LPS has been shown to inhibit LTP in perforant path granule cell synapses (Kelly *et al.*, 2003; Barry *et al.*, 2005) and stimulate immune activation, inflammation and deterioration in cell function (Kelly *et al.*, 2003). One of the main ways by which LPS induces these inflammatory pathways and detrimental effects is through activation of the pro-inflammatory cytokine IL-1β (Vereker *et al.*, 2000a). Increased concentration of IL-1β, has been reported in the aged brain (Griffin *et al.*, 2006) in response to γ-irradiation (Lynch *et al.*, 2003) and in response to stress (Vereker *et al.*, 2001). The results from this study revealed that peripheral administration of LPS induced a robust increase in IL-1β protein expression in the rat hippocampus in response to LPS, confirming earlier similar findings (Vereker *et al.*, 2000a; Kelly *et al.*, 2003). This LPS-induced increase in IL-1β was attenuated in hippocampal tissue prepared from LPS-treated rats which received IL-1F5.

In parallel with an increase in IL-1β concentration, the data indicate that LPS increases IL-6 and TNF-α activation in the rat hippocampus. This finding is comparable with other studies which show that the increase in IL-1β concentration in inflammatory conditions is often paralleled by increases in other pro-inflammatory cytokines (Waage *et al.*, 1989; Szczepanik *et al.*, 2001). For example traumatic brain injury is associated with sustained production and release of the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α leading an increase in the extracellular and cerebrospinal fluid (CSF) concentrations (Zhu *et al.*, 2004). Similarly concentrations of IL-1β and IL-6 concentrations were higher in the brain of aged mice after peripheral injection of LPS (Godbout *et al.*, 2005), and IL-6 is especially implicated in CNS immune diseases (Zhao and Schwartz, 1998). The data from this study reveal that IL-1F5 reversed the LPS-induced increase in IL-6 and TNF-α concentration in the rat hippocampus.

The diverse and detrimental effects exerted by IL-1β in the CNS have been attributed to its interaction with its receptor (O'Neill and Greene, 1998). Binding

leads to the formation of signalling complexes and ultimately the activation of the stress-activated protein kinase, JNK and p38 mitogen-activated protein kinase (Loscher *et al.*, 2000; Vereker *et al.*, 2000b; Lynch *et al.*, 2003; Jeong *et al.*, 2005). Associated with increased JNK phosphorylation is compromised glutamate release (Vereker *et al.*, 2000b), impaired LTP (Vereker *et al.*, 2000b; Martin *et al.*, 2002) and JNK activation has also been shown to trigger cell death cascades in neurons (Maroney *et al.*, 1998). Similarly there is a growing body of evidence implicating IL-6 in JNK activation (Jeong *et al.*, 2005). The data from the present study indicate that LPS induced a significant increase in JNK phosphorylation in the hippocampus and that IL-1F5 treatment significantly reversed this LPS-induced increase in JNK phosphorylation; these results support previous evidence which show that IL-4 another anti-inflammatory cytokine attenuated the LPS-induced increase in JNK phosphorylation in the hippocampus (Barry *et al.*, 2005). The ability of IL-1F5 to inhibit JNK phosphorylation is an important finding as inhibition of JNK activation was shown to prevent apoptosis in the hippocampus (Minogue *et al.*, 2003) and the inhibitory effects of LPS on LTP were blocked using a JNK inhibitor (Barry *et al.*, 2005).

JNK phosphorylation is induced in response to inflammatory challenge and is increased in the aged brain; this has been coupled with increased activation of another stress-activated protein kinase, p38 (Martin *et al.*, 2002). Activation of both of which are linked with apoptotic cell death (Xia *et al.*, 1995). Significantly, p38 activation has been linked with apoptotic changes in Alzheimer's Disease (Zhu *et al.*, 2000). Consistent with these reports, data from this study revealed that the LPS-induced increase in JNK phosphorylation was associated with a parallel increase in p38 phosphorylation in the rat hippocampus. Treatment with IL-1F5 abrogated this LPS-induced increase in p38 activation. Experiments carried out using inhibitors of p38, have revealed that blocking p38 reversed IL-1β-induced cytochrome c translocation and caspase-3 activation (Martin *et al.*, 2002). Since these are key initiators in apoptosis, this suggests a role for IL-1F5 in dampening down signalling cascades which may lead to cell death. It would be interesting to consider if IL-1F5 exerts any other effects downstream of both JNK and p38 such as blocking activation of the

transcription factor NFκB as studies have shown that inhibiting NFκB reversed the LPS-induced attenuation in glutamate release (Kelly *et al.*, 2003); this remains to be investigated.

Coupled with the inhibitory role of IL-1F5 on LPS-induced inflammatory changes is an analogous role for IL-1F5 on Aβ-induced changes. Treatment with Aβ initiates an inflammatory cascade in the CNS, including activation of microglial cells and release of free radicals (Tan *et al.*, 1999; Akiyama *et al.*, 2000; Wang *et al.*, 2004). Treatment with Aβ has also been shown to increase hippocampal IL-1β concentrations and to trigger JNK activation from cortical neurons (Morishima *et al.*, 2001; Minogue *et al.*, 2003). Similarly studies have shown that microglial cultures treated with Aβ produce IL-1β, IL-6 and TNF-α concentrations (Seabrook *et al.*, 2006). The evidence presented here concur with these past studies and indicate that Aβ stimulation induced a significant increase in IL-1β, IL-6 and TNF-α concentrations in hippocampal tissue. Significantly the data also show, for the first time, a modulatory role for IL-1F5 on Aβ-induced IL-1β, IL-6 and TNF-α concentration. Pre-treatment with IL-1F5 dramatically reduced the hippocampal concentrations of these pro-inflammatory cytokines. Curiously, injection of IL-1F5 alone induced an increase in the concentration of pro-inflammatory cytokines; this was an unexpected result and did not consistently occur, as evidenced by the presented results in Chapter 4. A possible explanation for this could be the fact that sometimes with an anti-inflammatory agent there needs to be an inflammatory insult in order for them to be protective. Here, IL-1F5 induced an increase in IL-1β, IL-6 and TNF-α concentration but when combined with Aβ, IL-1F5 had the capacity to reduce the production of these cytokines.

In this study there was also an age-related increase in IL-1β and TNF-α concentrations, this is in harmony with other studies which have shown that one of the manifestations associated with ageing is an increase in IL-1β and TNF-α concentrations in the brain (Macdonald *et al.*, 2000; Maher *et al.*, 2005; Nolan *et al.*, 2005). However surprisingly there was no age-associated increase in IL-6 concentration, which is inconsistent to results from previous studies that showed an age-related increase in IL-6 concentration in mouse brain (Ye and Johnson, 2001),

but consistent with findings from several investigators in this lab who have consistently failed to show an age-related increase in hippocampus of aged rats. These differences between these data and the data reported by Johnson's group may arise from analysis in whole brain (mouse) compared with hippocampus (rat) emphasising region specific changes which have been identified previously. Importantly, the results from this study show that IL-1F5 treatment attenuated this age-related increase in IL-1β and TNF-α concentration. The results presented here implicate IL-1F5 as a powerful anti-inflammatory and neuromodulatory agent.

Chapter 6

An investigation into the anti-inflammatory effects induced by IL-1F5; interaction with SIGIRR

6.1. Introduction

Microglial cells are the primary source of pro-inflammatory cytokines in the CNS (Hanisch, 2002). Although the results presented in Chapter 5. demonstrated that IL-1F5 could not exert its anti-inflammatory effects on LPS-induced pro-inflammatory signalling in isolated microglia cells, one of the aims of this study was to determine if IL-1F5 still had the capacity to modulate microglial activation, perhaps in an indirect manner.

The findings presented in Chapter 5. that IL-1F5 has the capacity to attenuate LPS-induced pro-inflammatory cytokine signalling in mixed glial cells and astrocytes and the findings that IL-1F5 antagonises LPS- and Aβ-induced and age-related inflammatory changes in the rat hippocampus, strongly suggests an antagonistic role for IL-1F5 in the CNS. The mechanisms by which IL-1F5 exerts its anti-inflammatory effects and the receptor upon which it acts are unknown; some studies have suggested that IL-1F5 antagonises IL-1F6, IL-1F8 and IL-1F9 signalling at IL-1Rrp2 (Debets *et al.*, 2001; Blumberg *et al.*, 2007). However, studies have shown that IL-1F5 is unable to bind to IL-1Rrp2 or other classical IL-1 receptors IL-1RI, AcP, IL-18R and AcPL (Born *et al.*, 2000; Smith *et al.*, 2000; Dunn *et al.*, 2003). Assuming that IL-1F5 is acting as a receptor antagonist for another unknown receptor then it can be inferred that IL-1F5 itself should only dampen down inflammatory responses and not provoke a biological response in its own right.

However results from out previous study showed that IL-1F5 induced an increase in IL-4 concentration in mixed glial cells and in rat hippocampus, suggesting that IL-1F5 is mediating its anti-inflammatory effects at an unknown receptor. In support of this idea, although IL-1F5 and IL-1ra share high amino acid sequence homology (Mulero *et al.*, 1999; Barton *et al.*, 2000; Smith *et al.*, 2000), IL-1F5 was found to be more similar to IL-1β in its loop region which confers agonistic properties (Dunn *et al.*, 2003). Moreover, results from our previous study demonstrated a role for the orphan receptor SIGIRR in IL-1F5 signalling (Costelloe *et al.*, 2008). The aims of this study were to establish if IL-1F5 has the capacity to iniate an anti-inflammatory signalling pathway in the brain, focusing in particular on the ability of IL-1F5 to up-regulate anti-inflammatory cytokines and to determine if

the anti-inflammatory effects induced by IL-1F5 are dependent on its interraction with SIGIRR.

6.2. Methods

Groups of young male Wistar rats were injected with sterile saline or IL-1F5 (5µl; 30ng/ml; diluted in sterile saline) 3 hours post-injections, rats were killed by cervical dislocation and tissue taken for analysis (see section 2.4 for specific details). Analysis of hippocampal tissue for microglial markers and cytokine mRNA expression was assessed by PCR, cytokine concentrations were assessed by ELISA and MAPKs by western immunoblotting (see sections 2.6. 2.8 and 2.9 for specific details).

Mixed glia cells, prepared from one-day old wildtype and SIGIRR$^{-/-}$ mice were pre-treated with IL-1F5 (3µg/ml) for 2 hours, following this they were treated with LPS (100ng/ml) for 24 hours. Supernatants were removed and analysed for cytokine concentrations by ELISA (see section 2.8 for specific details).

In another experiment, mixed glial cells prepared from one-day old male Wistar rats were treated with IL-1F5 (3µg/ml), supernatants were removed and cells were fixed in ethanol (70%) and fluorescently immunostained for SIGIRR and IL-1F5 (see section 2.10 for specific details). The binding of IL-1F5 with SIGIRR was determined by ELISA, briefly plates were incubated with or without a SIGIRR antibody (1µg/ml) and with increasing concentrations of IL-1F5 (3µg/ml, 10µg/ml, 30µg/ml, 100µg/ml, 300µg/ml) and binding was determined by the absorbance read at 450nm using a 96-well plate reader (see section 2.12 for specific details). Data are expressed as means ± standard error of the mean. A Student's t-test for unpaired means or a two-way ANOVA was performed to determine whether significant differences existed between conditions. Post hoc Student Newmann-Keuls test was used to determine where the significance lied (see section 2.13 for specific details).

6.3. Results

IL-1F5 attenuates CD40, CD11b, and MHC II mRNA expression in rat hippocampus

The results from the previous chapters show that IL-1F5 is unable to dampen down pro-inflammatory cytokine activation in isolated microglial cells, probably due to the absence of SIGIRR expression on these cells. However, in order to determine if IL-1F5 is still capable of modulating microglial activation in vivo, perhaps indirectly, CD40, CD11b and MHC II mRNA expression was examined in snap frozen hippocampal tissue obtained from control-treated and IL-1F5-treated rats. Figure 6.1. shows that CD40 mRNA expression was significantly decreased in snap frozen hippocampal tissue prepared from IL-1F5-treated rats (0.45 ± 0.08 RQ; n=6) compared with snap frozen hippocampal tissue obtained from saline-treated control rats (1.15 ± 0.16 RQ; n=6; **p<0.01; Student's *t* test). In parallel, CD11b mRNA expression was significantly decreased in snap frozen hippocampal tissue prepared from IL-1F5-treated rats (0.53 ± 0.12 RQ; n=6) compared with control-treated rats (1.57 ± 0.16 RQ; n=6; ***p<0.001; Student's *t* test). Moreover a similar decrease in MHC II mRNA expression was observed in tissue prepared from IL-1F5-treated rats (0.63 ± 0.05 RQ; n=6) compared with snap frozen hippocampal tissue obtained from saline-treated control rats (0.92 ± 0.03 RQ; n=6; ***p<0.001; Student's *t* test).

Does IL-1F5 decrease microglial activation by modulating CD200

CD200 is an important glycoprotein expressed on neurons which is capable of maintaining microglia in a quiescent state, Lyons and colleagues (2007) showed that CD200 was upregulated by IL-4. In order to assess whether IL-1F5, through it's induction of IL-4, was capable of inducing CD200, snap frozen hippocampal tissue obtained from IL-1F5 treated rats was analysed for CD200 mRNA and protein. Figure 6.2. illustrates that CD200 mRNA was decreased in hippocampal tissue prepared from IL-1F5-treated rats (0.70 ± 0.03 RQ; n=6) compared with controls (0.93 ± 0.07 RQ; n=6; *p<0.05; ANOVA).

Figure 6.3. (A) shows two sample immunoblots of CD200 and β-actin in control- and IL-1F5-treated hippocampal homogenates. Figure 6.3. (B) illustrates that

CD200 protein expression was similar in hippocampal tissue prepared from control-treated rats (0.13 ± 0.02 arbitrary units; n=6) and IL-1F5-treated rats (0.12 ± 0.01 arbitrary units; n=6).

Does IL-1F5 modulate microglial activation through induction of IL-1ra

Because IL-1F5 possesses anti-inflammatory properties and like IL-1ra can attenuates IL-1β-induced changes (Arend *et al*., 1998). It was considered that IL-1F5 might exert its effects by increasing IL-1ra. To determine if IL-1F5 was capable of inducing IL-1ra, the expression of IL-1ra mRNA was determined in snap frozen hippocampal tissue prepared from IL-1F5-treated rats. Figure 6.4. shows that IL-1ra mRNA was similar in hippocampal tissue from IL-1F5-treated rats (2.74 ± 1.05 RQ; n=6) compared with controls (3.55 ± 1.23 RQ; n=6).

Does IL-1F5 affect microglial activation through induction of IL-10

IL-10 is a potent anti-inflammatory cytokine induced by Th2 cells and is known to attenuate the production of pro-inflammatory cytokines. In order to assess whether the anti-inflammatory actions of IL-1F5 are mediated through IL-10 induction, its concentration was assessed in supernatants prepared from IL-1F5-treated cells and in hippocampal homogenates from IL-1F5-treated rats. Figure 6.5. illustrates that IL-10 concentration was similar in supernatants prepared from control-treated cells (274.4 pg/ml ± 6.76; n=6) and IL-1F5-treated cells (265 pg/ml ± 5.25; n=6). Similarly IL-10 concentration was similar in hippocampal homogenates prepared from control-treated rats (80.49 pg/mg ± 6.54; n=6) and IL-1F5-treated rats (75.02 pg/mg ± 2.34; n=6).

Does IL-1F5 modulate microglial activation through induction of IL-4

Previous studies have revealed that IL-4 can block the LPS-induced increase in IL-1β mRNA and protein (Nolan *et al*., 2005). Since IL-1F5 attenuated the LPS-induced increase in IL-1β concentration *in vivo* and *in vitro*, it was considered that IL-1F5 may exert its action by inducing an increase in IL-4. The effects of IL-1F5 administration on IL-4 mRNA expression was assessed in tissue from rats which

received IL-1F5. Figure 6.6. (A) shows two sample blots illustrating expression of IL-4 mRNA and the control gene 18S in tissue prepared from control- and IL-1F5-treated rats. Figure 6.6. (B) represents the mean data obtained from densitometric analysis showing a significant IL-1F5-induced increase in IL-4 mRNA expression (0.23 ± 0.06 arbitrary units; n=6) versus (0.07 ± 0.02 arbitrary units; n=6; *p<0.05; Student's *t* test) in controls. Figure 6.6. (C) shows data confirming the IL-1F5-induced increase in IL-4 mRNA by QPCR analysis (1.75 ± 0.54 RQ; n=6) versus (0.42 ± 0.12 RQ; n=6; *p<0.05; Student's *t* test) in controls.

IL-1F5 administration increased activation of ERK1/2 and STAT 6 in the hippocampus

IL-4 mediates its protective effects by binding to, and signalling through the IL-4 receptor. Among the downstream effects of IL-4 binding are increases in ERK phosphorylation (Nelms *et al.*, 1999) and in STAT dimerization and translocation to the nucleus. It was therefore considered that IL-1F5 may exert its action by inducing an increase in downstream IL-4 signalling. ERK activation was assessed in hippocampal lysates obtained from rats which were administered IL-1F5, using antibodies which specifically identifies the phosphorylated and total forms of ERK 1 (p44 subunit) and ERK 2 (p42 subunit) and activation is expressed as a ratio of phosphorylated ERK1/2 (p-ERK1/2) to t-ERK. STAT6 activation was assessed using an antibody which specifically identifies the phosphorylated form of STAT6 and activation was expressed as a ratio of phosphorylated STAT6 (p-STAT6) to β-actin.

Figure 6.7. (A) shows three sample immunoblots illustrating p-ERK 1, p-ERK 2 and t-ERK expressed in control and IL-1F5-treated rats. Figure 6.7. (B) represents the mean data obtained from densitometric analysis showing a significant increase in ERK 1 phosphorylation in hippocampal lysates prepared from IL-1F5-treated rats (1.03 ± 0.17 arbitrary units; n=6), compared with saline-treated controls (0.47 ± 0.12 arbitrary units; n=6; *p<0.05; Student's *t* test for independent means) and a significant increase in ERK 2 phosphorylation in hippocampal lysates prepared from IL-1F5-treated rats (0.86 ± 0.12 arbitrary units; n=6) compared with saline-treated controls (0.50 ± 0.08 arbitrary units; n=6; *p<0.05; Student's *t* test for independent means).

Figure 6.8. (A) shows two sample immunoblots illustrating p-STAT6 and β-actin expressed in control and IL-1F5-treated rats. Figure 6.8. (B) represents the mean data obtained from densitometric analysis showing a significant increase in p-STAT6 activity in hippocampal lysates prepared from IL-1F5-treated rats (0.95 ± 0.08 arbitrary units; n=6), compared with saline-treated controls (0.54 ± 0.09 arbitrary units; n=6; **p<0.01; Student's *t* test for independent means).

IL-1F5 attenuates IL-1β concentration in wildtype but not SIGIRR$^{-/-}$ mice

An established receptor for IL-1F5 has not yet been identified, however previous studies have suggested that the orphan receptor SIGIRR might mediate IL-1F5 signalling since the anti-inflammatory actions of IL-1F5 on LPS-induced signalling were absent in mixed glial cells prepared from SIGIRR$^{-/-}$ mice (Costelloe *et al.*, 2008).

To determine if IL-1F5 can induce its anti-inflammatory actions in mixed glial cells in the absence of SIGIRR, the concentration of the pro-inflammatory cytokine IL-1β was assessed in mixed glial cells prepared from LPS- and IL-1F5-treated wildtype and SIGIRR$^{-/-}$ mice. Figure 6.9. shows that LPS induced a significant increase in IL-1β concentration in mixed glial cells prepared from wildtype (19.63 pg/ml ± 5.16; n=6 versus 3.48 pg/ml ± 0.52; n=6; ***p<0.001; ANOVA; controls) and SIGIRR$^{-/-}$ mice (17.64 pg/ml ± 5.46; n=6 versus 7.85 pg/ml ± 1.45; n=6; *p<0.05, ANOVA; controls). Incubation of cells prepared from wildtype mice in the presence of LPS and IL-1F5 significantly decreased the LPS-induced change (5.15 pg/ml ± 1.37; n=6; $^{++}$p<0.01; ANOVA versus 19.63 pg/ml ± 6.16; n=6) but IL-1F5 failed to affect the LPS-induced increase in cells prepared from SIGIRR$^{-/-}$ mice (22.32 pg/ml ± 4.55; n=6 versus 17.64 pg/ml ± 5.46; n=6; LPS alone).

IL-1F5 induces IL-4 concentration in wildtype but not SIGIRR$^{-/-}$ mice

The findings from this study suggest that IL-1F5 mediates its anti-inflammatory actions through recruitment of intracellular signalling molecules resulting in the induction of IL-4. In order to determine if the anti-inflammatory effects induced by IL-1F5 are dependent on its interaction with SIGIRR, the

concentration of IL-4 was assessed in supernatants of mixed glial cells prepared from IL-1F5-treated wildtype and SIGIRR$^{-/-}$ mice. Figure 6.10. shows that incubation of cells prepared from wildtype mice in the presence of IL-1F5 significantly increased IL-4 concentration (30.46 pg/ml ± 6.38; n=6 versus 109.6 pg/ml ± 34.23; n=6; **$p<0.01$; ANOVA; in the absence and presence of IL-1F5). In contrast, IL-1F5 exerted no significant difference on IL-4 concentration in cells prepared from SIGIRR$^{-/-}$ mice (29.45 pg/ml ± 6.39; n=6; ANOVA versus 44.59 pg/ml ± 14.81; n=6; ANOVA; in the absence and presence of IL-1F5).

Co-localisation of IL-1F5 with SIGIRR on mixed glial cells

The findings from this study and our previous studies (Costelloe *et al.*, 2008), suggest a role for SIGIRR as a receptor for IL-1F5. In an attempt to assess this further, mixed glial cells were treated with IL-1F5 (3μg/ml), fixed in alcohol and double-immunostained for IL-1F5 and SIGIRR. The micrographs in Figure 6.11. are representative of 4 separate experiments and show double-immunofluorescent staining with anti-SIGIRR and anti-IL-1F5. Figure 6.11. (A) shows low (40X) and higher (60X) magnification images of SIGIRR staining on mixed glial cells indicated by the green Alexa staining. Figure 6.11. (B) shows low (40X) and higher (60X) magnification images of IL-1F5 staining on mixed glial cells indicated by the red Alexa staining. Figure 6.11. (C) shows an overlay image at low (40X) and higher (60X) magnifications which demonstrates co-localisation of SIGIRR and IL-1F5 indicated by the yellow merged photo.

Figure 6.12. (A) shows a scatter plot depicting the relationship between SIGIRR (green stain) on the x axis and IL-1F5 (red stain) on the y axis. The pixels are pseudocoloured blue and their colour represents the frequency of the green-red pixel overlap in the original image. This reveals very high co-localisation of SIGIRR and IL-1F5 which is also shown in Figure 6.12. (B) where the blue stain is evident on the cell surface.

Binding of IL-1F5 with SIGIRR

The binding of IL-1F5 to SIGIRR was assessed using an ELISA-based assay. Plates were incubated with or without SIGIRR (1μg/ml) and with increasing concentrations of IL-1F5 (3μg/ml, 10μg/ml, 30μg/ml, 50μg/ml, 100μg/ml, 150μg/ml and 300μg/ml). The red curve in Figure 6.13. shows that in the presence of SIGIRR, absorbance increases in a concentration-dependent manner and saturates at higher concentrations of IL-1F5 (100μg/ml, 300μg/ml) indicating specific binding. In the absence of SIGIRR (black curve) the absorbance of IL-1F5 and SIGIRR increases in a non-specific manner and never saturates indicating non-specific binding.

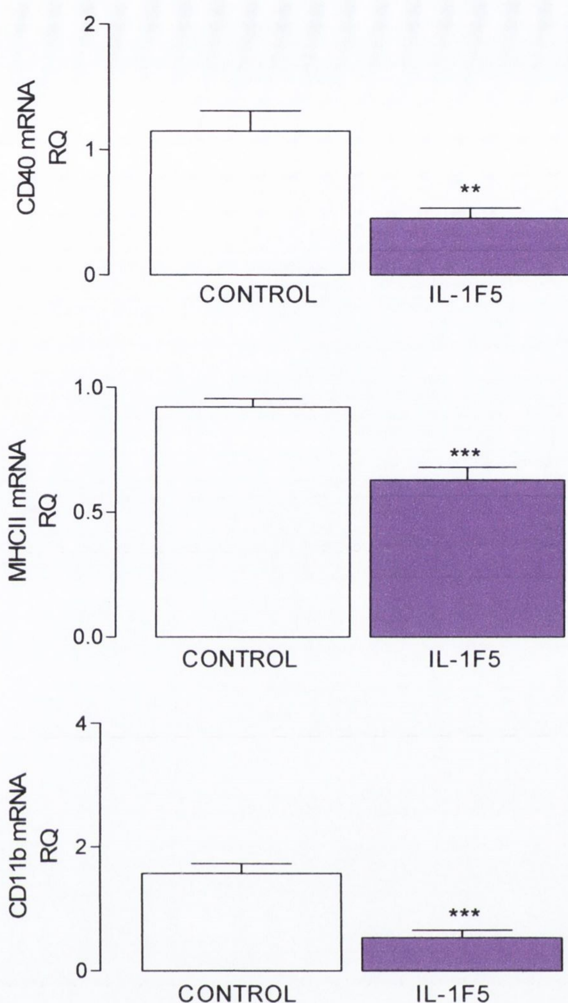


Figure 6.1. IL-1F5 attenuates microglial activation in rat hippocampus

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and CD40, MHC II and CD11b mRNA expression determined by QPCR. CD40, MHC II and CD11b mRNA expression was significantly decreased in tissue prepared from IL-1F5-treated rats compared with control-treated rats (**p<0.01; ***p<0.001; ***p<0.001; Student's t test for independent means). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of CD40, MHC II and CD11b mRNA to an endogenous control and are means ± SEM (n=6 in each group).

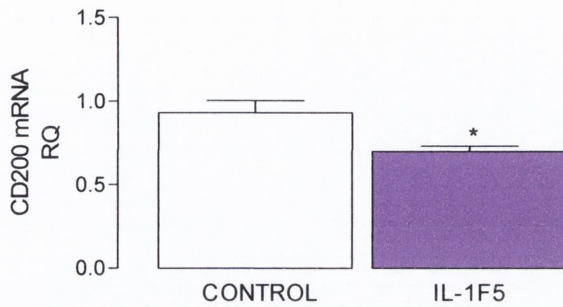


Figure 6.2. IL-1F5 decreases CD200 mRNA expression in rat hippocampus

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and CD200 mRNA expression determined by QPCR. CD200 mRNA expression was decreased in hippocampal tissue from IL-1F5-treated rats compared with controls (*$p<0.05$; Student's *t* test for independent means). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of CD200 mRNA to an endogenous control and are means ± SEM (n=6 in each group).

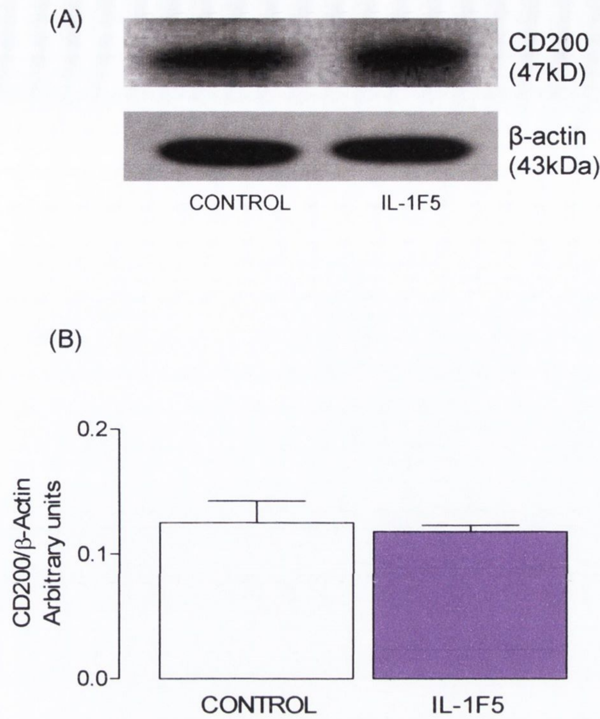


Figure 6.3. IL-1F5 did not increase CD200 expression in rat hippocampus

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and CD200 phosphorylation determined by Western immunoblotting (expressed as a ratio of phosphorylated CD200 to β-actin in arbitrary units). (A) Sample immunoblots indicating density of phosphorylated CD200 (p-CD200) and β-actin in tissue prepared from control- and IL-1F5-treated rats. (B) CD200 expression was similar in hippocampal homogenates obtained from IL-1F5-treated rats compared with control-treated rats. Values are expressed in arbitrary units obtained from calculating the ratio of CD200 to β-actin provided by densitometric analysis and are means ± SEM (n=6 values in all groups).

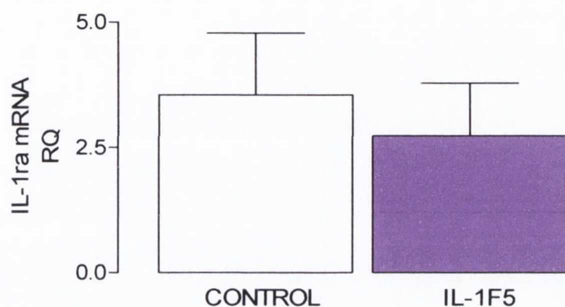


Figure 6.4. IL-1F5 did not increase IL-1ra mRNA expression in rat hippocampus

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-1ra mRNA expression determined by QPCR. IL-1ra mRNA was similar in hippocampal tissue prepared from control- and IL-1F5-treated rats. Values are expressed as relative quantities (RQ) obtained from calculating the ratio of IL-1ra mRNA to an endogenous control and are means ± SEM (n=6 in each group).

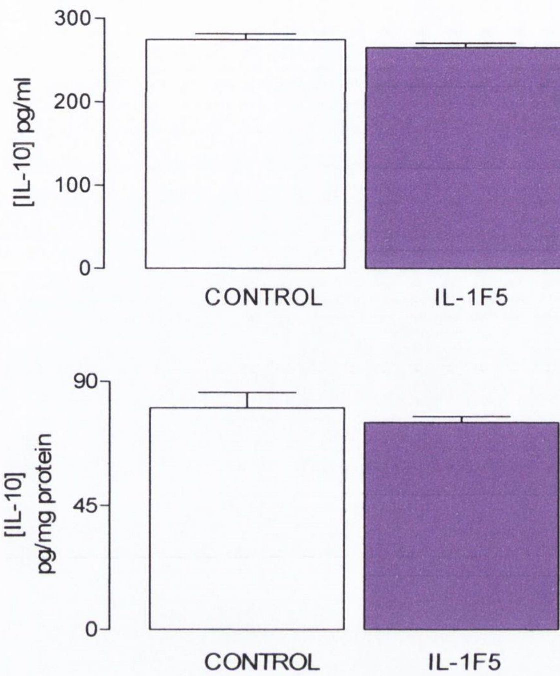


Figure 6.5. IL-1F5 did not increase IL-10 concentration

Rat mixed glial cells were cultured with IL-1F5 (3μg/ml). After 24 hours IL-10 concentrations in supernatants were quantified by ELISA. Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-10 concentration determined by ELISA. Mean IL-10 concentration was similar in supernatants obtained from mixed glial cells incubated in the presence and absence of IL-1F5. Mean IL-10 concentration was similar in hippocampal homogenates prepared from IL-1F5-treated rats compared with controls. Values are expressed as pg IL-10/ml and pg IL-10mg and are means ± SEM of 6 observations per group.

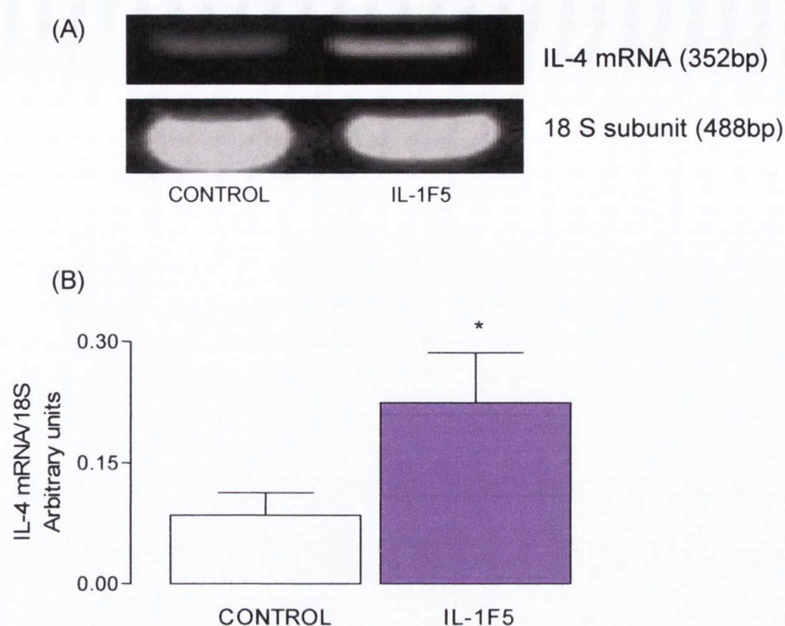


Figure 6.6. IL-1F5 increases IL-4 mRNA expression in rat hippocampus

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5µl) or IL-1F5 (30ng/ml; 5µl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-4 mRNA expression determined by RT-PCR (A) Sample blots indicating expression of IL-4 mRNA and 18S subunit in control-treated and IL-1F5-treated rats. (B) IL-4 mRNA expression was significantly increased in snap frozen hippocampal tissue prepared from IL-1F5-treated rats compared with controls (*$p < 0.05$; *$p < 0.05$; Student's t test for independent means). Values are expressed as arbitrary units obtained from calculating the ratio of IL-4 mRNA to the 18S subunit, data are means ± SEM (n=6 in each group).

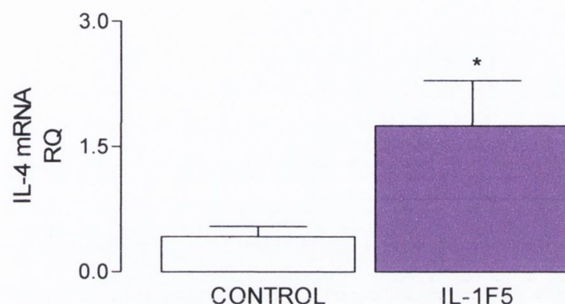


Figure 6.7. IL-1F5 increases IL-4 mRNA expression in rat hippocampus

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-4 mRNA expression determined by Q-PCR. IL-4 mRNA expression was significantly increased in snap frozen hippocampal tissue prepared from IL-1F5-treated rats compared with controls (*$p < 0.05$; *$p < 0.05$; Student's *t* test for independent means). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of IL-4 mRNA to an endogenous control, data are means ± SEM (n=6 in each group).

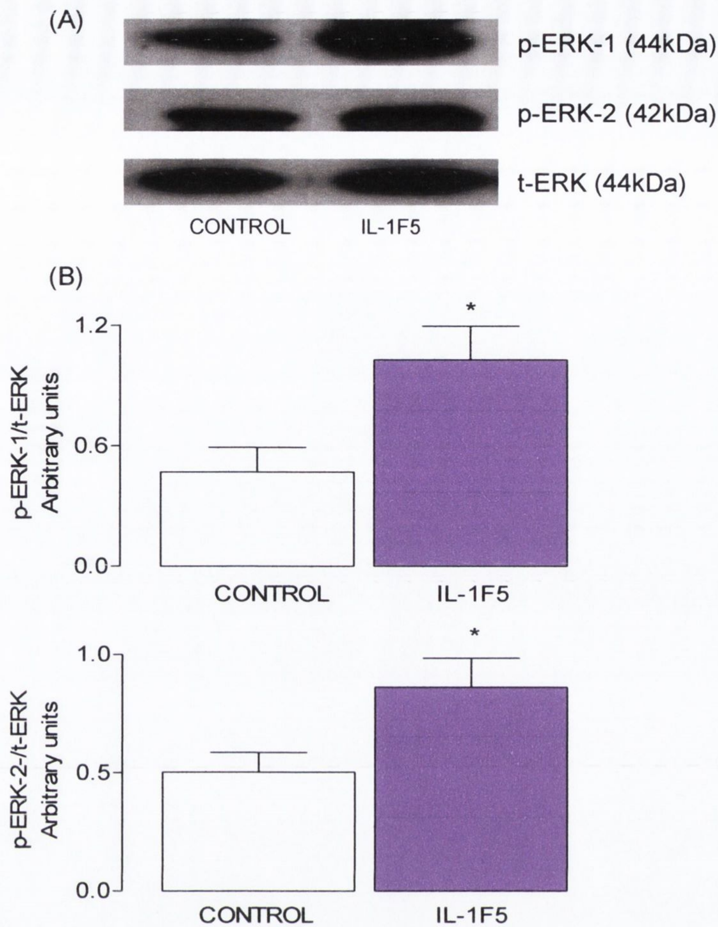


Figure 6.8. IL-1F5 increases ERK activation in rat hippocampus

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and ERK phosphorylation determined by Western immunoblotting (expressed as a ratio of phosphorylated ERK-1 and ERK-2 to total ERK in arbitrary units). (A) Sample immunoblots indicating density of phosphorylated ERK-1 and ERK-2 (p-ERK-1), (p-ERK-2) and total ERK (t-ERK) in tissue prepared from control- and IL-1F5-treated rats. (B) Phosphorylation of ERK 1 and ERK 2 was significantly increased in the hippocampus of IL-1F5-treated rats compared with controls (*$p<0.05$; Student's *t* test for independent means). Values are expressed in arbitrary units obtained from calculating the ratio of p-ERK-1 and p-ERK-2 to t-ERK provided by densitometric analysis and are means ± SEM (n=6 values in all groups).

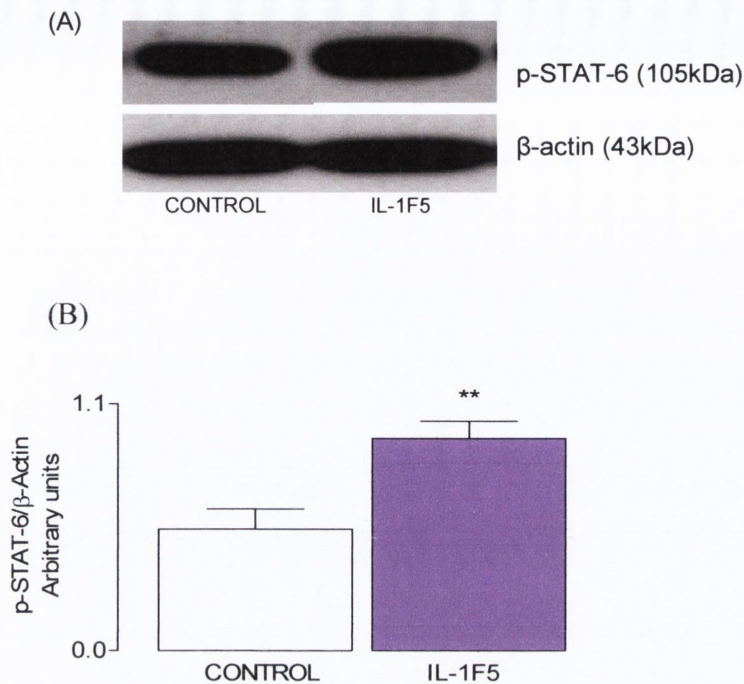


Figure 6.9. IL-1F5 increases STAT-6 activation in rat hippocampus

Young (2-3 months) male Wistar rats were injected i.c.v. with sterile saline (5μl) or IL-1F5 (30ng/ml; 5μl). After 3 hours, rats were killed by decapitation, hippocampi dissected free, homogenized and STAT-6 phosphorylation determined by Western immunoblotting (expressed as a ratio of phosphorylated STAT-6 and β-actin in arbitrary units). (A) Sample immunoblots indicating density of phosphorylated STAT-6 and β-actin in tissue prepared from control- and IL-1F5-treated rats. (B) Phosphorylation of STAT 6 was significantly increased in the hippocampus of IL-1F5-treated rats compared with controls (**p<0.01; Student's t test for independent means). Values are expressed in arbitrary units obtained from calculating the ratio of p-STAT6 to β-actin provided by densitometric analysis and are means ± SEM (n=6 values in all groups).

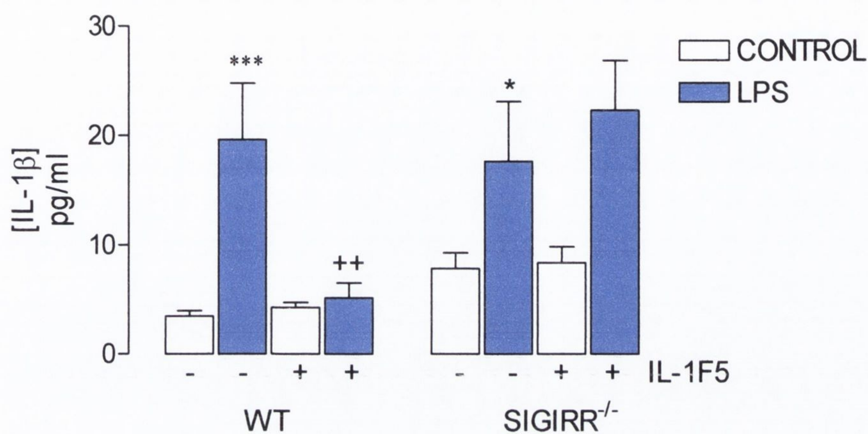


Figure 6.10. IL-1F5 attenuates LPS-induced IL-1β in wildtype but not SIGIRR$^{-/-}$ mice

Mice mixed glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice were cultured with LPS (100ng/ml) following 2 hour pre-treatment with IL-1F5 (3μg/ml). After 24 hours IL-1β concentrations in supernatants were quantified by ELISA. Treatment with LPS induced a significant increase in IL-1β concentration in supernatants of mixed glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice (*$p<0.05$; ***$p<0.001$; ANOVA). Pre-treatment with IL-1F5 significantly attenuated the LPS-induced increase in IL-1β concentration in mixed glia prepared from wildtype ($^{++}p<0.01$; ANOVA) but not SIGIRR$^{-/-}$ mice. Values are expressed as pg IL-1β/ml and are means ± SEM of 6 observations per group.

2-way ANOVA: Treatment $_{effect}$ F (3,124) = 46; p=0.0004, Strain $_{effect}$ F (1,124) = 6.11; p=0.0255, Interaction $_{effect}$ F (3,124) = 2.38; p=0.0732

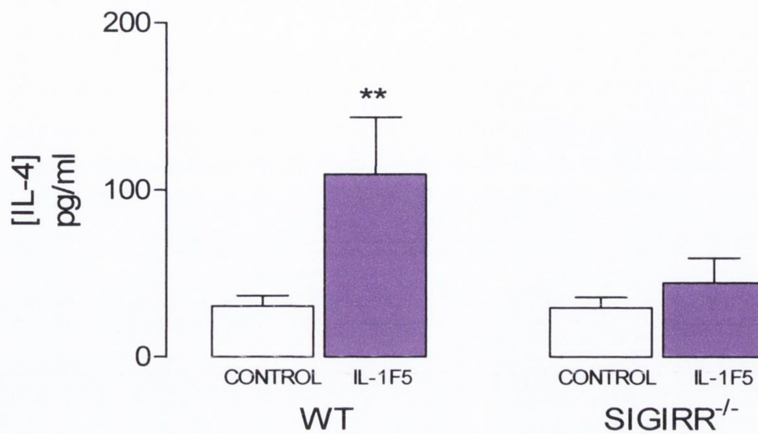


Figure 6.11. IL-1F5 increases IL-4 concentration in wildtype but not SIGIRR$^{-/-}$ mice Mice mixed glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice were cultured with IL-1F5 (3μg/ml) or medium. After 24 hours IL-4 concentrations in supernatants were quantified by ELISA. Incubation of mixed glial cells prepared from wildtype mice with IL-1F5 induced a significant increase in IL-4 concentration (**$p<0.01$; ANOVA). No effect of IL-1F5 was observed in mixed glia prepared from SIGIRR$^{-/-}$ mice. Values are expressed as pg IL-4/ml and are means ± SEM of 6 observations per group.

2-way ANOVA: IL-1F5 $_{effect}$ F (1, 25) = 7.92; p=0.0094, Strain $_{effect}$ F (1, 25) = 3.88; p=0.0600, Interaction $_{effect}$ F (1, 25) = 3.65; p=0.0676

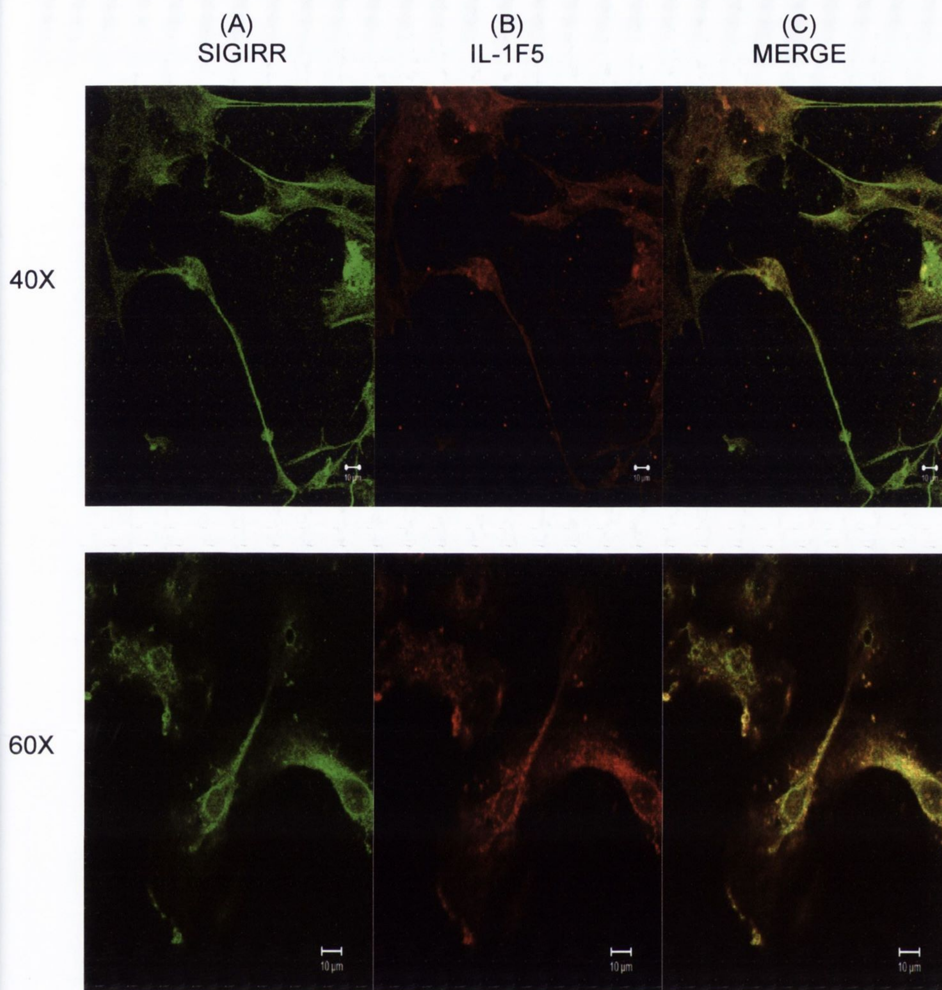


Figure 6.12. Co-localisation of IL-1F5 and SIGIRR in mixed glial cells

Rat mixed glial cells were cultured with IL-1F5 (3µg/ml) or medium. After 24 hours supernatants were removed, cells fixed with ethanol, immunostained with SIGIRR and IL-F5 and visualised by confocal microscopy. (A) SIGIRR immunostaining with an Alexa-labelled antibody (green stain) confirms SIGIRR expression on the cell surface of mixed glial cells. (B) IL-1F5 immunostaining with an Alexa-labelled antibody (red stain) confirms IL-1F5 staining on these cells. (C) A yellow signal is obtained on cells as a result of co-localisation of SIGIRR with IL-1F5. (Magnification 40X; 60X).

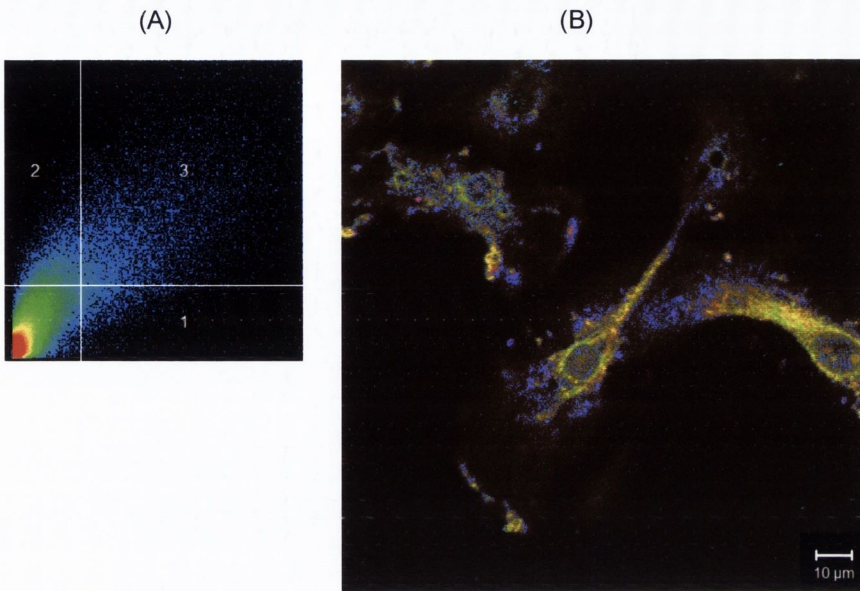


Figure 6.13. Co-localisation of SIGIRR and IL-1F5 in mixed glial cells

Rat mixed glial cells were cultured with IL-1F5 (3µg/ml) or medium. After 24 hours supernatants were removed, cells fixed with ethanol, immunostained with SIGIRR and IL-F5 and visualised by confocal microscopy. (A) A scatter plot illustrating the relationship between SIGIRR (quadrant 1) and IL-1F5 (quadrant 2) shows significant co-localisation between SIGIRR and IL-1F5. The frequency of SIGIRR and IL-1F5 overlap from the micrographs in Figure 6.11. is represented as the blue colour and the shift of blue pixels into quadrant 3 demonstrates co-localisation. (B) The blue stain on the surface of cells denotes co-localisation between SIGIRR and IL-1F5. (Magnification 60X; scale bar 10µm).

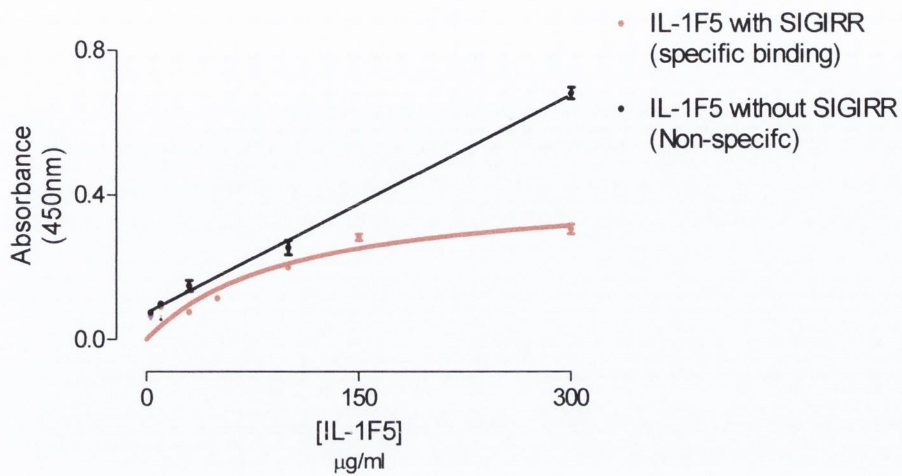


Figure 6.14. IL-1F5 binds to SIGIRR

ELISA plates coated with goat anti-human SIGIRR (1µg/ml) and recombinant human SIGIRR (1µg/ml) were incubated with increasing concentrations (10-300µg/ml) of recombinant IL-1F5 and absorbance was measured at 450nm. The red curve shows saturable binding of IL-1F5 with SIGIRR. The black curve shows that in the absence of SIGIRR the binding of IL-1F5 and SIGIRR is non-saturable.

6.4. Discussion

The evidence presented here, that IL-1F5 attenuates pro-inflammatory cytokine production in hippocampal tissue prepared from LPS- and Aβ-treated and aged rats, provides a definitive role for IL-1F5 as an anti-inflammatory cytokine in the CNS. Since microglial cells are the primary source of pro-inflammatory cytokines in the CNS (Van Dam *et al.*, 1995), it was considered that IL-1F5 might mediate its effects through dampening down microglial activation. Activated microglial cells have been reported in a variety of neurodegenerative diseases (Benveniste *et al.*, 2001; Tan *et al.*, 2002; Town *et al.*, 2005). Upon activation, microglial cells not only release neurotoxic molecules and pro-inflammatory cytokines but also express a variety of cell surface markers including MHC II, CD40 and CD80 which allow antigen presentation to T cells (Benveniste *et al.*, 2004). Moreover studies have shown that CD40 interacts with the CD40 ligand found on T cells, B cells and monocytes and these interactions promote up-regulation chemokines further enhancing inflammation (D'Aversa *et al.*, 2002). Increased expression of CD11b has been identified on microglial cells during inflammation associated with neurodegenerative diseases and this acts as a binding protein for ICAM-1 (Roy *et al.*, 2006). The data presented here indicate that icv injection of IL-1F5 significantly decreased CD40 mRNA, CD11b mRNA and MHC II mRNA expression in hippocampal tissue. These results reveal that IL-1F5 is capable of attenuating the production of pro-inflammatory cytokines possibly due to downregulating microglial activation.

Considering that the anti-inflammatory effects of IL-1F5 were absent in isolated microglial cells, it must be proposed that IL-1F5 mediates its inhibitory effects on microglial activation indirectly. Two hypotheses were examined in this study were that IL-1F5 dampened microglial activation through induction of CD200 or by increasing the anti-inflammatory cytokines IL-10, IL-1ra and IL-4. CD200 is a glycoprotein expressed on a variety of cells including neurons which binds to a receptor (CD200R) on myeloid cells regulating immune responses in these cells (Wright *et al.*, 2003). More recently studies have shown that the interaction between CD200 on neurons and the CD200R on microglia decreased MHC II mRNA

expression and pro-inflammatory cytokine production in hippocampus of aged rats and Aβ-treated mixed glial cells (Lyons *et al.*, 2007). The findings of the present study show that IL-1F5 decreased CD200 mRNA expression but had no effect on CD200 protein expression, suggesting that IL-1F5 is not mediating its attenuatory effects on microglial activation through induction of CD200.

It is now widely accepted that inflammatory changes, and in particular increases in pro-inflammatory cytokines, play a pivotal role in the pathophysiology associated with ageing and neurodegenerative diseases (Akiyama *et al.*, 2000; Martin *et al.*, 2002; Minogue *et al.*, 2003; Nolan *et al.*, 2005; Griffin *et al.*, 2006). Studies have shown that under these conditions there is an imbalance between pro- and anti-inflammatory cytokines, with the age-related up-regulation of the pro-inflammatory cytokine IL-1β associated with down-regulation of the anti-inflammatory cytokine IL-4 in the hippocampus (Maher *et al.*, 2005). On the basis of these findings, it seemed reasonable to propose that IL-1F5 may restore this balance by increasing anti-inflammatory cytokines. Therefore, in an effort to elucidate the underlying mechanisms by which IL-1F5 acts, the ability of IL-1F5 to induce production of the anti-inflammatory cytokines IL-1ra, IL-10 and IL-4 was investigated *in vitro* and *in vivo*.

The data presented here show that IL-1F5 failed to increase IL-1ra mRNA expression, previous studies on the ability of IL-1F5 to induce IL-1ra have shown that IL-1F5 induced an increase in IL-1ra concentration in hippocampus after 2 hours of treatment and in the cortex after 6 hours (Loscher unpublished). This suggests that the effects of IL-1F5 on IL-1ra production may be time-dependent and certainly merit further investigations.

The anti-inflammatory cytokine, IL-10, has been shown to modulate the expression of pro-inflammatory cytokines, in particular inhibiting the production of IL-1β and TNF-α (Fiorentino *et al.*, 1991). In addition to these effects, IL-10, also has been shown to enhance the production of IL-1ra (Jenkins *et al.*, 1994). Here IL-1F5 did not affect IL-10 production which is consistent with the lack of effect on IL-1ra. The results that IL-1F5 was unable to induce an increase in IL-1ra mRNA expression and IL-10 protein expression were surprising as these were likely candidates for

mediating the actions of IL-1F5 due to their known anti-inflammatory properties. It must be concluded that neither, IL-10 not IL-1ra play a role in mediating the effects of IL-1F5.

There is ample evidence from our laboratory and others to suggest that IL-4 is capable of dampening down the inflammatory response and antagonising IL-1β- and LPS-induced signalling. The data presented here reveal that IL-1F5 induced a significant increase in IL-4 mRNA expression and this provides a mechanism for the actions of IL-1F5. This concurs with results from a recent study from our laboratory that showed that IL-1F5 was capable of increasing IL-4 mRNA expression in mixed glial cells and in hippocampal tissue, furthermore the anti-inflammatory effects of IL-1F5 were absent in mixed glial cells prepared from IL-4 knockout (IL-4$^{-/-}$) mice, further corroborating the role of IL-4 in IL-1F5 signalling events (Costelloe *et al.*, 2008).

The anti-inflammatory capacity of IL-4 been previously identified, for instance, IL-4 has been shown to reduce LPS-induced sickness behaviour (Bluthé *et al.*, 2002). IL-4 down-regulated the expression of formyl peptide receptor (FPR2) on activated microglial cells (Iribarren *et al.*, 2005) and inhibited LPS activation of the MAPK cascade in microglial cells (Iribarren *et al.*, 2003). It has also been shown that treatment with IL-4 blocked microglial-mediated neuronal cell injury (Chao *et al.*, 1993). Furthermore IL-4 has been shown to suppress the induction of MHC class II antigens on rat microglial cells (Chao *et al.*, 1993; Suzumura *et al.*, 1994; Lovett-Racke *et al.*, 2000) and on astrocytes (Morga and Heuschling, 1996). The studies highlighted here demonstrates the ability of IL-4 to dampen microglial activation and, considering that IL-1F5 is capable of decreasing microglial activation and increasing IL-4, this points to a role for IL-4 in mediating the anti-inflammatory effects of IL-1F5 on microglial activation.

IL-4 signalling is mediated through interaction with the IL-4 receptor and activation of a variety of signalling pathways leading to gene expression and cell proliferation and survival pathways (Nelms *et al.*, 1999). IL-4 has been shown to activate PI-3-K (Nelms *et al.*, 1999), the Ras/MAP kinase including ERK (Wery *et al.*, 1996) and activation of the JAK/STAT pathway (Keegan *et al.*, 1994). Indeed

many actions of IL-4 seem to be dependent on activation of the JAK/STAT pathway, the IL-4-mediated suppression of CD40 gene expression in both macrophages and microglia was dependent on STAT-6 (Nguyen and Benveniste, 2000) and studies have shown that IL-4-mediated functions such as expression of IL-4 responsive genes are impaired in STAT-6-deficient mice (Takeda *et al.*, 1997). Similarly Nolan and colleagues (2005) showed an increase in JAK-1 and STAT 6 following treatment with IL-4. In this study, in addition to the increase in IL-4 mRNA expression in the hippocampus following treatment with IL-1F5, the data demonstrate that activation of ERK 1/2 and STAT 6 were similarly up-regulated in response to IL-1F5, suggesting that IL-1F5 might induce IL-4-like signalling.

To further examine whether IL-1F5 mediates its anti-inflammatory actions through interaction with SIGIRR, mixed glial cells were prepared from wildtype and SIGIRR$^{-/-}$ mice and treated with LPS in the presence or absence of IL-1F5, and the effects of this treatment on cytokine concentrations were assessed. The data indicate that IL-1F5 was incapable of antagonising the effects of LPS on IL-1β concentration in mixed glia prepared from SIGIRR$^{-/-}$ mice compared to wildtype mice. Another key finding of this study was that IL-1F5 did not induce an increase in IL-4 concentration in mixed glial cells prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice. These results further substantiate the hypothesis that SIGIRR is essential for IL-1F5 signalling and are in agreement with results from our previous study (Costelloe *et al.*, 2008). To strengthen the evidence that IL-1F5 mediates its actions through interaction with SIGIRR, co-localisation of IL-1F5 and SIGIRR was assessed in mixed glial cells. Here we present for the first time evidence of co-localisation between IL-1F5 and SIGIRR confirming interaction between the two molecules and suggesting that IL-1F5 binds to SIGIRR.

To further consolidate the hypothesis that IL-1F5 requires SIGIRR for full activation, binding of IL-1F5 to SIGIRR was determined using an ELISA-based binding assay. The most significant finding of this study is that IL-1F5 is the ligand for the previously described orphan receptor SIGIRR. This finding is novel, in that it is the only evidence to date to show a ligand for the anti-inflammatory receptor SIGIRR. The discovery that IL-1F5 can bind to SIGIRR is surprising as the first

studies by Thomassen and colleagues (1999) suggested the single Ig domain was too short to fold around an IL-like ligand. However in a later report by O'Neill (2003), it was suggested that even though SIGIRR only has a single Ig domain it could still bind to a ligand, which could be one of the IL-1 prologues. The reports to date on the mechanism of action of IL-1F5 appear to lack consistency. One study showed that IL-1F5 was unable to bind to the IL-1R family members: IL-1RI, AcP, IL-1Rrp 1, IL-1Rrp2, AcpL and T1/ST2 (Smith *et al.*, 2000), yet another study showed that IL-1F5 antagonised IL-1F9-induced NFκB in an IL-1Rrp2-dependent manner (Debets *et al.*, 2001). In contrast to this study, Towne and colleagues (2004) showed that IL-1F6, IL-1F8 and IL-1F9 induced activation of NFκB, but this time IL-1F5 failed to attenuate these effects.

It might be argued that the hypothesis that IL-1F5 and SIGIRR interact is logical, both IL-1F5 and SIGIRR are novel members of the IL-1 ligand and receptor super-families which have been shown not to induce IL-1-like signalling. The evidence from this study shows that IL-1F5 can antagonise LPS-and Aβ-induced and age-associated inflammation and others have shown it can antagonise the production of NFκB by IL-1F9 (Debets *et al.*, 2001). Similarly there is ample evidence to prove the negative regulatory capacity of SIGIRR on IL-1- and LPS-induced signalling (Wald *et al.*, 2003; Garlanda *et al.*, 2004; Qin *et al.*, 2005). The data presented here provides original evidence that IL-1F5 and SIGIRR is a novel ligand-receptor couple and this offers a potentially exciting new therapeutic target for CNS inflammation.

Chapter 7

Overview

7.1. General discussion

The most significant finding of this study is that IL-1F5 interacts with the previously characterised orphan receptor, SIGIRR. Unlike the other members of the IL-1R superfamily, there is ample evidence to suggest that the actions of SIGIRR are anti-inflammatory. Studies have shown that SIGIRR is a negative regulator of IL-1β- and LPS-induced signalling (Wald *et al.*, 2003; Lech *et al.*, 2007), and is capable of regulating inappropriate adaptive immune responses (Huang *et al.*, 2006; Lech *et al.*, 2008). The results from this study are in agreement with these previous observations and demonstrate for the first time a novel role for SIGIRR as a negative regulator of both LPS-induced and age-associated inflammation in the CNS. Moreover, the data presented here suggest a role for SIGIRR in maintaining immune homeostasis in the hippocampus, as SIGIRR$^{-/-}$ mice treated with and without LPS exhibited differential hippocampal-dependent behavioural deficits.

Exactly how SIGIRR mechanistically inhibits IL-1β- and LPS-induced signalling and dampens Th2 autoimmunity, and thus acts as a negative regulator was previously unknown. Studies showed that SIGIRR sequesters the TLR/IL-1R proximal signalling components IRAK and TRAF-6 and it was suggested that this might be how SIGIRR mediates its anti-inflammatory effects (Wald *et al.*, 2003; Qin *et al.*, 2005). Another study proposed that SIGIRR may directly interfere with TLR/IL-1R dimerization or that SIGIRR might recruit intracellular inhibitory molecules to these receptors such as IRAKM, IRAK2c, IRAK2d, MyD88s and Triad3A (Li and Qin, 2005). However these studies relied on the use of immunoprecipitation of SIGIRR with IRAK and TRAF6 and in the absence of a ligand, they do not represent the activated form of SIGIRR and cannot be considered definitive.

Another study proposed that the single Ig domain of SIGIRR might fold around and consequently bind to one of the new IL-1 ligands (O'Neill, 2003). In support of this, a previous study from our laboratory, identified the novel IL-1 family member, IL-1F5 as a possible ligand for SIGIRR, and it was demonstrated that the anti-inflammatory effects of this cytokine were dependent on the presence of SIGIRR (Costelloe *et al.*, 2008). The results from the present study are the first to show co-

localisation and binding of IL-1F5 with SIGIRR and are therefore the first to unequivocally identify IL-1F5 as a ligand for SIGIRR.

IL-1F5 structurally resembles IL-1ra, sharing 52% amino acid sequence homology (Smith *et al.*, 2000; Lin *et al.*, 2001) and similar folding patterns (Barton *et al.*, 2000), therefore it was originally suggested that IL-1F5 may act in a similar manner to IL-1ra. IL-1ra is the endogenous IL-1R antagonist, that inhibits IL-1β-mediated effects (Arend and Gabay, 2000), in particular it antagonises the inhibitory effects of IL-1β on LTP and glutamate release and reduces IL-1β-mediated JNK activation (Kelly *et al.*, 2003). Likewise, studies on the actions of IL-1F5, showed that IL-1F5 antagonised NFκB activation by IL-1F9 (Debets *et al.*, 2001), attenuated the inhibitory effects of IL-1β on LTP and reduced IL-1β-mediated IL-6 and JNK activation (Costelloe *et al.*, 2008). Concomitantly, the results from this study showed that IL-1F5 was also able to dampen Aβ-induced and age-associated inflammation and therefore IL-1F5 plays a multi-faceted role in the regulation of inflammatory changes in the CNS. A rational conclusion that can be made from the results presented here and previously would be that IL-1F5 like IL-1ra is a receptor antagonist. Nevertheless, on closer inspection although IL-1F5 and IL-1ra share similarities over their entire amino acid sequences, they differ markedly in their loop regions, sharing only 24% amino acid sequence homology in these regions (Smith *et al.*, 2000). In fact, IL-1F5 was found to be more similar to IL-1β in these regions (Smith *et al.*, 2000). Considering these loop regions are essential for IL-1β to mediate its agonistic properties, these findings were taken to suggest that IL-1F5 might be an agonist for an unspecified receptor (O' Neill, 2001).

Previous studies on the actions of IL-1F5 showed it did not act in similar manner to IL-1β. One study showed that IL-1F5 was unable to trigger IL-6 production from fibroblasts and endothelial cells and IFN-γ from myelomonocytes (Barton *et al.*, 2000). Similarly others showed IL-1F5 failed to activate NFκB in Jurkat T cells (Debets *et al.*, 2001; Towne *et al.*, 2004). The results from a previous study from this laboratory showed that IL-1F5 increased IL-4 mRNA expression and concentration *in vitro* and *in vivo*. In this study, evidence was presented to show that IL-4 and the IL-4 signalling components ERK and STAT-6 were increased in

hippocampal tissue from IL-1F5-treated rats. The data presented here, and previously, corroborated the idea that IL-1F5 was an agonist for a receptor and that it mediates its anti-inflammatory effects through up-regulation of IL-4. This prompted the formation of the hypothesis that IL-1F5 must be a ligand for a receptor that induces anti-inflammatory signalling.

Previous studies which aimed at identifying a receptor for the actions of IL-1F5 lack concordance. Several reports showed that IL-1F5 was unable to bind to or signal through any of the IL-1R family members (Smith *et al.*, 2000; Born *et al.*, 2000; Towne *et al.*, 2004). Interestingly, one study showed that IL-1F5 acted as an antagonist at IL-1Rrp2 (Debets *et al.*, 2001), but this antagonism of IL-1F5 on IL-1Rrp2 was not reproduced (Towne *et al.*, 2004). The existence of SIGIRR with its known anti-inflammatory capacity (Wald *et al.*, 2003; Garlanda *et al.*, 2004) and the identification that IL-1F5 induces anti-inflammatory effects, indirectly and circumstantially supports the hypothesis that IL-1F5 and SIGIRR interact and consistent evidence included the finding that the effects of IL-1F5 were dependent on SIGIRR (Costelloe *et al.*, 2008). Several lines of evidence presented here provide strong evidence that IL-1F5 is the ligand for SIGIRR.

Firstly, the expression of SIGIRR in the CNS is confined to astrocytes and here the regulatory effects of IL-1F5 on LPS-triggered events *in vitro* were evident only in astrocytes. Furthermore, IL-1F5 failed to induce IL-4 and could not impart its anti-inflammatory effects on LPS-induced signalling in mixed glial cells prepared from SIGIRR$^{-/-}$ mice. In addition, co-localisation of IL-1F5 and SIGIRR was observed in mixed glial cells, strongly suggesting interaction between the two. However, the most concrete evidence to consolidate an interaction between IL-1F5 and SIGIRR came from the binding ELISA, which revealed for the first time that IL-1F5 binds to SIGIRR.

The conclusions that can be drawn from the findings presented in this investigation are that IL-1F5 binds to SIGIRR expressed on astrocytes and initiates a signalling cascade which includes activation of ERK1/2, JAK (Costelloe, unpublished) and STAT-6. Activation of these signalling molecules causes them to translocate to the nucleus and initiate IL-4 transcriptional events as evidenced by the

increase in IL-4 mRNA and translation and release of IL-4 protein. IL-4 with its known anti-inflammatory properties (Szczepanik *et al.*, 2001; Iribarren *et al.*, 2005; Lyons *et al.*, 2007) then binds to the IL-4 receptor on microglia (Nolan *et al.*, 2004) and down-regulates production of pro-inflammatory cytokines. IL-4 has been previously shown to antagonise the effects of pro-inflammatory cytokines (Nguyen and Benveniste, 2000; Lyons et al., 2007), and it is possible that in this study, the IL-1F5-mediated attenuation of LPS- and Aβ-induced and age-associated pro-inflammatory cytokines is mediated by IL-4. IL-1F5 also down-regulates microglial activation *in vivo* and since IL-1F5 was incapable of ameliorating the effects of LPS in isolated microglial cells *in vitro*, the involvement of other cells in maintenance of microglia in a quiescent state might be concluded. Studies have shown interaction of CD200 ligand, expressed on neurons, with its receptor which is expressed on microglia maintains microglia in a quiescent state (Lyons *et al.*, 2007) and that up-regulation of CD200 ligand expression is ERK dependent (Peterman *et al.*, 2007). Although the expression of CD200 ligand was not altered in hippocampal tissue from IL-1F5-treated rats in this study, a possibility is that SIGIRR is expressed on neurons and that IL-1F5 could bind to SIGIRR and induce an increase in ERK phosphorylation (as evidenced in this study) and this could mediate an increase in CD200 ligand expression. CD200 ligand could then bind to its receptor on microglial cells and dampen microglial activation. It would have been interesting to determine the expression of SIGIRR on neurons and test this idea in an *in vitro* set up. It is also possible that fractalkine could be involved in dampening microglial activation by IL-1F5. Fractalkine is a chemokine expressed on neurons, microglia and astrocytes which binds to its receptor expressed on microglia (Harrison *et al.*, 1998; Mizuno *et al.*, 2003). Studies have demonstrated a neuroprotective role for fractalkine in human immunodeficiency virus (HIV) infection (Tong *et al.*, 2000) and other have shown that fractalkine suppressed the production of pro-inflammatory cytokines and NO by activated microglia (Mizuno *et al.*, 2003). Therefore it would be valuable to determine if IL-1F5 induces the expression of fractalkine and upon receptor binding, this dampens microglial activation. The findings presented here provides a greater understanding of the intricacies of the CNS immune system and therapeutic

opportunities for CNS inflammation may arise when SIGIRR agonists, IL-1F5 mimetics and procedures which can increase or decrease IL-1F5 and/or SIGIRR are made available.

An unexpected finding was that treatment with IL-1F5 alone induced an increase in pro-inflammatory cytokines in isolated microglial cells. Table 7.1. demonstrates the different effects of IL-1F5 on pro-inflammatory cytokine production in the different cell types in the CNS and in particular shows that IL-1F5 increases pro-inflammatory cytokine production in isolated microglia. In mixed glial cells (which comprise 70% astrocytes and 30% microglia) and astrocytes the concentration of the pro-inflammatory cytokines are similar in control- and IL-1F5-treated cells. In contrast, in isolated microglial cells, IL-1F5 treatment induced release of significantly more IL-1β, IL-6 and TNF-α concentration than control-treated cells. However, it is worthy of note that in mixed glial cells IL-1F5 induced a significant increase in IL-1β concentration compared with control-treated cells, in light of the findings here, this response is probably due to the effects of IL-1F5 on microglial cells, in the mixed glial cell population.

	IL-1β		IL-6		TNF-α	
	Control	IL-1F5	Control	IL-1F5	Control	IL-1F5
Mixed glia	50.64 pg/ml ± 10.89	135.4 pg/ml ± 33.37	189.2 pg/ml ± 6.11	202.3 pg/ml ± 7.52	29.51 pg/ml ± 24.40	0 pg/ml
Astrocytes	12.43 pg/ml ± 3.20	14.96 pg/ml ± 1.04	0 pg/ml	4.73 pg/ml ± 32.31	4.29 pg/ml ± 4.29	2.54 pg/ml ± 2.54
Microglia	2.78 pg/ml ± 2.02	**776.7 pg/ml ± 66.59**	0.45 ng/ml ± 0.18	**8.48 ng/ml ± 0.41**	4.75 pg/ml ± 4.84	**1525 pg/ml ± 56.42**

Table 7.1. Summary of the effect of IL-1F5 on pro-inflammatory cytokine production in vitro

Values are expressed as means ± SEM.

Accompanying the IL-1F5-induced increase in pro-inflammatory cytokines *in vitro* was an occasional increase in pro-inflammatory cytokines in response to IL-1F5 *in vivo*. In one study, in hippocampal tissue prepared from IL-1F5-treated rats there was a significant increase in the concentrations of IL-1β, IL-6 and TNF-α compared with tissue prepared from control-treated rats. Taken together these findings suggest two things, firstly that the actions of IL-1F5 are like a "double-edged sword", on one hand IL-1F5 imparts anti-inflammatory actions and is protective; whereas on the other hand, IL-1F5 is pro-inflammatory. Secondly, these findings also pose the question, what causes the differential effects of IL-1F5 on microglial cells?

One possibility, contrary to what was found in this study, is that SIGIRR is in fact expressed on microglial cells, which would be concordant with other studies (Andre *et al.*, 2005; Dimcheff *et al.*, 2006). It is possible that a modified form of SIGIRR, perhaps a glycosylated form, is expressed on microglia and that this was not revealed by the probes used here. This may explain the different effects in microglia and astrocytes and may account for the finding that IL-1F5 did not antagonise LPS-induced pro-inflammatory cytokines and that IL-1F5 itself induced their production in microglial cells. In support of this, several studies have suggested that the effects of SIGIRR are cell-type specific (Wald *et al.*, 2003; Lech *et al.*, 2007). It would be interesting to examine different forms of SIGIRR to determine if it is differentially glycosylated in different cell types.

Considering that LPS is a component of the gram negative bacteria E.*coli* and a potent stimulator of microglial cells (Qin *et al.*, 2005). It was considered, that since IL-1F5 was expressed in E.*coli*, that perhaps the IL-1F5 used in this study was contaminated with LPS and this was activating microglial cells to produce pro-inflammatory cytokines. However, LPS in the IL-1F5 was found to be less than 0.2EU/ug IL-1F5, which suggests no contamination.

An antagonistic role for IL-1ra (Hirsch *et al.*, 1996; Loddick *et al.*, 1997) has been consistently described and this has been shown to extend to hippocampus where IL-1β-induced changes in pro-inflammatory cytokines, stress-activated kinases and transmitter release were inhibited by IL-1ra (Loscher *et al.*, 2000). However Loscher and colleagues (2003) showed that, in hippocampal synaptosomes, IL-1ra also

mimicked the effects of IL-1β; it decreased glutamate release, inhibited LTP and induced an increase in JNK phosphorylation. Interestingly these effects of IL-1ra were independent of its effects on IL-1RI since they persisted in IL-1RI knockout (IL-1RI$^{-/-}$) mice (Loscher *et al.*, 2003) and were also evident in the presence of an IL-1RI antibody (Loscher *et al.*, pens. comm.), suggesting it was acting on an entirely different receptor. This finding suggested that the heightened inflammation observed in this study in response to IL-1F5 may be due to IL-1F5 up-regulating IL-1ra. Although the results from this study revealed that IL-1F5 was unable to induce IL-1ra mRNA expression in hippocampus within 3 hours of administration, results from a previously-unpublished study showed that IL-1F5 was able to induce IL-1ra concentration in hippocampal tissue 2 hours after treatment and in cortical tissue 6 hours after treatment (but did not induce IL-1ra concentration in J774 or BV2s at any time; Loscher, unpublished). Since IL-1ra concentration was detected 2 hours following treatment with IL-1F5, it is logical to suggest it would be up-regulated at the level of transcription long before 3 hours, which may explain the negative result observed here. Nonetheless, if IL-1F5 induced an increase in IL-1ra in the absence of an inflammatory stimulus and in isolated microglial cells, perhaps these are the conditions essential for IL-1ra to act as an agonist at an as yet unidentified receptor. Importantly IL-1F5 is capable of antagonising the IL-1ra-induced increase in activation of IRAK, JNK and p38 in IL-1RI$^{-/-}$ mice (Loscher, pens comm.) indicating that under these circumstances IL-1F5 acts as an IL-1ra antagonist. On the other hand IL-1F5 mimics the effect of IL-1ra in wildtype mice where both agents antagonise the effects of IL-1β (Loscher, penns comm.). Mulero and colleagues (1999) showed that IL-1F5 and IL-1ra were secreted together from the monocytic cells line THP-1 cells in response to LPS and phorbol esters, suggesting an associative role. In contrast, under conditions in which IL-1ra induces inflammation, IL-1F5, through its interaction with SIGIRR and induction of IL-4, can antagonise IL-1ra. The effects of IL-1F5 on IL-1ra may therefore act like an anti-inflammatory "trip switch". In support of this idea, Debets and colleagues (2001) showed that the concentration of IL-1F5 required to attenuate IL-1F9 activity is markedly less than IL-1ra, suggesting IL-1F5 is a more potent antagonist than IL-1ra.

It is now widely accepted that the effects of cytokines are often redundant and pleiotropic (Naka *et al.*, 2002), here IL-1F5 is inducing both anti- and pro-inflammatory signalling. A potential explanation for the disparate effects of IL-1F5 on microglial cells is that IL-1F5 induces agonist activity independent of SIGIRR, and is a ligand for another receptor which imparts pro-inflammatory signalling. Similarly, studies have shown that TNF-α imparts both deleterious and protective actions in the CNS and these effects have been explained by the existence of two distinct TNF-α signalling pathways mediated by two distinct TNF-α receptors of approximately 55kDa (TNFR1) and 75kDa (TNFR2) (Lucas *et al.*, 2006). Indeed studies have shown that the intracellular domains of the two receptors are structurally different suggesting they produce distinct signals (Bigda *et al.*, 1994). In support of this Tartaglia and colleagues (1991) showed that activation of TNFR1 induces cytotoxicity, whereas activation of TNFR2 induces T cell proliferation. Loscher and colleagues (2003) showed that IL-1ra is both antagonist at IL-1RI and agonist at a different receptor. Others have reported inconsistency with the effects of IL-6, showing at times that it is capable of being both protective and damaging (Gadient and Otten, 1997; Xing *et al.*, 1998; Naka *et al.*, 2002). Taken together these findings suggest that IL-1F5 might also initiate two distinct signalling pathways. Indeed, the characteristic determination of cytokines as either "pro-inflammatory" or "anti-inflammatory" should be reviewed and it is this author's opinion that it is the cytokines cognate receptor and not the cytokine itself that determines the "pro-inflammatory" or "anti-inflammatory" consequences.

The possibility exists that IL-1F5 binds to a receptor other than SIGIRR expressed on microglial cells, recruiting signalling molecules and inducing the production of pro-inflammatory cytokines and downstream inflammatory pathways. This is consistent with the fact that IL-1F5 is similar to IL-1β in its loop regions that impart agonistic activity (O'Neill, 2003). What are the likely candidate receptors that IL-1F5 might bind, to induce inflammation? Schmitz and colleagues (2005) recently revealed that IL-33 is the ligand for the previously orphan receptor ST2; they demonstrated that IL-33 by interacting with ST2 induced NFκB activation, MAPK phosphorylation and Th2 cytokines. Interestingly, these authors also suggested that

SIGIRR may act as an accessory protein for ST2, contrasting with studies that showed that SIGIRR is neither IL-1R-like nor AcP-like (Born *et al.*, 2000). It is possible that, in microglial cells ST2 and SIGIRR interact upon binding with IL-1F5 leading to the activation of MAPKs. However, with the data presented in this study, and the plethora of studies demonstrating an anti-inflammatory role for SIGIRR (Wald *et al.*, 2003; Huang *et al.*, 2006; Lech *et al.*, 2007; Lech *et al.*, 2008) it is unlikely that activation of SIGIRR induces pro-inflammatory signalling.

It has been suggested that IL-1F5 binds to IL-1Rrp2 (Debets *et al.*, 2001). This previous study showed that IL-1F5 antagonised IL-1F9 induced NFκB activation in cells transfected with IL-1Rrp2 (Debets *et al.*, 2001). In support of this, Blumberg and colleagues (2007) showed that inflammation was exacerbated in IL-1F6 transgenic mice combined with IL-1F5 deficiency, further suggesting that IL-1F5 acts antagonistically at this receptor. In contrast others showed that IL-1F5 exerted no pro-inflammatory or anti-inflammatory effects in cells transfected with IL-1Rrp2 (Towne *et al.*, 2004). Taken together these studies suggest that the increased inflammation observed in this study in response to IL-1F5, is unlikely to be due to its interaction with IL-1Rrp2.

Like IL-1β, IL-1F5 lacks a secretory signal peptide (Mulero *et al.*, 1999; Kumar *et al.*, 2000; Smith *et al.*, 2000). Due to this, it is believed that IL-1β is released from cells in an unconventional manner, by exocytosis of pre-terminal endocytic vesicles (Andrei *et al.*, 1999) and it is therefore possible that IL-1F5 is secreted through these processes also. Additionally, IL-1F5 lacks a pro-domain at its N-terminus and is released, without processing, as the active form (Kumar *et al.*, 2000). This is in contrast to IL-1β which when released, has a pro-domain that must be cleaved to produce its active form (Siegmund *et al.*, 2001). However studies have shown that although IL-1F5 lacks this pro-domain its coding regions matches with that of the cleaved form of IL-1β suggesting it still retains its tertiary structure and is therefore still active (Kumar *et al.*, 2000). In view of these findings an obvious candidate receptor for the pro-inflammatory effects of IL-1F5, is IL-1RI. Upon binding to IL-1RI, there is recruitment of an accessory protein AcP and activation of NFκB, MAPKs and synthesis and release of pro-inflammatory cytokines (Parker *et*

al., 2002). It is possible that, like IL-1β, IL-1F5 binds to IL-1RI and this interaction induces the production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α observed in this study. However the evidence suggests that IL-1F5 is unable to bind to the IL-1R family members (Born *et al*., 2000; Smith *et al*., 2000) and in particular the IL-1RI (Debets *et al*.,2001).

It is interesting to note that a splice variant of the IL-1 receptor accessory protein termed AcPb has been identified which is exclusively expressed in the brain (Sims, J.E. unpublished). Perhaps in the CNS, this accessory protein plays a fundamental role in the interactions of IL-1F5 with the IL-1RI and upon binding with these two molecules on microglial cells, IL-1F5 can induce pro-inflammatory signalling events. Apart from our previous work and the results presented here, the only other studies to date on the effects of IL-1F5 are in peripheral cells. These studies show that IL-1F5 failed to induce IL-6 production from fibroblasts and endothelial cells, IFN-γ production from myelomonocytes and NFκB activation in Jurkat T cells (Barton *et al*., 2000; Debets *et al*., 2001; Towne *et al*., 2004), suggesting that IL-1F5 does not act in a similar manner to IL-1β. Furthermore, results from our previous study showed that in peripheral macrophages and DCs treated with IL-1F5 there were no increases in IL-1β concentration (Costelloe *et al*., 2008). Since this is the only study to date, to identify a pro-inflammatory effect of IL-1F5, and since the data suggest that this effect of IL-1F5 is confined to its interaction with an as-yet unidentified receptor present on microglial cells and considering that the expression of AcPb is exclusive to the CNS, it might be proposed that, in microglial cells, IL-1F5 binds to IL-1RI, resulting in recruitment of the novel AcPb and this leads to downstream activation of NFκB, MAPKs and the production of pro-inflammatory cytokines. Therefore while IL-1F5 has predominantly anti-inflammatory effects in the brain, pro-inflammatory effects may occur in particular circumstances.

In chronic inflammatory states activation of inflammation may actually be beneficial (Lucas *et al*., 2006) and this is a possible explanation for the occasional inflammatory effects induced by IL-1F5; this certainly warrants further investigation. The data presented highlight the anti-inflammatory effects of SIGIRR in the CNS and

reveal that IL-1F5 exerts its anti-inflammatory effects by interacting with SIGIRR. The findings therefore reveal potential therapeutic value in exploring mechanisms by which SIGIRR expression and activation can be modulated.

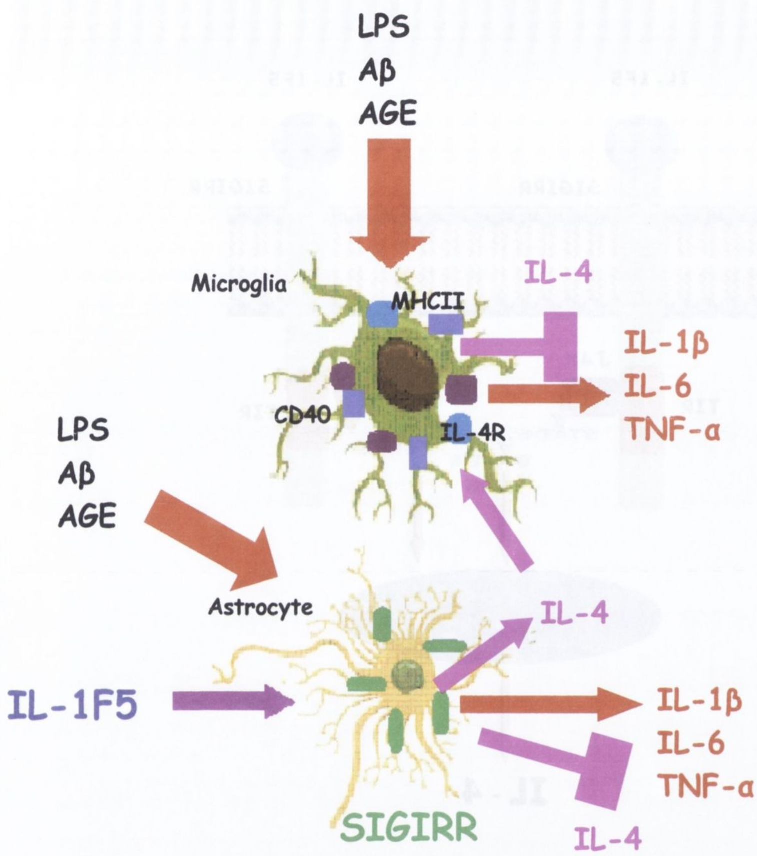


Figure 7.1. Schematic summarising the anti-inflammatory effects of IL-1F5 and SIGIRR

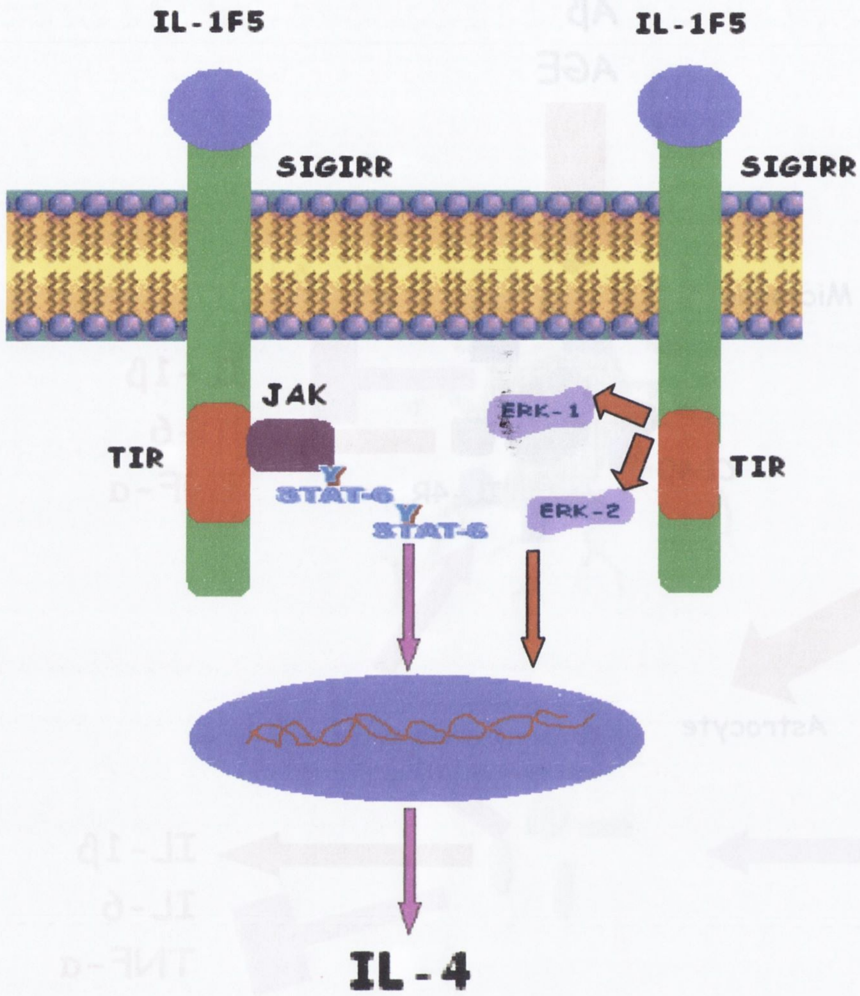


Figure 7.2. Schematic summarising IL-1F5 and SIGIRR signalling on astrocytes

7.2. Future studies

The conclusions drawn from this study are that IL-1F5 binds to SIGIRR on astrocytes and initiates a signalling cascade which includes activation of ERK and the JAK/STAT pathway, leading to IL-4 production. It is also possible that activation of these signalling molecules occurs in response to the IL-1F5-induced increase in IL-4, as these are characteristic downstream signalling cascades from the IL-4R. Therefore it is necessary to determine if activation of SIGIRR by IL-1F5 results in the recruitment of these signalling molecules or whether they are activated as a result of IL-4 signalling. This idea could be tested by treating mixed glia or astrocytes with IL-1F5 in the presence of an antibody to SIGIRR or the IL-4R and examining activation of ERK and the JAK/STAT pathway. It would also be interesting to determine if the TIR domain of SIGIRR interacts with ERK or JAK and this could be carried out using immunoprecipitation assays.

The major finding from this study that warrants further investigations is that IL-1F5 induces agonist activity in the CNS independent of SIGIRR. This suggests that IL-1F5 binds to another receptor on microglial cells. It is possible that, in microglial cells the receptors, ST2 and SIGIRR interact upon binding with IL-1F5 leading to the activation of MAPKs. It is also possible that IL-1F5 binds to IL-1RI and the CNS-specific AcPb. It would be beneficial to determine the cellular expression of ST2 and AcPb in the CNS, whether they are expressed on astrocytes or microglia. A binding study could be undertaken to determine if IL-1F5 binds to ST2 and SIGIRR or IL-1RI in the presence of AcPb. Alternatively cells could be transfected with ST2 and SIGIRR and IL-1RI and AcPb, treated with IL-1F5 and analysed for pro-inflammatory cytokine production. Similarly, the use of $ST2^{-/-}$, $IL\text{-}1RI^{-/-}$ and $Acpb^{-/-}$ mice treated with IL-1F5 would determine if the any of these receptors are essential for the pro-inflammatory effects induced by IL-1F5.

Considering the findings that IL-1F5 could not exert its anti-inflammatory effects in microglial cells *in vitro*, yet in hippocampal tissue from IL-1F5-treated rats, microglial markers were down-regulated, this suggests that there is involvement of other cells in maintenance of microglia in a quiescent state. A possible explanation is that IL-1F5 induces an increase in CD200 ligand expression on neurons and this

binds to its receptor on microglia causing a reduction in microglial activation. Another explanation is that IL-1F5 induces an increase in fractalkine (which is expressed on neurons, microglia and astrocytes) and this binds to its receptor on microglia, dampening microglial activation. It would be interesting to determine if SIGIRR is expressed on neurons and to establish if activation of SIGIRR by IL-1F5 induces an increase in CD200 or fractalkine ligand expression, this idea could be examined by treating neurons and mixed glia with IL-1F5 in the presence of an antibody to SIGIRR and examining activation of CD200 and fractalkine ligand expression.

One hypothesis tested here for the mode of action of IL-1F5 was its ability to induce anti-inflammatory cytokines. The data presented in this study, show that IL-1F5 induced an increase in IL-4 release. It would also have been interesting to assess the effects of IL-1F5 on other typical Th2-associated cytokines such as IL-5 and IL-13. The expression of IL-1F5 on T cells has not been fully characterised therefore it would be interesting to determine if IL-1F5 is expressed on T cells and, if so, to determine if IL-1F5 is capable of polarizing Th2 cells and thus whether it might play a role in Th2-associated immunity.

The findings from this study that SIGIRR is not expressed on microglia merits further investigations. Studies have shown that astrocytes and microglia express SIGIRR (Andre *et al.*, 2005; Dimcheff *et al.*, 2006), however in this study the expression of SIGIRR was undetectable on microglia in mixed glial cells and in isolated microglia. Furthermore the expression of SIGIRR was also undetectable in astrocytes and microglia by PCR analysis (data not shown). It would also be interesting to determine if SIGIRR is expressed on microglia, whether this SIGIRR is expressed as a modified form e.g. glycosylated differently. If this was the case it would explain the failure of IL-1F5 to ameliorate LPS-induced pro-inflammatory cytokine production. This idea could be carried out preparing mixed glia, isolated microglia, astrocytes and neurons and determining the expression of SIGIRR and the expression of glycosylated forms of SIGIRR by western immunoblotting.

Although the behavioural results from this study showed that SIGIRR$^{-/-}$ mice had altered hippocampal-dependent behaviour with or without LPS treatment, this

study relied on the hole-board test. It would be beneficial to utilise more stringent behavioural tests such as the Morris water maze or other markers of synaptic plasticity such as LTP. Furthermore it would be interesting to undertake these tests with IL-1F5$^{-/-}$ and SIGIRR$^{-/-}$ mice to fully elucidate the role of IL-1F5 and SIGIRR in hippocampal-dependent learning and behaviour.

In light of the anti-inflammatory actions induced by SIGIRR and the negative regulatory capacity of SIGIRR in autoimmune diseases in this study and in others. It would be an attractive hypothesis to suggest that SIGIRR would also be involved in regulating CNS autoimmune disorders such as MS or in a mouse model of AD and this requires further investigation. Similarly, in this study IL-1F5 was capable of ameliorating the inflammation caused by an acute injection of an Aβ peptide. While this has relevance to the pathology of AD it is not entirely clinically relevant, it would be interesting to study the anti-inflammatory effects of IL-1F5 in more a more relevant model of AD. Therefore it would be interesting to assess the effects of SIGIRR and IL-1F5 fusion proteins in EAE mice or APP transgenic mice. This would provide greater scope for investigating the actions of IL-1F5 and SIGIRR in attenuating inflammatory aspects of CNS autoimmune disorders and AD pathology.

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Appendix

VIII Appendix I: Mean data

Variable (units)	Wildtype mice		$SIGIRR^{-/-}$ mice	
	Control	LPS	Control	LPS
CD40 mRNA (RQ)	0.91 ± 0.22	16.35 ± 3.48	0.11 ± 0.01	27.02 ± 9.51
ICAM mRNA (RQ)	2.39 ± 0.65	66.55 ± 10.64	0.42 ± 0.06	213.1 ± 30.46
MCP-1 mRNA (RQ)	0.55 ± 0.15	1.38 ± 0.28	0.65 ± 0.13	1.93 ± 0.11
IP-10 mRNA (RQ)	1.31 ± 0.17	1625 ± 431.3	5.51 ± 3.96	1778 ± 178.0
IL-1 β mRNA (RQ)	0.43 ± 0.19	2.61 ± 0.79	0.05 ± 0.01	8.70 ± 3.35
IL-6 mRNA (RQ)	1.83 ± 0.37	452.4 ± 216.2	15.53 ± 14.91	1438 ± 420.6
TNF-α mRNA (RQ)	1.64 ± 0.56	849.5 ± 423.9	0.54 ± 0.14	2053 ± 641.7
IL-1β (pg/mg)	5.79 ± 1.62	11.08 ± 1.14	8.10 ± 2.31	17.34 ± 2.86
IL-6 (pg/mg)	28.60 ± 1.31	39.13 ± 2.28	30.44 ± 2.27	54.07 ± 5.09
TNF- α (pg/mg)	16.49 ± 2.73	16.24 ± 2.04	21.99 ± 3.47	59.99 ± 25.46
p-JNK (arbitrary units)	0.50 ± 0.09	0.60 ± 0.06	0.73 ± 0.05	1.06 ± 0.14

Table 1.1. Raw data from LPS-treated wildtype and $SIGIRR^{-/-}$ mice.
Values are expressed as means ± SEM.

Variable (units)	Wildtype mice		$SIGIRR^{-/-}$ mice	
	Control	**LPS**	**Control**	**LPS**
Head dipping (counts)	18.20 ± 0.97	12.83 ± 1.54	10.83 ± 1.08	3.83 ± 1.42
Rearing (counts)	9.20 ± 1.39	7.00 ± 1.55	12.40 ± 0.68	6.67 ± 1.31
Frequency of entries into centre (counts)	15.80 ± 1.24	9.83 ± 0.91	10.67 ± 0.99	2.60 ± 1.03
Time spent in the outer zone (% of total time)	63.13 ± 4.598	51.13 ± 4.39	81.45 ± 2.031	81.22 ± 7.34
Time spent in the central zone (% of total time)	36.87 ± 4.598	48.87 ± 4.39	18.55 ± 2.031	18.78 ± 7.34
Distance to the border (cm)	15.18 ± 1.01	16.31 ± 0.60	10.06 ± 0.25	8.84 ± 2.04
Total distance travelled (cm)	1831 ± 195	1128 ± 123.7	2049 ± 114.4	827.6± 23.9
Maximum distance travelled (cm)	13.18 ± 0.63	9.21 ± 0.78	12.53 ± 0.69	9.02 ± 1.08
Mean velocity travelled (cm/s)	18.31 ± 1.95	10.97 ± 1.28	20.51 ± 1.15	8.28 ± 2.24

Table 1.2. Raw data from behavioural study of wildtype and $SIGIRR^{-/-}$ mice treated with LPS.

Values are expressed as means ± SEM.

Variable (units)	Wildtype mice		SIGIRR$^{-/-}$ mice	
	Young	**Aged**	**Young**	**Aged**
CD40 (fold increase)	64.78 ± 15.72	82.55 ± 11.50	74.23 ± 9.10	747.0 ± 233.3
IL-1β mRNA (fold increase)	42.58 ± 19.19	317.4 ± 146.0	74.62 ± 9.63	1758 ± 485.9
IL-6 mRNA (fold increase)	51.55 ± 7.757	37.39 ± 5.64	6.23 ± 5.86	99.78 ± 19.78
TNF-α mRNA (fold increase)	59.05 ± 14.40	48.82 ± 13.52	57.60 ± 18.35	215.0 ± 57.26
IL-1β (pg/mg)	3.94 ± 1.27	51.12 ± 4.47	6.53 ± 2.75	61.90 ± 4.34
IL-6 (pg/mg)	28.60 ± 1.31	169.5 ± 59.24	30.44 ± 2.27;	711.3 ± 277.3
TNF-α (pg/mg)	20.05 ± 11.96	108.4 ± 18.38	31.59 ± 11.04	104.6 ± 5.02
	Aged	**Aged Aβ**	**Aged**	**Aged Aβ**
IL-1β mRNA (Fold increase)	54.34 ± 24.98	135.6 ± 70.09	44.88 ± 12.40	357.5 ± 257.5
IL-6 mRNA (Fold increase)	10.34 ± 2.91	36.19 ± 8.05	40.66 ± 20.17	45.39 ± 14.56
TNF-α mRNA (Fold increase)	62.42 ± 18.19	1266 ± 570.7	46.04 ± 11.87	13090 ± 10366
IL-1β (pg/mg)	51.12 ± 4.47	59.35 ± 8.92	65.20 ± 3.44	81.10 ± 19.53
IL-6 (pg/mg)	169.5 ± 59.24	137.1 ± 41.50	711.3 ± 277.3	34.07 ± 21.65
TNF-α (pg/mg)	126.7 ± 2.15	123 ± 5.37	104.6 ± 5.02	117 ± 7.67

Table 1.3. Raw data from Aβ-treated and aged wildtype and SIGIRR$^{-/-}$ mice. Values are expressed as means ± SEM.

Variable (units)	Rat mixed glial cells			
	Control	LPS	IL-1F5	LPS + IL-1F5
CD86/CD11b (% cells)	0.09 ± 0.06	1.145 ± 0.29	0.20 ± 0.07	0.32 ± 0.14
IL-1β (pg/ml)	50.64 ± 10.89	280.2 ± 16.32	135.4 ± 33.37	148.1 ± 19.61
IL-6 (pg/ml)	189.2 ± 6.11	675.2 ± 23.80	202.3 ± 7.52	597.4 ± 33.00
TNF-α (pg/ml)	29.51 ± 24.40	1512 ± 108	0	1067 ± 164.7

Variable (units)	R at astrocytes			
	Control	LPS	IL-1F5	LPS + IL-1F5
IL-1β (pg/ml)	12.43 ± 3.20	73.86 ± 10.48	14.96 ± 1.04	56.42 ± 3.56
IL-6 (pg/ml)	0	1574 ± 156.1	4.73 ± 32.31	1227 ± 97.98
TNF-α (pg/ml)	4.29 ± 4.29	228.7 ± 14.78	12.72 ± 0.0	188.8 ± 15.25

Variable (units)	Rat microglia			
	Control	LPS	IL-1F5	LPS + IL-1F5
IL-1β (pg/ml)	2.78 ± 2.02	130.6 ± 32.34	776.7 ± 66.59	832.9 ± 65.25
IL-6 (ng/ml)	0.45 ± 0.18	4.35 ± 0.22;	8.47 ± 0.30	8.48 ± 0.41
TNF-α (pg/ml)	4.75 ± 4.84	1198 ± 25.80	1525 ± 56.42	1562 ± 74.81

Table 1.4. Raw data from LPS and IL-1F5-treated rat mixed glia, isolated astrocytes and microglia.

Values are expressed as means ± SEM.

Variable (units)	Rat hippocampus			
	Control	**LPS**	**IL-1F5**	**LPS + IL-1F5**
IL-1β (pg/mg)	21.73 ± 9.03	96.89 ± 9.94	50.56 ± 15.51	40.61 ± 13.51
IL-6 (pg/mg)	100.4 ± 21.41	190.3 ± 36.09	107.1 ± 17.72	115.7 ± 8.86
p-JNK (arbitrary units)	2.15 ± 0.24	3.36 ± 0.33	2.581 ± 0.47	1.68 ± 0.24
p-p38 Arbitrary units	3.64 ± 0.28	6.09 ± 0.78	5.010 ± 0.61	4.49 ± 0.60

Variable (units)	Rat hippocampus			
	Control	**Aβ**	**IL-1F5**	**Aβ + IL-1F5**
IL-1β (pg/mg)	43.03 ± 8.24	164.9 ± 29.24	142.5 ± 26.54	82.08 ± 21.61
IL-6 (pg/mg)	699.9 ± 37.39	1121 ± 42.32;	1186 ± 145.2	891.8 ± 46.22
TNF-α (pg/mg)	315 ± 21.65	575.7 ± 22.29	565.0 ± 66.05	399.3 ± 29.36

Variable (units)	Rat hippocampus			
	Young	**Aged**	**IL-1F5**	**Aged + IL-1F5**
IL-1β (pg/mg)	129.1 ± 5.30	149.4 ± 6.09	110.9 ± 5.289	124.4 ± 7.46
IL-6 (pg/mg)	746.3 ± 108.5	803.7 ± 82.13	661.9 ± 85.31	661.9 ± 85.31
TNF-α (pg/mg)	157.3 ± 10.81	1612 ± 79.09	282.1 ± 32.98	1386 ± 66.81

Table 1.5. Raw data from the effects of IL-1F5 on LPS-, Aβ- and age-associated inflammatory changes in rat hippocampus.

Values are expressed as means ± SEM.

Variable (units)	Rat hippocampus	
	Control	IL-1F5
CD40 mRNA (RQ)	1.15 ± 0.16	0.45 ± 0.08
CD11b mRNA (RQ)	1.57 ± 0.16	0.53 ± 0.12
MHC II mRNA (RQ)	0.92 ± 0.03	0.63 ± 0.05
CD200 mRNA (RQ)	0.93 ± 0.07	0.70 ± 0.03
CD200 expression (arbitrary units)	0.13 ± 0.02	0.12 ± 0.01
IL-1ra mRNA (RQ	3.55 ± 1.23	2.74 ± 1.05
IL-10 (pg/mg)	80.49 ± 6.54	75.02 ± 2.34
IL-4 mRNA (arbitrary units)	0.07 ± 0.02	0.23 ± 0.06
IL-4 mRNA (RQ)	0.42 ± 0.12	1.75 ± 0.54
ERK-1 (arbitrary units)	0.47 ± 0.12	1.03 ± 0.17
ERK-2 (arbitrary units)	0.50 ± 0.08	0.86 ± 0.12
STAT-6 (arbitrary units)	0.54 ± 0.09	0.95 ± 0.08

Variable (units)	Rat mixed glial cells	
	Control	IL-1F5
IL-10 (pg/ml)	274.4 ± 6.76	265 ± 5.25

Table 1.6. Raw data from the effects of IL-1F5 in rat hippocampus and in mixed glia. Values are expressed as means ± SEM.

Variable (units)	Mixed glial cells							
	Wildtype mice				SIGIRR$^{-/-}$ mice			
	Control	LPS	IL-1F5	LPS + IL-1F5	Control	LPS	IL-1F5	LPS + IL-1F5
IL-1β (pg/ml)	3.48 ± 0.52	19.63 ± 5.16	4.252 ± 0.49	5.15 ± 1.37	7.85 ± 1.45	17.64 ± 5.46	8.37 ± 1.46	22.32 ± 4.55
IL-4 (pg/ml)	30.46 ± 6.38	-	109.6 ± 34.23	-	29.45 ± 6.39	-	44.59 ± 14.81	-

Table 1.7. Raw data from mixed glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice treated with LPS and IL-1F5.
Values are expressed as means ± SEM.

Variable (units)	Binding ELISA							
	Concentration of IL-1F5 (µg/ml)							
		3	10	30	50	100	150	300
Absorbance (450nm)	**IL-1F5 with SIGIRR**	0.07 ± 0.01	0.09 ± 0.002	0.08 ± 0.002	0.12 ± 0.002	0.20 ± 0.004	0.28 ± 0.01	0.30 ± 0.01
Absorbance (450nm)	**IL-1F5 without SIGIRR**	0.07 ± 0.01	0.10 ± 0.01	0.15 ± 0.01	-	0.26 ± 0.02	-	0.68 ± 0.02

Table 1.8. Raw data from IL-1F5 and SIGIRR binding ELISA
Values are expressed as means ± SEM.

IX. Appendix II: Company addresses

Alexis
Alexis Corporation (UK) Ltd.,
P.O. Box 6757,
Bingham,
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UK

Amersham
Amersham Biosciences Ltd.,
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Little Chalfont
Buckinghamshire HPA 9NA,
UK

Applied Biosystems,
Frankfurter Street 129b,
64293 Darmstadt,
Germany

BD Biosciences
Bio-Rad Laboratories Ltd.,BD Biosciences,
2350 Qume Drive,
Maylands Avenue,
San Jose,
CA,
USA

Biosource,
Biosource International,
542 Flynn Road,
Camarillo,
CA 93012,
USA

Bio-Rad
Bio-Rad House,
Hamel Hempstead,
Hertfordshire HP2 7TD,
UK

Calbiochem
Calbiochem-Novabiochem Corp.,
10394 Pacifica Centre Court
San Diego,
CA 92121,
USA

Canopus Corporation,
Grass Valley (UK) Ltd.,
Unit 1&2, The Duran Centre,
14 Arkwright Road,
Reading RG2 0LS
UK.

Carl Zeiss Ltd.,
15-20 Woodfield Road,
Welwyn Garden Centre,
Herfordshire,
AL7 1JQ,
UK

Cell Signalling
Cell Signalling
Technology Inc.,
166B Cummings Centre,
Beverly,
MA 0192590
USA

Chemicon International Inc.,
28820 Single Oak Drive,
Temecula,
CA 92590
USA

Dako Diagnostics Ireland Ltd.,
12 Camden Row,
Dublin 8

Gibco
Gibco Ltd.,
3 Fountain Drive,
Linchinnnan Drive,
Paisley PA4 9RF
Scotland

Harvard Apparatus Ltd.,
Fircroft way,
Edenbridge,
Kent TN8 6HE,
UK

Invitrogen,
Invitrogen Ltd.,
3 Fountain Drive,
Linchinnnan Drive,
Paisley PA4 9RF
Scotland

Kinematica Inc.,
1648 Locust Avenue,
Unit C,
Bohemia,
NY 1176
USA

Macherey-Nagel
Labquip (Ireland)Ltd.,
12 The Business Centre,
Fonthill Industrial Park
Clondalkin
Dublin 22

Maxim Biotechnology Inc.,
780 Dubuque Avenue,
So San Francisco,
CA 94080,
USA

Nanodrop Technologies Inc.,
3411 Silverside Rd,
Bancroft Bldg,
Willmington,
Delaware, USA

NUNC
Thermo Fisher Scientific,
Kamstrupvej 90,
Postbox 280,
DK-4000,
Roskilde,
Denmark

Pierce
Pierce Biotechnologies
3747 N. Meridian Road,
P.O. Box 117
Rockford IL 61105
USA

Promega,
Promega,
2800 Woods Hollow Road,
Madison WI 53711
USA

R&D Systems,
R&D Systems,
614 McKinley Place NE,
Minneapolis
MN 55413
USA

Roche,
Roche Diagnostics Ltd.,
Bell lane,A
Lewes,
East Sussex BN7 ILG,
UK

Santa Cruz

Santa Cruz Biotechnologies,
2161 Delaware Avenue,
Santa Cruz,
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USA

Serotec

Serotec Ltd.,
22 Bankside
South Approach
Kidlington,
Oxford OX5 1JE
UK

Sigma

Sigma-Aldrich Company Ltd.,
Fancy Road,
Poole,
Dorset BH12 4GH
UK

Startedt

Starstedt Ltd.,
Sinnottstown Lane,
Drinagh,
Wexford,
Ireland.

Ultra-Violet Products

Ultra-Violet Products Ltd.,
Unit 1
Trinity Hall Farm Estate,
Hullfield Road,
Cambridge CB4 1TG
UK

Vector

Vector Laboratories Inc.,
30 Ingold Road,
Burlingame,
CA 94010,
USA

Whatman

Whatman Plc
Whatman House,
St. Leonard's Road,
Maidstone,
Kent ME16 0LS
UK

X. Appendix III: Solutions

FACS buffer

pH 7.5

NaCl, 137 mM

KCl 2.7 mM

Na_2HPO_4 8.1 mM

KH_2PO_4 1.5 mM

5% FBS

0.1% NaN_3

Krebs buffer with $CaCl_2$

pH 7.5

NaCl 136 mM

KCl 2.54 mM

KH_2PO_4 1.18 mM

Mg_2SO_4 1.18 mM

$NaHCO_3$ 16 mM

Glucose 10 mM

$CaCl_2$ 2mM

Lysis buffer

pH 9.5

Tris-HCl 10

NaCl 50mM

$Na_4P_20_7.H_2O$ 10 mM

NaF 50mM

1% Igepal

Na_3VO_4 1 mM

PMSF 1 mM

Protease Inhibitor cocktail 1 mM

Phosphate buffered saline (PBS)

pH 7.5

NaCl 137 mM

KCl 207 mM

Na_2HPO_4 8.1 mM

KH_2PO_4 1.5 mM

PHEM buffer

pH 6.9

Pipes 60 mM

Hepes 25 mM

EDTA 10 mM

$MgCl_2$ 2 mM

Tris-buffered saline (TBS-T)

pH 7.4

Tris-HCl 20mM

NaCl 150mM

XI. List of Publications

Major publications:

Costelloe, C. *, **Watson, M**. *, Murphy, A. *, McQuillan, K., Loscher, C., Armstrong, M. E., Garlanda, C., Mantovani, A., O'Neill, L. A., Mills, K. H. & Lynch, M. A. (2008) IL-1F5 mediates anti-inflammatory activity in the brain through induction of IL-4 following interaction with SIGIRR/TIR8. *J Neurochem,* **105**, 1960-1969. (*Authors contributed equally to work).

Minor publications:

Watson, M. & Lynch, M.A. (2008) *9th International Congress of Neuroimmunology*, Texas. 'The interaction of SIGIRR and IL-1F5; an Important Neuromodulatory System'.

Watson, M., Costelloe, C. & Lynch, M.A. (2008) *Federation of European Neuroscience*, Switzerland. 'IL-1F5 a novel anti-inflammatory cytokine'

Watson, M., Costelloe, C. & Lynch, M.A. (2008) *International Neuroimmunology Symposium*, Ireland. 'A novel protective role for SIGIRR in endotoxic shock'

Watson, M., Costelloe, C., O'Connell, F. & Lynch, M.A. (2007) *TCD Postgraduate Research Day*, Ireland. 'An anti-inflammatory role for SIGIRR in the central nervous system?'

Watson, M., Lyons, A. & Lynch, M.A. (2007) *British Neuroscience Association,* UK. 'The Anti-inflammatory role of Interleukin-1F5 in the rat brain'

Journal of Neurochemistry JNC

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IL-1F5 mediates anti-inflammatory activity in the brain through induction of IL-4 following interaction with SIGIRR/TIR8

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Abstract

Similarity in structure and sequence homology has led to the identification of new members of the interleukin-1 (IL-1) ligand and receptor superfamilies. IL-1F6, IL-1F8 and IL-1F9 have been shown to signal through IL-1R-related protein 2 and IL-1 receptor accessory protein leading to activation of NFκB, while IL-1F7 and IL-1F10 interact with the IL-18 receptor and the soluble IL-1 receptor type I respectively. In contrast, identification of a biological role for IL-1F5 has remained elusive, with conflicting data relating to its possible ability to antagonize IL-1F9-stimulated activation of NFκB in Jurkat cells transfected with IL-1R-related protein 2. In this study, we set out to investigate a possible role for IL-1F5 in the brain and report that it antagonizes the inflammatory effects of IL-1β and lipopolysaccharide (LPS) *in vivo* and *in vitro* including the inhibitory effect on long-term potentiation (LTP) in rat hippocampus. We demonstrate that IL-1F5 induces IL-4 mRNA and protein expression in glia *in vitro* and enhances hippocampal expression of IL-4 following intracerebroventricular (i.c.v.) injection. The inhibitory effect of IL-1F5 on LPS-induced IL-1β is attenuated in cells from IL-4-defective ($IL–4^{-/-}$ mice). Our findings suggest that IL-1F5 mediates anti-inflammatory effects through its ability to induce IL-4 production and that this is a consequence of its interaction with the orphan receptor, single Ig IL-1R-related molecule (SIGIRR)/TIR8, as the effects were not observed in $SIGIRR^{-/-}$ mice. In contrast to its effects in brain tissue, IL-1F5 did not attenuate LPS-induced changes, or up-regulated IL-4 in macrophages or dendritic cells, suggesting that the effect is confined to the brain.

Keywords: cytokine, cytokine receptor, interleukin-1β, single Ig IL-1 receptor-related molecule, interleukin-4.

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The identification of new members of the interleukin-1 (IL-1) family, named IL-1F5–10 (Dunn *et al.* 2001; Sims *et al.* 2001; Sims 2002), and the expansion of the IL-1 receptor family to include the orphan receptors, IL-1R-related protein 2 (IL-1Rrp2), T1/ST2, three immunoglobulin domain-containing IL-1 receptor-related, IL-1 receptor accessory protein-like and single Ig IL-1 receptor-related molecule (SIGIRR), also called TIR8 (Sims 2002; Mantovani *et al.* 2004) presents a renewed challenge to understand the biological roles of IL-1. Recent studies have uncovered some of the actions of the newer members of the IL-1 family. Thus, IL-1F6 activates nuclear factor kappa B in keratinocytes through its interaction with IL-1Rrp2 (Debets *et al.* 2001), while both IL-1F8 and IL-1F9 also interact with IL-1Rrp2 to produce a similar effect (Towne *et al.* 2004). Significantly, antibodies to IL-1RAcP (IL-1 receptor accessory protein) blocked cytokine-induced activation of NFκB, suggesting that the accessory protein acts a coreceptor for signalling initiated by IL-1F6, IL-1F8 and IL-1F9 (Towne *et al.* 2004). In addition to their effect on NFκB, these cytokines have also been

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Abbreviation used: BSA, bovine serum albumin; DC, dendritic cell; DMEM, Dulbecco's modified Eagle's medium; i.c.v., intracerebroventricular; IL-1F, interleukin-1 family; IL-1RAcP, IL-1 receptor accessory protein; IL-1Rrp2, IL-1R-related protein 2; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LTP, long-term potentiation; pJNK, phospho-JNK; SIGIRR, single Ig IL-1 receptor-related molecule; T_{anneal}, annealing temperature; TBS, Tris-buffered saline; TBS–T, TBS containing 0.1% Tween 20; WT, wild-type.

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shown to activate c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (Towne *et al.* 2004). Current evidence suggests that IL-1F7 binds to IL-18R (Kumar *et al.* 2002; Bufler *et al.* 2004) and that IL-1F10 binds to the soluble IL-1 receptor (Lin *et al.* 2001), but the significance of these interactions remains to be established. Thus, while ligands have been identified for IL-1Rrp2, none have been identified for the other orphan receptors, including SIGIRR. However, activation of SIGIRR has been reported to play an inhibitory role in IL-1 signalling (Wald *et al.* 2003; Mantovani *et al.* 2004). Consistent with this anti-inflammatory action, SIGIRR-deficient mice exhibit exaggerated inflammatory responses to lipopolysaccharide (LPS) (Wald *et al.* 2003) and in dextran sodium sulphate-induced colitis (Garlanda *et al.* 2004).

Interleukin-1F5 has 44% sequence identity to IL-1ra, but it was concluded from analysis of the structure that it is unlikely to bind to IL-1RI (Dunn *et al.* 2003); this has been confirmed experimentally and IL-1F5 is similarly unable to bind IL-1RAcP, IL-18R, IL-18RAcP, IL-1Rrp2, T1/ST2, three immunoglobulin domain-containing IL-1 receptor-related or IL-1 receptor accessory protein-like (Born *et al.* 2000). Although it was reported that IL-1F9-induced activation of NFκB was antagonized by IL-1F5 in IL-1Rrp2-transfected Jurkat cells (Debets *et al.* 2001), others have failed to replicate this finding (Towne *et al.* 2004).

Because of its potential anti-inflammatory effects, we considered that IL-1F5 might modulate inflammatory responses in the brain and therefore assessed its effect on LPS-induced inhibition of long-term potentiation (LTP) in the hippocampus. LTP is a model for learning and memory and can be inhibited by inflammatory responses in the brain induced by LPS, which is associated with increased production of IL-1β and activation of JNK (Vereker *et al.* 2000; Lonergan *et al.* 2004; Barry *et al.* 2005). Our findings demonstrate that IL-1F5 abrogates LPS-induced inhibition of LTP and the associated increase in IL-1β concentration, suggesting that IL-1F5 has anti-inflammatory effects in the brain. In an effort to characterize these effects, we investigated the action of IL-1F5 on IL-4 production and report that it up-regulates expression of IL-4 in hippocampus. *In vitro* analysis indicated that IL-4 is produced by glia and the data suggests that this is dependent on the interaction of IL-1F5 with SIGIRR.

Experimental procedures

Animals

Male Wistar rats (3–4 months) were supplied by the Bioresources Unit, Trinity College Dublin, Ireland. C57BL/6 mice were obtained from Harlan UK, IL-4-defective (IL-$4^{-/-}$) mice were supplied by Bantham and Kingman (Hull, UK) and SIGIRR$^{-/-}$/TIR8$^{-/-}$ mice (Garlanda *et al.* 2004) were obtained from Istituto Clinico Humanitas IRCCS, Milan, Italy. All mice and rats were maintained in the Bioresources Unit under the guidelines of the EU, Irish Department of Health, and experiments were performed following approval by the local ethics committee.

Effects of IL-1F5 *in vivo*

To assess the effects of IL-1F5 *in vivo*, rats were anaesthetized with urethane (1.5 g/kg; 33% w/v), placed in a stereotaxic frame and injected intracerebroventricularly (i.c.v.; 2.5 mm posterior and 0.5 mm lateral to Bregma) with 5 μL saline or 5 μL IL-1F5 (30 ng/mL) (Dunn *et al.* 2003) and 5 min later were treated i.p. with LPS (100 μg/kg; *Escherichia coli* serotype 0111: B4; Sigma, Dorset, UK). In a second study, rats were injected i.c.v. with IL-1F5 (150 pg), or IL-1β (17.5 pg; R&D Systems, Abingdon, UK) or both in 5 μL saline. The IL-1F5 dose was chosen because previous experiments indicated that it mimicked the effect of IL-1ra (1500 pg) in attenuating the IL-1β-induced inhibition of LTP (Loscher *et al.* 2003). In some experiments, the effect of LPS in wild-type (WT) (C57BL/6) mice was compared with that in IL-$4^{-/-}$ and SIGIRR$^{-/-}$ mice. In this case, mice were anaesthetized by intraperitoneal injection of urethane (1.2 g/kg; 33% w/v) and treated i.c.v. with IL-1F5 or i.p. with LPS (200 μg/kg). In experiments to examine the effect of IL-1F5 on IL-4, WT and SIGIRR$^{-/-}$ mice were anaesthetized by i.p. injection of urethane (1.2 g/kg; 33% w/v) and treated i.c.v. with IL-1F5 (1 mm posterior and 0.6 mm lateral to Bregma) and after 1 h, were killed by decapitation. Hippocampi were dissected free, homogenized and stored at −80°C for later analysis.

Analysis of long-term potentiation

Rats were assessed for their ability to sustain LTP 4 h after administration of LPS or 1 h following IL-1β injection. Rats were anaesthetized with urethane and treated with saline, IL-1β, IL-1F5 and LPS as described above. To assess LTP, a bipolar stimulating electrode was positioned in the perforant path (4.4 mm lateral to lambda) and a unipolar recording electrode was positioned in the dorsal cell body region of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to Bregma; Martin *et al.* 2002). Test shocks were delivered at 30 s intervals, and after a stabilization period, responses were recorded for 10 min before and 40 min after, tetanic stimulation (three trains of stimuli; 250 Hz for 200 ms; 30 s inter-train interval). Rats were killed by decapitation at the end of the period of electrophysiological recording and the brains were rapidly removed for later analysis.

Glial cells

Glia were isolated from cerebral cortices of 1-day-old Wistar rats or from whole brain of C57BL/6, IL-$4^{-/-}$ or SIGIRR$^{-/-}$ mice as described (Nolan *et al.* 2005). Animals were decapitated; brain tissue was placed in 3 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL; Gibco, Uxbridge, UK). Samples were triturated (7×), passed through a sterile nylon mesh filter and centrifuged (2500 *g* for 3 min at 20°C). The pellet was resuspended in DMEM and resuspended glia were allowed to adhere to coverslips for 2 h in a humidified incubator containing 5% CO_2 : 95% air at 37°C before the addition of pre-warmed DMEM (400 μL). Cells were grown for 10 days prior to treatment and medium was replaced every 3 days. Cells were pre-treated with IL-

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1F5 (3 μg/mL) for 2 h, LPS was added (100–1000 ng/mL; Sigma), and incubation continued for 24 h. This concentration of IL-1F5 was chosen because it mimicked the effect of IL-1ra (300 ng/mL) in blocking IL-1β-induced inhibition of glutamate release *in vitro* (Loscher *et al.* 2003), while preliminary experiments which analysed concentration-dependent effects, indicated that it attenuated the LPS-induced increase in IL-1β as effectively as higher concentrations. In certain experiments, cultured rat glia were pre-treated for 4 h with anti-SIGIRR antibody (20 μg/mL; R&D Systems, Minneapolis, MN, USA) prior to IL-1F5 treatment. To investigate SIGIRR staining on glia, cells were fixed in alcohol, permeabilized with 0.1% Triton X-100, blocked and incubated overnight with anti-SIGIRR antibody (20 μg/mL; R&D Systems, USA). Coverslips were washed and incubated in secondary antibody (anti-goat IgG conjugated to FITC) and washed. Cells were viewed under 40× magnification (excitation 490 nm and emission 520 nm).

Dendritic cells and macrophages

Bone marrow-derived dendritic cell (DC) were prepared by culturing bone marrow cells with granulocyte macrophage colony-stimulating factor and adherent peritoneal macrophages were prepared by peritoneal lavage as described (Higgins *et al.* 2006). DCs or macrophages were cultured (200 μL/well; $1–2 \times 10^6$ cells/mL) in the presence or absence of IL-1F5 (1–3 μg/mL) for 2 h, LPS (100–1000 ng/mL) was added and was incubation continued for 24 h. Supernatants were removed for analysis of cytokine concentrations by ELISA.

Analysis of IL-1β, IL-6 and IL-4 mRNA

cDNA synthesis was performed on 1 μg total RNA using oligo(dT) primer (superscript reverse transcriptase; Life Technologies Ltd., Strathclyde, UK). Equal amounts of cDNA were used for PCR amplification and primers were tested in preliminary experiments through and increasing number of cycles to ensure that PCR products were obtained in the appropriate range. The following primer pairs were used to measure the target gene expression [product size and primer annealing temperature (T_{anneal}) in parenthesis]; rat IL-4 mRNA: upstream 5′-TCCATGCACCGAGATGTTTGTACC-3′ and downstream 5′-CGTAGGATGCTCCCTTTATGAACG-3′ (299 bp, $T_{anneal} = 60°C$); rat β-actin: upstream 5′-AGAAGAGCTATGAGC-TGCCTGACG-3′ and downstream 5′-CTTCTGCATCCTGTCAGC-GATGC-3′ (236 bp, $T_{anneal} = 65°C$); mouse IL-6 mRNA: upstream 5′-GACAAAGCCAGAGTCCTTCAG-3′ and downstream 5′-CTAGGTTTGCCGAGTAGATCTC-3′ (277 bp, $T_{anneal} = 60°C$); mouse IL-4: upstream 5′-GCACCTTCTTTTCCTTCATC-3′ and downstream 5′-CTGATGTACCAGTTGGGGAA-3′ (299 bp, $T_{anneal} = 60°C$); and mouse β-actin mRNA: upstream 5′-AGAA-GAGCTATGAGCTGCCTGACG-3′ and downstream 5′-CTTCT-GCATCCTGTCAGCGATGC-3′ (236 bp, $T_{anneal} = 65°C$). Equal volumes of PCR product from each sample was loaded onto 1.5% agarose gels, bands were separated by application of 90 V, photographed and quantified using densitometry (Labworks, UVP BioImaging Systems, Cambridge, UK). The target gene was normalized to mRNA expression of the β-actin housekeeping gene, with the exception of IL-4 mRNA in mouse tissue which was normalized with respect to 18S gene using a Dual PCR kit (Maxim Biotechnology, San Francisco, CA, USA).

Analyses of cytokine concentrations

Interleukin-1β, IL-6 and IL-4 concentrations were analysed by ELISA in hippocampal homogenates and in supernatants from cultured cells. Values are expressed as pg/mL (supernatants) or pg/mg (homogenates; after correcting for protein concentrations).

Analysis of JNK phosphorylation and IL-1F5

Hippocampal tissue was homogenized in Krebs solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.2 mM $NaHCO_3$, and 10 mM glucose, pH 7.4) containing $CaCl_2$ (22 mM) and assessed for expression of phospho-JNK (pJNK), total JNK and IL-1F5 by gel electrophoresis and immunoblotting. Tissue samples were equalized for protein concentration and diluted to a final protein concentration of 1 mg/mL. Aliquots (10 μL) were added to NuPAGE–LDL sample buffer (Invitrogen, Paisley, UK) containing NuPAGE reducing agent, heated at 70°C for 10 min and loaded onto 10% Nu polyacrylamide gel electrophoresis-low density lipoprotein Novex Bis–Tris gels (Invitrogen) in the case of JNK and 12% gels in the case of IL-1F5. Proteins were separated (200 V constant for 45 min) and transferred onto nitrocellulose membrane (30 V constant for 1 h). Membranes were blocked for 1 h in Tris-buffered saline (TBS) 0.1% Tween 20 (TBS–T) and 5% bovine serum albumin (BSA) for JNK, or overnight in 5% Marvel in TBS–T for IL-1F5. Membranes were incubated overnight at 4°C with primary antibody (pJNK 1 : 1000; total JNK 1 : 1000; diluted in TBS–T with 2% BSA; Cell Signaling Technology Inc., Danvers, MA, USA) at 4°C. In the case of IL-1F5, incubation with the primary antibody (AF1275; R&D Systems, UK; 1 : 100 in TBS–T with 1% BSA) continued for 2 h. Membranes were washed three times in TBS–T, incubated with horseradish peroxidase-linked anti-rabbit antibody (1 : 1000 in TBS–T with 2% BSA for JNK) or anti-goat antibody (1 : 1000 in TBS–T with 5% BSA) for 1 h and washed again in TBS–T. Immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) for JNK and Supersignal (Pierce, Northumberland, VA, USA) for IL-1F5. Values are expressed as the ratio of pJNK : total JNK or IL-1F : actin.

Statistical analysis

Data were analysed, as appropriate, using either Student's *t*-test for independent mean or a one-way ANOVA followed by *post hoc* Student–Newman–Keuls test to determine which conditions were significantly different from each other. Data are expressed as mean with standard errors and deemed statistically significant when $p < 0.05$.

Results

IL-1F5 attenuates IL-1 and LPS-induced inflammatory responses in glial cells and in the brain and IL-1 or LPS-induced LTP

The homology with IL-1ra has led to the proposal that IL-1F5 might, like IL-1ra, antagonize the effects of IL-1β and to assess this, we analysed the modulatory effect of IL-1F5 on IL-1β-induced IL-6. We demonstrate that stimula-

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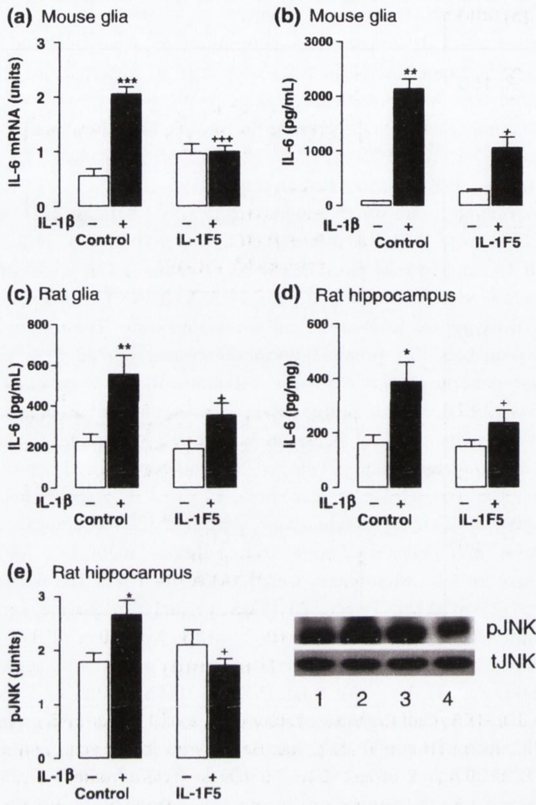

Fig. 1 IL-1F5 antagonizes IL-1β-induced IL-6 in brain tissue *in vitro* and *in vivo*. Mouse glia (a and b) or rat glia (c) cells were cultured with IL-1β (3.5 ng/mL), following 2 h pre-treatment with IL-1F5 (3 μg/mL). After 24 h, IL-6 protein concentrations in supernatants were quantified by ELISA or IL-6 mRNA expression in cell pellets was estimated by PCR. (d and e) Rats were injected i.c.v. with 5 μL IL-1F5 (30 ng/mL) or IL-1β (3.5 ng/mL) or both. After 3 h, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-6 concentrations (d) determined by ELISA and JNK phosphorylation (e) determined by western blotting (expressed as a ratio of phosphorylated to total JNK in arbitrary units). Sample blots indicating density of phosphorylated and total JNK (pJNK and tJNK) for hippocampal tissue prepared from control- (lane 1), IL-1β- (lane 2), IL-1F5- (lane 3) and IL-1β + IL-1F5- (lane 4) treated rats are presented. Data are mean ± SEM (n = 4–6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, with versus without IL-1β; $^{+}p < 0.05$ and $^{+++}p < 0.001$, IL-1F5 + IL-1β versus IL-1β alone by ANOVA.

tion of murine mixed glial cells with IL-1β significantly increased IL-6 mRNA and protein (p < 0.01–0.001; Fig. 1a and b) and that co-incubation with IL-1F5 significantly attenuated the IL-1β-induced increase in IL-6 (p < 0.05–0.001). IL-1β similarly increased IL-6 in rat glia ($p < 0.05$; Fig. 1c) and IL-1F5 significantly attenuated this effect ($p < 0.05$). We then investigated this effect *in vivo* and report that i.c.v. injection of IL-1β significantly increased IL-6 concentration in rat hippocampus ($p < 0.05$; Fig. 1d) and this was significantly inhibited by co-administration of IL-1F5 ($p < 0.05$; Fig. 1d). It is interesting to note that IL-1F5 did not completely block these IL-1β-induced changes. In parallel with IL-1β-induced increase in hippocampal IL-1β concentration, we observed that phosphorylation of JNK was significantly increased in hippocampal tissue prepared from IL-1β-injected rats ($p < 0.05$; Fig. 1e) and that this effect was significantly abrogated by i.c.v. injection of IL-F5 ($p < 0.05$; Fig. 1e). We investigated IL-1F5 in hippocampal tissue prepared from the same rats in which these assessments were made and found that relative density of IL-1F5 (expressed as a ratio of IL-1F5/actin) was unchanged in samples prepared from IL-1β-treated rats [0.114 ± 0.06 (mean ± SEM; n = 5) compared with 0.233 ± 0.11 (control)] but was increased approximately eightfold in tissue prepared from rats treated with IL-1F5 alone (1.82 ± 0.24) or in combination with IL-1β (1.14 ± 2.47; n = 5). We next assessed the modulatory effect of IL-1F5 on LPS-induced changes *in vitro* and *in vivo* and show that the LPS-induced IL-1β production in mixed glial cultures prepared from mice and rats was significantly (albeit incompletely) attenuated by IL-1F5 ($p < 0.001$; Fig. 2a and b). IL-1F5 also attenuated the LPS-induced increases in IL-6 and tumour necrosis factor-α, although the attenuation was partial, as in the case of IL-1β. In contrast, whereas LPS significantly increased IL-1β concentration in macrophages and DC (p < 0.05–0.001), IL-1F5 exerted no modulatory effect in these cells (Fig. 2c and d). Injection (i.p.) of LPS significantly increased IL-1β and IL-6 concentrations in the hippocampus ($p < 0.05$), which was completely attenuated by i.c.v. injection of IL-1F5 ($p < 0.05$; Fig. 2e and f). Similarly, while JNK activation was significantly increased in hippocampal tissue prepared from LPS-treated rats ($p < 0.05$), this increase was abrogated by i.c.v. injection of IL-1F5 (Fig. 2g).

It has been shown that an increase in IL-1β expression in the hippocampus has a negative impact on its function. This area of the brain plays a pivotal role in learning and memory and consequently an inverse relationship between IL-1β and cognitive function has been described (Maher *et al.* 2005). LTP is considered to be a biological substrate for learning and memory and therefore we assessed the effect of IL-1F5 on IL-1β and LPS-induced changes in LTP. We found that IL-1β significantly decreased LTP ($p < 0.001$; Fig. 3a), but this decrease was significantly attenuated by IL-1F5 ($p < 0.001$). We also report that LPS significantly inhibited LTP and that LTP was restored in LPS-treated rats which received IL-1F5 ($p < 0.001$; Fig. 3b), and although mean excitatory post-synaptic potential slope was slightly increased in the IL-1F5-treated (138.8 ± 11; SEM), compared with the control-treated (118.6 ± 5) group, this difference was not statistically significant because of the greater variability in response in the IL-1F5-treated animals.

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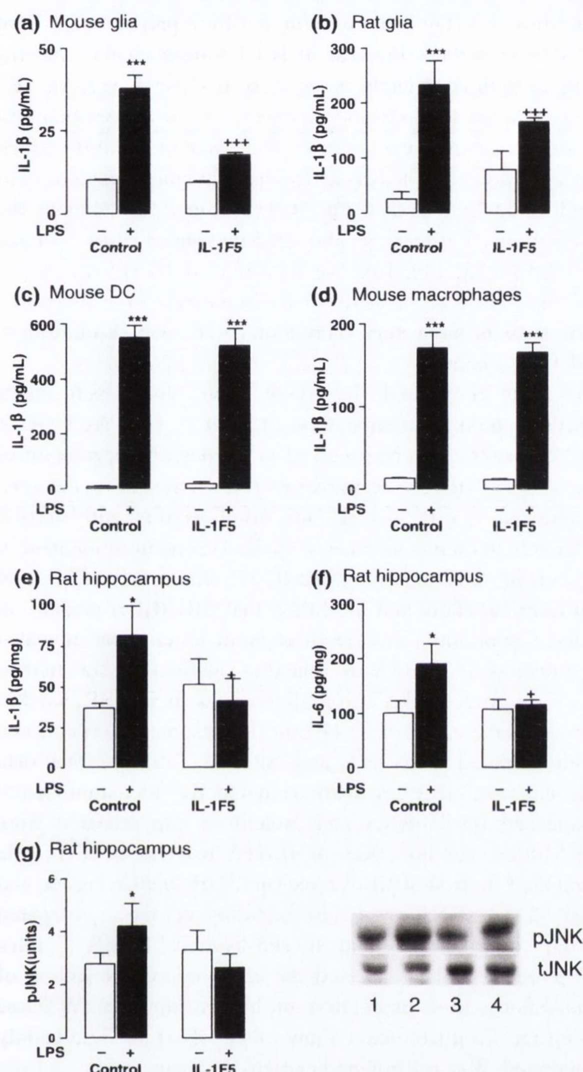

Fig. 2 IL-1F5 antagonizes LPS-induced IL-1β in brain tissue *in vitro* and *in vivo.* Mouse glia (a), rat glia (b), mouse DC (c) or mouse macrophages (d) were cultured in the presence or absence of LPS (100 ng/mL), following 2 h pre-treatment with IL-1F5 (1 and 3 μg/mL in the case of mouse and rat cells respectively) or medium only (control). After 24 h, IL-1β protein concentrations in supernatants were quantified by ELISA. (e and f) Rats were injected i.c.v. with 5 μL saline or 30 ng/mL of IL-1F5 and 5 min later were treated i.p. with LPS (100 μg/mL, 200 μL). After 3 h, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-1β (e) or IL-6 (f) concentrations determined by ELISA and JNK phosphorylation (g) determined by western blotting (expressed as a ratio of phosphorylated to total JNK in arbitrary units). Sample blots indicating density of phosphorylated and total JNK (pJNK and tJNK) for hippocampal tissue prepared from control- (lane 1), LPS- (lane 2), IL-1F5- (lane 3) and LPS + IL-1F5- (lane 4) treated rats are presented. Data are mean ± SEM ($n = 4$–6). *$p < 0.05$ and ***$p < 0.001$, with versus without LPS; $^{+}p < 0.05$ and $^{+++}p < 0.001$, IL-1F5 versus control by ANOVA.

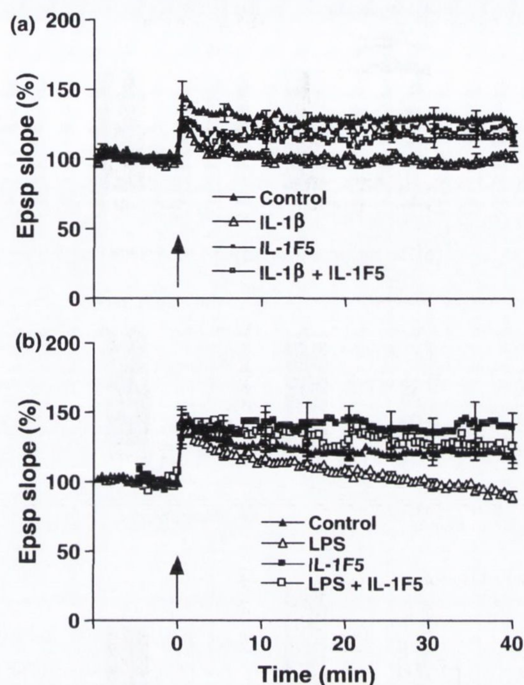

Fig. 3 IL-1F5 reverses the inhibitory effects of IL-1β or LPS on tetanus-induced LTP in dentate gyrus. Rats were injected i.c.v. with 5 μL saline or 30 ng/mL of IL-1F5 and 5 min later were treated i.c.v. with (3.5 ng/mL; 5 μL) IL-1β (a) or i.p. with 100 μg/kg LPS (b). Rats were assessed for their ability to sustain LTP 1 h following i.c.v. injection of IL-1-β injection or 4 h after i.p. administration of LPS. The values are mean percentage changes in EPSP slope following tetanic stimulation (arrow) compared with the values in the 5 min prior to stimulation compared ($n = 5$ or 6 rats per treatment group). SEM are included for every tenth response.

IL-1F5 mediates its anti-inflammatory effects through induction of IL-4

Our findings demonstrate an anti-inflammatory effect of IL-1F5 and, because of previous reports which indicated that IL-4 attenuated the LPS-induced increase in IL-1β and the LPS- and IL-1β-induced inhibition of LTP (Barry *et al.* 2005; Nolan *et al.* 2005), we considered that IL-1F5 may exert its action by inducing IL-4 production. We found that treatment of glia, prepared from mouse or rat, with IL-1F5 induced significant IL-4 production ($p < 0.01$; Fig. 4a and b). In parallel, i.c.v. injection of IL-1F5 significantly increased IL-4 mRNA and protein expression in rat hippocampus ($p < 0.05$–0.01; Fig. 4c and d). In contrast, IL-1F5 did not induce IL-4 production by DC or macrophages (Fig. 4e and f), although treatment of these cells with phorbol myristic acid and ionomycin (as a positive control) significantly increased IL-4 production (not shown).

These data suggest that the action of IL-1F5 might be mediated by IL-4 and to address this, we prepared glia from WT and IL-4$^{-/-}$ mice. The data show that LPS significantly

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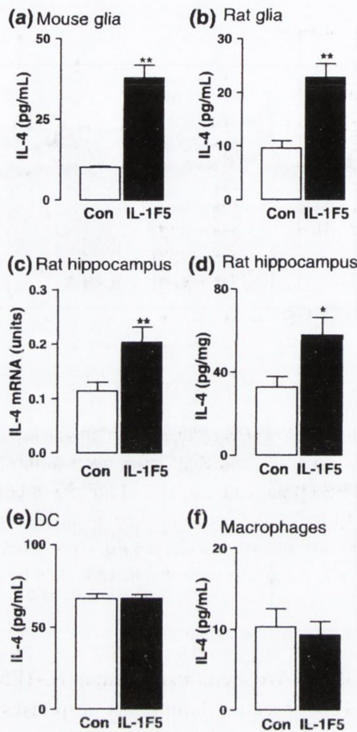

Fig. 4 IL-1F5 increases in IL-4 in brain tissue. Mouse glia (a), rat glia (b), mouse DC (e) or mouse macrophages (f) were cultured with IL-1F5 (1 and 3 μg/mL in the case of mouse and rat cells respectively) or medium only (control). After 24 h, IL-4 protein concentrations were quantified in supernatants by ELISA. (c and d) Rats were injected i.c.v. with 5 μL saline or 30 ng/mL of IL-1F5. After 3 h, rats were killed by decapitation, hippocampi dissected free, homogenized and IL-4 mRNA (c) and IL-4 protein (d) determined by PCR and ELISA respectively. (d) Values are expressed as mean ± SEM ($n = 3$ for DC and macrophages; $n = 6$–14 in all other experiments). $^{*}p < 0.05$ and $^{**}p < 0.01$, IL-1F5 versus control by Student's *t*-test for independent mean.

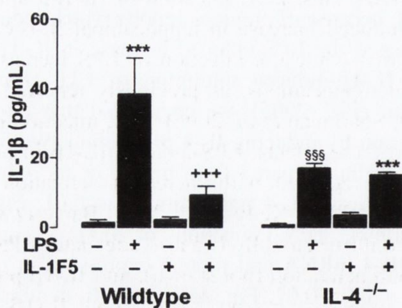

Fig. 5 IL-1F5 abrogates the LPS-induced increase in IL-1β in glia from WT but not IL-4$^{-/-}$ mice. Glial cells from WT and IL-4$^{-/-}$mice stimulated with medium only, LPS (100 ng/mL) or IL-1F5 (3 μg/mL) or both and IL-1β concentration were determined in the supernatants 24 h later. Values are mean ± SEM ($n = 10$). $^{*}p < 0.05$ and $^{***}p < 0.001$, IL-1F5 versus control; $^{+++}p < 0.001$, with versus without IL-1F5; $^{§§§}p < 0.001$, WT versus IL-4$^{-/-}$ by ANOVA.

increased IL-1β concentration in glial preparations from WT mice ($p < 0.001$) and that IL-1F5 significantly inhibited this LPS-induced increase in IL-1β ($p < 0.001$; Fig. 5). LPS also induced a significant increase in IL-1β concentration in cultured glia prepared from IL-4$^{-/-}$ mice ($p < 0.001$), albeit to a significantly lesser extent than in glia prepared from WT mice ($p < 0.001$), but IL-1F5 failed to attenuate the LPS-induced effect in glia prepared from IL-4$^{-/-}$ mice (Fig. 5).

The anti-inflammatory effects of IL-1F5 are abrogated in SIGIRR$^{-/-}$ mice

We next considered the possible receptor which might mediate the anti-inflammatory effect of IL-1F5. As SIGIRR has been shown to be involved in the negative regulation of IL-1 signalling, we examined its role in mediating the anti-inflammatory effects of IL-1F5. Addition of an anti-SIGIRR antibody to rat glia attenuated the IL-1F5-induced increase in IL-4 (Fig. 6a), suggesting that IL-1F5 interacts with SIGIRR to exert its effect and indicating that SIGIRR is present on glia. Importantly immunohistochemical analysis revealed expression of SIGIRR on glia (Fig. 6b). To further investigate our proposal that IL-1F5 interacts with SIGIRR, we first analysed the effect of IL-1F5 on IL-4 expression in cultured glia prepared from WT and SIGIRR$^{-/-}$ mice. The data demonstrate that incubation with IL-1F5 significantly increased IL-4 mRNA and protein in glia prepared from WT mice, but no effect of IL-1F5 was observed in glia prepared from SIGIRR$^{-/-}$ mice ($p < 0.05$–0.001; Fig. 6c and d). IL-4 mRNA was undetectable in tissue prepared from control-treated and IL-1F5-treated SIGIRR$^{-/-}$ mice (Fig. 6c). We then assessed the effect of i.c.v. injection of IL-1F5 on IL-4 expression in hippocampus of WT and SIGIRR$^{-/-}$ mice and show that IL-1F5 significantly increased IL-4 mRNA and protein in tissue prepared from WT mice ($p < 0.05$; Fig. 6e and f). However, neither IL-4 mRNA nor protein was increased in IL-1F5-treated tissue prepared from SIGIRR$^{-/-}$ mice (Fig. 6e and f) and these values were significantly reduced compared with those in C57BL/6 mice.

Finally, we analysed the effect of IL-1F5 on the LPS-induced IL-1β in WT and SIGIRR$^{-/-}$ mice. LPS significantly increased IL-1β concentration in supernatants of glial cells prepared from WT and SIGIRR$^{-/-}$ mice ($p < 0.05$; Fig. 7), but while co-incubation with IL-1F5 significantly attenuated the LPS-induced increase in IL-1β concentration in glial cells from WT mice ($p < 0.01$), it exerted no significant effect in cells from SIGIRR$^{-/-}$ mice. These data suggest that expression of SIGIRR is required to mediate the effects of IL-1F5 in the brain. Although IL-1β concentration was increased in control-treated and IL-1F5-treated cells prepared from SIGIRR$^{-/-}$, compared with WT, mice, analysis by ANOVA revealed that the increase did not reach statistical significance.

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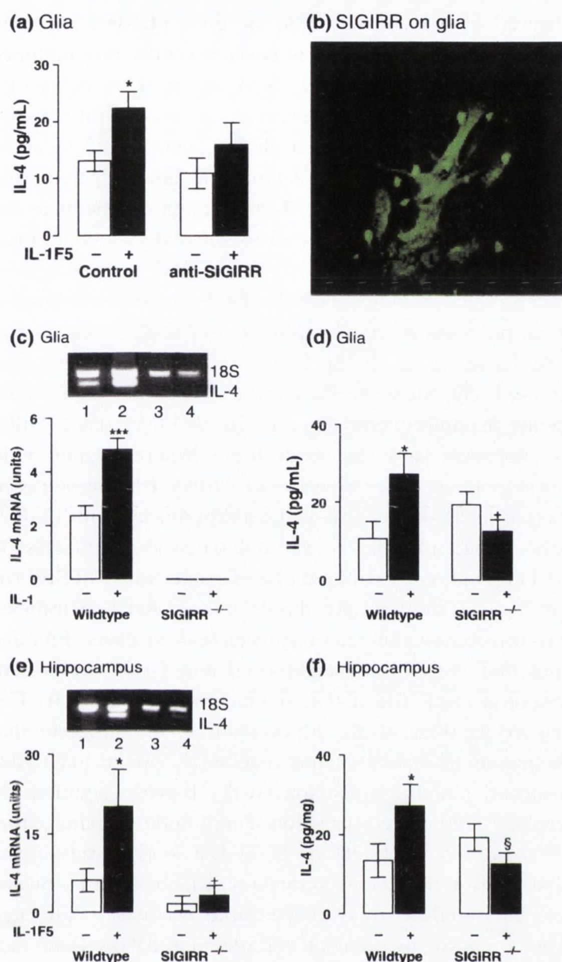

Fig. 6 IL-1F5 fails to induce IL-4 in SIGIRR$^{-/-}$ mice. (a) Mouse glial cells were cultured with IL-1F5 (3 μg/mL) or medium only in the presence or absence of anti-SIGIRR antibody (20 μg/mL). (b) SIGIRR is expressed on glia. (c and d) Mouse glial cells from WT or SIGIRR$^{-/-}$ mice were cultured with IL-1F5 or medium only. After 24 h, IL-4 concentrations in the supernatants were determined by ELISA (a and d) or IL-4 mRNA (and 18S subunit) by PCR (c). Bands on representative gel: wild-type, saline-treated (1); wild-type IL-1F5-treated (2); SIGIRR$^{-/-}$ saline-treated (3); and SIGIRR$^{-/-}$, IL-1F5-treated (4). WT or SIGIRR$^{-/-}$ mice were injected i.c.v. with 5 μL saline or 30 ng/mL of IL-1F5. After 3 h, rats were killed by decapitation, hippocampi were dissected free, homogenized and IL-4 mRNA (e) and IL-4 protein (f) were determined by PCR and ELISA respectively. Bands on representative gel (e) as described for (c). Data are mean ± SEM; $n = 5$–10. $^{*}p < 0.05$ and $^{***}p < 0.001$, IL-1F5 versus control by ANOVA and $^{+}p < 0.05$ and $^{\S}p < 0.05$ versus C57 by ANOVA and Student's *t*-test respectively.

Discussion

The significant new finding of this study is that IL-1F5 mediates anti-inflammatory activity in the brain through the induction of IL-4 following interaction with the orphan receptor, SIGIRR. We demonstrate that IL-1F5 suppresses IL-1β- and LPS-induced inflammatory responses in the brain and highlight an action on hippocampal function *in vivo*. As the effects of IL-1F5 are absent in IL-4$^{-/-}$ mice, it can be concluded that IL-4 mediates this action. The data suggest that interaction of IL-1F5 with SIGIRR is necessary to induce IL-4, because IL-1F5 fails to exert this action in SIGIRR$^{-/-}$ mice.

The evidence presented indicates that IL-1F5 antagonizes the effects of IL-1β and LPS, albeit incompletely, *in vivo* and *in vitro*; this is the first demonstration of such effects. One consistently reported effect of IL-1β is to increase IL-6 (Molina-Holgado *et al.* 2000) and here we show that IL-1β-induced production of IL-6 by cultured mouse and rat glia is attenuated by IL-1F5. This effect was mirrored by a similar change *in vivo*; thus, i.c.v. injection of IL-1F5 antagonized the IL-1β-induced increase in hippocampal IL-6 concentration. Similarly, while i.p. injection of LPS increased IL-1β and IL-6 in hippocampus, as previously reported (Vereker *et al.* 2000; Sparkman *et al.* 2006), i.c.v. injection of IL-1F5 antagonized these effects. LPS-induced IL-1β in hippocampus has been coupled with increased activation of JNK (Vereker *et al.* 2000; Kelly *et al.* 2003; Barry *et al.* 2005), which we confirmed and IL-1F5 also attenuates LPS-induced JNK and p38 activation (not shown), and IL-1β production.

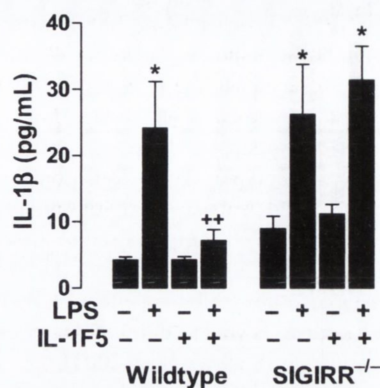

Fig. 7 IL-1F5 attenuates LPS-induced IL-1β in WT but not SIGIRR$^{-/-}$ mice. Glia cells from WT and SIGIRR$^{-/-}$ mice were stimulated with medium only, LPS (100 ng/mL), IL-1F5 (3 μg/mL) or LPS and IL-1F5. After 24 h, IL-1β concentration were determined in supernatants by ELISA. Values are mean ± SEM; $n = 10$. $^{*}p < 0.05$, with versus without LPS; $^{++}p < 0.01$, with versus without IL-1F5 by ANOVA.

We and others have reported that both IL-1β and LPS inhibit LTP in the hippocampus and the evidence indicates that these changes are dependent on downstream activation of JNK and p38 (Curran *et al.* 2003; Kelly *et al.* 2003; Barry *et al.* 2005). Here, we found that i.c.v. injection of IL-1F5 antagonized the IL-1β- and LPS-induced inhibition of LTP. This effect of IL-1F5 is similar to the reported antagonistic effect of IL-1ra on IL-1β-induced inhibition of LTP (Coogan

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and O'Connor 1997; Loscher *et al.* 2000), with which IL-1F5 shares significant homology (Dunn *et al.* 2003). IL-1ra has been shown to attenuate the effects of LPS *in vivo* and *in vitro* in other experimental paradigms. For example, it attenuates the LPS-induced increase in GABA-ergic transmission in organotypic slices (Hellstrom *et al.* 2005) and the LPS-induced enhancement of proinflammatory cytokines in animals which were exposed to inescapable tail shock (Johnson *et al.* 2004). Similarly, IL-1ra has been shown to antagonize the inhibitory effect of IL-1β on LTP and glutamate release, as well as the stimulatory effect of IL-1β on JNK activation (Loscher *et al.* 2003).

It is important to note that IL-1ra attenuates the effects of LPS and IL-1β in cells other than those derived from brain, for example, endothelial cells, hepatocytes, chondrocytes and osteoblasts (Chole *et al.* 1994; Kitade *et al.* 1996; Matsukawa *et al.* 1998; Fernandes *et al.* 2002). However, we were unable to obtain evidence that the antagonistic effect of IL-1F5 extended beyond cells derived from brain tissue. Thus, we observed no effect of IL-1F5 on LPS-induced IL-1β in DC or macrophages, or on the LPS-induced IL-1β or IL-6 production by spleen cells (not shown) although IL-1F5 inhibited the LPS-induced increases in IL-1β (shown here) and similarly inhibited the LPS-induced increases in IL-6 and tumour necrosis factor-α in mixed glia. We must conclude from these findings that the action of IL-1F5 is confined to brain-derived cells and a recent study which assessed the effect of IL-1F5 on LPS-induced IL-1β release in pure cultures of microglia and astrocytes suggest that the effect of IL-1F5 appears to be confined to astrocytes (Watson *et al.*, unpublished data). The present finding that the effects of IL-1F5 are confined to brain may account for the fact that, to date, there are few reports of any action of IL-1F5 and a lack of consistency in the findings of different groups. For example, it was reported that IL-1F5 antagonized IL-1Rrp2-dependent IL-1F9-induced activation of NFκB in Jurkat cells (Debets *et al.* 2001). However, although another group showed that IL-1F9, as well as IL-1F6 and IL-1F8, can activate NFκB, these authors failed to replicate the antagonistic effect of IL-1F5 which they described as inconsistent and incomplete (Towne *et al.* 2004). Interestingly, IL-1F5 also failed to affect the IL-1F8-induced IL-6 production by human articular chondrocytes and synovial cells, which also depends on IL-1Rrp2 activation (Magne *et al.* 2006).

Recent evidence from this laboratory has highlighted the importance of IL-4 in modulating the neuroinflammation associated with age and LPS treatment and IL-4 has been shown to attenuate the deficit in LTP, which is a feature of aged, IL-1β-treated and LPS-treated rats (Barry *et al.* 2005; Maher *et al.* 2005; Nolan *et al.* 2005; Lynch *et al.* 2007); however, we have not measured IL-4 concentrations in hippocampal tissue prepared from these rats following i.c.v. injection. Here, we found that IL-4 mediates the anti-inflammatory effects of IL-1F5. Injection of IL-1F5 increased IL-4 mRNA and protein in hippocampus and stimulation of cultured rat glial cells with IL-1F5 induced IL-4 mRNA and protein expression. In contrast, and consistent with the lack of its antagonist action in peripheral cells, IL-1F5 failed to increase IL-4 production in DC, macrophages or spleen cells, although these cells secreted IL-4 in response to stimulation with phorbol myristic acid and ionomycin. These findings add further support to the thesis that IL-1F5 acts specifically in brain. The data indicate that IL-4 concentrations in brain is low as previously reported (Maher *et al.* 2005; Nolan *et al.* 2005; Lynch *et al.* 2006; Clarke *et al.* 2007) even when increased by eicosapentaenoic acid or atorvastatin, or secretory/excretory products of *Fasciola Hepatica*, which also increases IL-4 (to about the same extent) in bone marrow-derived DCs (Nolan *et al.* 2005). Further evidence of a role for IL-4 was provided by experiments with IL-$4^{-/-}$ mice. We found that IL-1F5 attenuated the LPS-induced IL-1β production by cultured glia prepared from WT, but not IL-$4^{-/-}$ mice. Interestingly, the data show that LPS induced a lesser effect in glia prepared from IL-$4^{-/-}$ mice. We have found that the LPS-induced IL-1β and CD86 and CD40 expression were attenuated in glia prepared from IL-$4^{-/-}$ mice (McQuillan *et al.*, unpublished). This suggests that endogenous IL-4 may exert a regulatory role on microglial activation, inhibiting LPS-induced IL-1β production and co-stimulatory molecule expression. These findings indicate that the anti-inflammatory effects of IL-1F5 in brain rely on its ability to stimulate IL-4 production, which in turn, antagonizes IL-1β-induced and LPS-induced changes. These conclusions are consistent with our previous observations that IL-4 decreases IL-1β and IL-1R1 mRNA and protein (Nolan *et al.* 2005) and that IL-4 mimics the effect of IL-1F5 in blocking IL-1β- or LPS-induced inhibition of LTP, as well as the associated increase in IL-1β-induced signalling (Barry *et al.* 2005; Nolan *et al.* 2005; Lynch *et al.* 2006).

The ability of IL-1F5 to induce anti-inflammatory effects in brain, through induction of IL-4, appears to rely on its interaction with SIGIRR. IL-1F5-induced IL-4 production was blocked by an anti-SIGIRR antibody and IL-1F5 failed to induce IL-4 mRNA or protein in $SIGIRR^{-/-}$ mice *in vivo*. Furthermore, IL-1F5 induced IL-4 mRNA and protein expression in cultured glial cells prepared from WT, but not from $SIGIRR^{-/-}$, mice. In addition, IL-1F5 robustly blocked LPS-induced IL-1β production in glia from WT, but not from $SIGIRR^{-/-}$, mice. These findings provide evidence that the effects of IL-1F5 are mediated through activation of SIGIRR. It should be noted that IL-4 mRNA and protein expression in brain and in glia are low, and the evidence indicates that IL-4 mRNA expression is further reduced in hippocampal tissue prepared from $SIGIRR^{-/-}$ mice (and below detectable levels in the case of glia prepared from mouse brain), although IL-4 protein remains at resting concentrations in preparations obtained from $SIGIRR^{-/-}$

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mice. Expression of SIGIRR in peripheral tissues is widespread (Thomassen *et al.* 1999) and has been observed in brain, albeit to a lesser extent than in other tissues (Wald *et al.* 2003); analysis of 11 discrete brain areas indicated that, although SIGIRR is expressed in hippocampus, its expression is higher in frontal and temporal cortices (data not shown). We also found that SIGIRR is expressed on glia as reported by others (Andre *et al.* 2005) although a previous study failed to detect SIGIRR mRNA in a microglial cell line (Dimcheff *et al.* 2006) and immunohistochemical analysis has indicated SIGIRR staining on these cells. Comparative analysis by others (Garlanda *et al.* 2004) indicated that SIGIRR expression was similar in macrophages and (activated) DCs but these cells do not release IL-4 in response to IL-1F5, although they are capable of releasing IL-4 in response to ionomycin. It is possible that, like the binding of other members of the IL-1 family to their respective receptors, binding of IL-IF5 to SIGIRR requires an accessory protein, which may be expressed on brain cells but not on peripheral cells. Interestingly spliced variants of IL-IRAcP have been recently identified and have been found to be expressed in brain (Jensen and Whitehead 2003; Lu *et al.* 2008).

An anti-inflammatory role has already been ascribed to SIGIRR. Over-expression of SIGIRR in Jurkat and HepG2 cells has been associated with a reduction in IL-1- and IL-18-mediated activation of NFκB (Thomassen *et al.* 1999; Wald *et al.* 2003) suggesting, like other reports (Mantovani *et al.* 2004), that SIGIRR may function as a negative regulator of IL-1 and IL-18 signalling; whereas only 10% of SIGIRR-deficient mice, compared with 70% of WT mice survived a lethal LPS challenge (Wald *et al.* 2003), this was not observed in mice of a different genetic background (Garlanda *et al.* 2004). SIGIRR$^{-/-}$ mice showed exacerbated colitis (Garlanda *et al.* 2004) and were more susceptible to colitis-associated cancer irrespective of the genetic background (Garlanda and Mantovani, unpublished observations). Similarly SIGIRR$^{-/-}$ mice were hyperresponsive to IL-1 injection, with marked induction of chemokines in lung (though not liver), compared with WT, mice (Wald *et al.* 2003). The evidence therefore suggests that SIGIRR negatively regulates LPS- and IL-1-driven events, specifically through its interaction with the receptor complexes (Polentarutti *et al.* 2003; Qin *et al.* 2005).

The present findings suggest that the anti-inflammatory effect of SIGIRR extends to the brain and its importance is highlighted by the observation that the inhibitory effect of IL-1F5 on the LPS-induced IL-1β is dependent on the presence of SIGIRR. The evidence is consistent with the suggestion that a significant downstream effect of SIGIRR activation in brain is induction of IL-4 production. We propose that IL-1F5 is a potent anti-inflammatory cytokine and that its action may be brain-specific and dependent on SIGIRR-mediated induction of IL-4.

Acknowledgements

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1 IL-1F5 is expressed in rat hippocampus. Rats were injected i.c.v with 5 μL IL-1F5 (30 ng/mL) or IL-1β (3.5 ng/mL) or both.

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