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Interaction of T cells and glia in the Central Nervous System

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A Thesis submitted to
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for the degree of
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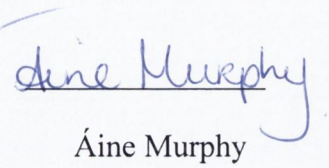

Áine Murphy

Abstract

The central nervous system (CNS) was thought of as an immune privileged site, however, it is now known that the CNS is neither isolated nor passive in its interaction with the immune system. Diseases such as multiple sclerosis (MS), and the animal model for MS, experimental autoimmune encephalomyelitis (EAE), develop through inappropriate immune responses in the CNS. MS is a chronic inflammatory disease, caused by demyelination of axons mediated by IFN-γ-secreting Th1 cells and IL-17-secreting Th17 cells. It is characterised by CNS infiltrates composed of T cells, macrophages and dendritic cells (DC) and associated with the activation of endogenous glia.

This study has examined the interaction of encephalitogenic Th1 and Th17 cells with CNS resident glia *in vitro.* Co-culture of antigen-specific Th1 and Th17 cells with glia leads to the differential production of the proinflammatory cytokines, TNF-α, IL-1β and IL-6 and enhanced expression of MHC class II, CD40, CD80 and CD86 on microglia. This inflammatory response was predominantly mediated by cell-contact between the T cells and glia. However, CD40 and CD80 were found not to mediate this interaction. Peripherally-derived Th2 cells and brain-derived neurons were capable of suppressing the inflammatory response in co-cultures of glia with antigen-specific T cells secreting both IFN-γ and IL-17 termed Th1/Th17 cells. Th2 cells significantly decreased IL-6, IL-1β and IL-17 production in cultures of glia with Th1/Th17 cells and significantly attenuated the expression of CD40, CD80 and CD86 on microglia. The suppressive effect of Th2 cells was partially mediated by IL-4 and IL-10. Neurons also attenuated production of IL-17, TNF-α, IL-1β and IL-6 in co-cultures of Th1/Th17 cells and glia via CD200 ligand-receptor interactions, however, they did not significantly alter the APC function of microglia.

This study demonstrated that infiltration of macrophages into the CNS and the expression of MHC class II, CD40, CD80 and CD80 on resident microglia and infiltrating macrophages was greatest at the onset of clinical symptoms of EAE. Production of TNF-α, IL-6 and IL-1β was enhanced at the onset of clinical symptoms but decreased as phagocytic activity of microglia and macrophages increased. In later stages of disease, irreversible neuronal damaged was found to have occurred.

Antigen presenting cells (APC) such as macrophages and DC infiltrate the CNS during EAE. This study assessed the ability of glia, macrophages and DC to induce T cell cytokine production and stimulate T cell proliferation and found that all APC were able to act as competent APC, albeit at different T cell: APC ratios. In addition, DC, macrophages and glia were found to respond differently to the novel IL-1 family member IL-1F5 demonstrating that the anti-inflammatory effects of IL-1F5 are confined to CNS resident APC.

An inflammatory phenotype exists in the CNS during EAE, therefore, different approaches were used to address the role of anti-inflammatory proteins. Using knock out mice, the immunomodulatory receptor, SIGIRR, and the anti-inflammatory cytokine IL-4, were found to have no endogenous role in controlling EAE. Treatment with rosiglitazone, a stimulator of IL-4 production in the brain, failed to attenuate EAE. However, there appears to be a protective role for IL-4 during EAE in aged animals.

In conclusion, both resident cells and infiltrating peripheral immune cells participate in inflammation in the CNS during EAE. Immunomodulatory cells from the periphery, such as Th2 cells, and endogenous CNS cells, such as neurons, have the potential to ameliorate immune responses in the brain. Further research is required to exploit these findings for therapeutic benefit during EAE.

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Abbreviations The following abbreviations have been used:

ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cells
APC	Allophycocyan
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BCR	B cell receptor
BDNF	Brain derived neurotrophic factor
BMDC	Bone Marrow derived Dendritic Cell
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CD200Fc	CD200 fusion protein
CDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's Adjuvant
CIA	Collagen Induced Arthritis
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
CpG	Cytosine-guanine dinucleotide
CSF	Cerebrospinal Fluid
CTLA-4	Cytotoxic T lymphocyte associated-4
dATP	Deoxyadenosine triphosphate
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACs	Fluorescent activated cell sorting
FCS	Foetal bovine serum
FcR	Fc Receptor

FITC	Fluoresecein isothiocyanate
GABA	Gamma-aminobutyric acid
GDGF	Glial derived growth factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HRP	Horse radish peroxidase
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneally
IRAK	Interleukin-1 receptor associated kinase
JNK	c-jun N-terminal kinase
KLH	Keyhole Limpet Hemocyanin
LFA	Leukocyte function associated antigen
LPS	Lipopolysaccharide
LTA	Lipoteichnoic acid
MAC	Macrophage antigen
MACS	Magnetic cell sorting
MBP	Myelin basic protein
MCP	Monocyte chemoattractant protein
mg	milligram
MHC	Major histocompatability complex
MIP	Macrophage Inflammatory protein
ml	millilitre
MMP	Matrix metalloproteinases
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
Mφ	Macrophage

NBM	Neurobasal medium
NFκB	Nuclear factor-κ B
ng	nanogram
NGF	Nerve growth factor
NK	Natural killer cell
NLR	Nucleotide oligomerisation domain-like receptors
NO	Nitric oxide
NOD	Nucleotide oligomerisation domain
OPD	Ortho-Phenylenediamine
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PGE2	Prostaglandin E2
PLP	Proteolipid protein
PMA	Phorbyl Myristate Acetate
PNS	Peripheral nervous system
PPRs	Pattern recognition receptors
PT	Pertussis Toxin
QPCR	Quantitative Polymerase Chain Reaction
STAT	Signal Transducer and Activator of Transcription
ROS	Reactive Oxygen species
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription- Polymerase Chain Reaction
s.c	subcutaneous
SEM	Standard error of mean
SIGIRR	Single Ig containing IL-1 related receptor
STAT	Signal transducer and activator of transcription
Strep-HRP	Streptavidin-Horseradish peroxidase linked
Tbet	T box expressed in T cells
TCR	T cell receptor

TGF-β	Transforming Growth Factor-β
TIMP-1	Tissue inhibitor of matrix proteinases-1
Th	Helper T cell
TIR	Toll-IL-1-like receptor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
Treg	regulatory T cell
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molcule-1
VLA-4	Very late antigen-4
μg	microgram
μl	microlitre

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Chapter 5 The role of immunomodulatory molecules in EAE

Chapter 1
Introduction

1.1. The Central Nervous System and the Immune system and their interaction

The nervous system and the immune system are two of the most complex and vital systems in the human body. In the 1940s, the central nervous system (CNS) was characterised as an immunologically privileged site. It was thought to be isolated from the immune system by the presence of the blood brain barrier (BBB) suggesting an absence of immunosurveillance within the CNS. Furthermore, the CNS is almost completely devoid of a lymphatic system, and expression of major histocompatibility complex (MHC) class I and II, molecules necessary for antigen presentation, is low in the CNS (Merrill & Jonakait, 1995). The CNS can be infected with pathogens and therefore clearly must have immunological mechanisms to provide defense against pathogens. It is now known that the CNS is neither isolated nor passive in its interactions with the immune system.

Cells of the immune system interact with each other via immunological synapses, in a similar way to neurons in the CNS, and even share some of the same molecules at these synaptic junctions. For example, semaphorins, originally identified as repulsive axon guidance factors functioning in the developing nervous system, have immunological functions. SEMA4D, a class IV semaphorin, is involved in the activation and maturation of innate immune cells and subsequent generation and enhancement of adaptive immune responses (Kikutani & Kumanogoh, 2003). MHC class I, an important molecule for antigen presentation in the immune system, is expressed on developing neurons that are

undergoing structural changes and synaptic modifications (Huh *et al.*, 2000). Studies in mice with deficiency of MHC class I have demonstrated a role for MHC molecules in remodelling and plasticity in the developing and mature mammalian CNS (Huh *et al.*, 2000). In addition, neurotrophins, small molecules released by neurons, have the ability to regulate MHC molecule expression in the CNS (Collawn & Benveniste, 1999). Furthermore, another highly conserved molecule, Toll, long known for its role in the development of the early nervous system in the fruit fly Drosophila *melangoster*, has been recognized as conferring resistance to fungal infection in adult Drosophila *melangoster* (Lemaitre *et al.*, 1996). Toll homologues, known as Toll-like receptors (TLRs), have since been identified in the CNS, and have become one of the most intensely studied families of receptors in both host response to infection and autoimmune disease research.

1.2. The Immune System

The immune system is a vital network of cells, tissues and organs that function to protect an organism from invading pathogens. It has the ability to distinguish between the body's own cells and those of foreign pathogens. The immune system can specifically recognise and respond to these pathogens and the resulting immune response functions to eliminate pathogen from the body. The immune system has evolved into non-specific and specific components, the innate and the adaptive immune system respectively.

Innate immune mechanisms are the body's first line of defence and act immediately by initiating a generalised and rapid response upon encountering a

pathogen. However, recognition is not specific to a particular pathogen and immunological memory is not generated. If a pathogen breaches the early lines of defence, the adaptive immune response is activated, antigen-specific effector cells are generated that target antigen on the pathogen and memory cells are generated, preventing reinfection with the same micro-organism.

1.2.1. The Innate Immune System

Physical barriers such as intact skin and mucous membranes of the body afford a high degree of protection against pathogens. The sebaceous secretions and sweat of the skin contain bactericidal and fungicidal fatty acids and confer protection against many potential pathogens. The mucus membranes of the respiratory tract trap inhaled particles and cilia sweep the secretions, containing the foreign material, toward the oropharynx where they are swallowed; acidic stomach secretions can then destroy the micro-organisms present. Nasal secretions and saliva contain mucopolysaccharides, capable of blocking some viruses, while tears and the mucous secretions of the respiratory, alimentary and genitourinary tracts contain lysozyme that is particularly effective against bacteria. Commensal micro-organisms in the gut and bacteria normally present on the skin provide a further layer of constitutive innate immunity. This normal bacterial flora produce various antimicrobial substances, such as bacteriocines and acids, while at the same time compete with potential pathogens for essential nutrients (Brock, 1998).

Once micro-organisms have breached the immune system's physical barriers of skin and epithelium, the inducible innate immune system is activated and an inflammatory response is initiated. Inflammation contributes to tissue homeostasis by removing dead tissue, promoting the rapid death of damaged cells and wound healing. Inflammation contributes to tissue defense by the production of cytotoxic substances, chemokines for immune cell recruitment and cytokines for coordinating the immune response.

The innate immune system confers protection against a broad spectrum of pathogens, and is activated via pathogen recognition receptors (PRRs), which recognise conserved microbial components, called pathogen associated molecular patterns (PAMPs) (Medzhitov & Janeway, 2002). The principal functions of PRRs include opsonization, activation of complement, phagocytosis, activation of proinflammatory signalling pathways, and induction of apoptosis (Medzhitov & Janeway, 2002). The best-known PRRs are the TLR family and the intracellular nucleotide-binding oligomerization domain (NOD) like receptors (NLR). The NLR family of PRRs are expressed in the cytosol where they detect intracellular pathogens such as viruses and bacteria, and their products, that have gained access to intracellular compartments. The TLRs are a large family of PRRs that detect pathogens either at the cell surface or at lysosome / endosome membranes (Akira, 2006).

Some of the better known examples of PAMPs include lipopolysaccharide (LPS) from Gram-negative bacteria which bind TLR4, lipoteichoic acid (LTA) from Gram-positive bacteria which bind TLR2 and products of which bind NLR1 or NLR2, cytosine-guanine dinucleotide (CpG) motif which bind TLR9, lipoproteins that bind TLR2 and double stranded (ds) RNA, produced by viruses

during their infection cycle, which bind TLR3. These structures allow the innate immune system to distinguish between self and non-self molecules since only particular classes of micro-organisms express PAMPs. PAMPs are often shared by large groups of micro-organisms, this allows a limited number of germline-encoded PRRs to recognize a vast variety of potential pathogens (Medzhitov, 2001). Recognition of PAMPs by PRRs activates conserved host defense signalling pathways that induce activation of inflammatory and antimicrobial innate immune responses (Medzhitov, 2001).

1.2.2. Cells of the Immune System

Cells of the immune system originate from pluripotent haematopoietic stem cells that can be subdivided into myeloid and lymphoid lineages. The myeloid lineage includes granulocytes, mast cells, macrophages and dendritic cells (DC) of the innate immune system.

Polymorphonuclear leukocytes consist of three types of short-lived cells, neutrophils, eosinophils and basophils that are also known as granulocytes due to the presence of cytoplasmic granules. Neutrophils are phagocytic cells that engulf pathogens and internalise them into phagosomes and destroy them. They have membrane bound granules called lysosomes, which contain substances that mediate an antimicrobial response. The phagosome fuses to one or more lysosomes and the lysosomal contents are released to destroy the pathogen. Neutrophils are the most abundant type of phagocyte, accounting for 50-60% of all circulating leukocytes. Most eosinophils are found in tissues and upon

activation they release highly toxic granule proteins, such as RNase, DNase and lipase, and free radicals, which can kill micro-organisms. They also release prostaglandins, leukotrienes and cytokines, which amplify the proinflammatory response. Basophils are present in low numbers in the circulation and have a similar role to eosinophils in defense against pathogens. They release histamine upon activation causing vasodilation allowing for recruitment of other immune cells to the site of infection. Mast cells also differentiate in the tissues, reside near small blood vessels and when activated release substances that affect vascular permeability. They are believed to play a role in protecting mucosal surfaces against pathogens.

Natural killer (NK) cells account for up to 15% of blood lymphocytes and recognise changes on virus-infected cells and destroy them via an extracellular killing mechanism. On binding to infected cells, NK cells produce membrane-disrupting molecules that lead to the destruction of the target cell. This innate defence mechanism occurs without prior exposure to the infectious agent. Using a similar mechanism, NK cells are also thought to play a role in host defence against tumours, recognising changes in the cell membrane of transformed cells (Diefenbach & Raulet, 2002).

The induction of the adaptive immune response begins with the ingestion of a pathogen or its product by an immature DC. DC are specialised phagocytic cells, however, their primary function is not the elimination of pathogen but the presentation of antigen to cells of the adaptive immune system known as T cells. Immature DC internalise pathogens, process them and present antigenic peptides

via MHC class II molecules. Following phagocytosis of a pathogen, or binding of a PAMP to a PRR, DC become activated, migrate to a local lymph node and mature into highly effective antigen presenting cells (APC), expressing high levels of MHC class II and co-stimulatory molecules. DC secrete cytokines that direct the induction of distinct T cell subtypes. DC also induce tolerance by presenting self antigens, in the absence of co-stimulation, which results in incomplete T cell activation.

Monocytes circulate in the blood and differentiate into macrophages upon entering tissues. Macrophages are primarily phagocytic cells that engulf pathogens and destroy them in intracellular vesicles, a function they perform in both innate and adaptive immune responses. Macrophages can also be stimulated to express costimulatory molecules and MHC class II. In this regard, both DC and macrophages, functioning as APC, link the innate and adaptive immune responses.

1.2.3. The Adaptive Immune System

While the innate immune system provides the first line of defence against a wide variety of pathogens, it cannot always eliminate infectious micro-organisms. The lymphocytes of the adaptive immune system have developed a more complex means of antigen specific defence that help to clear infection and provide increased protection against subsequent re-infection with the same pathogen. The cells of the innate immune system, macrophages and DC, play a crucial part in the initiation and direction of adaptive immune responses in

addition to participating in the removal of pathogens targeted by the adaptive immune response.

A common lymphoid progenitor gives rise to lymphocytes of adaptive immunity. There are two major types of lymphocytes, B cells and T cells. These adaptive immune cells mount a targeted and precise attack against a specific invader. B cells and T cells are distinguished by their sites of differentiation, the bone marrow and thymus respectively, as well as the mechanism of their effector response.

B cells protect the extracellular spaces of the body by secretion of antibodies, which target extracellular pathogens. B cells produce immunoglobulins that are inserted into the cell surface membrane where they act as antigen-specific B cell receptors (BCR). Bound antigen is then internalised. Signals from bound antigen, coupled with stimulation from helper T cells, induce the B cells to differentiate into plasma cells and proliferate. Furthermore, B cells can present antigenic peptides via MHC class II to helper T cells. Plasma cells, which are normally restricted to the secondary lymphoid organs and tissues, secrete antibodies that can bind to and neutralise the toxic effects of a pathogen. Antibodies can coat pathogens making them easier to phagocytose, a process known as opsonization, in addition they activate the complement system leading to the destruction of bacterial cells. Finally, antibodies can neutralise virus by preventing binding to their receptors. Most plasma cells only survive for a few days before dying by apoptosis.

T cells are divided into two main classes, cytotoxic $CD8^+$ T cells that kill virus-infected cells and helper $CD4^+$ T cells that activate other cells including B cells and macrophages. $CD4^+$ T cells recognise their specific antigen in association with MHC class II molecules, whereas $CD8^+$ T cells recognise antigen in association with MHC class I molecules.

T cell receptor (TCR) and CD4 or CD8 bind to antigen/MHC class II complexes on the surface of APC but this signal alone does not stimulate naïve T cells to proliferate and differentiation into effector T cells. A second signal is required through binding of costimulatory molecules such as CD80 and CD86 expressed on APC. Binding of CD28 to CD80/CD86 results in clonal expansion of the T cell. Once a naïve T cell is activated, it expresses proteins, such as CD40 ligand, which contribute to sustaining or modifying the costimulatory signal that drives clonal expansion and differentiation. The subsequent cytokine profile of the $CD4^+$ T cell is determined by the cytokine environment that exists during antigen presentation, to which the APC is a major contibutor (Gutcher & Becher, 2007).

Classically, effector $CD4^+$ T cells have been divided into two lineages based on their cytokine profile. T helper (Th) 1 cells, characterised by their production of interferon-gamma (IFN-γ), have evolved to eliminate intracellular pathogens and are potent activators of cell-mediated immunity. Interleukin-12 (IL-12), a proinflammatory cytokine released by DC and macrophages, induces the production of IFN-γ by NK cells and favours the differentiation of naïve T cells into Th1 cells (Trinchieri *et al.*, 2003). Typically, autoimmune diseases of the CNS such as Multiple sclerosis (MS) and the animal model of MS,

experimental autoimmune encephalomyelitis (EAE) were thought to be Th1-mediated diseases since administration of antibodies to IL-12p40 and blocking of Th1 differentiation and proliferation prevented relapse of EAE (Constantinescu *et al.*, 1998). However this hypothesis has been reviewed with the recent discovery of other T cell lineages.

Th2 cells, characterised by IL-4, IL-5 and IL-13 production, eliminate parasitic infections via activation of eosinophils (Greenfeder *et al.*, 2001) and are potent activators of B cell immunoglobulin production (Janeway *et al.*, 2001). The anti-inflammatory cytokine, IL-4 promotes induction of Th2 cells from naïve T cells. Up until the 1990s it was thought that dysregulated Th1 and Th2 responses mediate autoimmunity and allergy respectively, more recently additional $CD4^+$ T cell subtypes have been identified which may contribute to these diseases. These include IL-10 and transforming growth factor-beta (TGF-β) producing regulatory T cells (Treg) and IL-17-producing T cells (Th17).

Tregs can downregulate immune responses via secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. One subset of Tregs are Tr1 cells which secrete IL-10. Development of Tr1 cells from naïve cells is dependent upon IL-10 and IFN-α. IL-10 suppresses T cell responses directly by decreasing T cell production of IL-2, tumour necrosis factor-alpha (TNF-α) and IL-5 and indirectly by inhibiting antigen presentation via decreased MHC class II and costimulatory molecules expression on APC (Mills, 2004). Another subset of Tregs are known as natural Tregs, which are $CD4^+CD25^+$; and comprise 10-15% of $CD4^+$ T cells in human circulation. Natural Treg cells elicit their effects in a

contact dependent antigen-nonspecific manner. They are profoundly immunosuppressive and block $CD4^+$ and $CD8^+$ T cell proliferation (Mills, 2004). The third set of immunosuppressive T cells are Th3 cells, these are mainly muscosal in origin and are activated by mucosal presentation of antigen. Th3 cells secrete TGF-β that can block T cell cytokine production and proliferation. Alterations in function of Treg cells can result in the development of autoimmunity (Viglietta *et al.*, 2004).

Th17 cells are a distinct memory T cell population characterised by production of IL-17 (Aggarwal *et al.*, 2003; Mills, 2008). IL-17, secreted by both $CD4^+$ and $CD8^+$ T cells, has been classed as a proinflammatory cytokine due to its ability to induce many mediators of inflammation including those involved in the proliferation, maturation and chemotaxis of neutrophils (Witowski *et al.*, 2004). Increased levels of IL-17 have been associated with several inflammatory and autoimmune conditions including rheumatoid arthritis (Chabaud *et al.*, 1999) and inflammatory bowel disease (Fujino *et al.*, 2003). The role of Th1 and Th17 cells in the pathogenesis of EAE will be discussed in more detail below.

1.2. The Central Nervous System

The human nervous system is the most complex and versatile achievement of the process of evolution. The nervous system of all animals functions to detect changes in the external and internal environment and to execute the appropriate response in muscles, organs and glands. The capacity for higher functions such as

cognition, learning, memory, self awareness and ultimately intellect and personality increases with ascent up the evolutionary scale.

The nervous system is divided into the peripheral nervous system (PNS) and the central nervous system (CNS). The PNS constitutes the link between the CNS and the periphery of the body, from which it receives sensory information and to which it sends controlling impulses.

There are two main classes of cells in the CNS: neurons and glia. Although they share similarities with all cells, neurons have unique morphological and physical properties, which allow them to perform their specialised function i.e. the transmission of chemical signals between cells by means of chemical transmitters.

Astrocytes are the most numerous type of glia in the brain and play a number of roles including maintenance of tissue homeostasis, provision of metabolic support to neurons and are critical components of the blood brain barrier (BBB). Microglia are the principle immune cells in the brain and share phenotypic characteristics with macrophages. Oligodendrocytes are glial cells responsible for the formation and maintenance of myelin in the CNS. Disruption to the myelin sheath results in neuronal dysfunction as seen in diseases such as MS and EAE.

1.3.1. The innate immune system in the CNS

The innate immune system of the CNS is comprised of astrocytes and microglia that are capable of regulating the initiation and progression of immune responses. They act as both sentinel cells and the first line of defence by

responding to non-specific "danger" signals (Carson & Sutcliffe, 1999). In the normal healthy brain astrocytes play essential roles in providing glia-neurons contact, maintaining ionic homeostasis, buffering excess neurotransmitters (Hansson & Ronnback, 1995), secreting neurotrophic factors and serving as critical cell components of the blood brain barrier (Farina *et al.*, 2007). As components of the BBB, they are in close proximity to endothelial cells and can secrete cytokines. Although not as prominent as microglia, astrocytes play a significant role during infectious and autoimmune diseases in the CNS. Astrocytes become activated in response to immunological challenge (Aloisi, 1999), increase their expression of MHC class I and II in response to IFN-γ (Kraus *et al.*, 1992) and are capable of presenting antigen to T cells (Fontana *et al.*, 1984). Cultured astrocytes have been shown to secrete a range of neurotrophic factors crucial for the survival of neurons such as nerve growth factor (NGF), glial-derived growth factor (GDGF) and ciliary neurotrophic factor (CNTF) (Merrill & Jonakait, 1995). However, activated astrocytes become hypertrophic, increase production of glial fibrillary acidic protein (GFAP) and form glial scars, hindering axonal regeneration (Block & Hong, 2005).

1.3.1.1. Microglia- principal immune cells in the CNS

Microglia are considered to be the resident macrophages of the brain, derived from the myeloid lineage of immune cells and comprise approximately 12% of all brain cells. Microglia express on their surface many of the common macrophage antigens, such as F4/80, CD11b and CD45 (Sedgwick *et al.*, 1991). In mature brains, resting microglia exhibit a ramified morphology, are responsible

for immune surveillance and in contrast to macrophages and DC do not express MHC class I and II in their quiescent state (Perry *et al.*, 1993; Kreutzberg, 1996). Parenchymal microglia can become activated in response to immunological stimuli and brain injury and this activation is accompanied by a morphological change to an amoeboid structure. There are at least two states of microglial activation, activated non phagocytic microglia found in the inflamed CNS and reactive phagocytic microglia observed in areas of brain trauma (Minagar *et al.*, 2002) but it is possible and even likely that transitional states exist. Microglia isolated from the healthy CNS constitutively express the costimulatory molecule CD86, as well as the adhesion molecules, CD40 and intracellular adhesion molecule-1 (ICAM-1) (Carson *et al.*, 1998). Activation of microglia leads to enhanced expression of MHC class II and CD80, molecules necessary for antigen presentation by microglia (Shrikant & Benveniste, 1996). While macrophages express CD45 at high levels, microglia express CD45 at low levels and CD45 has been correlated with the ability to present antigen to naïve T cells in the normal CNS and under pathological conditions (Carson *et al.*, 1998) but this view is not widely held. Thus, the low expression of CD45 on microglia may lead to antigen presentation by microglia being less efficient when compared with macrophages or DC. Therefore, presentation of antigen by microglia may depend upon expression of MHC class II and costimulatory molecules as well as sufficient CD45 expression (Carson & Sutcliffe, 1999).

There is significant debate in the literature relating to whether microglia exert neuroprotective or neurotoxic actions when activated. On the one hand they produce cytotoxic factors, such as superoxide (Colton & Gilbert, 1987) and nitric

oxide (NO) (Chao *et al.*, 1993); and proinflammatory cytokines, such TNF-α (Si *et al.*, 2004) and IL-1β (Liu *et al.*, 2005), in response to immunological stimuli. However, microglia support neuronal survival through release of neurotrophic factors, such as brain derived neurotrophic factor (BDNF) (Elkabes *et al.*, 1996), and the anti-inflammatory cytokine TGF-β (Hurley *et al.*, 1999).

However the production of proinflammatory mediators may be essential in limiting and directing immune responses in the CNS. For example, inhibition of NO production by microglia increased the susceptibility of rats to EAE (Cowden *et al.*, 1998), this was attributed to enhanced proliferation of T cells as a result of NO inhibition. Microglia also release prostaglandins, such as PGE2, when activated. PGE2 can decrease the expression of co-stimulatory and MHC molecules on infiltrating APC, inhibit T cell proliferation and skew the T cell phenotype toward a Th2 phenotype(Hilkens *et al.*, 1995; Lenschow *et al.*, 1996). Even supposedly protective cytokines can have deleterious effects in the CNS, transgenic overproduction of TGF-β increased the severity of inflammation in the CNS due to its chemoattractant properties on macrophages and T cells(Wyss-Coray *et al.*, 1997). Furthermore, Luo and colleagues (2007) demonstrated that production of TGF-β in the CNS creates a permissive environment for the induction of EAE. Microglia release neurotrophins, which promote the development and normal function of neurons and glia (Elkabes *et al.*, 1996). In addition, Elkabes and colleagues (1996) show that neurotrophins promote the proliferation and phagocytic activity of microglia. Phagocytic microglia can exhibit benefical functions by the removal of myelin debris during demyelinating disease, while limited or dysfunctional phagocytosis by microglia has been implicated in neurodegenerative diseases (Napoli & Neumann, 2008). Moreover,

microglia may exert neuroprotective properties during antigen presentation to T cells in the CNS. Microglia express Fas ligand and thus can trigger apoptosis in activated T cells (Bonetti *et al.*, 1997). Therefore, activated microglia can potentially control and limit T cell activation within the CNS.

1.4. Multiple Sclerosis- an autoimmune disease of the CNS

MS is a chronic inflammatory disease of the CNS, caused by demyelination of axons, either directly or indirectly, by cells of the immune system. It is characterised by CNS infiltrates composed of T cells, B cells and macrophages and focal demyelinating plaques within the CNS (Stromnes & Goverman, 2006). MS is a highly heterogenous disease, manifesting in various forms due to complex genetic traits that translate into different immune anomalies and/or increased vulnerability of CNS tissue to inflammatory insult or reduced ability to repair damage (Sospedra & Martin, 2004).

It can develop in many forms, the most common is relapsing-remitting MS which is characterised by isolated disease attacks, followed by remissions (Keegan & Noseworthy, 2002). Over time, the disease can develop into secondary progressive MS, characterised by a slow and continuous course of disease progression and the gradual accumulation of irreversible disability. Primary progressive MS is a third form of MS in which disease progresses from the onset with no remissions and the patient suffers from a steady decline in function (Keegan & Noseworthy, 2002). The fourth type, progressive-relapsing MS, is defined by a progressive disease course from the onset although these patients also experience occasional acute attacks (Noseworthy *et al.*, 2000).

The age of onset of MS is highly variable, but onset of symptoms usually occurs between 20 and 40 making it the most common CNS disorder of young people. MS affects roughly 2.5 million people worldwide (McKeown *et al.*, 2003). The most common clinical symptoms are: monocular visual loss, due to optic neuritis; weakness of the lower limbs, with or without upper limb weakness; sensory loss or paraesthesia of the limbs or trunk; sensory or cerebellar ataxia; cranial nerve symptoms and signs, such as diplopia, facial sensory disturbance, oscillopsia and nystagmus, due to brain stem involvement; bladder and bowel disturbance; and memory and cognitive impairment (Pender, 1995).

The disease develops in genetically-susceptible individuals. The concordance rate for MS in monozygotic twins was found to be 25.9%, much higher than that in dizygotic twins (2.3%) or non-twin siblings (1.9%) (Ebers *et al.*, 1996). The strongest genetic risk factor for MS is associated with some histocompatibility leukocyte antigen (HLA) class II molecules, presumably due to their role as antigen-presenting molecules (Haines *et al.*, 1998). Environmental factors such as viral and bacterial infections have been shown to trigger MS (Goverman *et al.*, 1993). Women with the disease outnumber men by 1.6-2.0:1 suggesting that hormonal variables may be risk factors (Coo & Aronson, 2004). Prevalence rates of the disease have increased with socioeconomic development and have been related to industrialisation, urban living, pollution and changes in diet (Sospedra & Martin, 2004). Another possible factor is the "hygiene hypothesis" which states that delayed exposure to or overall reduction in childhood infections in developed countries leads to skewed immune

responsiveness within the population and an increased propensity to develop autoimmunity and allergy (Sospedra and Martin, 2004).

1.4.1. Inflammation in the CNS

Naïve T cells cannot penetrate the BBB. However, activated T cells migrate across the BBB into the CNS, but only those that recognise CNS antigen persist (Hickey, 1991). Activated T cells express high levels of very late antigen-4 (VLA-4) and leukocyte-function-associated-antigen-1 (LFA-1) that facilitate their transmigration across the BBB (Carson *et al.*, 2006). Activated CNS-specific $CD4^+$ T cells can then chaperone naïve non-CNS-specific T cells across the BBB into the CNS (Krakowski & Owens, 1997). In addition, activation of the peripheral immune system leads to leakiness of the BBB. Systemic production of IFN-γ and TNF-α compromise the integrity of the BBB as a result of their cytotoxic properties mediated by nitric oxide (Chavarria & Alcocer-Varela, 2004). This allows additional lymphocytes, activated macrophages and DC to permeate the CNS. Molecules, such as matrix metalloproteinases (MMPs), facilitate entry of lymphocytes into the CNS by further compromising the BBB (Leppert *et al.*, 2001). MMPs degrade extracellular matrix molecules and are involved in the demyelination process. The development of EAE was found to be associated with a 3-fold increase in total MMP activity in cerebrospinal fluid (CSF), which could be inhibited by a broad MMP inhibitor (Clements *et al.*, 1997). MMP-7 expression was increased 500-fold at the onset of clinical symptoms and in later stages of disease, while the expression of MMP-9 was increased 5 fold at the onset of disease (Clements *et al.*, 1997). Attempts have been made to link the

onset of CNS inflammation with changes in MMP levels. One study showed that MS patients had higher levels of MMP-9 and higher ratios of MMP-9 to its natural inhibitor, tissue inhibitor of matrix proteinases-1 (TIMP-1) than healthy controls (Boz *et al.*, 2006).

1.5. Experimental Autoimmune Encephalomyelitis

Insights into the mechanisms of the complex pathogenesis of MS rely heavily on animal models of CNS demyelination, the most commonly used animal model for MS is EAE. EAE is induced by stimulating an immune response directed against CNS antigens, such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). EAE can be induced by immunisation with these myelin antigens in adjuvant or by adoptive transfer of myelin-specific T cells, both of which result in inflammatory infiltrates into the CNS and demyelination (Stromnes & Goverman, 2006).

Active or passive induction of EAE activates peripheral myelin specific $CD4^+$ T cells which can cross the BBB more efficiently than naïve T cells (Hickey, 1991). Once within the CNS, T cells are stimulated by antigen presented by both CNS resident and infiltrating APC. The myelin-specific T cells are reactivated and an inflammatory cascade ensues. Proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IFN-γ and IL-17 and chemokines, such as MIP-1α, MIP-1β, RANTES, TCA-3, IP-10, MCP-1 are released, further propagating immune cell infiltration, particularly macrophage infiltration, into the CNS leading to disease exacerbation and ultimately demyelination (Sospedra & Martin, 2004).

EAE models have been developed in efforts to mimic the diverse clinical and pathological aspects of MS. The location of the infiltrates within the CNS determines the clinical symptoms manifested in different EAE models (Sobel, 2000). In the majority of mouse EAE models, disease is manifested clinically as ascending flaccid paralysis beginning with limp tail and progressing to hindlimbs then forelimbs (Stromnes & Goverman, 2006). This is known as "classic" EAE, reflecting localised inflammation restricted to the spinal cord. Inflammation during MS takes place in both the brain and the spinal cord and this represents a limitation of this "classic" EAE model (Stromnes & Goverman, 2006). Atypical EAE models vary from the classical model due to differences in genetic background of the animals and different combinations of immunogens, display increased inflammation in the brain, as well as the spinal cord, and thus better resemble MS.

The applications of the EAE model include the elucidation of CNS inflammation, mechanisms of cellular infiltration across the BBB, demyelination and axonal damage within the CNS and actions of immunoregulatory agents. Importantly, EAE models can be used to identify therapeutic targets for MS, however, caution must be employed when extrapolating results to humans since success for these targets is rare in clinical trials (Friese *et al.*, 2006). For example, anti-CD40 ligand was shown to suppress relapsing-remitting EAE by blocking Th1 differentiation and effector function (Howard *et al.*, 1999). Despite its promise in EAE, anti-CD40 ligand was not developed as a potential therapy for MS since human platelets express CD40L and it lead to thromboembolic complications in humans (Kawai *et al.*, 2000). Similarly, blockade of TNF-α by

antibodies to soluble TNF receptors prevents and reverses EAE, however, this approach was discontinued in MS patients as it worsened disease. (Selmaj, 2000). The differences between the animal models and MS patients have been attributed to immune system variations due to different pathogenic challenges and different size, fecundity and longevity (Friese *et al.*, 2006). In contrast to the failure of anti CD40 and TNF-α in MS trials, glatiramer acetate, a synthetic amino acid copolymer, was initially designed to mimic encephalitogenic MBP epitopes, but was found to suppress EAE in several species and it reduces relapses in MS patients by 30% (Johnson *et al.*, 1995).

1.6. Pathogenic T cells in MS and EAE

IL-12 is a heterodimeric molecule comprised of p35 and p40 subunits. IL-12 is secreted by activated macrophages and is a proinflammatory cytokine that activates NK cells to produce IFN-γ. IL-12 and IFN-γ induce the differentiation of $CD4^+$ T cells into Th1 cells. Traditionally MS and EAE have been thought of as $CD4^+$-Th1-mediated diseases. This hypothesis has recently been reconsidered as mounting evidence suggests otherwise. Interestingly, $CD4^+$ T cells are not dominant in MS lesions (Booss *et al.*, 1983; Babbe *et al.*, 2000), in fact $CD8^+$ T cells outnumber $CD4^+$ T cells at all stages of lesion development. In the model of Theiler's murine virus-induced demyelinating disease, $CD8^+$ T cells mediate clinical disease and tissue injury (Rossi *et al.*, 1998). $CD4^+$ Th1 cells may not be purely pathogenic in MS since IFN-$\gamma^{-/-}$ mice display more severe clinical disease compared to wild type mice (Chu *et al.*, 2000). In contrast, mice with impaired IFN-γ production, signal transducer and activator of transcription (STAT)$4^{-/-}$ mice,

are resistant to EAE with minimal inflammatory infiltrates into the CNS (Chitnis *et al.*, 2001). However STAT6-deficient mice, which have a predominantly Th1 phenotype, experience a more severe clinical course of EAE compared with wild type or STAT4$^{-/-}$ mice (Chitnis *et al.*, 2007). As the *in vivo* data from IFN-$\gamma^{-/-}$ mice suggest, treatment of PLP-immunised mice with neutralising antibodies against IFN-γ resulted in exacerbated disease leading to >75% mortality, suggesting that the loss of IFN-γ leads to worsened disease (Langrish *et al.*, 2005). These data suggest that CD4^{+} Th1 cells can both contribute to inflammation but may also have a regulatory role during disease via production of IFN-γ, by suppressing expansion of activated CD4^{+} T cells (Ferber *et al.*, 1996; Krakowski & Owens, 1996)

IL-23, a cytokine composed of the p40 subunit of IL-12 but a distinct p19 subunit, has been implicated as a critical cytokine for autoimmunity in the CNS (Langrish *et al.*, 2005). Cua and colleagues (2003) suggested that the perceived central role for IL-12 in autoimmune inflammation was in fact due to the action of IL-23, which is the crucial factor in this response. Previously IL-12 was thought to mediate disease as administration of antibodies to IL-12p40 prevented relapse of EAE in (PL/J x SJL/J)F1 mice (Constantinescu *et al.*, 1998). In contrast, IL-12p35-deficient mice were susceptible to EAE with similar mononuclear cell infiltration in the spinal cord and demyelination as observed in controls (Becher *et al.*, 2002; Gran *et al.*, 2002). These experiments highlighted the redundancy of IL-12 in the induction of EAE and caused a shift in emphasis to the related cytokine IL-23. Using mice deficient in IL-23 (p19$^{-/-}$), IL-12 (p35$^{-/-}$) and both IL-23 and IL-12 (p40$^{-/-}$), Cua and colleagues (2003) found that only IL-23p19$^{-/-}$ mice and IL-

$12p40^{-/-}$ mice were resistant to EAE and concluded that IL-23 and not IL-12 is essential for the development of CNS autoimmune inflammation.

IL-23 promotes a T cell population from memory T cells characterised by the production of IL-17 and therefore known as Th17 cells (Aggarwal *et al.*, 2003). IL-17, secreted by both $CD4^+$ and $CD8^+$ T cells, has been classed as a proinflammatory cytokine due to its ability to induce many mediators of inflammation, including those involved in the proliferation, maturation and chemotaxis of neutrophils (Witowski *et al.*, 2004). Th17 cells are highly encephalitogenic and are responsible for disease pathology in EAE (Langrish *et al.*, 2005). This was shown in a study in which PLP-specific T cells, expanded in the presence of IL-23 or IL-12, were transferred into naïve mice. The data indicate that both Th1 and Th17 cells were capable of trafficking to the CNS. However, mice that were treated with IL-23-driven Th17 cells developed severe EAE whereas mice that received IL-12-driven Th1 cells did not. The number of Th17 cells in the CNS was directly correlated with disease severity. Administration of anti-IL-17 antibodies resulted in partial protection from EAE, consistent with findings that IL-17-deficient mice are resistant to EAE (Horai *et al.*, 2000). In MS, increased expression of IL-17 has been detected in brain lesions (Lock *et al.*, 2002) and in mononuclear cells isolated from blood and CSF (Matusevicius *et al.*, 1999).

While IL-23 is known to induce IL-17 production from memory T cells (Aggarwal *et al.*, 2003), it is not sufficient to induce the differentiation of Th17 cells from naïve T cells *in vitro*, implying a role for other factors in the

development of this T cell subset (Veldhoen *et al.*, 2006). Veldhoen and colleagues (2006) established that TGF-β and IL-6 were involved in the differentiation of naïve $CD4^+$ T cells to IL-17 producing cells, a process that is amplified by IL-1β and TNF-α. Furthermore, IL-21, a cytokine produced by Th17 cells, in combination with TGF-β, can induce the differentiation of Th17 cells (Korn *et al.*, 2007). Other studies show that IL-1 and IL-23 also promote IL-17 production by T cells (Sutton *et al.*, 2006). Consistent with a requirement for IL-1 in the induction of IL-17 is the finding that IL-23 alone did not induce IL-17 production from T cells in $IL\text{-}1R^{-/-}$ mice (Sutton *et al.*, 2006). Bettelli and colleagues (2008) recently proposed that the role of IL-23 is to stabilise and maintain the Th17 phenotype.

There are conflicting reports in the literature as to whether Th17 cells represent a subset of T cells distinct from Th1 cells. Bettelli and colleagues (2005) proposed that Th1 and Th17 cells are of the same T cell lineage (Bettelli & Kuchroo, 2005) and that, unlike Th1 cells, Th17 cells are reported to produce low levels of IFN-γ (Aggarwal *et al.*, 2003). Based on the idea that IL-23 was the main promoter of Th17 cells, Bettelli and colleagues (2005) argued that since IL-23 does not activate naïve T cells to induce Th17 cell differentiation and the IL-23R is only expressed on activated and memory T cells (Langrish *et al.*, 2005), it is unlikely that Th17 cells arise from unique precursors contained in a naïve T cell population but emerge from previously activated T cells. In contrast, Harrington and colleagues (2006) report that Th17 cells develop from a distinct lineage of T cells to Th1 cells. Previously, they found that IL-17-producing cells can be induced from naïve precursors by the addition of IL-23 with concurrent

neutralisation of IFN-γ (Harrington *et al.*, 2005), suggesting that Th1 cells could inhibit Th17 cell development and were unlikely to be of the same lineage. TGF-β promotes the development of Th17 cells (Veldhoen *et al.*, 2006) while inhibiting Th1 and Th2 cell generation (Li *et al.*, 2006). Harrington and colleagues (2006) propose that TGF-β establishes Th17 lineage commitment and prevents Th1 development, confirming that Th17 cells are distinct from Th1 cells. T-bet is a key regulator of the Th1 lineage and although T-bet$^{-/-}$ mice are resistant to EAE, generation of Th17 cells in these mice was unimpaired (Park *et al.*, 2005). This finding suggests that, although T-bet is a factor critical for development of Th1 cells, it is not required for Th17 cell generation and implies that Th1 cells and Th17 cells develop independently. In contrast, another study reported that antigen re-stimulation of Th17 cells upregulated T-bet expression in these cells, which resulted in a progression from a Th17 to a Th1 cytokine profile (Mathur *et al.*, 2006). Mathur and colleagues (2006) concluded that Th17 cells are transient in nature and revert to a Th1 phenotype when re-stimulated. Therefore, whether Th17 cells are a subset of T cells distinct from Th1 cells requires further elucidation.

IFN-γ has a dual pathogenic and protective role in EAE. IFN-γ may be protective due to its ability to inhibit Th17 cell development (Harrington *et al.*, 2005) since co-culturing of IFN-γ-deficient APC and CD4^{+} T cells enhances production of IL-17. Harrington and colleagues (2005) assessed the effect of IFN-γ on IL-23R expression on Th17 cells and found that IFN-γ treatment reduced IL-23R expression on Th17 cells by 41% but neutralising IFN-γ during IL-23

stimulation of $CD4^+$ T cells resulted in a 2.5-fold increase in IL-23R expression. Therefore, IFN-γ can alter the responsiveness of Th17 cells to IL-23.

Most recently, it has been demonstrated that both Th1 and Th17 cells may have distinct pathogenic roles in EAE. Kroenke and colleagues (2008) found that adoptive transfer of IL-12p70- or IL-23-polarised T cells resulted in clinically indistinguishable disease. However, it was observed that IL-12p70-driven disease was characterised by macrophage-rich infiltrates and enhanced inducible nitric oxide (iNOS) expression, whereas neutrophils and granulocyte-colony-stimulating factor were prominent in IL-23 driven lesions. In addition, it was found that monocyte-attracting chemokines CXCL9, 10 and 11 were preferentially expressed in the CNS of mice injected with IL-12p70-driven T cells, whereas the neutrophil-attracting chemokines CXCL1 and CXCL2 were enhanced in the CNS of mice given IL-23-polarised T cells (Kroenke *et al.*, 2008). Consistent with a role for both Th1 cells and Th17 cells in EAE, Stromnes and colleagues (2008) report that inflammation during EAE is focused in the brain when Th17/Th1 ratios are high, whereas a wide range of Th17/Th1 ratios induced inflammation in the spinal cord. An additional study by O'Connor and colleagues (2008) suggests that Th1 cells are capable of permeating the non-inflamed CNS, initiating inflammation and facilitating the entry of Th17 cells. Interestingly, Kroenke and colleagues (2008) propose that IL-12p70-driven EAE more closely resembles relapsing remitting MS, owing to the prominence of macrophages and the absence of neutrophils in the CNS infiltrating population. While the relative role of Th1 and Th17 cells in EAE is still being debated, the most likely scenario is that both T cell subsets contribute to CNS inflammation and autoimmunity.

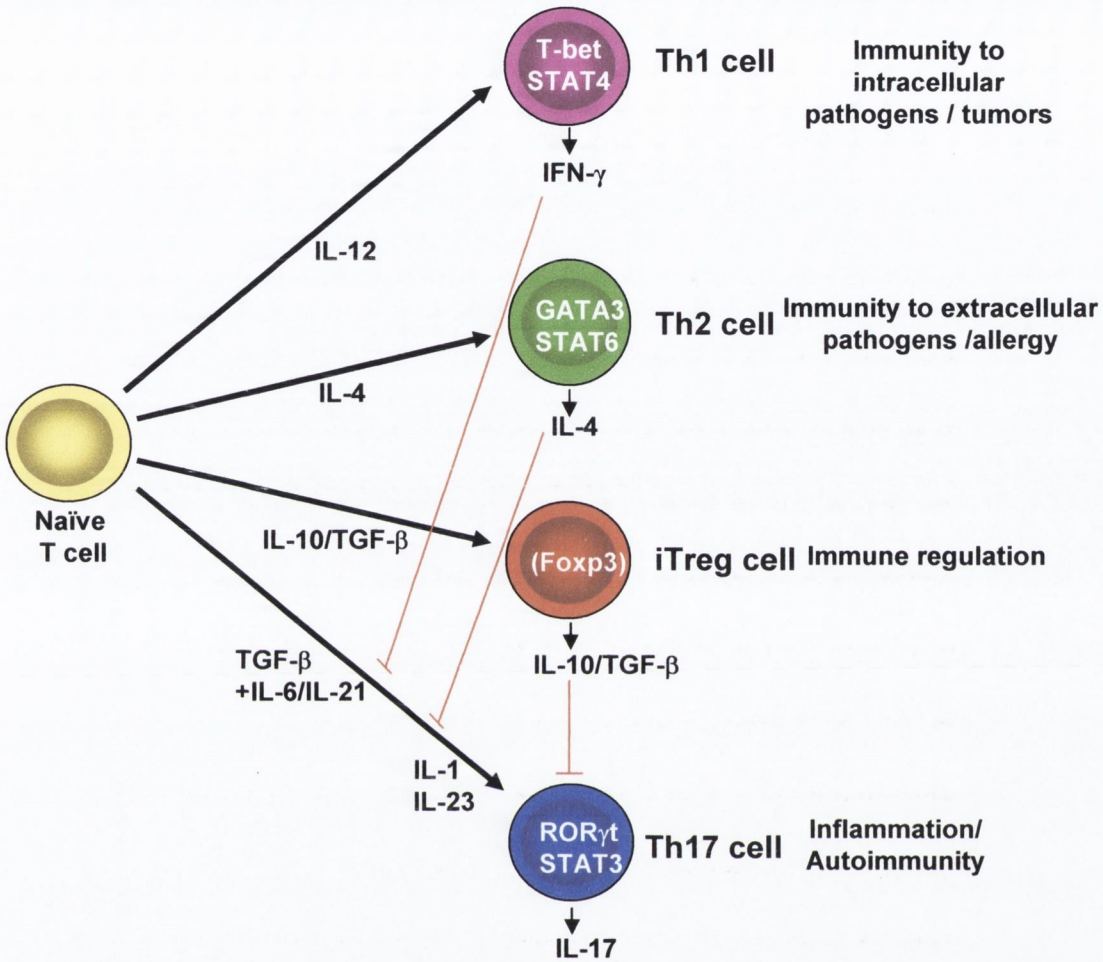

Figure 1. Subtypes of Th cells- their differentiation and function.
Adapted from Mills, K. H. G. (2008). IL-12 enhances the expression of T bet and STAT-4 in naïve T cells and promotes the development of Th1 cells, which secrete IFN-γ and mediate immunity to intracellular pathogens and tumours. IL-4 enhances expression of GATA-3 and STAT-6, which promotes the development of Th2 cells, which secrete IL-4, IL-5, IL-10 and IL-13 and mediate immunity to extracellular pathogens, but also enhances allergy. IL-10 and TGF-β promote induction of inducible Treg (iTreg) cells, which may or may not express Foxp3. TGF-β with IL-6 or IL-21 enhances the expression of RORγt and promote development of Th17 cells, their expansion is enhanced by IL-1β and IL-23. Th17 cells secrete IL-17, as well as IL-21 and IL-22, and are pathogenic in many organ specific autoimmune diseases. IL-4 and IFN-γ control the development of Th17 cells.

1.7. Antigen presenting cells in the CNS

A population of cells, displaying a $CD45^{hi}$ phenotype, permeate the CNS and locate in the perivascular regions, meninges, choroid plexus and the subarachnoid spaces. This population consists of pericytes, macrophages and DC, which can be rapidly replaced by bone marrow derived cells (Carson *et al.*, 2006). Hickey and Kimura (1988) found that induction of EAE required the expression of MHC class II on invading peripheral APC and suggested that MHC class II expression on microglia was not necessary for the initiation of EAE. This was the first study to suggest that peripheral and not CNS endogenous APC were necessary and sufficient for CNS autoimmunity.

Using a mouse germline in which MHC class II expression is restricted to $CD11c^+$ cells, a population mainly consisting of DC, Greter and colleagues (2005) found that antigen presentation by CNS-infiltrating DC was sufficient to initiate development of EAE. At the onset of EAE, $CD11c^+CD11b^+$ cells within the CNS have been found to express high levels of MHC class II, CD80 and CD86, consistent with a mature phenotype and competence as an APC (Deshpande *et al.*, 2007). $CD11c^-$ $CD11b^+$ macrophages and microglia express MHC class II and other co-stimulatory molecules but not to the same extent as $CD11c^+$ cells. A comparison of the ability of $CD11c^-$ microglia, $CD11c^-$ macrophages and $CD11c^+$ DC to act as APC to promote proliferation of CNS antigen specific T cells revealed that DC were the most effective. Microglia were less effective than infiltrating DC and macrophages in driving T cell proliferation and this has been linked to microglial production of prostaglandins and NO (Carson & Sutcliffe,

1999). Prostaglandins and NO inhibit T cell proliferation, but also decrease the expression of MHC class II and co-stimulatory molecules on APC infiltrating the CNS. Prostaglandins can also shift T cell differentiation toward a Th2 phenotype (Hilkens *et al.*, 1995). Thus, it has been suggested that microglia may be more effective at promoting short self limiting T cell responses (Carson *et al.*, 2006). Microglia also play a role in terminating T cell responses within the CNS and in promoting clinical remission during EAE. Magnus and colleagues (2005) found that exposure of microglia to IFN-γ enhanced expression of the inhibitory co-stimulatory molecule B7-H1 thus inhibiting proinflammatory T cell responses.

It is clear that both resident and CNS infiltrating cells have vital roles in mediating neuroinflammation and that CNS autoimmunity is the result of integration of numerous and potentially conflicting effector functions. A study of Huitinga and colleagues (1993) demonstrated that blockade of immune cell infiltration into the CNS and inhibition of their interactions with CNS resident cells had a protective effect in EAE. In this study, intravenous administration of antibodies to MAC-1, β2 integrins involved in the transmigration of monocytes across the BBB, significantly suppressed disease severity in EAE. The proposed therapeutic effect appeared to be mediated by blockade of macrophage infiltration into CNS lesions (Huitinga *et al.*, 1993).

1.8. T cell interaction with microglia

Once peripherally primed $CD4^+$ T cells have crossed the BBB and invaded the CNS, the degree of their activation and polarisation is likely to be influenced

by the CNS microenvironment. In active lesions of MS, microglia exhibit more activated morphology with increased expression of MHC class II and the adhesion/co-stimulatory molecules CD54, CD40, CD80 and CD86 (Cannella et al., 1995; Cannella & Raine, 1995) indicating a role for microglia as APC. The activated state of microglia suggests that they may interact with invading autoreactive T cells.

Soluble molecules secreted by T cells can affect microglial activation (Seguin *et al.*, 2003). Seguin and colleagues (2003) found that treatment of human microglia with supernatants from MBP-specific Th1 cells increased the expression of MHC class II, CD80, CD86, CD40 and CD54 and increased secretion of the cytokines TNF-α and IL-6 and the chemokine CXCL10/IP-10. However these effects were not mediated by IFN-γ alone, as treatment of microglia with IFN-γ was insufficient to induce these changes and an anti-IFN-γ antibody did not block the effects of Th1 supernatant. Seguin and colleagues (2003) also found that supernatant from Th2 cells did not alter co-stimulatory/adhesion molecule expression, cytokine/chemokine production and failed to abrogate the effects of Th1 supernatants on microglia. In a complementary study in rodents by Aloisi and colleagues (2000), it was found that Th1 cells, but not Th2 cells, enhanced expression of MHC class II, CD40 and CD54 molecules on microglia. In contrast to the study by Seguin and colleagues (2003), it was found that exposure of microglia to IFN-γ *in vitro* enhanced expression of MHC class II, CD40 and CD54 (Aloisi *et al.*, 2000a). Substantial Th2 activation by microglia was only triggered after microglia were exposed to IFN-γ (Aloisi *et al.*, 2000a). Granulocyte macrophage colony stimulating factor

(GM-CSF) is another T cell derived mediator of microglial activation. GM-CSF production by encephalitogenic T cells was found to upregulate CD45, MHC class II, CD40 and CD86 expression on microglia (Ponomarev *et al.*, 2007). This study illustrated that production of GM-CSF by T cells and the subsequent activation of microglia was required for the induction of EAE. Taken together, these data indicate that signals delivered from Th1 $CD4^+$ T cells induce the activation of microglia into mature and competent APC that can sustain T cell responses within the CNS.

Cell-cell contact between T cells and microglia may be required for activation of cytokine production from microglia and enhanced expression of activation markers on microglia (Chabot & Yong, 2000; Dasgupta *et al.*, 2002; Giuliani *et al.*, 2005). Chabot and colleagues (2001) found that the interaction of T cells and microglia *in vitro* results in significant production of TNF-α, IL-12, IL-10 and IL-4 compared with either cell population in isolation, with the greatest increase observed in TNF-α production. Cell contact was required for significant production of TNF-α and IL-10 with soluble factors mediating a minor, if any, role in cytokine induction. This report demonstrated that production of TNF-α generated from microglia–T cell co-cultures was dependent on interactions that involve at least three arms, namely VLA-4–VCAM-1, CD40L–CD40, and CD28-CTLA-4-B7 (Chabot *et al.*, 2001).

Dasgupta and colleagues (2002) found that MBP-primed T cells triggered the expression of iNOS in microglia in a cell contact dependent manner mediated by VLA-4. Pretreatment of MBP-primed T cells with IFN-β, a cytokine used in

the treatment of MS, was found to decrease T cell expression of VLA-4 and thereby inhibited the ability of the T cells to induce iNOS expression in microglia. In the CNS, CD40 is expressed on a variety of cells, including vascular endothelial cells, smooth muscle cells, astrocytes and microglia. Interaction between CD40L present on infiltrating T cells and CD40 on microglia and other resident CNS cells triggers a series of intracellular signalling events that promote the production of a wide array of cytokines, chemokines and neurotoxins (Chen *et al.*, 2006). Minocycline can attenuate T cell mediated microglial activation via its ability to downregulate expression of CD40L on T cells (Giuliani *et al.*, 2005). Pretreatment of T cells with minocycline before co-culture with microglia resulted in decreased TNF-α production, with an increase in IL-10 production, while decreasing clustering of T cells around microglial cells. Previously, CD40 was demonstrated to have a role in EAE and MS (Gerritse *et al.*, 1996). Activated CD40L expressing T cells were found in the brain of MS patients and $CD40L^+$ cells were colocalised with $CD40^+$ cells in active lesions. Furthermore, studies *in vivo* showed that administration of an anti-CD40L monoclonal antibody prevented the development of EAE (Gerritse *et al.*, 1996). Ponomarev and colleagues (2006) found that in the presence of microglia lacking CD40 expression, activated T cells cannot enhance the expression of CD45, MHC class II and CD86 on microglia *in vivo*. Fewer T cells and peripheral macrophages accumulated in the CNS due to the lack of CD40 expression on microglia. Th1 cells induce CD80 expression on microglia but downregulate constitutive expression of CD86 (Wolf *et al.*, 2001). This study suggested that T cells are able to modulate CD80 and CD86 expression on microglial cells in the brain. Taken together, these data suggest an important role for T cell-microglial cell interaction in MS and EAE disease pathology.

1.9. Neuronal interaction with immune cells

Neurons are not merely passive targets that are vulnerable to attack from activated immune cells such as microglia and encephalitogenic T cells. Neurons also have the ability to regulate microglial and T cell activation. Thus a variety of neuronal signalling molecules actively control microglial function suggesting that neurons may be key immune modulators in the brain.

Activated microglia can mediate both neurotoxicity and neuroprotection. Glutamate serves as a signal for microglial activation (Farber & Kettenmann, 2005) and activation of glutamate receptors on the surface of microglia triggers the release of TNF-α (Noda *et al.*, 2000). Excessive neuronal release of glutamate leads to the activation of microglia, excitotoxicity and ultimately neurodegeneration (Farber & Kettenmann, 2005). An example of the neuroprotective effect of activated microglia is provided in a study, where selective ablation of proliferating microglia during middle cerebral artery occlusion resulted in an increase in infarct size and in the number of apoptotic neurons (Lalancette-Hebert *et al.*, 2007).

There is cumulative evidence to suggest that neuronal-microglial communication does occur following neuronal damage, which initiate activation of microglia. Peripheral transection of the facial nerve has profound effects on glia in the CNS at the corresponding innervation site of the lesioned nerve. After transection of the facial nerve, microglia, but not astrocytes, proliferate, become hypertrophic, express MHC class I and II and CD80, and secrete TNF-α

(Kreutzberg, 1996). Microglia can also respond to neuronal activity and express receptors for neurotransmitters, such as GABA, noradrenaline and dopamine, allowing neurotransmitters to regulate microglial function (Farber *et al.*, 2005).

Healthy neurons constitutively express high levels of fractalkine, a chemokine also known as CX3CL1 that exists as a membrane-bound and soluble protein. Its corresponding receptor CX3CR1 is expressed on microglia. In rat microglial cultures, LPS-induced TNF-α secretion was partially blocked by fractalkine (Zujovic *et al.*, 2000). The neurotoxic effect of activated microglia on cultures of neuronal hippocampal cells was enhanced by the addition of an anti-fractalkine antibody (Zujovic *et al.*, 2000). Activation of the fractalkine receptor CX_3CR1, before or after glutamate insult, confers neuroprotection against excitotoxicity (Limatola *et al.*, 2005). These results suggest that fractalkine can act as an anti-inflammatory chemokine through its ability to control and suppress certain aspects of microglial activation. *In vivo* data also demonstrates a neuroprotective role for fractalkine. Cardona and colleagues (2006) found that CX3CR1 deficiency was associated with neuronal cell death after systemic LPS challenge in $CX3CR1^{-/-}$ mice. Additionally, these mice displayed exacerbated neuronal cell loss in a toxin-induced model of Parkinson disease and in a model of genetic motor neuron disease (Cardona *et al.*, 2006).

It has been shown that interaction of CD200, a membrane glycoprotein, with its receptor, modulates myeloid cell activity by direct cell-cell contact. CD200 and its receptor are structurally related, with the receptor having a longer cytoplasmic tail containing three tyrosine residues (Wright *et al.*, 2003). The

CD200R is expressed on the surface of human and mouse myeloid cells, such as microglia, macrophages, DC, neutrophils and mast cells as well as T cells (Wright *et al.*, 2003). Neurons express CD200 and have the potential to interact with and regulate CD200R-expressing microglia. Hoek and colleagues (2000) found that microglia in wild type mice are quiescent and express MHC class I and II, CD11b and CD45 at low or negligible levels, whereas, microglia derived from $CD200^{-/-}$ mice exist in a more activated state exhibiting a less ramified morphology, shorter glial processes and a disordered arrangement. This morphology was accompanied by increased expression of CD11b and CD45 on microglia, especially in the spinal cord. Microglia were also shown to aggregate in $CD200^{-/-}$ mice, a phenomenon not seen in the healthy CNS but observed in times of inflammation and neurodegeneration. Hoek and colleagues (2000) also investigated the role of microglia in $CD200^{-\backslash-}$ mice in autoimmune disease and found that onset of EAE occurred 3 days earlier in the $CD200^{-/-}$ mice compared to the wild type controls. $CD200^{-/-}$ mice displayed greater microglial and macrophage activation throughout the CNS during EAE.

CD200 also has an important role in other autoimmune diseases. Collagen induced arthritis (CIA), a model for rheumatoid arthritis, is an inflammatory autoimmune disease sharing the characteristic of tissue specific influx of T cell, macrophages and granulocytes seen in EAE. C57BL/6 mice are resistant to CIA after a single immunisation with collagen, whereas a single immunisation induced disease in 50% of $CD200^{-/-}$ mice (Hoek *et al.*, 2000). Consistent with this finding, Gorczynski and colleagues (2002) found that infusion of anti-CD200R and/or CD200 fusion protein (CD200Fc) resulted in the amelioration of CIA with a

concomitant decrease in serum levels of TNF-α and IFN-γ and anti-collagen IgG1 and IgG2a. Similiarly *Toxoplasma encephalitis* in $CD200^{-/-}$ mice led to increased proliferation and activation of microglia, as illustrated by an increase in MHC class II expression, TNF-α secretion and iNOS induction (Deckert *et al.*, 2006). This increased microglial activation resulted in reduced intracerebral parasite plasma burden and increased survival.

There is evidence to suggest that T cells can act directly on neurons and mediate neurotoxicity. Giuliani and colleagues (2003) demonstrate that activated T cells align along the axons and soma of cultured human neurons leading to substantial neuronal death. T cells must be activated for this process to occur and cell contact dependent mechanisms involving FASL, LFA-1 and CD40 are employed to mediate neurotoxicity (Giuliani *et al.*, 2003). $CD4^+$ and $CD8^+$ T cells were found to be equally neurotoxic and this vulnerability to cell death was neuron-specific, since T cells did not kill oligodendrocytes and astrocytes. The direct targeting of neurons by T cells could therefore result in irreversible damage in the CNS.

Neurons are not without their own protective mechanisms against encephalitogenic T cells. Intracellular adhesion molecule (ICAM)-1, expressed on endothelial, epithelial and glial cells (Lee & Benveniste, 1999), has been shown to promote the activation of T cells through binding of its receptor, LFA-1. ICAM-5, another member of the immunoglobulin superfamily, is only expressed on neurons, and activated T cells promote the cleavage of ICAM-5 from the surface of neurons, which results in the formation of shedded ICAM-5 (sICAM-5) (Tian

et al., 2008). sICAM-5 suppressed the TCR-mediated activation of T cells by decreasing the expression of CD69, CD40L and CD25 on naïve T cells. Furthermore, sICAM-5 promoted the expression of TGF-β and IFN-γ, but not TNF-α. This data suggests that neurons can restrict the activation of antigen-specific T cells that infiltrate the brain.

The crucial role of neurons in regulating the T cell response in the CNS is illustrated by the finding that neurons can induce the proliferation of activated $CD4^+$ T cells that have entered the CNS through CD80/CD86-CD28 interactions and TGF-β signalling pathways (Liu *et al.*, 2006). The interaction between neurons and T cells results in the generation of $CD25^+TGF\text{-}\beta1^+CTLA\text{-}4^+FoxP3^+$ T regulatory cells from committed encephalitogenic T cells. These Treg cells are capable of suppressing encephalitogenic T cells and ameliorating EAE. Interestingly, the neuron-T cell interaction leads to increased neuronal survival, although the mechanism remains to be elucidated (Liu *et al.*, 2006).

1.10. The role of cytokines in the pathogenesis of MS and EAE

Activation of microglia, macrophages, DC and encephalitogenic T cells within the CNS during EAE leads to the production of both proinflammatory cytokines, such as TNF-α, IL-6, IL-1β, IFN-γ, IL-12 and IL-23 and the anti-inflammatory cytokines TGF-α, IL-10 and IL-4.

1.10.1. TNF-α

TNF-α is produced in two biologically active forms, soluble and membrane-associated, both of which are made up of three identical subunits and are able to bind two receptors, TNF-R1 (p55) or TNF-R2 (p75). TNF-α is strongly produced by activated macrophages but is also expressed by T cells, NK cells, mast cells, endothelial cells, fibroblasts, astrocytes and microglia (Chung & Benveniste, 1990; Vassalli, 1992). TNF-α potently induces CNS inflammation by increasing antigen presentation, promoting astrocytic proliferation and affecting chemokine and adhesion molecule expression, thereby regulating cell trafficking to the CNS (Sedgwick *et al.*, 2000; Selmaj, 2000). TNF-α may also directly mediate demyelination, as it has been shown that TNF-α can induce apoptosis of oligodendrocytes *in vitro* (Selmaj & Raine, 1988). The exact role of TNF-α in MS and EAE has proven difficult to elucidate. TNF-$\alpha^{-/-}$ mice displayed a significant delay in onset and duration of EAE (Korner *et al.*, 1997). The number of infiltrating cells into the CNS was unaffected, however, the pattern of infiltration was changed dramatically by TNF-α deficiency with leukocytes clustered in perivascular regions and failing to extend into the brain parenchyma. This suggested that TNF-α has a critical role in trafficking of cells into the CNS. MS patients have elevated numbers of peripheral blood mononuclear cells (PBMCs) that secrete TNF-α (Ozenci *et al.*, 2000). Consistent with this, TNF-α concentrations are elevated in actively demyelinating lesions compared with inactive or remyelinating lesions (Bitsch *et al.*, 2000). Surprisingly, treatment with a soluble TNF-α receptor Ig fusion protein or anti-TNF-α exacerbates MS (van Oosten *et al.*, 1996). Deficiency in either TNF-α or TNF-R2 prevented

oligodendrocyte progenitors from repairing myelin damage, this finding points to an additional role for TNF-α in promoting remyelination (Arnett *et al.*, 2001). With such conflicting data, it appears that the role of TNF-α in MS and EAE is multifaceted and further work is required to define its precise involvement.

1.10.2. IL-6

IL-6 is a pleiotropic cytokine that contributes significantly to inflammation and autoimmunity. IL-6 is significantly upregulated in the blood, CSF and brain tissue of MS patients (Maimone *et al.*, 1991). Studies of EAE carried out in IL-$6^{-/-}$ mice demonstrate that IL-6 is important in the initiation of the disease (Okuda *et al.*, 1999). Consistently, IL-$6^{-/-}$ mice are resistant to MOG_{35-55} induced EAE. VCAM-1 and ICAM-1 expression is not enhanced on CNS endothelial cells in IL-$6^{-/-}$ mice with EAE, suggesting that IL-6 is an important factor in encephalitogenic T cells gaining access to the CNS (Eugster *et al.*, 1998). In addition, lymph node cells from IL-$6^{-/-}$ mice produce significantly less MOG-specific IFN-γ and more IL-4 and IL-10 demonstrating that a lack of IL-6 results in immune deviation toward a regulatory phenotype (Okuda *et al.*, 1999). The role of IL-6 in EAE is restricted to the initiation phase of disease, with little influence on the effector phase, as IL-$6^{-/-}$ mice are susceptible to passive transfer of EAE with wild type donor cells (Okuda *et al.*, 1999).

1.10.3. IL-1β

IL-1β is a proinflammatory cytokine that is secreted by activated macrophages and microglia. It activates T cells, vascular endothelium and increases the access of effector cells to the CNS. IL-1β is involved in local tissue destruction and systemically it induces fever and IL-6 production (Janeway *et al.*, 2001). In MS, IL-1β levels in the CSF correlate with disease activity and the balance between IL-1β and IL-1 receptor antagonist (IL-1ra) is associated with disease severity, susceptibility and/or progression (de Jong *et al.*, 2002). Blocking the effects of IL-1β reduced neuronal loss and inflammation induced by experimental insults (Loddick *et al.*, 1997). Chronic administration of IL-1 into the striatum resulted in marked recruitment of neutrophils, vasodilation, breakdown of the BBB followed by microglial and astrocyte activation and extensive reversible demyelination (Ferrari *et al.*, 2004).

1.10.4. IFN-γ

There are conflicting data in the literature regarding the role of IFN-γ in the pathogenesis of MS and EAE. IFN-γ is the prototypic Th1 cytokine, produced by T cells and NK cells, primarily affecting macrophage and DC function via upregulation of MHC class II, co-stimulatory and adhesion molecules. IFN-γ can also activate NK cells and inhibit Th2 cell proliferation thereby promoting a Th1 phenotype (Janeway *et al.*, 2001). IFN-γ drives the production of proinflammatory cytokines and NO which is known to be directly involved in destruction of myelin in MS and EAE (Sherman *et al.*, 1992). IFN-γ also exerts its affects on microglia

and astrocytes by upregulating their antigen presenting capabilities and cytokine and chemokine production (Aloisi *et al.*, 1998; Olson *et al.*, 2001). Patients with MS have higher numbers of PBMCs expressing IFN-γ messenger ribonucleic acid (mRNA) than normal individuals (Hohnoki *et al.*, 1998). In EAE, IFN-γ mRNA levels in the CNS correlate with disease severity, the highest levels are observed just before peak of acute disease and at the height of subsequent relapses (Begolka *et al.*, 1998). Therefore, it was hypothesised that blocking IFN-γ would be therapeutic in EAE and possibly in MS. However, treatment with a neutralising antibody to IFN-γ increased the severity of EAE (Billiau *et al.*, 1988; Lublin *et al.*, 1993), while intrathecal injection of a nonreplicating viral vector encoding IFN-γ was protective as a result of increased apoptosis of $CD4^+$ T cells within the CNS (Furlan *et al.*, 2001a). Similar results have been demonstrated in knockout mice, with IFN-$\gamma^{-/-}$ mice developing more severe EAE compared with wild type controls (Ferber *et al.*, 1996). Furthermore, IFN-$\gamma R^{-/-}$ mice are highly susceptible to induction of EAE and cells from these mice transfer an exacerbated form of disease in wild type mice (Willenborg *et al.*, 1996).

1.10.5. IL-12 and IL-23

IL-12 and IL-23 have deleterious effects in the CNS. Importantly, microglia produce IL-12, in response to immune signals mediated by CD40 engagement (Becher *et al.*, 2000). Numerous studies have found a correlation between CNS inflammation and elevated levels of IL-12 in CSF and PBMCs (Fassbender *et al.*, 1998; van Boxel-Dezaire *et al.*, 1999). IL-12 expression in the CNS has been detected in relapsing-remitting EAE at preclinical stages of EAE; it

decreases during remission and becomes elevated again at relapse, suggesting that IL-12 mediates pathogenic immune responses within the CNS (Issazadeh *et al.*, 1998). Mice that were immunised with T cells that had been incubated with IL-12, displayed more severe clinical disease due to increased production of TNF-α and IFN-γ by the T cells (Leonard *et al.*, 1995). Furthermore, inhibition of endogenous IL-12 *in vivo* during EAE prevented paralysis (Leonard *et al.*, 1995). T cells from anti-IL-12 antibody treated mice induced mild disease on adoptive transfer, however co-treatment with anti-IL-10 restored normal disease. Additionally, anti-IL-12 treatment during EAE significantly increased IL-10 production in naïve and primed animals suggesting IL-12-mediated suppression of IL-10 contributes significantly to disease pathogenesis (Segal *et al.*, 1998). A study by Becher and colleagues (2002) demonstrated that local production of IL-12p40 in the CNS is important in promoting and maintaining disease. Taken together, these data suggest that endogenous IL-12 plays a pivotal role in the pathogenesis of autoimmune disease. However, contrary to expectations, IL-12p35 mice are fully susceptible to EAE and can exhibit more severe clinical disease (Gran *et al.*, 2002). This inconsistency may be explained following the discovery of IL-23 which shares the IL-12p40 subunit. IL-23 is predominantly secreted by APC such as DC, macrophages and microglia and the IL-23R is expressed on many cell types, particularly memory T cells. Expression of p19 mRNA is enhanced in CNS-derived macrophages and microglia early in disease (Li *et al.*, 2003). Interestingly, only macrophages acquire IL-23R expression after EAE onset, demonstrating that microglia and macrophages differ in their ability to respond to the cytokine (Cua *et al.*, 2003). IL-23p19$^{-/-}$ mice lacking IL-23, but not IL-12, are resistant to MOG-induced EAE (Cua *et al.*, 2003). While T cell and macrophage

infiltrate into the CNS in IL-23p19$^{-/-}$ mice, these infiltrating cells fail to expand and microglia remain in a quiescent state. Collectively, these data suggest that IL-12 is clearly integral to disease initiation and priming of CD4 response, while IL-23 is important in promoting and maintaining chronic inflammation within the CNS.

1.10.6. TGF-β

TGF-βs are members of a superfamily of multifunctional cytokines, with TGF-β1 being the predominant isoform expressed in the immune system (Schmid et al., 1991). TGF-β1 has potent immunosuppressive properties and can abrogate IL-12-induced signaling, resulting in decreased T cell proliferation and IFN-γ production, and increased apoptotic cell death (Bright & Sriram, 1998). TGF-β1 also inhibits APC function by decreasing MHC class II expression (Schluesener, 1990). Within the CNS, TGF-β1 prevents the induction of chemokine gene expression in microglia exposed to Th1 cytokines including IFN-γ and TNF-α. The data suggest that the effect of TGF-β1 is to impair cell entry into the CNS and to hinder migration of microglia in the CNS parenchyma (Paglinawan *et al.*, 2003). There is also evidence to suggest that TGF-β1 can play a regulatory role in MS as increased amounts of TGF-β1 are detectable in the CSF and serum of patients that are clinically stable (Carrieri *et al.*, 1998). During EAE, endogenous levels of TGF-β1 have been shown to correlate with the onset of remission and administration of anti-TGF-β1 at disease onset leads to a worsening of the clinical course of EAE and more extensive pathological lesions (Racke *et al.*, 1992). Further support for the disease-limiting role of TGF-β1 during autoimmunity

comes from a report where incubation of TGF-β1 with encephalitogenic T cells reduced their potential to induce EAE by inhibiting the production of TNF-α and lymphotoxin (Stevens *et al.,* 1994). In contrast to the data suggesting a protective role for TGF-β1 in EAE, Luo and colleagues (2007) report that local TGF-β1 production by glial cells early in disease, results in TGF-β1 signalling in neurons and later in infiltrating T cells in lesions. It was hypothesized that this production of TGF-β1 within the CNS creates a permissive environment for the initiation of autoimmune inflammation. Systemic treatment with a TGF-β1 inhibitor ameliorated paralytic disease and decreased T cell infiltration and IL-6 production in the CNS (Luo *et al.,* 2007).

1.10.7. IL-10

IL-10 is a product of Th2 cells, macrophages, B cells, DC, astrocytes and microglia that inhibits Th1 cell proliferation. It is also a potent inhibitor of APC function through its ability to decrease MHC class II expression on microglia (O'Keefe *et al.*, 1999) and CD80 and CD86 expression on macrophages (Ding *et al.*, 1993). Thus IL-10 is classed as an anti-inflammatory cytokine. It has been reported that MS patients have decreased numbers of PBMCs secreting IL-10 and lower serum levels of IL-10 (Huang *et al.*, 1999). A study by Cua and colleagues (2001) showed that EAE could be ameliorated by administration of an IL-10-expressing adenovirus to the CNS. The authors propose that for optimum therapeutic benefit, IL-10 must either access the CNS from the peripheral circulation or be delivered directly to it. IL-$10^{-/-}$ mice display an enhanced susceptibility to EAE induction and develop exacerbated clinical disease,

demonstrating that IL-10 has an endogenous anti-inflammatory role during EAE (Bettelli *et al.*, 1998). Furthermore, T cells from IL-10 deficient mice exhibit stronger antigen-specific proliferation, produce increased levels of IFN-γ and TNF-α when stimulated with an encephalitogenic peptide, and induce very severe EAE when transferred into wild-type mice (Bettelli *et al.*, 1998).

1.10.8. IL-4

IL-4 is another anti-inflammatory cytokine believed to play an important role in immunomodulation during EAE. IL-4 is released from Th2 cells and in a positive feedback mechanism promotes the induction of Th2 cells from naïve T cells, along with the DC-derived factors, monocytic chemotactic protein (MCP)-1 and OX40 ligand (Ohshima *et al.*, 1998). In the CNS, IL-4 induces microglial activation and proliferation but decreases IFN-γ-induced MHC class II expression on microglia (Suzumura *et al.*, 1994). *In vivo* studies have shown that IL-4 is an important anti-inflammatory cytokine involved in the amelioration of EAE. The use of the therapeutic agent atorvastatin attenuated chronic and relapsing EAE due to the promotion of the Th2 cells from naïve T cells and IL-4 production (Youssef *et al.*, 2002). Furthermore, intrasplenic electrotransfer of IL-4 encoding plasmid DNA decreased inflammation in the spinal cord and was effective in both the prevention and modulation of EAE (Ho *et al.*, 2006a).

The presence of anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β in the CSF or MS brain parenchyma may reflect ongoing immunoregulatory mechanisms that are initiated after disease exacerbation and are necessary for disease resolution in EAE (Issazadeh *et al.*, 1995). Therefore a

potential therapeutic strategy is to upregulate these anti-inflammatory cytokines in the hope of ameliorating the disease.

1.11. Aims of this project

The aims of this project were to

- characterise the differential interactions of Th1 cells and Th17 cells with glia *in vitro*
- assess the effect of immunomodulatory cells on the immune response induced by T cell: glial interactions
- compare the antigen presenting capabilities of CNS resident glia and peripheral immune cells such as macrophages and DC
- analyse the infiltration of macrophages into the CNS and the expression of antigen presenting molecules on resident microglia and infiltrating macrophages during the progression of EAE
- analyse the phagocytic activity and the production of cytokines by microglia and macrophages in the CNS during the development of EAE.
- assess the roles of immunomodulatory proteins in EAE

Chapter 2
Materials and Methods

2.1 Materials

2.1.1. Cell culture media

Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera, UK) was supplemented with 8% heat activated (56ºC for 60min) foetal calf serum (FCS) (Biosera, UK), 100mM L-Glutamine (GIBCO, UK), 100 µg/ml penicillin/streptomycin (GIBCO, UK).

Dulbecco's Modified Eagles Medium (DMEM) (GIBCO, UK) was supplemented with 10% heat activated (56°C for 60min) FCS, 100mM L-Glutamine, 100 µg/ml penicillin/streptomycin.

Neurobasal medium (NBM) was supplemented with 10% heat activated (56°C for 60min) FCS, 100mM L-Glutamine, 100 µg/ml penicillin/streptomycin.

2.1.2. Phosphate-buffered saline (PBS) 20X

320g Sodium chloride (NaCl, 1.4M)

46g di-Sodium hydrogen phosphate (Na_2HPO_4, 0.08M)

8g Potassium dihydrogen phosphate (KH_2PO_4, 0.01M)

8g Potassium chloride (KCl, 0.03M)

Dissolved in 2L dH_2O, pH 7.0

2.1.3. Ammonium chloride lysis solution (0.87%)

4.35g Ammonium Chloride NH_4Cl

Dissolved in 500mls ddH_2O

2.1.4. ELISA wash buffer

500 ml 20X PBS

9.5 L dH_2O

5 ml Tween 20

2.1.5. Phosphate Citrate Buffer

10.2g Citric acid anhydrous ($C_6H_8O_7$)

36.9g di-Sodium hydrogen orthophosphate dodecahydrate ($NaHPO_4.12H_2O$)

Dissolved in 1L of dH_2O, pH 5.0

2.1.6. ELISA substrate solution

25 ml Phosphate Citrate Buffer

7 µl H_2O_2

1 o-Phenylenediamine dihydrochloride (OPD) tablet (Sigma)

2.1.7. ELISA stopping solution (1M)

26.6 mls 18.8M (H_2SO_4)

473.4 mls dH_2O

2.1.8. FACS Buffer

PBS (Dulbeccos)

1% FCS

0.1% sodum azide (NaN_3)

2.1.9. MACs buffer

PBS (Dulbeccos)

0.5% BSA

2 mM EDTA

2.1.10. Krebs solution containing calcium

2.1.10.1. Krebs solution

3.975g NaCl

0.095g KCl

0.08g KH_2PO_4

0.135g $MgSO_4$

0.67g $NaHCO_3$

0.9g Glucose

Make up to 500 ml with dH_2O and pH to 7.3

2.1.10.2. Calcium (stock)

2.94g $CaCl_2$ in 20 ml dH_2O

Store at 4°C

2.1.10.3. Krebs solution containing calcium

Add 200 µl $CaCl_2$ to 100 ml Krebs solution (1:500 dilution) just before use, for washing or short term storage.

For long term storage, add 10% DMSO to Krebs/$CaCl_2$, snap freeze tissue in liquid N_2 and store at -80°C.

2.2. Methods

2.2.1. Preparation of primary glial cell culture

Glial cultures were prepared from 1-day old C57BL/6 neonatal mice (BioResources Unit, Trinity College, Dublin). Mice were decapitated, the brain dissected and the meninges removed. Brains were chopped with a sterile scalpel (Schwann-Mann, UK), placed into 2 ml of prewarmed Dulbecco's Modified Eagle's medium (DMEM; GIBCO, UK) supplemented with 10% heat inactivated horse serum (Gibco, UK), penicillin (100 U/ml; Gibco, UK), streptomycin (100 U/ml; Gibco, UK), and incubated for 25 min at 37°C with 5% CO_2: 95% air in a Nuaire Flow CO_2 incubator. The solution was triturated (X5) using a sterile Pasteur pipette before being passed through a sterile nylon mesh filter (40 μm; Becton Dickinson Labware, France). The suspension was centrifuged (1200 rpm, 5 min, 20°C). The pellet was resuspended in warmed DMEM, cells were counted and seeded at $1x10^6$ cells/ml (100 μl/well) in 24 well plates and incubated at 37°C for 2 h. Wells were then flooded with prewarmed DMEM (500 μl) and cells were grown for 10-14 days. The medium was changed every 3 days.

2.2.2. Preparation of primary neurons

Primary neurons were cultured from 1-day old C57BL/6 neonatal mice (BioResources Unit, Trinity College, Dublin). The neonates were decapitated, the brain dissected and chopped with a sterile scalpel (Schwann-Mann, UK) and then incubated in 2 ml phosphate citrate buffer (PBS; Biosera), which contained 0.3% trypsin (Sigma, UK) for 25 min at 37°C with 5% CO_2: 95% air in a Nuaire Flow CO_2 incubator. Brain tissue was gently triturated (X5) in PBS containing 0.1%

trypsin inhibitor (Sigma, UK), DNase (0.2 mg/ml; Sigma, UK) and $MgSO_4$ (0.1M). Cell suspensions were passed through a sterile nylon mesh filter and pelleted by centrifugation (1200 rpm; 5 min; 20°C) The pellet was resuspended in neurobasal medium (NBM) supplemented with 10% heat inactivated horse serum (Gibco, UK), penicillin (100 U/ml), streptomycin (100 U/ml), glutamax (2mM; Gibco, UK) and B-27 (1:50 dilution; Gibco, UK). The resuspended neurons were counted, equalised to a density of $1x10^6$ cells and plated (50 µl/well) on circular 10mm diameter coverslips, coated in poly-L-lysine (60 µg/ml) in 24 well plates and incubated at 37°C for 2 h before addition of supplemented NBM (500 µl) to each well. Cells were grown for 48 h. The medium was then replaced with NBM containing cytosine arabino-furanoside (5 µg/ml; Sigma, UK) to prevent proliferation of non-neuronal cells. Arabino-furanoside supplemented media was removed after 24 h and all subsequent medium changes were with complete NBM.

2.2.3. Preparation of bone marrow-derived Dendritic Cell culture

Bone marrow-derived immature dendritic cells (BMDC) were prepared by culturing bone marrow cells removed from the femurs and tibiae of C57BL/6 mice (Harlan, Bicester, Oxon, U.K.). Cells were cultured in RPMI 1640 medium (Biosera, UK) supplemented with 10% FCS and 10% supernatant from a GM-CSF-expressing cell line (J558-GM-CSF) at 37°C with 5% CO_2: 95% air in a CO_2 incubator. Naïve mice were sacrificed by cervical dislocation and the femurs and tibiae were removed and dissected from surrounding muscle and tissue. The bone marrow was then flushed from the bones using a 25G needle attached to a 20 ml syringe containing complete RPMI-1640. A single cell suspension of flushed

bone marrow was prepared using a 19G needle attached to an empty 20 ml syringe. The single cell suspension was centrifuged (1200 rpm, 5 min, 20°C) and the cells re-suspended in 1 ml of heated 0.87% ammonium chloride for 2 min, to lyse contaminating red blood cells. The cells were washed in complete RPMI-1640 and pelleted by centrifugation (1200 rpm, 5 min, 20°C). The washed mononuclear cells were then re-suspended in 2ml complete RPMI-1640 and counted using trypan blue. The immature BMDC were cultured at $1x10^6$ cells/ml of complete RPMI supplemented with 10% GM-CSF with no more than $50x10^6$ cells in total in a T175 tissue culture flask (Greiner, UK). Medium (RPMI-1640 supplemented with 10% FCS and 10% GM-CSF) was replaced every 3 days. On day 6, cells were washed and recultured in RPMI-1640 supplemented with 10% FCS and 5–10% GM-CSF for a further 4 days. On day 10, loosely adherent cells were removed by gentle pipetting, cells were pelleted by centrifugation (1200 rpm, 5 min, 20°C) and seeded in RPMI-1640 supplemented with 10% FCS and 5% GM-CSF at $1x10^6$ cells/ml in round bottom 96-well plates (200 µl/well).

2.2.4. Preparation of spleen cell culture

C57BL/6 mice were sacrificed by cervical dislocation; spleens were removed, homogenized and passed through a sterile nylon mesh filter (40 µm; Becton Dickinson Labware, France) to obtain a single cell suspension. Red blood cells were lysed with ammonium chloride solution (0.87%). Cells were pelleted by centrifugation (1200 rpm, 5 min, 20°C), counted with trypan blue and added to a round bottomed 96-well plates (200 µl/well, $2x10^6$ cells/ml).

2.2.5. Preparation of peritoneal lavage

Naïve C57BL/6 mice were sacrificed by cervical dislocation. The peritoneal cavity was exposed taking care not to puncture the peritoneal membrane. A 25G needle was used to inject RPMI-1640 (5 ml, Biowest, UK) into the cavity. An air bubble was then injected as a reference point for re-insertion of the needle. The body of the mouse was then gently shaken to move the peritoneal macrophages into the medium. The needle was then re-inserted and the maximum amount of the injected medium was removed, taking care not to pierce the intestines. The cells were then centrifuged (1200 rpm, 5 min, 20°C). The resulting pellet was resuspended in complete RPMI and the cells were counted.

2.2.6. Cell count using haemocytometer

Cell counts were performed by diluting cells (normally 1:10) in trypan blue (Sigma, UK). A 10µl volume of the cell suspension was then loaded onto a disposable haemocytometer (Hycor Biomedical, UK). Viable cells, which did not stain and appear light under a light microscope, were counted. The cell number was multiplied by 10^4 and the dilution factor.

2.2.7. Animals

Specific pathogen-free C57BL/6 (H-2^b) mice were purchased from Harland UK Ltd., Bicester, Olac, UK, maintained according to the local regulations and guidelines and under licence from Department of Health and Children, Ireland with the approval of the local Ethics Committee. All mice used were female and 6-12 weeks old at the initiation of the experiments.

2.2.8. Generation of irradiated APC

Whole spleens from C57BL/6 mice were placed in 50 ml tubes (Greiner, UK) containing ice cold complete RPMI. Tubes were placed into the irradiation chamber of a Nordian Gammcell 3000 Elan irradiator and irradiated at a dose of 30Gy, which prevents subsequent cell proliferation. A single cell suspension was prepared, the red blood cells were lysed and the cells were counted.

2.2.9. Subcutaneous immunisations into the footpad

C57BL/6 mice were immunized with KLH (20 μg) and LPS (10 μg) by s.c. injection into the footpad using a total volume of 50μl/mouse (25 μl/foot). Mice were sacrificed 7 days post immunisation.

2.2.10. Generation of KLH- specific T cell lines

Seven days after immunisation, the popliteal lymph nodes were harvested and re-stimulated with KLH (25 μg/ml) and specific cytokines and antibodies depending on the type of T cell line being developed. A Th1 cell line was developed by culturing cells with IL-12 (1 ng/ml) and a Th17 cell line was generated by culturing cells with IL-23 (10 ng/ml). After 4 days, IL-2 (10 U/ml) was added to the T cell lines. After a further 7 days, the T cell lines ($1x10^5$ cells/ml) were divided into two groups; one group was re-stimulated with irradiated APCs ($2x10^6$ cells/ml) and KLH (25 μg/ml). The other group was used for co-culture with mixed glial cells. T cell lines were cultured at $1x10^5$ cells/ml with irradiated APC ($2x10^6$ cells/ml) and KLH (25 μg/ml) for 4 days followed by 7 days of culture with IL-2 (10 U/ml).

2.2.11. Induction of EAE using MOG_{35-55}

To induce EAE, 8-10 week C57BL/6, $SIGIRR^{-/-}$ and $IL\text{-}4^{-/-}$ mice were injected subcutaneously (s.c.) with 150μg MOG_{35-55} peptide (Cambridge Biosciences, UK) in complete freund's adjuvant (CFA; DIFCO, UK) supplemented with 5 mg/ml H37RA (*Mycobacteria tuberculosis*, DIFCO, UK) and injected intraperitionally (i.p.) with 500 ng pertussis toxin (Kakasukin) on day 0 and day 2 post immunisation. EAE was scored according to a 0-5 scale as follows: no clinical symptoms 0; limp tail, 1; wobbly gait, 2; hind limb weakness, 3; hind limb paralysis, 4; tetra paralysis, 5. Mice were killed by cervical dislocation; spinal cord, cerebellum and striatum were removed and snap frozen in liquid nitrogen for mRNA analysis or stored in Krebs solution containing calcium at -80°C for protein analysis. Alternatively whole brain was removed for isolation of mononuclear cells.

2.2.12. Generation of MOG-specific T cell lines

EAE was induced as described above. At 7 days post immunisation, the mice were sacrificed and spleens and inguinal lymph nodes were harvested. The spleen cells and lymph node cells were re-stimulated *ex vivo* with MOG (25 μg/ml) and specific cytokines. A Th17 cell line was developed using spleen cells and lymph node cells ($2x10^6$ cells/ml) cultured in the presence of IL-23 (10 ng/ml, R&D Systems), IL-1β (20 ng/ml, R&D systems), and in some cases anti-IFN-γ (10 μg/ml, BD Pharmingen). Cells ($2x10^6$ cells/ml) were cultured with IL-12 (1 ng/ml, R&D Systems) to develop a Th1 cell line. A Th2 cell line was developed from spleen cells ($2x10^6$ cells/ml) cultured in the presence of IL-4 (10 ng/ml, R&D Systems) and Dexamethasone (1×10^{-8} M, SIGMA, UK). In the case of Th1 and

Th2 cell lines, IL-2 (10 U/ml, R&D Systems) was added after 4 days and all cell lines were cultured until day 7.

2.2.13. Co-culture of T cells, glia and neurons

Following culture of MOG-specific T cells or KLH-specific T cells, T cells were pooled and washed in complete RPMI. Cells were then counted and added to glia at a ratio of 0.5:1 (5×10^4 T cell: 1×10^5 glia). When transwells were used, T cells were placed in transwells with irradiated APC at a ratio of 1:5 (5×10^4 T cell: 2.5×10^5 APC) and antigen. When neutralising antibodies were used, cells were incubated with the antibody (10 μg/ml) for 1 h before addition to the co-culture. Neutralising antibody was then added to the co-culture to maintain a concentration of 10 μg/ml. For experiments that involved the addition of neurons, neurons were removed from the plates by Trypsin-EDTA, pooled and washed in complete NBM. Neurons were counted and added to the glia at the same time as T cells at a ratio of 1:8 (12.5×10^3 neurons: 1×10^5 glia). The cells were cultured overnight and supernatant was removed for cytokine analysis and cell surface marker expression on microglia was assessed by flow cytometry.

2.2.14. Mononuclear cell isolation from CNS tissue

Control mice and mice with EAE were anaesthetised with pentobarbital sodium (40 μl, Euthethal, Merrial), perfused intracardially with ice cold PBS (20 ml) and the brain and spinal cord isolated to Hank's balanced salt solution containing 3% FCS (GIBCO, UK) (HBSS/FCS). Tissue was dissociated through a sterile nylon mesh filter (70 μm), washed with HBSS/FCS, centrifuged (170 x g, 10 min, 20°C) and then enzymatically digested in Collagenase D (1 mg/ml, Roche Applied

Sciences) and DNAse I (10 μg/ml, SIGMA) for 1 h at 37°C. Cells were washed in HBSS/FCS and resuspended in 1.088 g/ml Percoll (9ml, SIGMA, UK). This was underlayed with 1.122 g/ml Percoll (5ml); and overlayed with 1.072 g/ml Percoll (9ml), 1.030 g/ml Percoll (9ml) and 1X PBS (9ml). Percoll gradients were centrifuged at 1250 x g for 45min at 20°C. Mononuclear cells were removed from the 1.088:1.072 and 1.072:1.030 g/ml interfaces and washed twice in HBSS/FCS. Cells were then stained for flow cytometry.

2.2.15. Flow cytometry

Expression of cell surface markers was assessed on microglia prepared *ex vivo* from brain and and spinal cord tissue of control mice and mice with EAE and on cultured mixed glial cells by flow cytometry using a DAKO $CyAN_{ADP}$ flow cyometer, calibrated using Flow-Check Fluorospheres (Beckman Coulter, Ireland). Mononuclear cells were resuspended in FACS buffer (100 μl). Cultured mixed glial cells were harvested from wells by addition of Trypsin-EDTA (100 μl/well) and incubated at 37°C for 5-10 min. Cells were transferred to FACS tubes, centrifuged (1200 rpm, 5 min) and resuspended in FACS buffer (100 μl). The low-affinity IgG receptors (FcγRIII) were blocked by incubating with CD16/CD32 FcγRIII block (1: 100 dilution, BD Pharmingen, US) for 10 min at room temperature. Cells were washed in FACS buffer (3 ml/$3x10^6$ cells), resuspended in FACS buffer (100 μl) and incubated with the appropriate FACS antibodies or isotype control antibodies for 20 min at room temperature in the dark. Excess antibody was removed by washing each tube twice in FACS buffer (3 ml/tube); samples were centrifuged (1200 rpm, 5 min) between washes.

Immunofluorescence analysis was then performed using Summit software and analysed using FlowJo software.

For intracellular staining, co-cultured mixed glial cells and T cells were harvested from wells of tissue culture plates by addition of Trypsin-EDTA (100 µl/well) and incubated at 37°C for 5-10 min Cells were transferred to FACS tubes, centrifuged (1200 rpm, 5 min) and resuspended in FACS buffer (100 µl). The low-affinity IgG receptors (FcγRIII) were blocked by incubating with CD16/CD32 FcγRIII block (1: 100 dilution, BD Pharmingen, US) for 10 min at room temperature followed by incubation with the appropriate FACS antibodies specific for cell surface molecules for 20 min at room temperature in the dark. Excess antibody was removed by washing each tube twice in FACS Buffer (3 ml/tube) and centrifugation (1200 rpm, 5 min) between each wash. The cells were then prepared for intracellular staining using a cell permeabilization kit (Caltag). 100 µl of fixation buffer A was added to each tube and the cells were gently resuspended. The cells were incubated for 15 min at room temperature in the dark. The cells were washed twice in FACS Buffer, (3 ml/tube) and centrifuged (1200 rpm, 5 min) between each wash. Permeabilisation buffer B (100 µl) was then added to each tube along with the intracellular antibodies. The cells were again incubated for 15 min at room temperature in the dark. The cells were then washed twice in FACS buffer (3 ml/tube) and centrifuged (1200 rpm, 5 min) between each wash. Immunofluorescence analysis was performed using Summit software and analysed using FlowJo software.

Table 2.1. Fluorochrome labelled antibodies used in this study

Cell surface marker	Fluorescent Label	Dilution factor	Supplier
CD11b	Alexa Fluor 647	1/400	BD PharMingen
I-A/I-E	FITC/PE	1/500	BD PharMingen
CD40	FITC/PE	1/200	BD PharMingen
CD80	PE	1/200	BD PharMingen
CD86	FITC	1/200	BD PharMingen
CD4	PE-Cy7	1/200	eBioscience
CD3	APC-Alexa Fluor 750	1/200	BD PharMingen
TNF-α	FITC	1/100	eBioscience
IL-6	PE	1/20	eBioscience
IL-1β	FITC	1/5	eBioscience
IFN-γ	FITC	1/200	BD PharMingen
IL-17	PE	1/200	BD PharMingen

2.2.15.1. Cell counts using flow cytometry

To determine the absolute number of cells infiltrating the spinal cord or brain in each animal, a 50 µl sample of digested tissue was removed to a FACS tube both before and after percoll separation. These cells were washed, incubated with 1 µg/ml anti- CD16/CD32 (1:100, Fcγ Block; BD Pharmingen) for 15 min at room temperature abd then stained with anti-CD45 (PE-Cy7) and anti-CD11b (Alexa-Fluor 647) for 15 min in the dark. Cells were washed with FACS buffer and resuspended in 400 µl FACS buffer for analysis. Cytocount beads (20 µl, DakoCytomation) were added to each tube just prior to FACS analysis and the contents vortexed. Cytocount beads are manufactured at a concentration of 1100 beads/µl. Thus, the absolute number of $CD11b^+$ and $CD45^+$ cells can be determined by multiplying :

$$\frac{\#\ CD11b^+CD45^+\ \text{cells acquired}}{\#\ \text{beads acquired}} \times \text{Cytocount concentration} \times \text{Volume dilution} = \text{Total cell \#/ sample}$$

2.2.16. $CD4^+$ T cell isolation from spleens and inguinal lymph nodes

$CD4^+$ T cells were isolated from the spleens and inguinal lymph nodes of C57BL/6 mice with EAE. A $CD4^+$ T cell isolation kit was used according to manufactures instructions (Miltenyi Biotec, Germany). Spleens and inguinal lymph nodes were dissected, homogenized and passed through a sterile nylon mesh filter (40 µm; Becton Dickinson Labware, France) to obtain a single cell suspension. Red blood cells were lysed with ammonium chloride solution (0.87%). Cells were pelleted by centrifugation (1200 rpm, 5 min, 20°C), counted

with trypan blue. Cells were centrifuged (1200 rpm, 5 min, 20°C) and resuspended in 40µl of MACS buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) per 10^7 cells. Biotin-antibody cocktail (10 µl/10^7 cells) was added, mixed and incubated for 10 min at 4°C. MACS buffer (30 µl/10^7 cells) and anti-biotin microbeads (20 µl/10^7 cells) were then added and incubated for an additional 15 min at 4°C. Cells were washed in buffer (20x labelling volume) and centrifuged (300 x g, 10 min). Supernatant was removed completely and the pellet was resuspended in MACS buffer (500 µl/10^8 cells). Cells were then isolated using the autoMACS™ separator. The fraction containing $CD4^+$ T cells was collected, counted and cells were cultured with ascending ratios of APC ($2x10^5$ $CD4^+$ T cell: $3.2x10^3$, $1.6x10^4$, $8x10^4$, $4x10^5$ and $2x10^6$ DC, macrophage or glia) for 5 d in the presence of MOG_{35-55}. Supernatants were collected for ELISA.

2.2.17. Measurement of proliferation by [^{3}H] thymidine incorporation

Antigen induced T cell proliferation was assessed by monitoring [^{3}H] thymidine incorporation into newly synthesized DNA. Briefly, 5 days after stimulation in a 96 well plate, cells were pulsed with 0.5µCi [^{3}H] Thymidine/well (Amersham) diluted in fresh, warm, complete medium. After 6 h incubation at 37°C, plates were frozen at -20°C. DNA from each well was directly harvested onto glass filter mats (Wallac) using a Tomtec harvester. Once completely dry, the filter mats were sealed in plastic bags with 5 ml scintillation fluid, and [^{3}H] was detected by liquid scintillation counting using a micro-β-counter (Wallac). Relative proliferation was expressed in the form of counts per min (CPM).

2.2.18. Protein assay

Protein concentrations of CNS tissue isolated from control mice and mice with EAE were determined using a BCA assay kit (Pierce). 25 ml reagent 1 was mixed with 500 µl reagent 2 to form a working solution. A standard curve was prepared using a range of concentrations of BSA in the same buffer as the samples to be tested. Each sample, standard and blank (25 µl) were added to designated wells of a 96 well plate in triplicate, followed by 200 µl working solution. The plate was incubated at 37°C for 30min to develop the colour, after which the absorbance was read at 562nm.

2.2.19. Determination of concentration IL-10, IL-1β, IL-17, TNF-α, IFN-γ, IL-5, IL-4 and IL-6 cytokine by ELISA

Concentrations of the cytokines IL-10, IL-1β, IL-17 and TNF-α were measured using commercially available ELISA kits (R&D Systems, UK) and concentrations of IFN-γ, IL-4 and IL-6 were detected using commercially available paired antibodies (BD Pharmingen, US). High-binding certified 96-well microtitre plates (Greiner Bio-one, UK) were coated overnight at 4°C with 50 µl/well of rat anti-mouse IL-1β (4 µg/ml), IL-10 (2 µg/ml), IL-17 (2 µg/ml), TNF-α (0.8 µg/ml), IL-6 (1 µg/ml), IL-4 (1 µg/ml, IL-5 (1 µg/ml) and IFN-γ (1 µg/ml) capture antibody in PBS. Plates were then washed in wash buffer (PBS/0.05% Tween 20) and non-specific binding sites were blocked by adding 200 µl of blocking buffer (1% BSA in PBS or 5% dried milk (Marval) w/v in PBS) for 2 h at room temperature. After this, plates were washed again in wash buffer and 50 µl/well of test supernatant/tissue homogenate as well as serially diluted standard recombinant proteins for each cytokine, IL-1β (0-1000 pg/ml), IL-10 (0-2000

pg/ml), IL-17 (0-1000 pg/ml) and TNF-α (0-2000 pg/ml) all diluted in 1% BSA (Sigma, UK)/PBS and IL-6 (0-5000 pg/ml), IL-4 (0-2500 pg/ml), IL-5 (2500pg/ml) and IFN-γ (0-10 ng/ml) all diluted in PBS were added. Plates were incubated overnight at 4°C. Plates were then washed in wash buffer and incubated with 50 µl/well of biotinylated goat anti-mouse IL-1β (100 ng/ml), IL-10 (400 ng/ml), IL-17 (200 ng/ml) and TNF-α (150 ng/ml) diluted in 1% BSA and biotinylated rat anti-mouse IL-6 (1 µg/ml), IL-4 (1 µg/ml), IL-5 (1 µg/ml) and IFN-γ (1 µg/ml) diluted in PBS. The plates were incubated for 2 h at room temperature before washing and incubation with 50 µl/well of horseradish peroxidise (HRP)-conjugated streptavidin (1:200 in 1% BSA or 1:200 in PBS) for 30 min at room temperature in the dark. The plates were washed and cytokine concentrations were determined after addition of 50 µl/well Ortho-Phenylenediamine (OPD; SIGMA, UK) in phosphate citrate buffer (0.4 mg/ml). The enzyme reaction was quenched using the stop solution H_2SO_4 (50 µl/well) and the plates were read at 492 nm, once standard curves for each cytokine had developed sufficiently. Cytokine concentrations contained in the test samples were evaluated with reference to the standard curve prepared using recombinant mouse IL-1β, IL-10, IL-17, TNF-α, IL-6, IL-4, IL-5 and IFN-γ of known concentration.

Table 2.2. Origin and specificities of antibodies for ELISA

Antibody specificity	Clone	ELISA	Purified/ conjugate	Supplier
Mouse IFN-γ	R4-6A2	cIFN-γ	Purified	BD Pharmingen
Mouse IFN-γ	XMG1.2	dIFN-γ	Biotin	BD Pharmingen
Mouse IL-4	11B11	cIL-4	Purified	BD Pharmingen
Mouse IL-4	BVD6-24G2	dIL-4	Biotin	BD Pharmingen
Mouse IL-5	TRFK5	cIL-5	Purified	BD Pharmingen
Mouse IL-5	TRFK4	dIL-5	Biotin	BD Pharmingen
Mouse IL-6	MP5-20F3	cIL-6	Purified	BD Pharmingen
Mouse IL-6	MP5-32C11	dIL-6	Biotin	BD Pharmingen
Mouse IL-10	-	cIL-10	Purified	R&D Systems
Mouse IL-10	-	dIL-10	Biotin	R&D Systems
Mouse IL-1β	-	cIL-1β	Purified	R&D Systems
Mouse IL-1β	-	dIL-1β	Biotin	R&D Systems
Mouse TNF-α	-	cTNF-α	Purified	R&D Systems
Mouse TNF-α	-	dTNF-α	Biotin	R&D Systems
Mouse IL-17	-	cIL-17	Purified	R&D Systems
Mouse IL-17	-	dIL-17	Biotin	R&D Systems

c= capture

d=detection

2.2.20. Real-time Polymerase Chain Reaction (PCR)

2.2.20.1. Preparation of tissue for RNA isolation

Cells from *in vitro* studies and tissue from *in vivo* studies were homogenised in 350 µl of cell lysis mastermix (Nucleospin RNA II, Macherey-Nagel) for extraction of RNA. Lysate was filtered using NucleoSpin Filter, collected in an Eppendorf and centrifuged (11,000 x g, 1 min). Ethanol (70%, 350 µl) was added to the filtrate, mixed and loaded onto NucleoSpin RNA II columns. Tubes were centrifuged (8,000 x g, 30 sec) and the RNA binds to the column. The silica membrane was desalted by adding membrane desalting buffer (350 µl) and centrifuged (11,000 x g, 1 min) to dry the membrane. To digest the DNA, DNase reaction mixture (95 µl) was added to the column and incubated at room temperature for 15 min. The silica membrane was washed and dried. RNA was eluted by adding RNase free H_20 and centrifugation (11,000 x g, 1 min) and RNA concentration was quantified using a NanoDrop Spectrophotometer ND-1000 v3.5 (NanoDrop Technologies, Inc. USA).

2.2.20.2. Reverse Transcription for cDNA synthesis

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

followed by 2 h at 37°C on a thermocycler (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences Ireland).

2.2.20.3. Real-time PCR

Real-time PCR primers and probes were delivered as "TaqMan® Gene Expression Assays" for the mouse genes listed in Table 1 (Applied Biosystems, Darmstadt, Germany). Real-time PCR was performed on Applied Biosystems ABI Prism

7300 Fast Track Sequence Detection System v1.3.1 in 96-well format and 10 µl reaction volume per well. cDNA (200 pg/well) were mixed with Taqman Universal PCR Fast Mastermix (Applied Biosystems, Darmstadt, Germany) and the respective target gene assay. Either mouse β-actin RNA (# 4352341E, Applied Biosystems, Darmstadt, Germany) or eukaryotic 18S (# 4319413-E, Applied Biosystems, Darmstadt, Germany) were used as reference. Each sample was measured in duplicate in a single RT-PCR run. Forty cycles were run with the following conditions: 20 sec at 95°C and for each cycle 3 sec at 95 °C for denaturation and 30 sec at 60°C for transcription (x40). Analysis of gene expression values was performed using the efficiency-corrected comparative CT method, determining target gene expression relative to beta-actin or 18S endogenous control expression and relative to the control sample.

Table 2.3. Primers used in this study

Gene name	Gene Description	Taqman Gene expression Assay number	GenBank accession number
IL-1β	Interleukin-1 beta	Mm00434228_m1	NM_008361
IL-6	Interleukin-6	Mm00446191_m1	NM_031168.1
TNF-α	Tumour Necrosis Factor alpha	Mm00443258_m1	NM_013693.2
IFN-γ	Interferon-gamma	Mm00801778_m1	NM_008337.3
IL-17A	Interleukin-17 A	Mm00439619_m1	NM_010552.3
CD200L	CD200 ligand	Mm00487740_m1	NM_010818.3
IL-4	Interleukin-4	Mm00445259_m1	NM_021283
CD40	CD40	Mm00441891_m1	NM_170701.2
CD80	CD80	Mm00711660_m1	NM_009855.2
CD86	CD86	Mm00444543_m1	NM_019388.3

2.2.21. Statistical Analysis

Statistical analysis was performed using the computer based statistical package GraphPad Prism. Data is expressed as mean ± standard error of the mean (SEM). Student's *t* test and One-way ANOVA using the Newman Keuls post-hoc test were used where appropriate to determine statistical differences. EAE studies were analysed by Two-way repeated measures ANOVA

Chapter 3
Interaction of T cells and glia *in vitro*

3.1. Introduction

Multiple sclerosis, and an animal model for MS, EAE, are chronic demyelinating diseases of the CNS. During active disease, demyelination within the CNS is associated with inflammatory reactions that are orchestrated by infiltrating T cells and endogenous glia. Both IFN-γ secreting Th1 cells and IL-17-secreting Th17 cells has been shown to have a pathogenic role in EAE (Kroenke *et al.*, 2008; Stromnes *et al.*, 2008). Kroenke and colleagues (2008) found that adoptive transfer of IL-12p70- or IL-23-polarised T cells resulted in clinically indistinguishable disease, however, IL-12p70-driven disease was characterised by macrophage-rich infiltrates and enhanced NOS_2 expression, whereas neutrophils and GM-CSF were prominent in IL-23-driven lesions. Further evidence that both Th1 and Th17 cells contribute to disease was reported by Stromnes and colleagues (2008) who found that EAE was most intense in the brain when Th17/Th1 ratios are high, whereas a wider range of Th17/Th1 ratios induced inflammation in the spinal cord (Stromnes *et al.*, 2008). More recently, O'Connor and colleagues (2008) reported that Th1 cells have the capacity to infiltrate the noninflamed CNS, induce inflammation and subsequently facilitate the entry of Th17 cells into the CNS (O'Connor *et al.*, 2008). In contrast, a regulatory role for Th1 cells has been previously been described, since IFN-$\gamma^{-/-}$ mice suffer from more severe EAE (Chu *et al.*, 2000). This study suggests that Th1 cells have a protective role during disease, by suppressing expansion of activated $CD4^+$ T cells (Chu *et al.*, 2000). However, the exact role of Th1 and Th17 cells in the brain during pathogenesis remains unresolved.

The hallmark of brain inflammation in MS and EAE is the activation of glial cells. The presence of activated glia in the CNS suggests that glia may interact with invading encephalitogenic T cells. Activation of astrocytes and microglia during disease leads to production of proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β, as well as chemokines, which contribute to inflammation and myelin damage within the CNS (Benveniste, 1997). In addition, activated microglia enhance their expression of MHC class II, CD80, CD86 and CD40, molecules critical for antigen presentation, and thus become professional APC capable of reactivating infiltrating T cells (Matyszak *et al.*, 1999). Astrocytes can also act as APC and have been shown to upregulate IL-17 and IFN-γ gene expression and protein synthesis in T cells (Miljkovic *et al.*, 2007).

Inflammation in the CNS can be attenuated by neuroprotective mechanisms, mediated by both infiltrating peripheral immune cells and endogenous cells in the CNS. Th2 cells, characterised by IL-4, IL-10, IL-5 and IL-13 production, have a protective role in EAE (Cua *et al.*, 1995; Stohlman *et al.*, 1999; Kirwin *et al.*, 2006). The mechanisms of this Th2 mediated neuroprotection have not been fully elucidated, however, treatment with Th2 cells reduced inflammatory infiltrates into the CNS and decreased expression of MHC class II on microglia and infiltrating macrophages in mice with EAE (Kirwin *et al.*, 2006). This suggests that Th2 cells can attenuate activation of APC within the CNS during EAE.

Neurons can regulate the activation of microglia via CD200 ligand-receptor interactions. CD200, a membrane glycoprotein expressed on neurons, binds to its receptor on microglia and macrophages and attenuates myeloid cell activity (Hoek *et al.*, 2000). Microglia from $CD200^{-/-}$ mice exist in a more

activated state, exhibiting a less ramified morphology, shorter glial processes and a disordered arrangement (Hoek *et al.*, 2000). The exacerbated activation of microglia in $CD200^{-/-}$ mice leads to increased susceptibility to CNS autoimmunity. Earlier onset of EAE in $CD200^{-/-}$ mice was attributed to greater microglial and macrophage activation throughout the CNS (Hoek *et al.*, 2000), highlighting the importance of CD200 mediated inhibition of microglia activation.

Thus the aims of this chapter were to:

- investigate the differential effects of Th1 and Th17 cells on cytokine production and expression of MHC class II and co-stimulatory molecules on microglia in co-cultures of glia and T cells.
- assess the ability of Th2 cells to attenuate cytokine production and APC function of microglia in co-cultures of Th1/Th17 cells and glia.
- elucidate the effect of neurons on cytokine production and APC function of microglia in co-cultures of Th1/Th17 cells and glia.

3.2. Results

3.2.1. IL-17 and IFN-γ expressing $CD3^+$ $CD4^+$ T cells are present in the brain during EAE.

It has been reported that both IFN-γ secreting Th1 cells and IL-17 secreting Th17 cells traffic to the CNS and initiate disease in the EAE model (Stromnes *et al.*, 2008). In adoptive transfer studies, EAE was induced by transfer of cells that secreted IL-17 but not by T cells that secreted IFN-γ, although both types of cells could cross the blood-brain barrier and infiltrate the CNS (Langrish *et al.*, 2005). However, more recent studies have suggested that both Th1 and Th17 cells mediate pathology in EAE by distinct mechanisms (Kroenke et al., 2008), and that Th1 cells can traffic to the CNS, initiate inflammation and facilitate Th17 cell entry into the CNS (O'Connor *et al.*, 2008). To verify the presence of Th1 and Th17 cells in the brain during EAE, mononuclear cells were isolated from the brain of control mice or mice with EAE. Cells were restimulated overnight with PMA (10ng/ml) and ionomycin (1μg/ml), and incubated with Brefeldin A (10μg/ml). Cells were stained with anti-CD4 and anti-CD3, fixed and permeabilized, stained intracellularly with anti-IFN-γ and anti-IL-17 and analysed by flow cytometry. The data in Figure 3.1 show that the percentage of $CD3^+CD4^+$ T cells in the brain increase as a result of EAE induction (**$p<0.01$, Figure 3.1A). There is a significant increase in the percentage of IL-17 and IFN-γ expressing $CD3^+CD4^+$ cells in the brain of mice with EAE compared to control mice (*$p<0.05$, Figure 3.1B and C). Representative dotplots of one control mouse and one mouse with EAE at the peak of disease are shown. There was a significant increase in the percentage of $CD3^+CD4^+$ T cells in the brain during disease

progression (Figure 3.1D). Within the population of $CD3^+CD4^+$ cells significant production of IL-17, IFN-γ or both cytokines was seen in the mouse with EAE and the percentage of these cells was greater than that observed in control mice (Figure 3.1E).

3.2.2. Th17 cells expressed high levels of IL-17; little IFN-γ and Th1 cells secrete high levels of IFN-γ.

It has previously been shown that antigen-specific T cells interact with microglia inducing activation and expression of proinflammatory cytokines (Dasgupta *et al.*, 2005). The aim of this study was to investigate the ability of Th17 cells to activate glia and to compare this with the effect of Th1 cells. Spleen cells and lymph node cells isolated from C57BL/6 mice immunised with KLH were polarised into Th17 cells by incubation with antigen and IL-23 (10ng/ml), IL-1β (10ng/ml) and anti-IFN-γ (10μg/ml) and to Th1 cells by incubation with antigen and IL-12 (1ng/ml). Th17 polarised cells secreted high levels of IL-17, very low amounts of IFN-γ and IL-4, and some IL-10 while Th1 cells secrete high levels of IFN-γ, no IL-17 or IL-4 and some IL-10 (Figure 3.2).

3.2.3. Co-culture of glia and KLH-specific Th17 cells induces significant TNF-α and IL-6 production and alters co-stimulatory molecule expression on microglia.

KLH-specific Th17 cells were co-cultured with glia at a ratio of 0.5:1 for 24 h. Cells were either in direct contact or a transwell system was used. Co-culture of KLH-specific Th17 cells with glia results in significant TNF-α production compared to medium-treated glia, regardless of whether the cells were

in contact or a transwell system was used. (***$p<0.001$; Figure 3.3A). Culturing of KLH-specific Th17 cells with glia induces IL-6 production, which was significantly enhanced when cells were in contact (***$p<0.001$; Figure 3.3B) and when a transwell system was used (**$p<0.01$; Figure 3.3B). However direct contact between the cells stimulates significantly greater IL-6 production compared to when the cells were separated by a semi-permeable membrane ($^{++}p<0.01$; Figure 3.3B).As a result of Th17 cell: glial cell interaction, ICAM-1 expression was significantly enhanced on the surface of microglia. For significant upregulation of CD86 on microglia, cell contact was required between the Th17 cells and glia (Figure 3.4 and 3.5).

3.2.4. Co-culture of glia and KLH-specific Th1 cells induces significant TNF-α and IL-6 production in a cell contact dependent manner and alters co-stimulatory molecule expression on microglia.

The role of Th1 cells in the pathogenesis of EAE is still unclear. The disease is more severe in IFN-$\gamma^{-/-}$ mice (Chu *et al.*, 2000), while Th1 cells can contribute to inflammation, they may also have a regulatory role during disease by suppressing expansion of activated $CD4^+$ T cells (Chu *et al.*, 2000). In the present study, Th1 cells and their secreted cytokines were assessed for their ability to activate glia. KLH-specific Th1 cells were added to glia at a ratio of 0.5:1. Cells were either in direct contact or in a transwell. When cells were in direct contact, TNF-α and IL-6 production was significantly increased in the co-culture, however, the interaction of the cells with soluble factors failed to induce release of TNF-α and IL-6 (***$p<0.001$; $^{+++}p<0.001$; Figure 3.6 A and B).

Contact between KLH-specific Th1 cells and glia significantly elevated the expression of ICAM-1 on microglia, as does the interaction of microglia with T cell secreted factors (Figure 3.7). In contrast, there was a requirement for direct contact between the Th1 cells and glia to induce an upregulation of CD80 and CD86 on microglia (Figure 3.8).

3.2.5. Co-culture of glia with KLH-specific Th1 cells induces significantly greater TNF-α and IL-6 production compared to co-culturing of glia with KLH-specific Th17 cells.

The activation of glia by Th1 cells was compared to that induced by Th17 cells in the same experiment. Glia were cultured with KLH-specific Th1 cells and Th17 cells for 24 h. While the interaction of glia with KLH-specific Th17 cells induced significant TNF-α (*$p<0.05$, Figure 3.9A) and IL-6 (***$p<0.001$, Figure 3.9B) production compared to glia treated with medium, the interaction of glia with Th1 cells induced significantly greater levels of TNF-α (***$p<0.001$; $^{+++}p<0.001$, Figure 3.9A) and IL-6 (***$p<0.001$; $^{+++}p<0.001$, Figure 3.9B).

3.2.6. Cytokine production by KLH-specific Th1 and Th17 cells after a second round of antigen re-stimulation *in vitro*.

Th17 cells and Th1 cells were re-stimulated with KLH (25 μg/ml) and irradiated APC in the presence of Th17 polarising cytokines IL-23 (10 ng/ml) and IL-1β (10 ng/ml) and anti-IFN-γ (10 μg/ml) and Th1 polarising cytokine IL-12 (1 ng/ml). The Th17 cell line expressed high levels IL-17 and low levels of IFN-γ, IL-10 and IL-4. The Th1 cell line secreted low levels of IFN-γ, no IL-17, IL-10 and IL-4 (Figure 3.10).

3.2.7. Co-culture of glia and re-stimulated KLH-specific Th17 cells induces significant TNF-α and IL-6 production and upregulates MHC class II and co-stimulatory molecule expression on microglia.

KLH-specific Th17 cells that had been cultured for two rounds of antigen stimulation *in vitro* were co-cultured with glia at a ratio of 0.5:1. Th17 cells were either in direct contact with glia or in a transwell. Direct contact between glia and Th17 cells induced significant TNF-α production (**$p<0.01$; Figure 3.11A), yet the interaction of cells with soluble factors stimulated significantly greater amounts of TNF-α (***$p<0.001$; $^{+++}p<0.001$; Figure 3.11A). Conversely, IL-6 production was greater when glia and Th17 cells were in contact (***$p<0.001$; Figure 3.11B), than when Th17 cells were in a transwell (**$p<0.001$; $^{+++}p<0.001$; Figure 3.11B). Th17 cells were assessed for their ability to induce the expression of the activation markers MHC class II, CD80, CD86 and CD40 on microglia. The data presented in Figure 3.12 and Figure 3.13 suggest that cell contact is required for Th17 cells to significantly enhance the expression of MHC class II, CD80 and CD86 on microglia. In contrast, CD40 expression on microglia was increased by cell contact with Th17 cells and by Th17 cell-secreted factors.

3.2.8. TNF-α and IL-6 production and activation marker expression on microglia is significantly increased in co-cultures of glia and antigen re-stimulated KLH-specific Th1 cells.

KLH-specific Th1 cells that had been cultured for two round of antigen stimulation *in vitro* were added directly to glia or were placed in a transwell. The cells were cultured for 24 h after which cytokine production was assessed by ELISA and activation marker expression on microglia was analysed by flow

cytometry. Despite low IFN-γ production from the antigen re-stimulated Th1 cell line, TNF-α and IL-6 production was enhanced by contact between glia and Th1 cells compared to medium treated glia and glia treated with Th2 cells in a transwell (***$p<0.001$; $^{+++}p<0.001$; Figure 3.14A and B). While cell secreted soluble factors stimulated the release of TNF-α compared with medium treated glia (*$p<0.05$; Figure 3.14A), they failed to elicit the production of IL-6. Consistent with the cytokine data, T cell: glial cell contact was necessary for significant upregulation of MHC class II expression and the co-stimulatory molecules CD80, CD86 and CD40 expression on microglia (Figure 3.15 and Figure 3.16).

3.2.9. Glial: KLH-specific Th17 cell co-cultures induced greater IL-6 production and similar TNF-α production compared with glia cultured with KLH-specific Th1 cells.

Antigen re-stimulated KLH-specific Th17 cells and Th1 cells induced significantly increased, but comparable, concentrations of TNF-α production when cultured with glia (***$p<0.001$; Figure 3.17A). Although supernatants from cultures of glia with Th1 cells had significantly higher IL-6 levels compared to glia alone (***$p<0.001$; 3.17B), IL-6 production was greatest in glia and Th17 cell co-cultures ($^{+++}p<0.001$; Figure 3.17B).

3.2.10. Effect of IL-17 and IFN-γ on cytokine production and expression of activation molecules on glia.

In order to elucidate the role of IL-17 and IFN-γ in the modulatory effect of KLH-specific Th1 and Th17 cells on glia, mixed glial cells were treated for 24

h with IL-17 (10 ng/ml) and IFN-γ (10 ng/ml). Neither IL-17 nor IFN-γ induced the release of TNF-α and IL-6 from mixed glial cultures (Figure 3.18). IL-17 treatment had no effect of the expression of activation markers on microglia; in contrast IFN-γ increased MHC II and CD40 expression on microglia (Figure 3.19).

3.2.11. Cytokine production from MOG-specific Th1 and Th17 cells.

To more accurately mimic the *in vivo* interactions of Th1 and Th17 cells in the brain during EAE after immunisation with MOG_{35-55}, MOG-specific T cell lines were generated for co-culture with glia. EAE was induced in C57BL/6 mice as described in method 2.2.11. After 7 days, spleen and inguinal nodes were removed and the cells stimulated with MOG_{35-55} (25 μg/ml) and Th1 polarising cytokine IL-12 (1 ng/ml) and Th17 polarising cytokines IL-23 (10 ng/ml) and IL-1β (10 ng/ml). Cells were cultured for 7 days, supernatants were removed and IFN-γ, IL-17, IL-10 and IL-4 concentrations were quantified by ELISA. Th1 cells secreted high levels of IFN-γ and IL-10 and no IL-17 or IL-4. Whereas Th17 cells released high levels of IL-17 and IFN-γ, comparable to Th1 cell secreted levels and little or no IL-10 and IL-4. The data show that the MOG-specific Th17 cell line was contaminated with Th1 cells, as indicated by high levels of both IFN-γ and IL-17, for this reason these cells were termed Th1/Th17 cells (Figure 3.20).

3.2.12. Addition of MOG-specific Th1 cells does not enhance cytokine production in co-cultures of glia and MOG-specific Th1/Th17 cells.

This experiment assessed the ability of MOG-specific Th1 and Th1/Th17 cells to induce cytokine production when cultured with glia. Co-cultures of glia

with Th1/Th17 cells only and Th1 cells only significantly increased TNF-α production (**$p<0.01$; ***$p<0.001$; Figure 3.21A) with the addition of Th1/Th17 cells to glia resulting in the greatest amount of TNF-α release. Glia and Th1/Th17 cell co-cultures significantly enhanced IL-6 and IL-1β production when compared with glia alone or glia treated with Th1 cells (***$p<0.001$; $^{+++}p<0.001$; Figure 3.21B and 3.21C).The function of Th1 cells in EAE remains unclear with Th1 cells displaying both pathogenic (Kroenke *et al.*, 2008) and regulatory roles (Chu *et al.*, 2000) during disease. To investigate the potential enhancing or inhibitory effect of Th1 cells on cytokine production, MOG-specific Th1 cells were added to co-cultures of glia and Th1/Th17 cells. Interestingly, the addition of Th1 cells to Th1/Th17 cell: glia co-culture neither increased nor decreased TNF-α or IL-6, but did significantly attenuate IL-1β production ($^{\delta\delta\delta}p<0.001$; Figure 3.21C).

3.2.13. CD40 ligand and CD80 do not mediate cytokine production and upregulation of activation molecules in glial: Th1/Th17 co-cultures.

The data above show that T cells can induce activation of glial cells in a contact dependent manner, here the mechanisms of that action were investigated. A role for the adhesion molecules CD40 and CD80 has been found in T cell mediated microglial activation. Guiliani and colleagues (2005) reported that minocycline decreased TNF-α production by T cell activated microglial cells which corresponded with a decrease in CD40 ligand expression on the surface of T cells, implicating CD40-CD40L interaction as a mechanism for T cell mediated microglial activation. It is also thought that the ligation of the costimulatory molecule CD80 on microglia with CD28 or CTLA-4 on T cells is crucial to the onset and disease progression of EAE (Vanderlugt et al., 2000). Neutralising

antibodies to CD40L and CD80 were used to ascertain the role of CD40L and CD80 in co-cultures of MOG-specific Th1/Th17 cells and glia. Mixed glia and Th1/Th17 cells were co-incubated in the presence or absence of neutralising antibodies to CD40L and CD80. Microglial stimulated IL-17 production from Th1/Th17 cells was not attenuated by co-incubation with CD40L and CD80 (***$p<0.001$; Figure 3.22A). In fact, addition of CD40L and CD80 antibodies significantly enhanced IL-17 production in the co-culture ($^{+}p<0.05$; $^{++}p<0.01$; Figure 3.22A). Consistent with the IL-17 data, IL-6 production in the glia: T cell co-culture was not decreased but in fact was significantly increased as a result of blocking CD40L and CD80 interactions (***$p<0.001$; $^{++}p<0.01$, $^{+++}p<0.001$; Figure 3.22B). Conversely, IL-1β release was unaffected by addition of CD40L and CD80 neutralising antibodies to the co-culture of Th1/Th17 cells and glia (***$p<0.001$; Figure 3.22C).

The effect of blocking CD40L and CD80 on the expression of activation markers on $CD11b^{+}$ microglia was assessed by flow cytometry. Th1/Th17 cells significantly increased the percentage of cells expressing MHC class II, CD40, CD80 and CD86 (Figure 3.23 and 3.24). However, addition of neutralising antibodies to CD40L and CD80 did not significantly alter the percentage of microglia expressing MHC class II, CD40, CD80 and CD86.

3.2.14. Cytokine production from MOG-specific Th2 cells and Th1/Th17 cells.

The effect of MOG-specific Th2 cells on glial activation was assessed since Th2 cells have been shown to be protective in EAE (Stohlman *et al.*, 1999). Spleens and lymph nodes were removed from mice 7 days after EAE induction

and stimulated with MOG_{35-55} (25 μg/ml) and Th1/Th17 polarising cytokines IL-23 (10 ng/ml) and IL-1β (10 ng/ml) or the Th2 polarising proteins dexamethasone (10^{-8}M) and IL-4 (20 ng/ml). After 7 days, supernatants were removed and IFN-γ, IL-17, IL-10 and IL-4 concentrations were quantified by ELISA (Figure 3.25). Th1/Th17 cells secreted high levels of IFN-γ and IL-17, low levels of IL-5 and IL-10. Th2 cells secreted high levels of IL-10, low levels of IL-5 and no IFN-γ and IL-17.

3.2.15. MOG-specific Th2 cells did not significantly alter cytokine production when co-cultured with glia.

Th2 cells are reported to have the ability to diminish Th1-induced inflammatory reactions and actively support the resting state of microglia. This has been suggested as one mechanism of Th2-mediated remission of neuroinflammation during EAE (Gimsa *et al.*, 2001). The ability of MOG-specific Th2 cells to alter cytokine production in T cell: glia co-cultures was compared to Th1/Th17 cells. MOG-specific Th2 and Th1/Th17 cells were co-cultured with glia for 24 h after which cytokine production was assessed by ELISA. Addition of Th2 cells did not significantly alter IL-1β or IL-6 production in the co-culture. In contrast, Th1/Th17 cell: glia co-cultures induced significantly elevated levels of IL-1β and IL-6 (***$p<0.001$; Figure 3.26).

3.2.16. The attenuating effect of MOG-specific Th2 cells on Th1/Th17 cell: glia co-cultures is mediated in part by IL-4 and IL-10.

Th2 cells are protective in EAE (Kirwin et al., 2006). Simultaneous transfer of Th2 cells and Th1 cells reduced the day of onset, disease severity, peak

clinical disease as well as cumulative disease score in mice with EAE. Therefore, we hypothesised that MOG-specific Th2 cells could ameliorate cytokine production and activation marker expression in Th1/Th17 cell: glia co-cultures. To investigate this hypothesis, MOG-specific Th2 cells were added to co-cultures of mixed glia and MOG-specific Th1/Th7 cells. As previously demonstrated, glia and Th1.Th17 cultures induce significant release of IL-1β, IL-6 and IL-17 (***$p<0.001$; Figures 3.27, 3.28 and 3.29). Addition of Th2 cells to glia and Th1/Th17 cell cultures significantly decreased the production of IL-1β ($^{+++}p<0.001$; Figure 3.27), IL-6 ($^{+++}p<0.001$; Figure 3.28) and IL-17 ($^{++}p<0.01$; Figure 3.29). Next, the potential mediators of the attenuating effects of Th2 cells in the co-cultures were investigated. Kirwin and colleagues (2006) demonstrated that Th2 protection from PLP-induced EAE was reversed by inhibiting IL-10, suggesting that IL-10 has an important role in the protective effect of Th2 cells in EAE. A study by Shaw and colleagues (1997) showed that IL-4 has a critical role in reducing disease onset and severity. Encephalitogenic T cells transduced with a retroviral gene construct expressing IL-4 can delay disease onset and reduce disease severity in MBP-immunised mice. Consequently, the role of IL-4 and IL-10 in Th2 mediated inhibition of cytokine production in cultures of Th1/Th17 cells and glia was assessed. Neutralising antibodies to IL-4 and IL-10 were added to co-cultures of mixed glia, Th1/Th17 cells and Th2 cells. Inhibition of IL-4 and IL-10 significantly reversed the attenuating effect of Th2 cells on IL-1β production ($^{\delta\delta}p<0.01$; Figure 3.27). However, neutralisation of IL-4 but not IL-10 partially reversed the inhibitory effect of Th2 cells on IL-6 production ($^{\delta\delta}p<0.01$; Figure 3.28). Interestingly, addition of IL-4 and IL-10 neutralising antibodies did not reverse the attenuating effect of Th2 cells on IL-17 production (Figure 3.29).

The effect of Th2 cells on the expression of antigen presenting molecules on microglia was assessed by flow cytometry. Th1/Th17 cells significantly upregulated MHC class II, CD40, CD80 and CD86 expression on microglia (Figure 3.30 and 3.31). The percentage of microglia expressing CD40, CD80 and CD86 was attenuated by co-culture with Th2 cells (Figure 3.30 and 3.31). Neutralising Th2 derived IL-4 and IL-10 did not reverse the attenuating effect of Th2 cells on CD40 expression on microglia. Conversely, the Th2 attenuation of the expression of CD80 and CD86 on microglia was overcome by the addition of neutralising antibodies to IL-4 and IL-10 (Figure 3.30 and 3.31).

3.2.17. Neurons attenuate cytokine production from Th1/Th17 cell: glia co-cultures.

It has been reported that neurons can regulate microglial activation via CD200 ligand-receptor interactions (Lyons *et al.*, 2007). The effect of neurons on cytokine production in co-cultures of Th1/Th17 cells and glia was assessed. The Th1/Th17 cell line secreted high levels of IFN-γ and IL-17, low levels of IL-10 and no IL-4 (data not shown). Mixed glia (1 x 10^5 cells/well) were co-cultured with MOG-specific Th1/Th17 cells (5 x 10^4 cells/well) and neurons (12.5 x 10^3 cells/well) for 24 h. Co-cultures of glia and Th1/Th17 cells significantly induced the production of IL-17, TNF-α, IL-6 and IL-1β (***$p<0.001$; Figure 3.32). The addition of neurons significantly attenuated cytokine production from the co-cultures ($^{++}p<0.01$; $^{+++}p<0.001$; Figure 3.32). To determine a role for CD200 in mediating the inhibitory effect of neurons on cytokine production from T cell: glial cultures, mixed glia were cultured with Th1/Th17 cells and neurons for 24 h in the presence or absence of a neutralising antibody to CD200R1. Neutralising

the CD200R1 on glia significantly reversed the inhibitory effect of neurons on TNF-α ($^{\delta}p<0.05$; Figure 3.32B), IL-6 ($^{\delta\delta\delta}p<0.001$; Figure 3.32C) and IL-1β ($^{\delta}p<0.05$; Figure 3.32D) production, implicating CD200 ligand-receptor interactions in neuron mediated regulation of cytokine production in cultures of Th1/Th17 cells and glia. The addition of an anti-CD200R1 antibody to glia alone had no effect on proinflammatory cytokine production. In contrast, blocking the CD200R1 did not reverse the effect of neurons on IL-17 production (Figure 3.32A).

Since neurons could regulate cytokine production in Th1/Th17 cell: glial cell cultures, their effect on the expression of activation markers on microglial cells was analysed by flow cytometry. Th1/Th17 cells significantly increased the expression of MHC class II, CD40, CD80 and CD86 on microglia compared with medium-treated microglia (Figures 3.33 and 3.34). However, the addition of neurons with or without the CD200R1 neutralising antibody did not significantly alter Th1/Th17 cell-induced upregulation of activation marker expression on microglia (Figure 3.33 and 3.34).

To more conclusively confirm the role for CD200 in neuron mediated attenuation of cytokine production in co-cultures of Th1/Th17 cells and glia and to remove a level of complexity in the culture system, neurons were replaced with CD200 fusion protein (CD200Fc). Glia were cultured with MOG-specific Th1/Th17 cells in the presence or absence of CD200Fc protein. Culture of Th1/Th17 cells with glia significantly enhanced TNF-α, IL-6 and IL-1β concentration in the supernatant (***$p<0.001$; Figure 3.35). Consistent with the effect of neurons in T cell: glia cultures, addition of CD200Fc significantly inhibited TNF-α, IL-6 and IL-1β production in the co-cultures ($^{+}p<0.05$; $^{++}p<0.01$;

Figure 3.35). The data suggest that the interaction of CD200 to its receptor on glia leads to a decrease in cytokine production in co-cultures of Th1/Th17 cells and glial cells.

3.3. Discussion

The significant new findings of this study are that cytokine production and enhanced expression of antigen presenting molecules on microglia in co-cultures of T cells and glial cells is mainly dependent upon cell-cell contact. Furthermore, the findings demonstrate that MOG-specific Th2 cells suppress cytokine production in Th1/Th17 cell-glial co-cultures via downregulation of the antigen presenting capabilities of microglia; this effect was partially mediated by IL-4 and IL-10. In addition, this study illustrates a novel role for neurons in immune regulation; neurons inhibit cytokine production in cultures of glia with Th1/Th17 cells via CD200 ligand-receptor interactions.

The infiltration of antigen-specific Th1 and Th17 cells into the CNS is considered a key event in the pathogenesis of MS and EAE (Stromnes *et al.*, 2008). The data presented here show the presence of infiltrating $CD4^+$ T cells in the CNS of mice with EAE. The $CD4^+$ T cells secrete IL-17, IFN-γ or IL-17 plus IFN-γ. The data also suggest that T cells interact with CNS resident glia. These interactions result in enhanced production of proinflammatory cytokines and upregulation of antigen presenting molecules on microglia and predominantly depend on cell-cell contact between the T cells and the glia. Cell-contact between KLH-specific Th1 cells and glia significantly increased the production of TNF-α and IL-6, and enhanced the expression of MHC class II, CD40, CD80 and CD86 on microglia, whereas treatment of glia with Th1 cells in a transwell did not. Only the expression of ICAM-1 on microglia was increased by cell contact with Th1 cells and Th1 cell secreted factors. These results are consistent with findings from

Dasgupta and colleagues (2003) who showed that MBP-primed T cells induced the production of IL-1β, IL-1α, TNF-α and IL-6 from microglia in a cell contact dependent manner. In addition, a study by Aloisi and colleagues (2000) showed that antigen-specific interaction of Th1 cells with microglia increased the expression of MHC class II, CD40 and CD54 on microglia. In the present study, cell contact between KLH-specific Th17 cells and glia increased the production of IL-6 and the expression of MHC class II, CD80 and CD86 on microglia, however, TNF-α production was elevated in response to T cell-glia contact and incubation of glia with T cell-derived factors. The data suggest there may be a role for both cell contact and Th17 derived cytokines in the induction of TNF-α by glia in response to Th17 cells.

To further investigate the role of Th1 and Th17 derived cytokines in the induction of TNF-α and IL-6 production by glia, glia were treated with the signature cytokines secreted by these T cell subsets namely IL-17 and IFN-γ. Neither IL-17 nor IFN-γ treatment induced the production of TNF-α or IL-6 from glia. In contrast, treatment of glia with KLH-specific Th17 cells in a transwell induced significant TNF-α production. This suggests that other secreted factors from KLH-specific Th17 cells may contribute to glial activation. One potential candidate is IL-22, which is known to act synergistically with IL-17A and IL-17F to enhance expression of antimicrobial peptides (Liang *et al.*, 2006). In contrast to IL-17, IFN-γ upregulated MHC class II and CD40 expression on microglia. This is consistent with a previous report that microglia exposed to IFN-γ *in vitro* had enhanced expression of MHC class II, CD40 and CD54 (Aloisi *et al.*, 2000a). The data indicate that cell contact is important for the production of proinflammatory

cytokines and upregulation of co-stimulatory and adhesion molecules on microglia in co-cultures of T cells and glia, especially Th1 cells.

The role of Th1 and Th17 cells in the pathogenesis of EAE is currently unresolved. Both cells types have been shown to induce disease, albeit through distinct mechanisms (Kroenke *et al.*, 2008; O'Connor *et al.*, 2008). Co-culture of glia with KLH-specific Th1 cells generated greater production of TNF-α and IL-6 compared to co-culture with Th17 cells. After antigen re-stimulation of the T cells, Th17 cells induce TNF-α production to the same extent as Th1 cells and greater production of IL-6 when cultured with glia. It has previously been reported that secondary antigen stimulation of Th17 cells results in the upregulation of T bet expression and the progression of Th17 cells to a Th1 phenotype (Mathur *et al.*, 2006). The data presented here show that Th17 cells were stable after secondary antigen stimulation, with increased IL-17 expression, however, there was an increase in the expression of IFN-γ. Therefore, significant progression of Th17 cells to a Th1 cytokine profile may require more repeated re-stimulation with antigen. MOG-specific T cell lines were generated to further address T cell-glial interaction with relevance to EAE, pathogenic T cells are specific for myelin antigens including MOG. Using a protocol involving re-stimulation with MOG and IL-23 and IL-1β, MOG-specific T cells were found to produce both IFN-γ and IL-17 and were termed Th1/Th17 cells. This is consistent with the cytokine profiles of many $CD4^+$ T cells in the brain during EAE which co-produce IL-17 and IFN-γ (Suryani & Sutton, 2007). MOG-specific Th1/Th17 cells consistently induced greater production of TNF-α, IL-6 and IL-1β compared to Th1 cells when cultured with glia. This is in contrast to the results observed

with KLH-specific Th1 and Th17 cells which showed that Th1 cells were more effective than Th17 cells at stimulating cytokine production after only one round of antigen stimulation. A study by O'Connor and colleagues (2008) showed that Th1 cells have the capacity to infiltrate the non-inflamed brain, where they promote inflammation, and facilitate the entry of Th17 cells into the brain resulting in the progression of EAE. In co-cultures of T cells with glia, Th1/Th17 cells induce greater production of proinflammatory cytokines compared to Th1 cells and this may account for their increased encephalitogenic potential in the brain during EAE. A recent study has demonstrated the importance of Th17:Th1 ratios in disease pathology, with differences in ratios accounting for the disparity in the sites of inflammation in the CNS (Stromnes *et al.*, 2008). Inflammation in the brain occurs when Th17:Th1 ratios are high, whereas a wider range of Th17:Th1 ratios induce inflammation in the spinal cord. Stromnes and colleagues (2008) propose that Th1 cells inhibit the infiltration of T cells into the brain parenchyma. Moreover, Chu and colleagues (2000) previously reported a regulatory role for Th1 cells during EAE, mediated by the ability of IFN-γ to suppress and induce apoptosis of antigen-specific T cells. However, the present study found that addition of Th1 cells did not alter TNF-α, IL-6 and IL-1β production in cultures of glia with Th1/Th17 cells. Perhaps the differential cytokine production induced by Th1 cells and Th1/Th17 cells in T cell-glial cultures, adds to the complexity of their distinct pathogenic pathways during EAE.

Having demonstrated the requirement for contact between glia and T cells for the induction of proinflammatory cytokines and increased expression of

antigen presenting molecules on microglia, CD40 and CD80 were thought to be likely mediators of this T cell-glial interaction, as both molecules have been found to be important in T cell mediated microglial activation (Giuliani *et al.*, 2005) (Chang *et al.*, 1999; Becher *et al.*, 2001). Giuliani and colleagues (2005) reported that decreased expression of CD40L on T cells, as a result of minocycline treatment; lead to decreased TNF-α production by activated microglial cells, implicating CD40-CD40L interactions in T cell mediated activation of microglial cells. Furthermore, Becher and colleagues (2001) reported that lack of CD40 expression on CNS-resident cells diminished the intensity and duration of MOG-induced EAE. This was attributed to reduced activation of T cells by microglia. Despite the importance of CD40L and CD40 in T cell-microglial interactions, addition of a neutralising antibody to CD40L did not attenuate TNF-α, IL-6 or IL-1β production in co-cultures of glia and Th1/Th17 cells. However, blocking CD40L-CD40 interaction significantly increased the production of TNF-α and IL-6 and the expression of MHC class II, CD40, CD80 and CD86 on microglia. The data suggest a redundancy of CD40 function. The inhibition of CD40L-CD40 interactions may be overcome by as yet unidentified co-stimulatory molecules. Previously, Chang and colleagues (1999) reported that the ligation of CD80 on microglia with CD28 or CTLA-4 on T cells was crucial to the onset and disease progression of EAE. Mice lacking both CD80 and CD86 or CD28 suffered from markedly less severe EAE due to reduced infiltration of inflammatory cells into the brain and spinal cord. Interestingly, addition of a neutralising antibody to CD80 expressed on glia did not decrease TNF-α, IL-6 or IL-1β production in cultures of glia with Th1/Th17 cells. In fact, inhibition of CD80 interaction significantly increased the production of TNF-α and IL-6, and increased the

expression of MHC class II, CD40 on microglia. This suggests that the effects of neutralising CD80 may be overcome by the expression of other co-stimulatory on microglia. Chang and colleagues (1999) reported that mice lacking either CD80 or CD86 alone developed clinical and pathologic EAE that was comparable to EAE in wild-type mice, however, mice lacking both CD80 and CD86 displayed minimal clinical symptoms of EAE suggesting an overlap in the functions of CD80 and CD86. Therefore, the interaction of CD86 on microglia with its ligand may be capable of overcoming the neutralisation of CD80 interaction.

It has previously been shown that Th2 cells are protective in EAE and are capable of modifying the cytokine profile of encephalitogenic T cells and the antigen presenting function of CNS resident and infiltrating APC (Stohlman *et al.*, 1999; Kirwin *et al.*, 2006) (Falcone & Bloom, 1997). In contrast to Th1/Th17 cells, the data presented here show that MOG-specific Th2 cells fail to induce production of IL-1β and IL-6 when co-cultured with glia. This is consistent with a study by Seguin and colleagues (2003), who found that treatment of microglia with Th2 cell supernatant did not alter TNF-α, IL-6, and CXCL10 production by microglia or induce expression of MHC class II, CD80, CD86, CD40, and CD54 on microglia (Seguin *et al.*, 2003). In fact, the present study showed that addition of MOG-specific Th2 cells to cultures of glia and Th1/Th17 cells significantly decreased production of IL-1β, this effect was partially mediated by IL-4 and IL-10. Th2 cells also had an attenuating effect on the production of IL-6 in cultures of glia with Th1/Th17 cells and this effect was mediated by IL-4. Consistent with the anti-inflammatory effects of the Th2-derived cytokines, delivery of IL-4 or IL-10 into the CNS inhibits EAE (Mathisen *et al.*, 1997; Shaw *et al.*, 1997; Furlan *et*

al., 2001b). The addition of Th2 cells to cultures of glia and Th1/Th17 cells also significantly attenuated IL-17 production but this effect was not mediated by IL-4 or IL-10. Th2 cells decreased the expression of CD40, CD80 and CD86 but not MHC class II on microglia treated with Th1/Th17 cells. The data suggest that Th2 cells and their cytokines directly inhibit APC function of microglia, which leads to decreased production of IL-17 by Th1/Th17 cells and attenuated production of IL-1β and IL-6 in cultures of Th1/Th17 cells and glia. Expression of CD80 and CD86 on microglia was increased by the addition of neutralising antibodies to IL-4 and IL-10 demonstrating a role for the Th2-derived cytokines in the regulation of CD80 and CD86 expression on microglia. It has previously been shown that IL-4 and IL-10 are potent downregulators of microglial activation, inhibiting expression of MHC class II, production of NO, and secretion of TNF-α and IL-12 (Chao *et al.*, 1993; Aloisi *et al.*, 1997). Therefore, infiltration of Th2 cells into the CNS during EAE could potentially play a role in limiting inflammation by inhibiting the antigen presenting function of glia and decreasing proinflammatory cytokine production.

The MOG-specific Th2 cells used for these experiments secrete high levels of IL-10, some IL-5 and no IL-17 or IFN-γ. This is a similar cytokine profile to Tr1 cells that are reported to secrete high levels of IL-10, with low levels of IL-5 and TGF-β and are also capable of inhibiting antigen-specific T cell responses (Groux *et al.*, 1997). It is possible that the "Th2" cell line may actually be a Tr1 cell line. IL-4 production, indicative of a Th2 cell line, could not be measured since exogenous IL-4 was added to promote the expansion of a Th2 cell line. However, it is unlikely that these cells are Tr1 cells as ability of the cells to

attenuate cytokine production in cultures of Th1/Th17 cells and glia are partially mediated by IL-4. This provides indirect evidence of production of IL-4 from the cells, in addition to IL-10, and this is indicative of a Th2 phenotype.

In addition to the neuroprotective effects of Th2 cells, the data show that neurons can attenuate TNF-α, IL-1β and IL-6 production in cultures of glia with MOG-specific Th1/Th17 cells. This is consistent with a report by Lyons and colleagues (2007) demonstrating that addition of neurons to amyloid-β treated glia significantly inhibited production of IL-1β, TNF-α and IL-6, suggesting that neurons are capable of suppressing activation of microglia. Addition of a neutralising antibody to CD200R1 significantly reversed the attenuating effect of neurons on proinflammatory cytokine production in co-cultures of Th1/Th17 cells and glia, suggesting CD200L-CD200R interaction plays a key role in the ability of neurons to decrease proinflammatory cytokine production in cultures of glia with Th1/Th17 cells. Although CD200R is expressed on $CD4^+$ T cells (Rijkers *et al.*, 2008) addition of neurons to Th1/Th17 cells alone did not significantly alter IL-17 or IFN-γ production (data not shown). IL-17 production from Th1/Th17 cells was decreased by neurons only in the presence of glia. The data suggest a specific role for neuronal–glial interaction, mediated by CD200, in attenuating production of TNF-α, IL-6 and IL-1β in cultures of glia and Th1/Th17 cells. Contrary to expectations, neurons did not significantly alter the expression of antigen presenting molecules on microglia. The Th1/Th17 cell-induced expression of MHC class II, CD40, CD80 and CD86 expression on microglia was not altered by the addition of neurons to the culture. In contrast, addition of neurons to

amyloid-β treated glia resulted in a decrease in MHC class II mRNA (Lyons *et al.*, 2007). The data suggest that neurons directly inhibit microglia resulting in attenuated production of TNF-α, IL-1β and IL-6 and this is not mediated by decreased expression of antigen presenting molecules on microglia. To further address the role for CD200 ligand-receptor interactions in neuronal inhibition of cytokine production, cultures of Th1/Th17 cells and glia were incubated with CD200 fusion (CD200Fc) protein. CD200Fc significantly decreased TNF-α, IL-6 and IL-1β production in the co-cultures of glia with Th1/Th17 cells, confirming that CD200 engagement of its receptor downregulates inflammation. This finding is consistent with previous *in vivo* studies that found that CD200Fc is effective at suppressing induction of another autoimmune disease, collagen induced arthritis, by decreasing TNF-α and IFN-γ production (Gorczynski *et al.*, 2002). However, the CD200Fc protein achieves a smaller percentage attenuating effect than that of neurons, suggesting that other neuronal factors may be involved in neuronal attenuation of proinflammatory cytokine production in co-cultures of glia with Th1/Th17 cells.

The data presented here illustrate that Th1 and Th17 cells have differential effects on cytokine production and expression of antigen presenting molecules on microglia, this may contribute to differences in inflammation observed after infiltration of Th1 and Th17 cells into the CNS. Furthermore, the data demonstrate that peripheral immune cells as well as CNS resident cells can modulate T cell-glial interactions. Strategies that promote Th2 cell differentiation and infiltration into the CNS and upregulate the expression of CD200 on neurons

may be of therapeutic value and may initiate neuroprotective mechanisms during inflammation in the CNS.

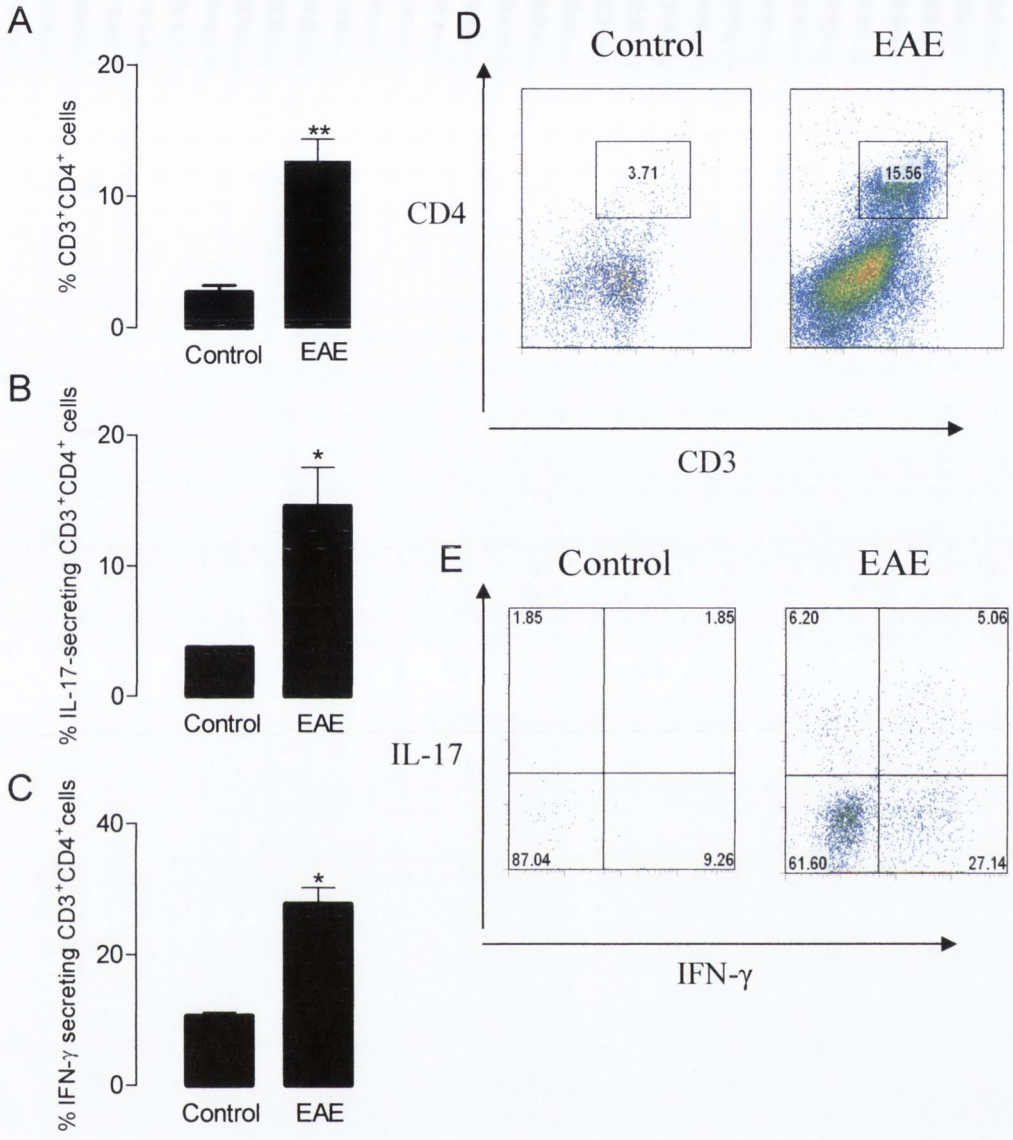

Figure 3.1. IL-17 and IFN-γ expressing $CD3^+CD4^+$ T cells are present in the brain during EAE. Mononuclear cells were isolated from the brain of control mice or mice with EAE 12 days post immunisation. Cells were restimulated overnight with PMA (10 ng/ml) and ionomycin (1 μg/ml), and incubated with Brefeldin A (10 μg/ml). Cells were stained with anti-CD4 and anti-CD3, fixed and permeabilised, stained intracellularly with anti-IFNγ and anti-IL-17 and analysed by flow cytometry. (A) Percentage of $CD3^+CD4^+$ cells in the brain of control mice and mice with EAE, secreting IL-17 (B) and IFN-γ (C). *$p<0.05$; **$p<0.01$, by Student's *t* test. Representative dotplots of one control mouse and one mouse with EAE. (D) $CD3^+CD4^+$ T cells in the brain of a control mouse and a mouse with EAE. (E) IFN-γ and IL-17 secretion from $CD3^+CD4^+$ T cells in the brain of a control mouse and a mouse with EAE. (n=3)

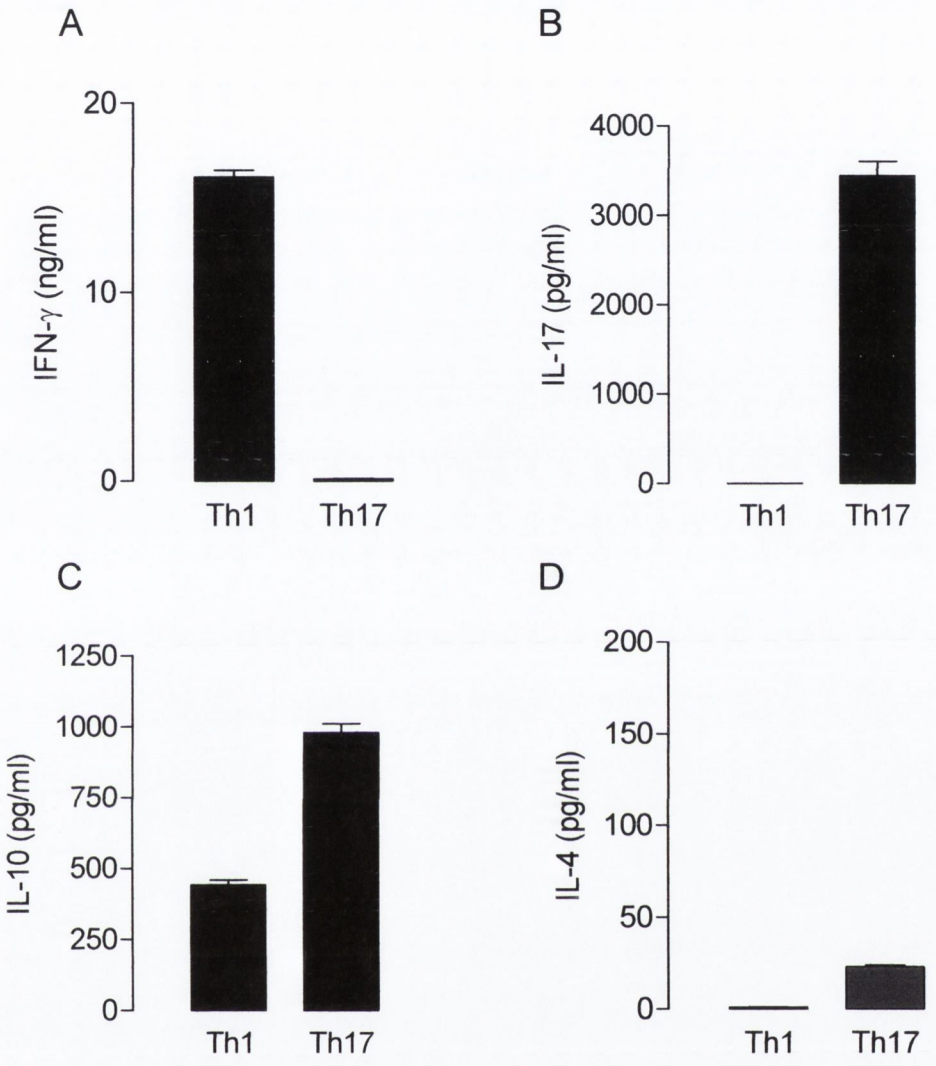

Figure 3.2. Cytokine production by short term KLH-specific Th1 and Th17 cell lines. C57BL/6 mice were immunised with KLH (10 μg) and LPS (10 μg) into the footpad. After 7 days, the popliteal lymph nodes were removed and the cells stimulated with KLH (25 μg/ml) and Th1 polarising cytokine IL-12 (1 ng/ml) or Th17 polarising cytokines IL-23 (10 ng/ml), IL-1β (10 ng/ml) and anti-IFN-γ (10 μg/ml). After 4 days, supernatants were removed and IFN-γ (A), IL-17 (B), IL-10 (C) and IL-4 (D) concentrations were quantified by ELISA.

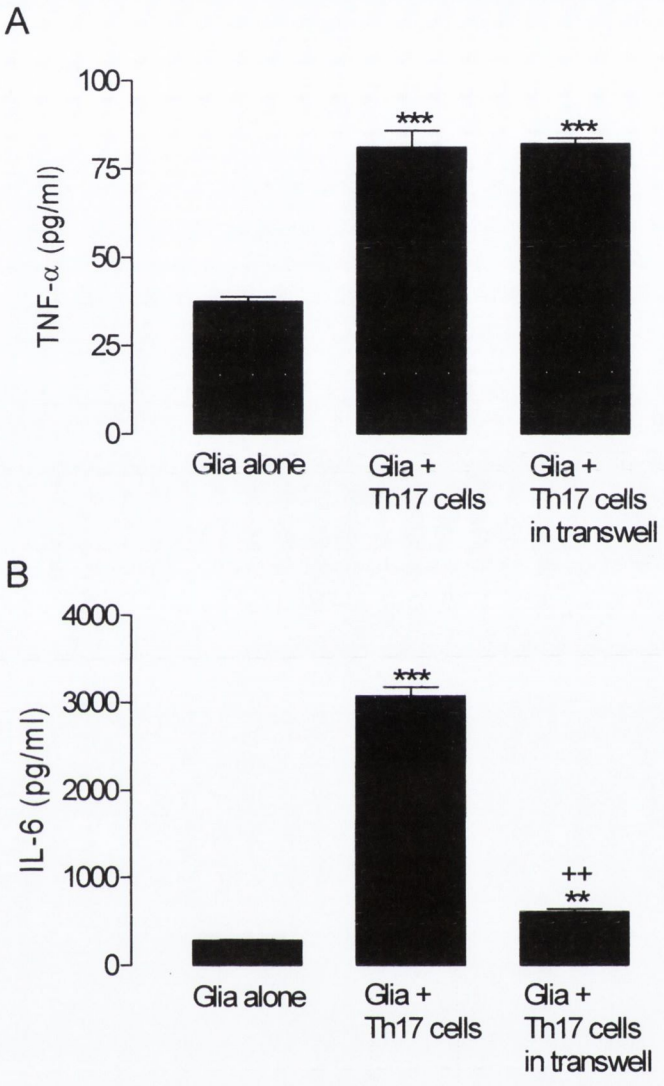

Figure 3.3. Co-culture of glia and KLH-specific Th17 cells induces significant TNF-α and IL-6 production. Mixed glial cells were cultured with KLH-specific Th17 cells at a ratio of 0.5:1 (T cell: glia) or with medium only. Cells were either in direct cell-cell contact or in a transwell which allowed soluble factors secreted by the T cells to interact with the glia. After 24 h, TNF-α (A) and IL-6 (B) concentration was assessed by ELISA. ***$p<0.001$; **$p<0.01$; versus glia alone; $^{++}p<0.01$; versus glia incubated with Th17 cells that were in direct contact, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments (n=2).

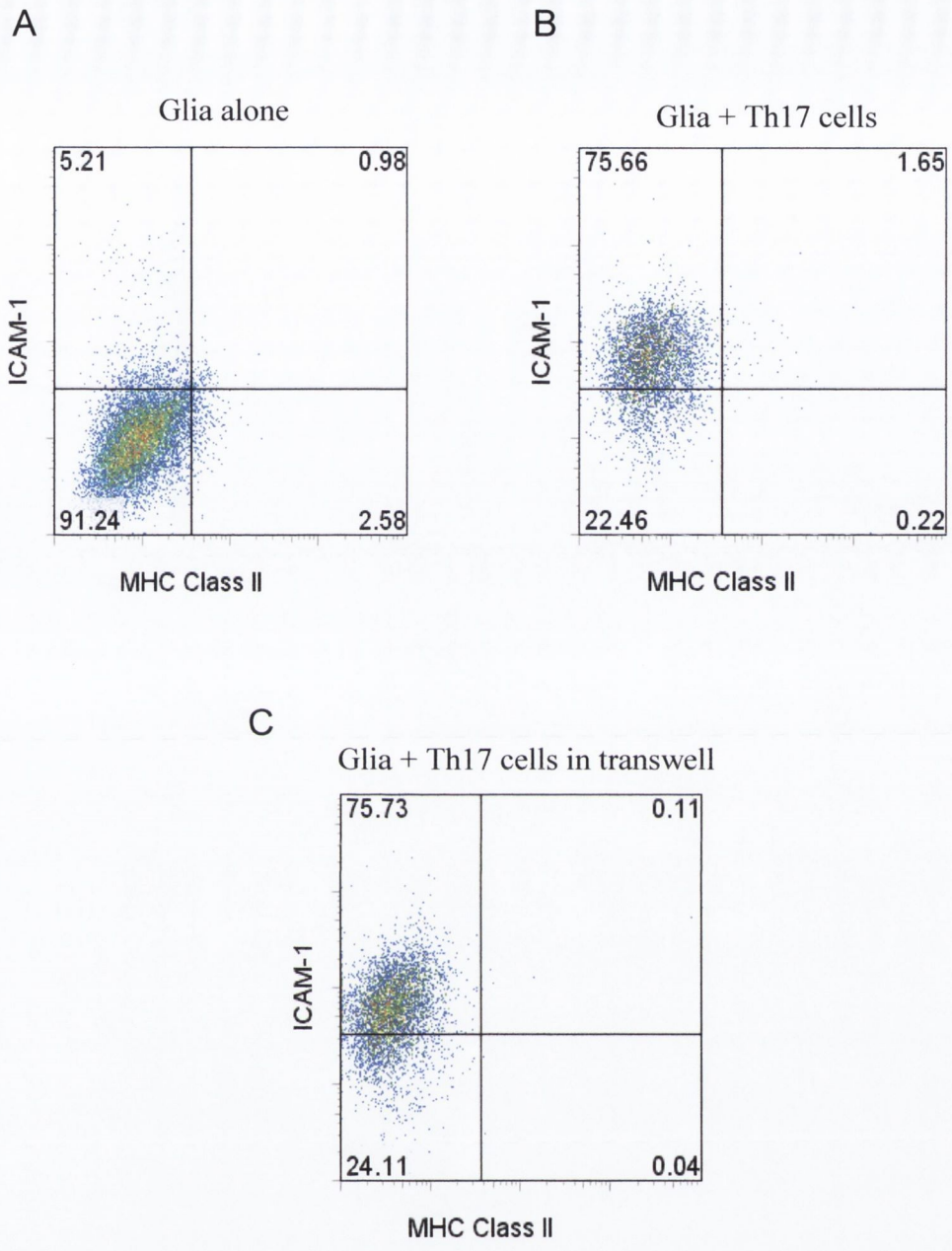

Figure 3.4. Co-culture of glia with KLH-specific Th17 cells results in a significant increase in ICAM-1 expression on microglia. Glial cells were treated with KLH-specific Th17 cells at the ratio of 0.5:1 (T cell: glial cell) or with medium alone. Cells were in direct contact or in a transwell system. After 24 h, MHC class II and ICAM-1 expression was assessed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th17 cells in direct contact, (C) microglia treated with Th17 cells in a transwell. Numbers represent the percentage of cells in the gate.

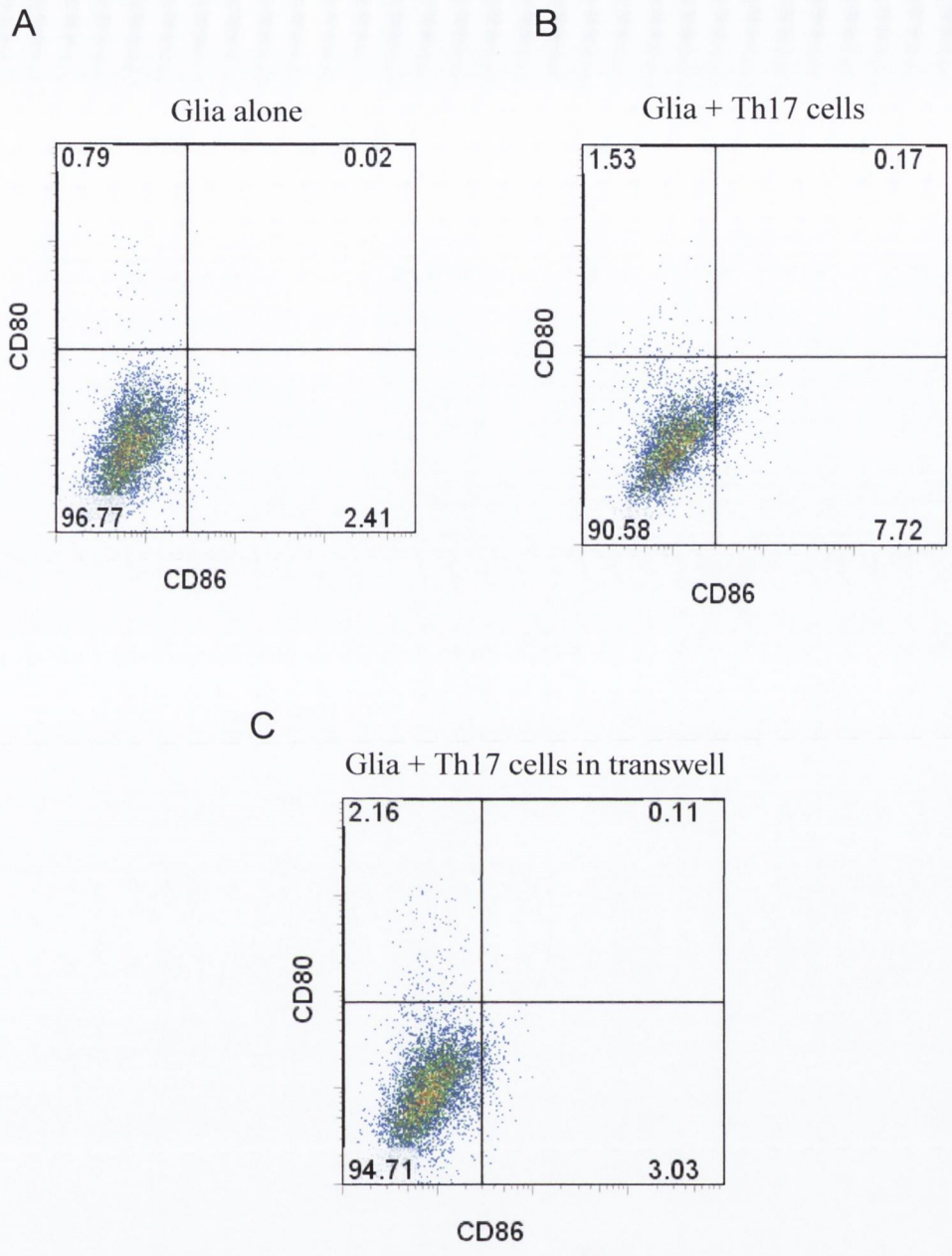

Figure 3.5. T cell: glial cell contact is required for significant upregulation of CD86 on microglia. Glial cells were treated with KLH-specific Th17 cells at the ratio of 0.5:1 (T cell:glial cell) or with medium alone. Cells were contact or the Th17 cells were in a transwell. After 24 h, CD80 and CD86 expression was assessed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th17 cells in direct contact, (C) microglia treated with Th17 cells in a transwell. Numbers represent the percentage of cells in the gate.

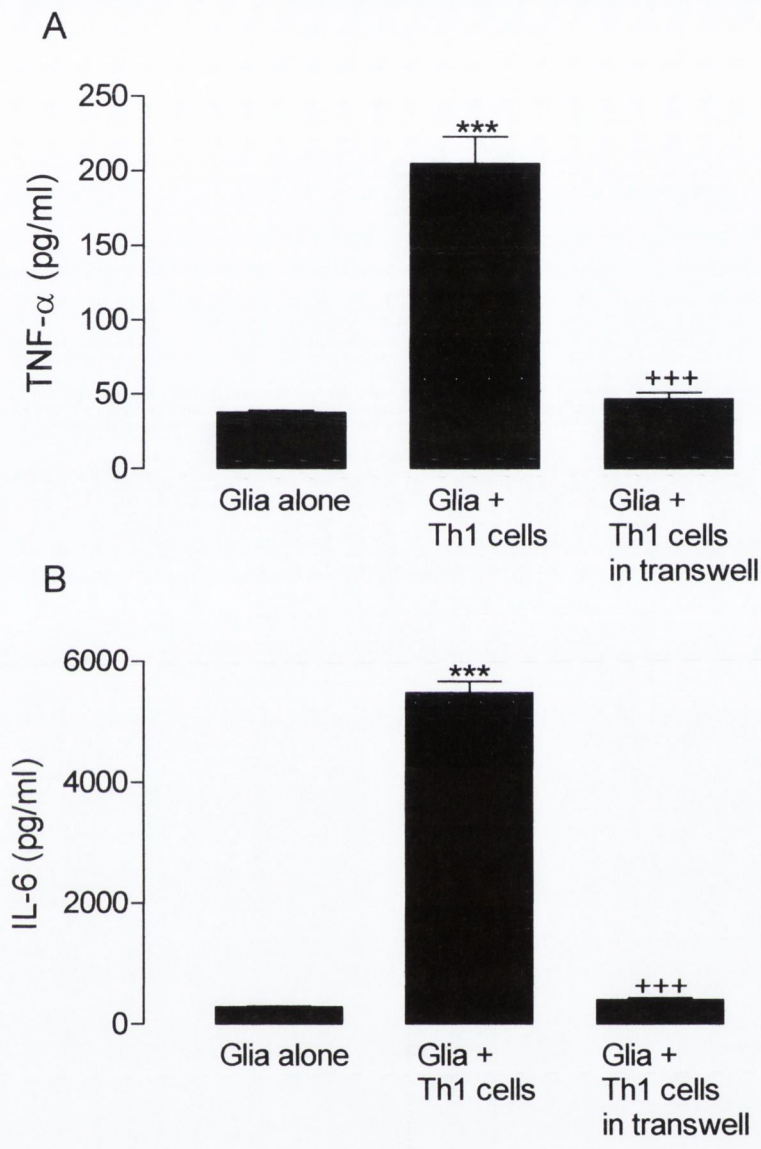

Figure 3.6. Co-culture of glia and KLH-specific Th1 cells induces significant TNF-α and IL-6 production in a cell contact dependent manner. Glia were treated with KLH-specific Th1 cells at a ratio of 0.5:1 (T cell: glia) or with medium only. Cells were either in direct contact or a transwell system was used. After 24 h, TNF-α (A) and IL-6 (B) production was assessed by ELISA. ***$p<0.001$; versus glia alone, $^{+++}p<0.001$; versus glia that were in direct contact with Th1 cells, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments (n=2).

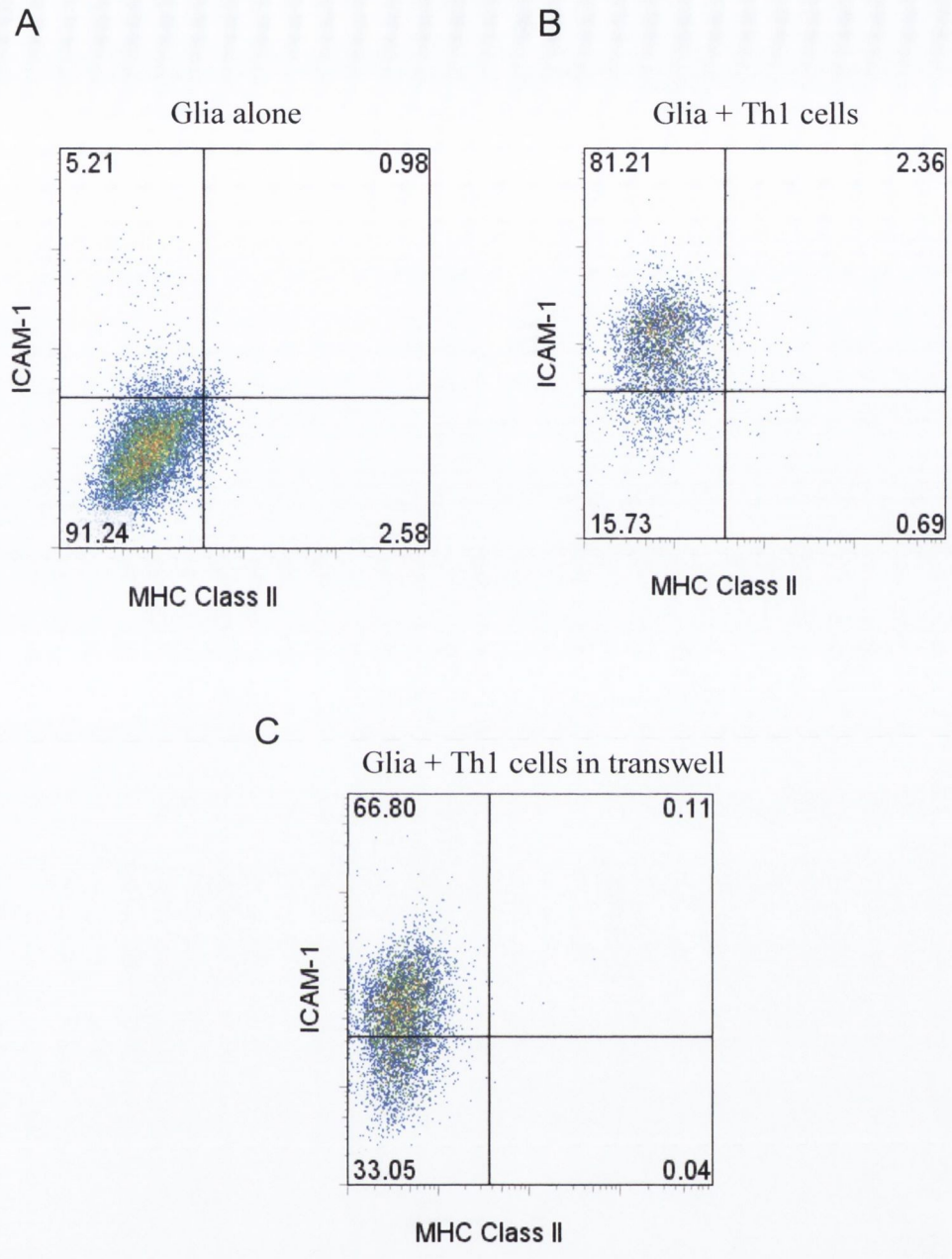

Figure 3.7. Co-culture of glia with KLH-specific Th1 cells results in a significant increase in ICAM-1 expression on microglia. Th1 cells were co-cultured with glial cells at the ratio of 0.5:1 (T cell:glial cell). Cells were in contact or in a transwell. After 24 h, MHC class II and ICAM-1 expression was analysed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th1 cells in direct contact, (C) microglia treated with Th1 cells in a transwell. Numbers represent the percentage of cells in the gate.

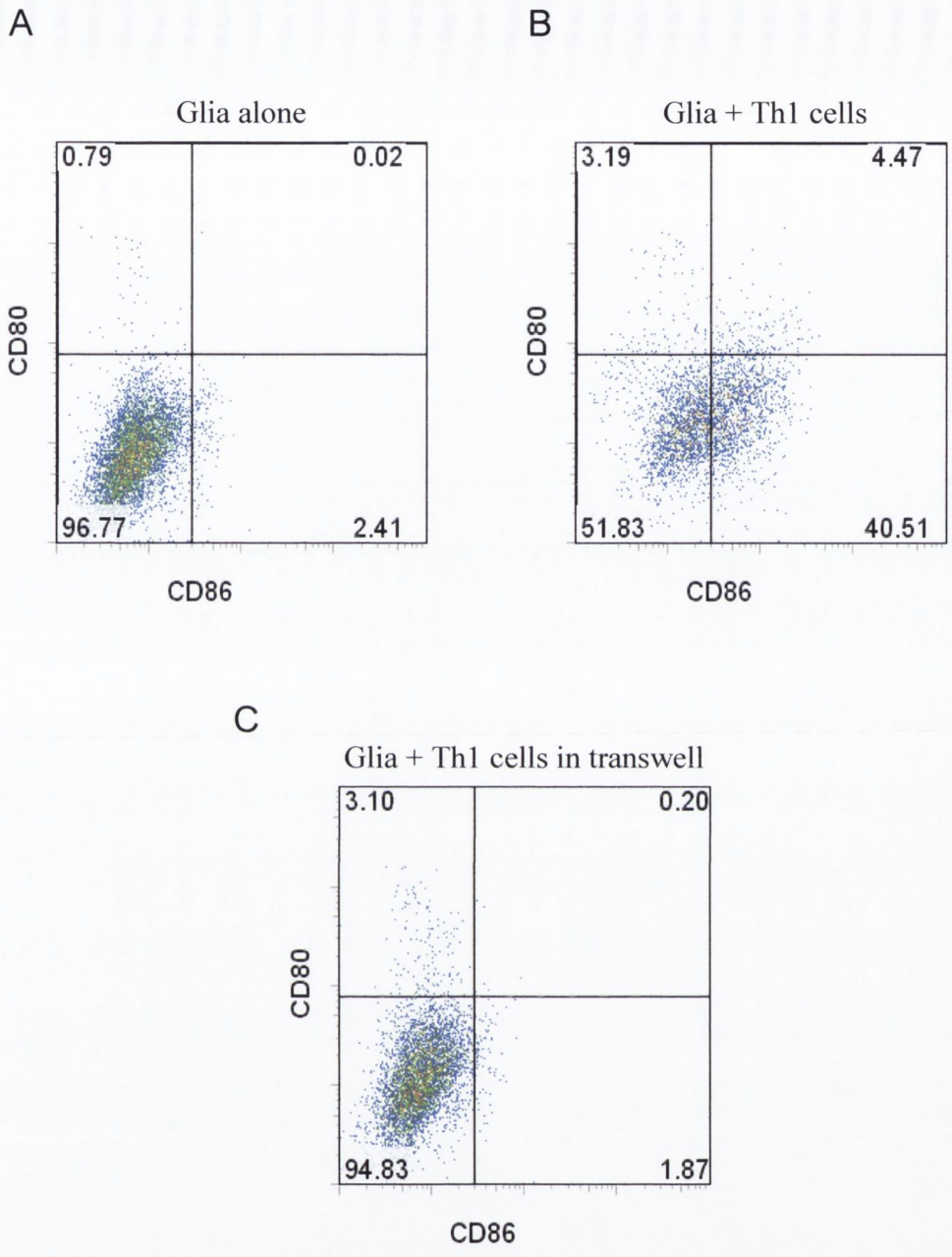

Figure 3.8. T cell: glial cell contact is required for a significant upregulation of CD80 and CD86 on microglia. Glial cells were treated with KLH-specific Th1 cells at the ratio of 0.5:1 (T cell:glial cell) or with medium alone. Cells were in direct contact or a transwell system was used. After 24 h, CD80 and CD86 expression was assessed on $CD11b^+$ cells by flow cytometry. (A) medium treated microglia, (B) microglia treated with Th1 cells in direct contact, (C) microglia treated with Th1 cells in a transwell. Numbers represent the percentage of cells in the gate.

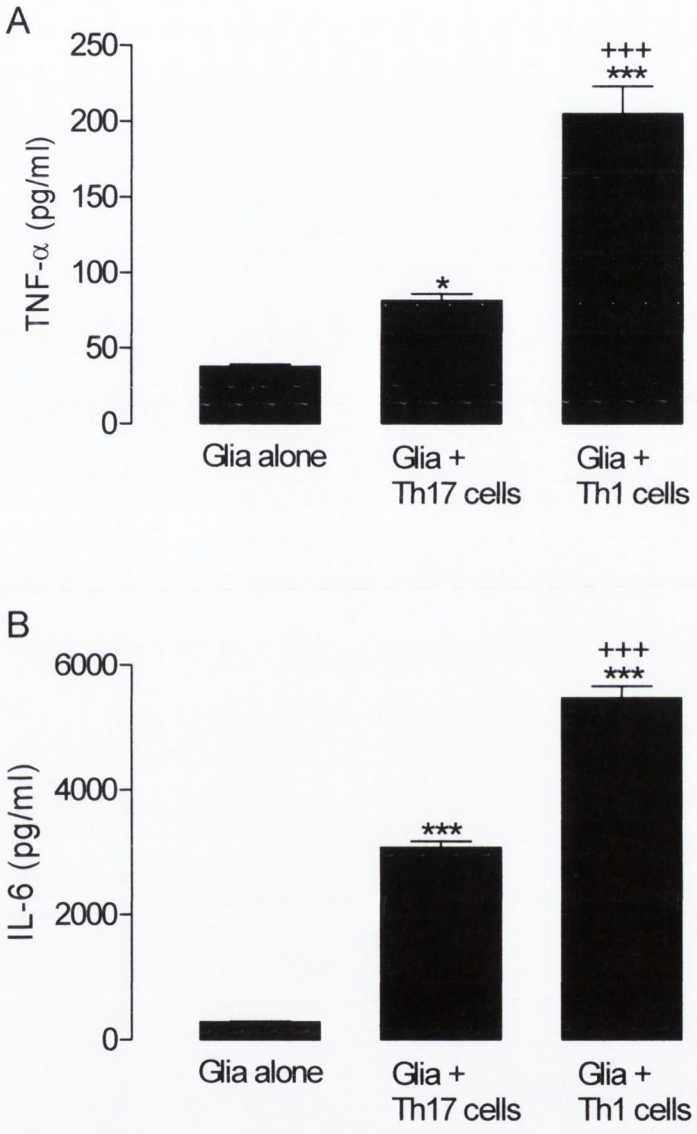

Figure 3.9. Co-culture of glia with KLH-specific Th1 cells induces significantly greater TNF-α and IL-6 release compared to KLH-specific Th17 cells. This Figure is constructed from data in Figures 3.3 and 3.6. Mixed glia cultured from C57BL/6 neonatal mice were treated with KLH-specific Th1 cells and Th17 cells. After 24 h, TNF-α (A) and IL-6 (B) concentration was determined by ELISA. ***$p<0.001$; *$p<0.05$; versus medium-treated glia, $^{+++}p<0.001$; versus glia treated with Th17 cells, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments (n=2).

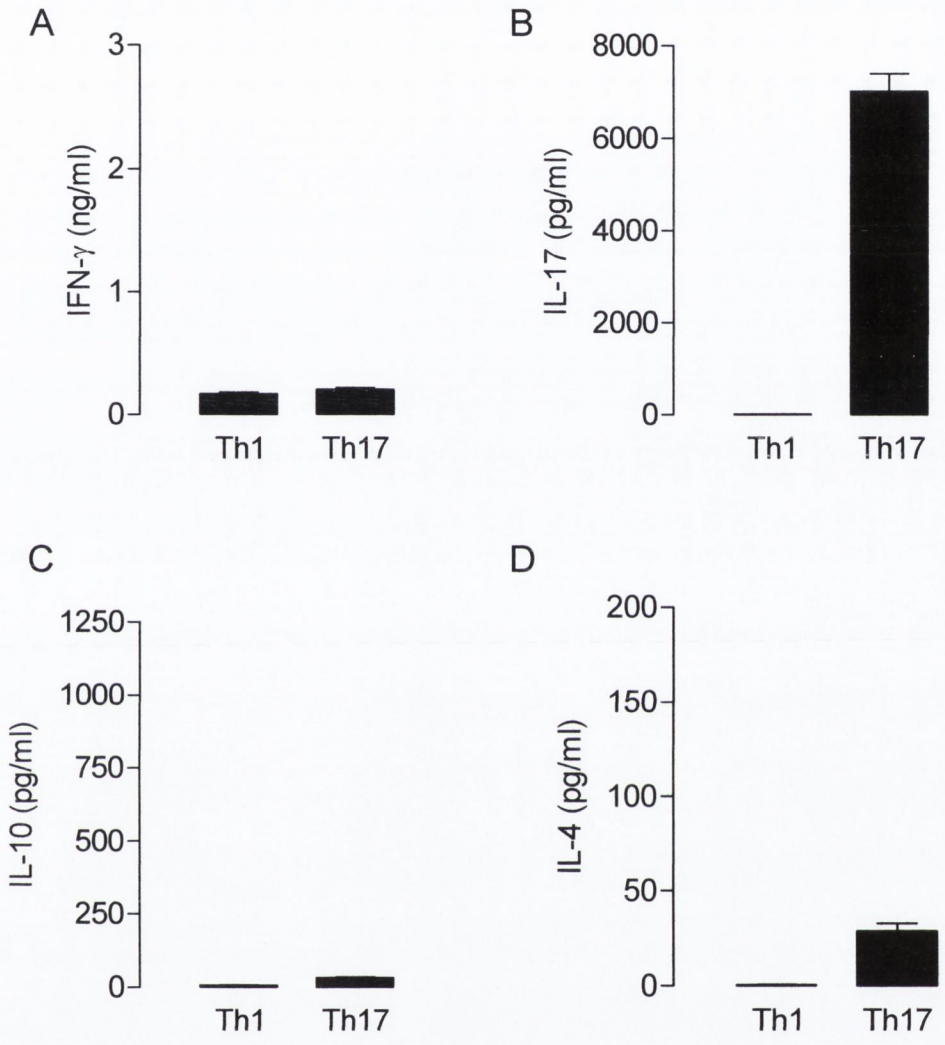

Figure 3.10. Cytokine production from KLH-specific Th1 and Th17 cells lines after a second round of antigen stimulation. C57BL/6 mice were immunized with KLH (10 μg) and LPS (10 μg) into the footpad. After 7 days, the popiteal lymph nodes were removed and the cells stimulated with KLH (25 μg/ml) and Th1 polarising cytokine IL-12 (1 ng/ml) and Th17 polarising cytokines IL-23 (10 ng/ml), IL-1β (10 ng/ml) and anti-IFN-γ (10 μg/ml) . After 11 days of culture, cells were restimulated with irradiated APC, KLH and polarising cytokines. After a further 4 days, supernatants were removed and IFN-γ (A), IL-17 (B), IL-10 (C) and IL-4 (D) concentrations were quantified by ELISA.

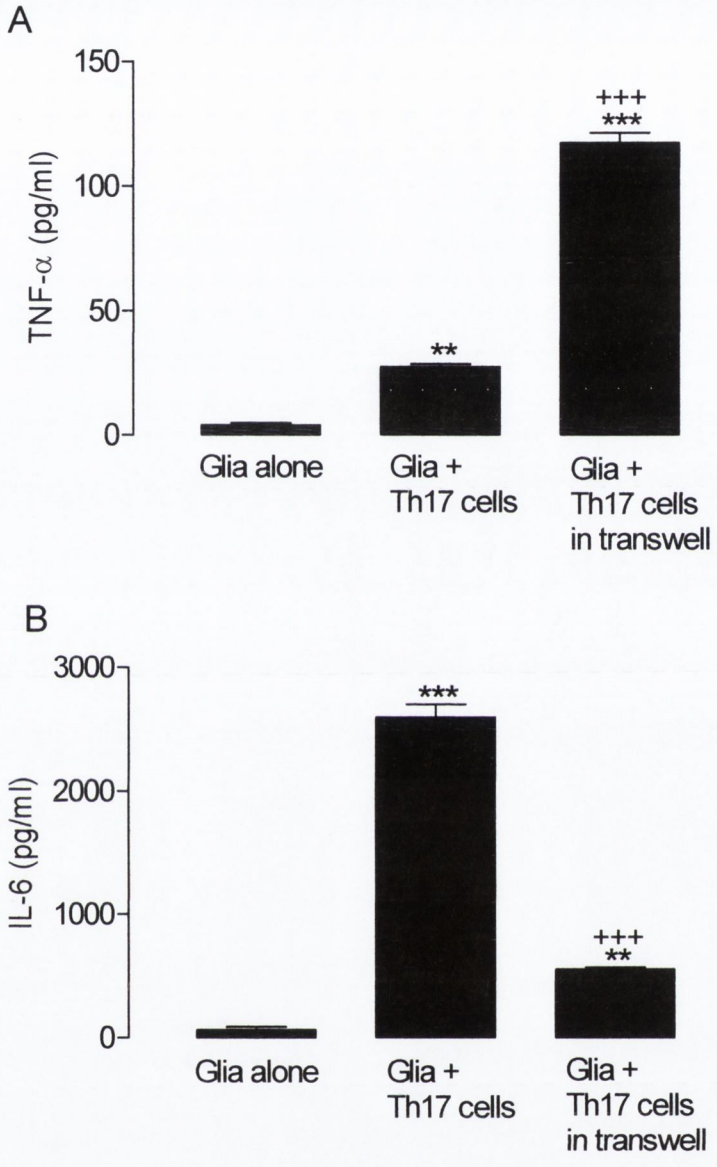

Figure 3.11. Co-culture of glia and re-stimulated KLH-specific Th17 cells induces significant TNF-α and IL-6 production. After a second round of antigen stimulation, KLH-specific Th17 cells were co-cultured with mixed glial cells at the ratio of 0.5:1 (T cell: glial cell). Cells were either in direct cell-cell contact or in a transwell system. After 24 h, production of TNF-α (A) and IL-6 (B) was assessed by ELISA. ***$p<0.001$,**$p<0.01$, versus medium-treated glial cells, $^{+++}p<0.01$; versus glia incubated with Th17 cells that were in direct contact, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments (n=2).

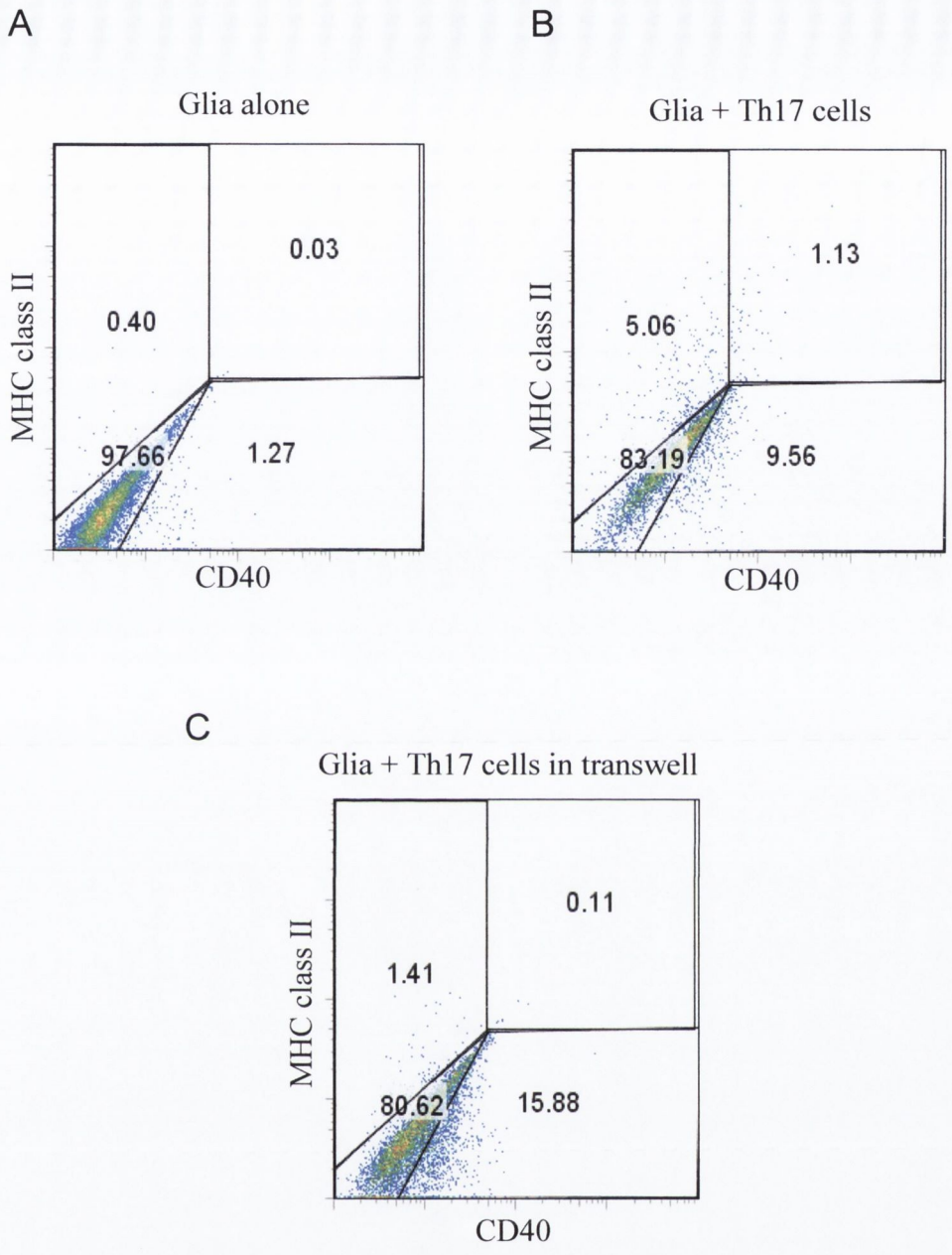

Figure 3.12. T cell:glial cell contact is required for a significant upregulation of MHC class II expression on microglia. Glia were treated with medium only or were co-cultured with antigen re-stimulated KLH-specific Th17 cells. Cells were either in direct cell contact or a transwell system was used. After 24 h, MHC class II and CD40 expression on $CD11b^+$ cells was analysed by FACS. (A) medium-treated microglia, (B) microglia treated with Th17 cells in direct contact, (C) microglia treated with Th17 cells in a transwell. Numbers represent the percentage of cells in the gate.

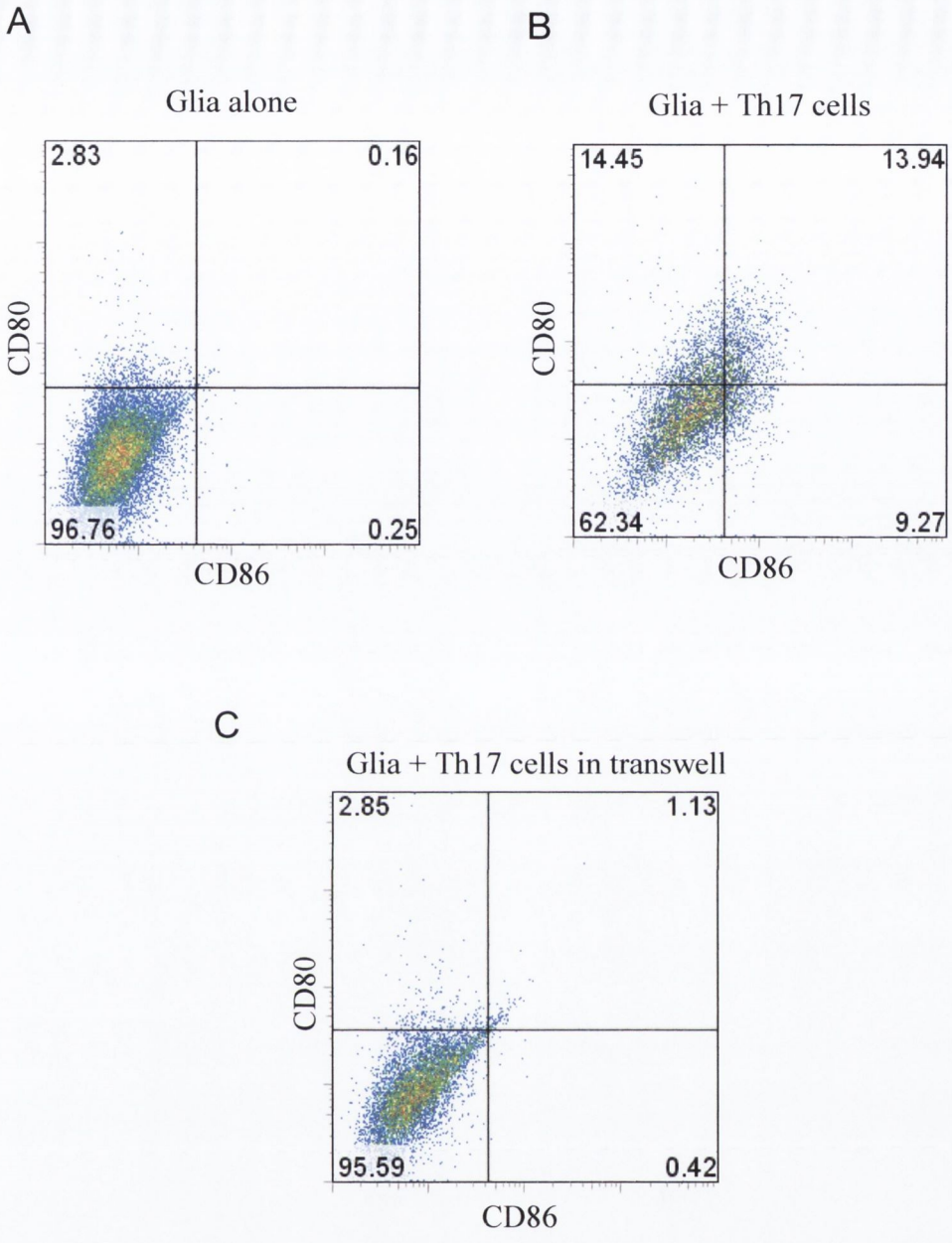

Figure 3.13. T cell:glial cell contact is required for a significant upregulation of CD80 and CD86 on microglia. Antigen re-stimulated KLH-specific Th17 cells were co-cultured with glial cells at the ratio of 0.5:1 (T cell:glial cell). Cells were in contact or Th17 cells were in a transwell. After 24 h, CD80 and CD86 expression was assessed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th17 cells in direct contact, (C) microglia treated with Th17 cells in a transwell. Numbers represent the percentage of cells in the gate.

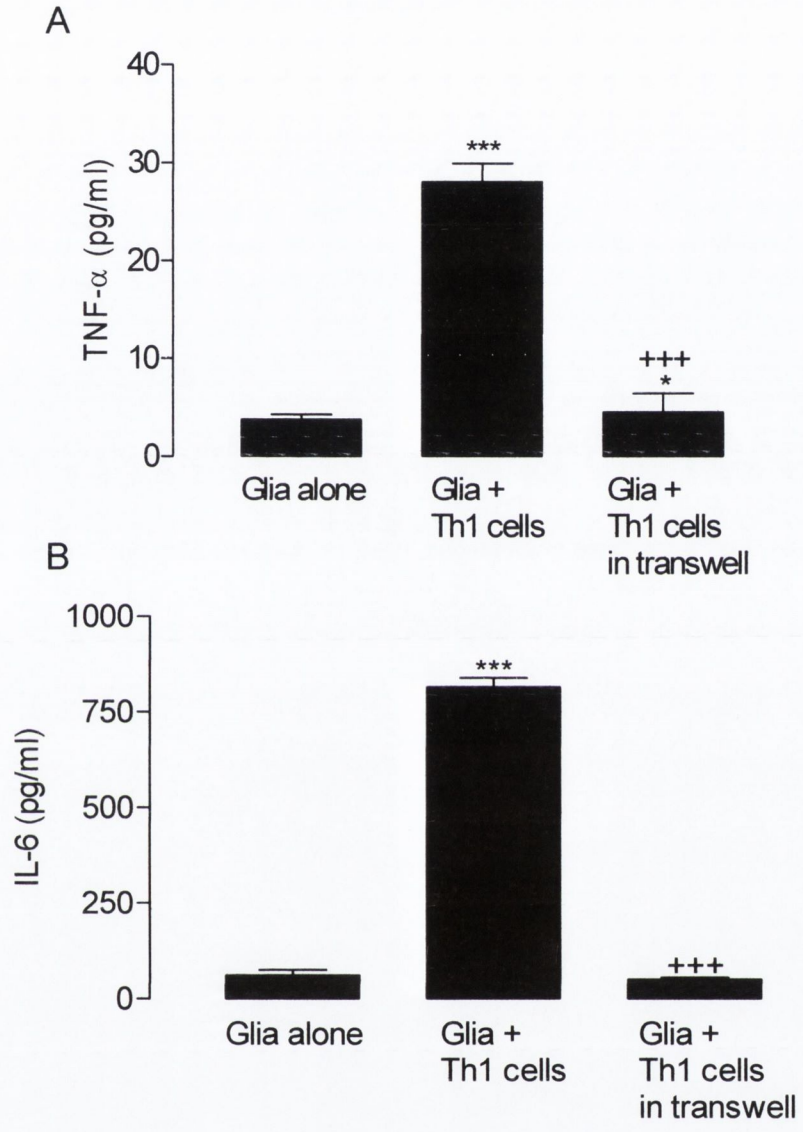

Figure 3.14. Co-culture of glia and antigen re-stimulated KLH-specific Th1 cells induces significant TNF-α and IL-6 production in a cell contact dependent manner. Glia were treated with second round antigen re-stimulated KLH-specific Th1 cells at a ratio of 0.5:1 or with medium alone. Th1 cells were in a transwell or were in contact with the glia. After 24 h, TNF-α (A) and IL-6 (B) concentration was quantified by ELISA. *$p<0.05$; ***$p<0.001$; versus medium-treated glia, $^{+++}p<0.001$; versus glia that were in direct contact with Th1 cells, by one-way ANOVA. Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments (n=2).

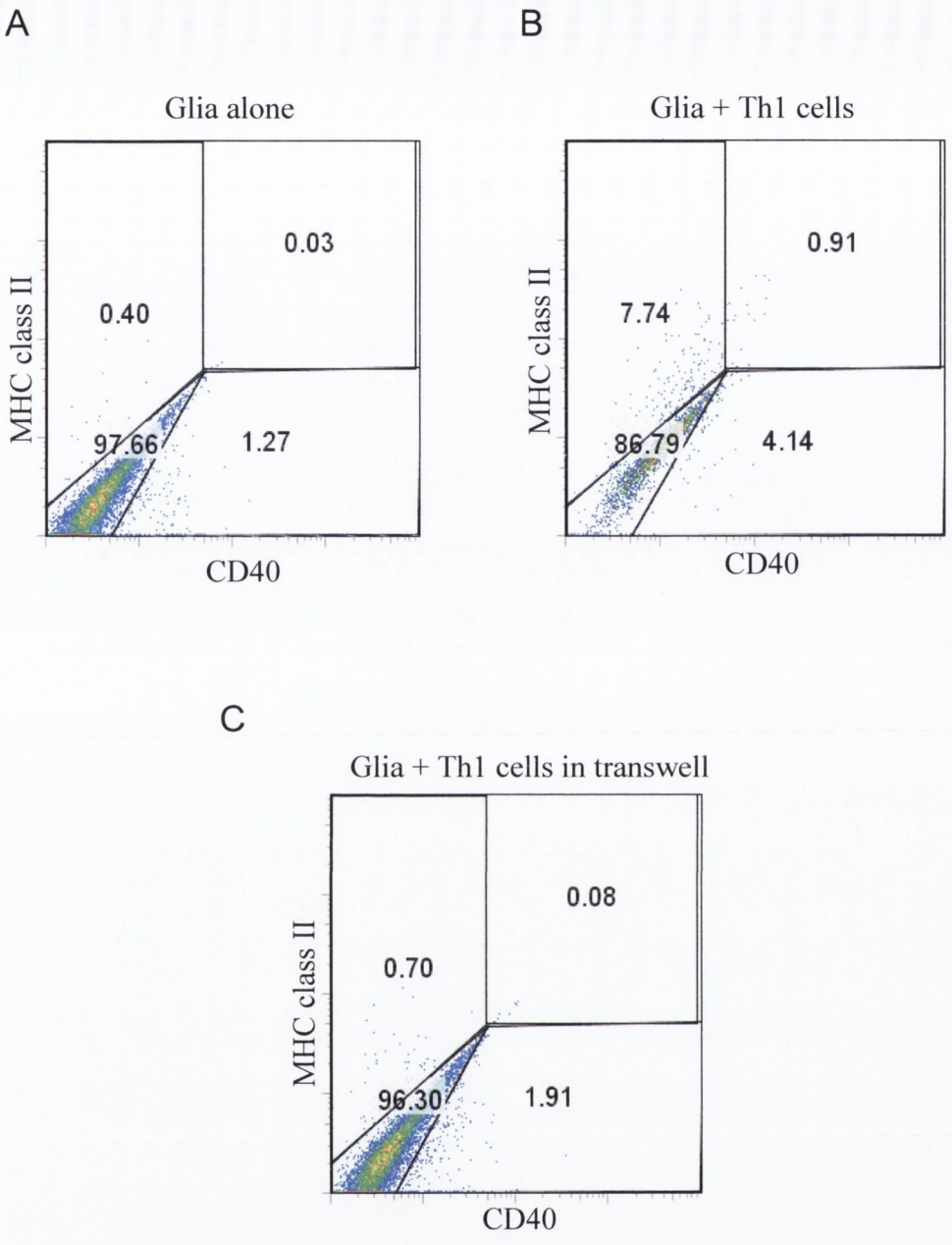

Figure 3.15. T cell:glial cell contact is required for a significant upregulation of MHC class II and CD40 expression on microglia. Glia were treated with medium only or were co-cultured with second round antigen re-stimulated KLH-specific Th1 cells at the ratio of 0.5:1 (T cell:glial cell). Th1 cells were in contact with glia or were in a transwell. After 24 h, MHC class II and CD40 expression on $CD11b^+$ cells was analysed by ELISA. (A) medium-treated microglia, (B) Th1 cells in direct contact with microglia, (C) microglia treated with Th1 cells in a transwell. Numbers represent the percentage of cells in the gate.

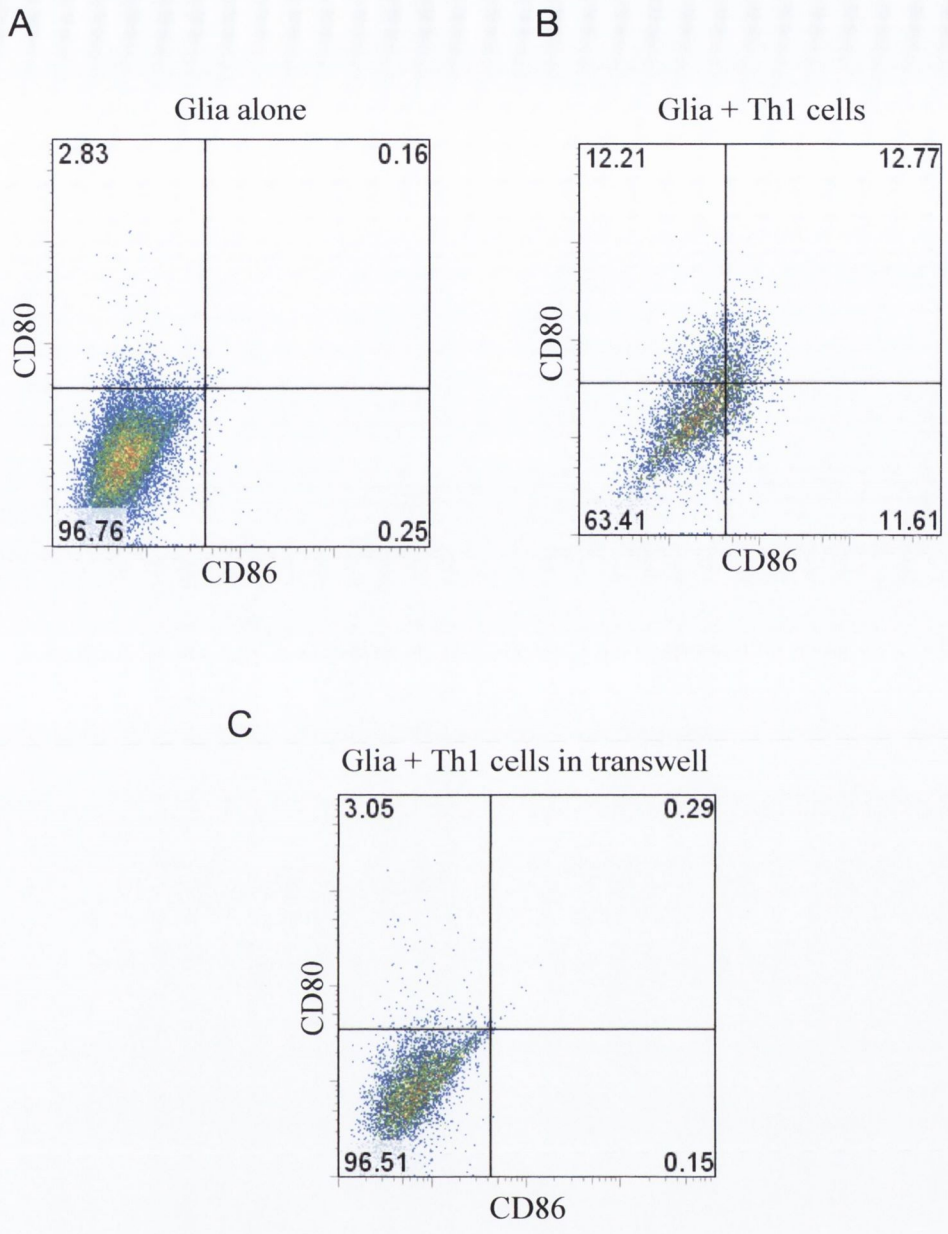

Figure 3.16. T cell:glial cell contact is required for a significant upregulation of CD80 and CD86 on microglia. Second round antigen re-stimulated KLH-specific Th1 cells were co-cultured with glial cells at the ratio of 0.5:1 (T cell:glial cell). Cells were in contact or Th1 cells were in a transwell. After 24 h, CD80 and CD86 expression was assessed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th1 cells in direct contact, (C) microglia treated with Th1 cells in a transwell. Numbers represent the percentage of cells in the gate.

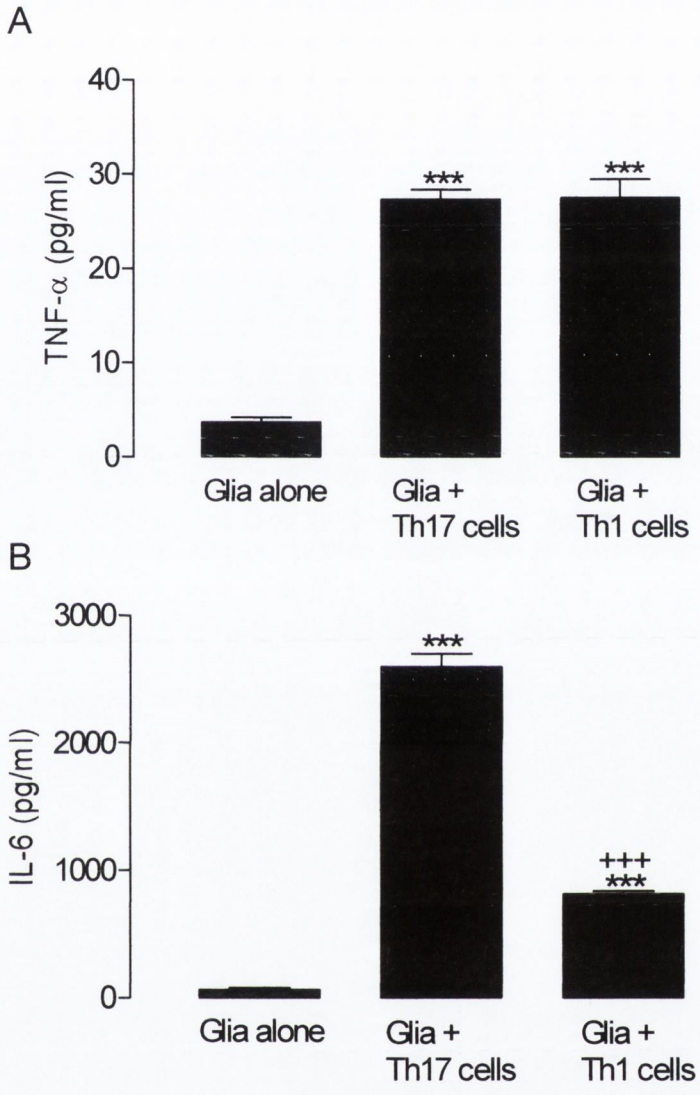

Figure 3.17. Co-culture of glia with antigen re-stimulated KLH-specific Th17 cells induces greater IL-6 but equal TNF-α production compared with co-culture of glia and KLH-specific Th1 cells. This Figure is constructed from data in Figures 3.11 and 3.14. After a second round of antigen re-stimulation, KLH-specific Th1 and Th17 cells were incubated for 24 h with glia. Supernatants were removed and TNF-α (A) and IL-6 (B) production was assessed by ELISA. ***$p<0.001$; versus medium-treated glia; $^{+++}p<0.001$; versus glia treated with Th17 cells, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments (n=2).

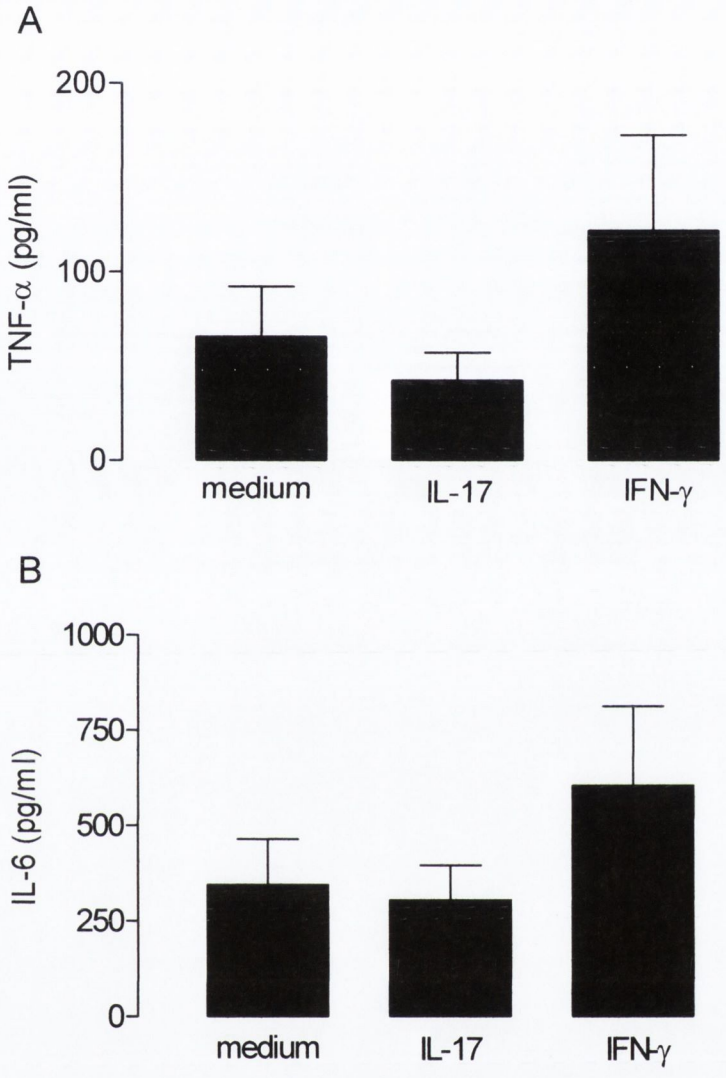

Figure 3.18. IL-17 and IFN-γ do not stimulate TNF-α and IL-6 production from glia. Mixed glia were cultured from C57BL/6 neonatal mice for 10 days and treated with IL-17 (10 ng/ml) and IFN-γ (10 ng/ml) for 24 h. Supernatants were removed and TNF-α (A) and IL-6 (B) production was assessed. Data presented as mean ± SEM. (n=4).

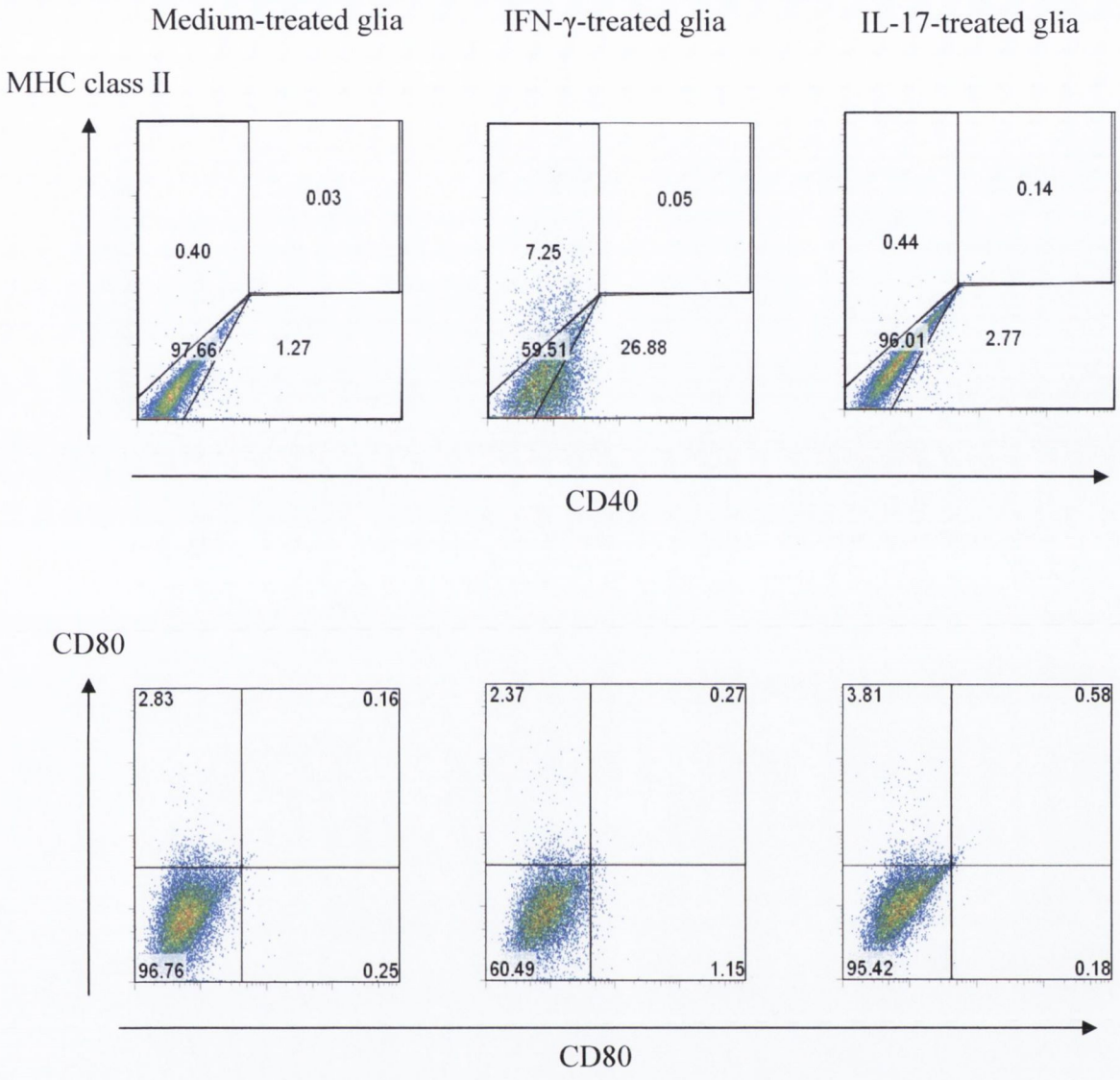

Figure 3.19. IFN-γ upregulates MHC class II and CD40 expression on microglia. Glia were cultured from 1 day-old C57BL6 neonatal mice. After 10 days in culture, glia were treated with medium, IFN-γ (10 ng/ml) and IL-17 (10 ng/ml) for 24 h. MHC class II, CD40, CD80 and CD86 expression was analysed on $CD11b^+$ microglia by flow cytometry. Numbers represent the percentage of cells in the gate. (n=4)

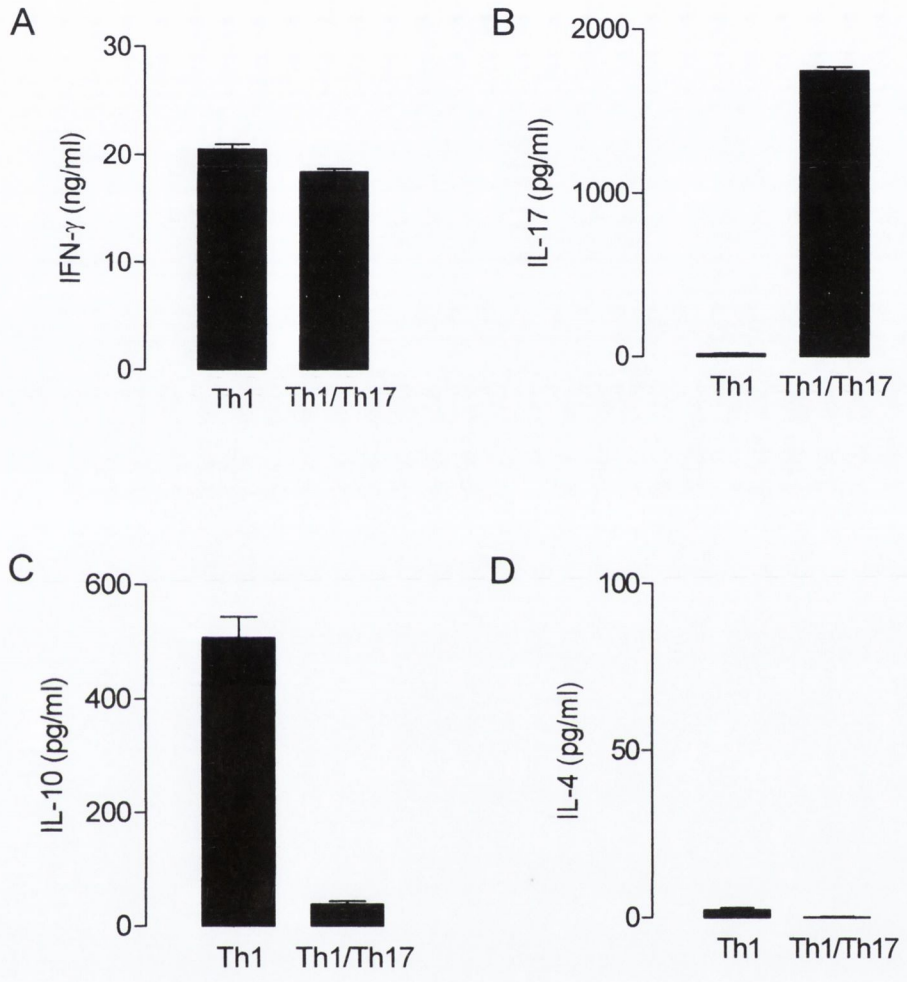

Figure 3.20. Cytokine production from MOG-specific Th1 and Th1/Th17 cells. EAE was induced in C57BL/6 mice as described in method 2.2.11. After 7 days, spleen and inguinal nodes were removed, the cells were stimulated with MOG_{35-55} (25 μg/ml) and Th1 polarising cytokine IL-12 (1 ng/ml) and Th1/Th17 polarising cytokines IL-23 (10 ng/ml) and IL-1β (10 ng/ml). After 7 days, supernatants were removed and IFN-γ (A), IL-17 (B), IL-10 (C) and IL-4 (D) concentrations were quantified by ELISA.

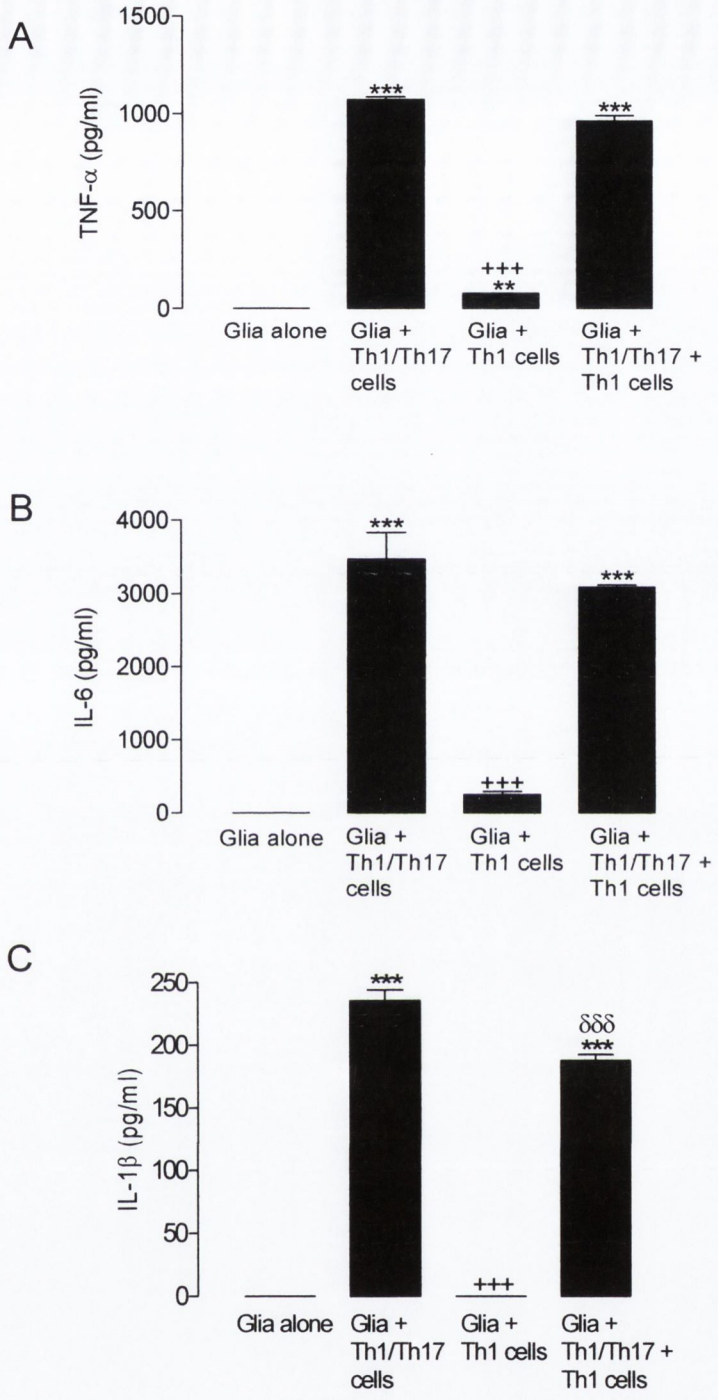

Figure 3.21. Addition of MOG-specific Th1 cells does not enhance cytokine production in co-cultures of glia and MOG-specific Th1/Th17 cells. MOG-specific Th1 cells and Th1/Th17 cells were added to cultured glial cells for 24 h at a ratio of 0.5:1. Supernatant was removed and TNF-α (A), IL-6 (B) and IL-1β (C) concentrations were quantified by ELISA. **$p<0.01$ ***$p<0.001$; versus medium treated glia, $^{+++}p<0.001$; versus glia treated with Th1 cells $^{\delta\delta\delta}p<0.001$; versus Th1/Th17 alone treated glia, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 4 independent experiments.

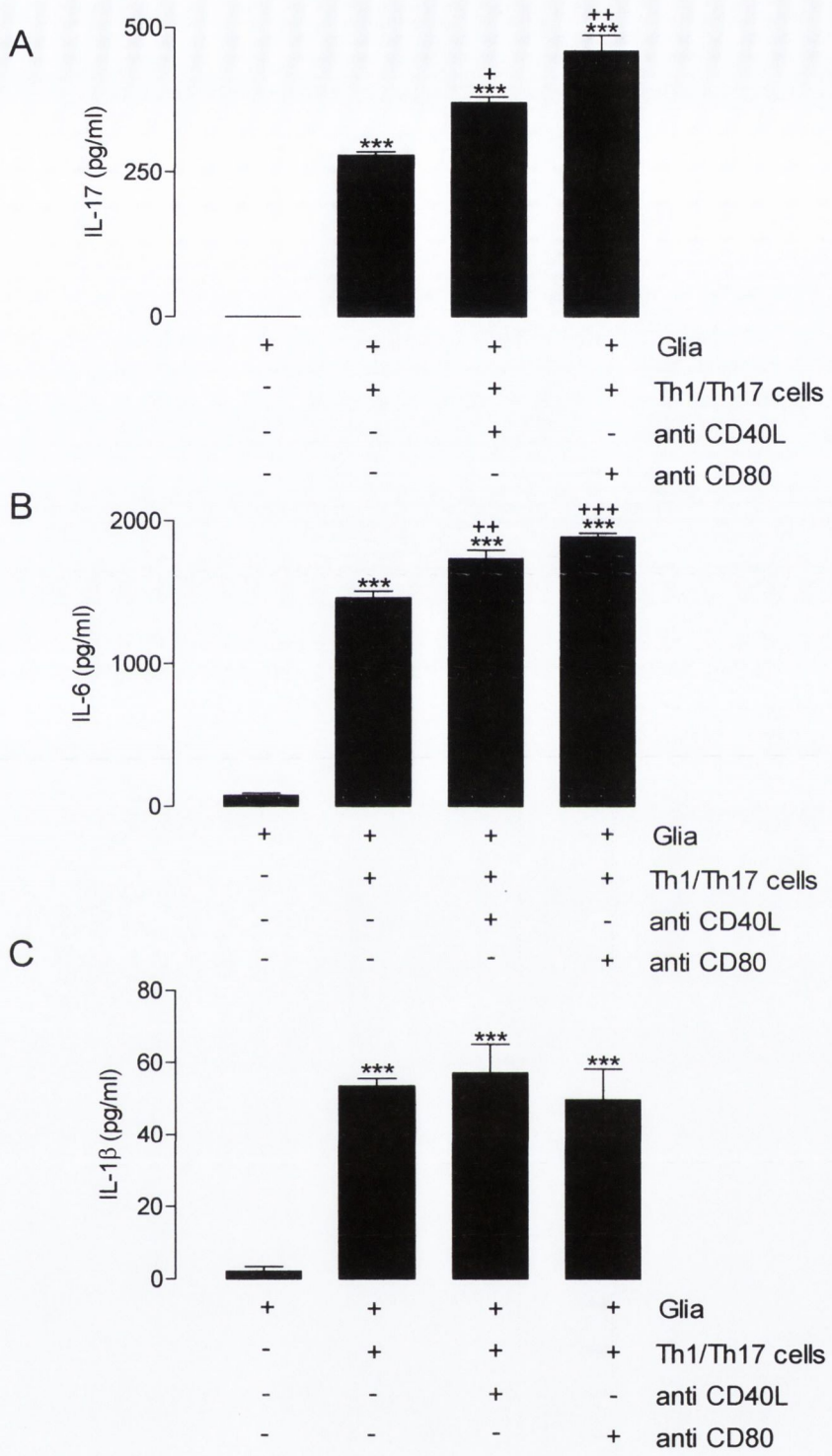

Figure 3.22. CD40 ligand and CD80 are not required for induction of proinflamamatory cytokines in co-cultures of glia and MOG-specific Th1/Th17 cells. MOG-specific Th1/Th17 cells were co-cultured with mixed glial cells at the ratio of 0.5:1 in the presence or absence of neutralising antibodies to CD40L (10 μg/ml) and CD80 (10 μg/ml). After 24 h, supernatant was removed and IL-17 (A), IL-6 (B) and IL-1β (C) production was assessed by ELISA. ***$p<0.001$; versus medium-treated glia, $^{++}p<0.01$; $^{+++}p<0.001$; versus glia treated with Th1/Th17 cells, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments (n=4).

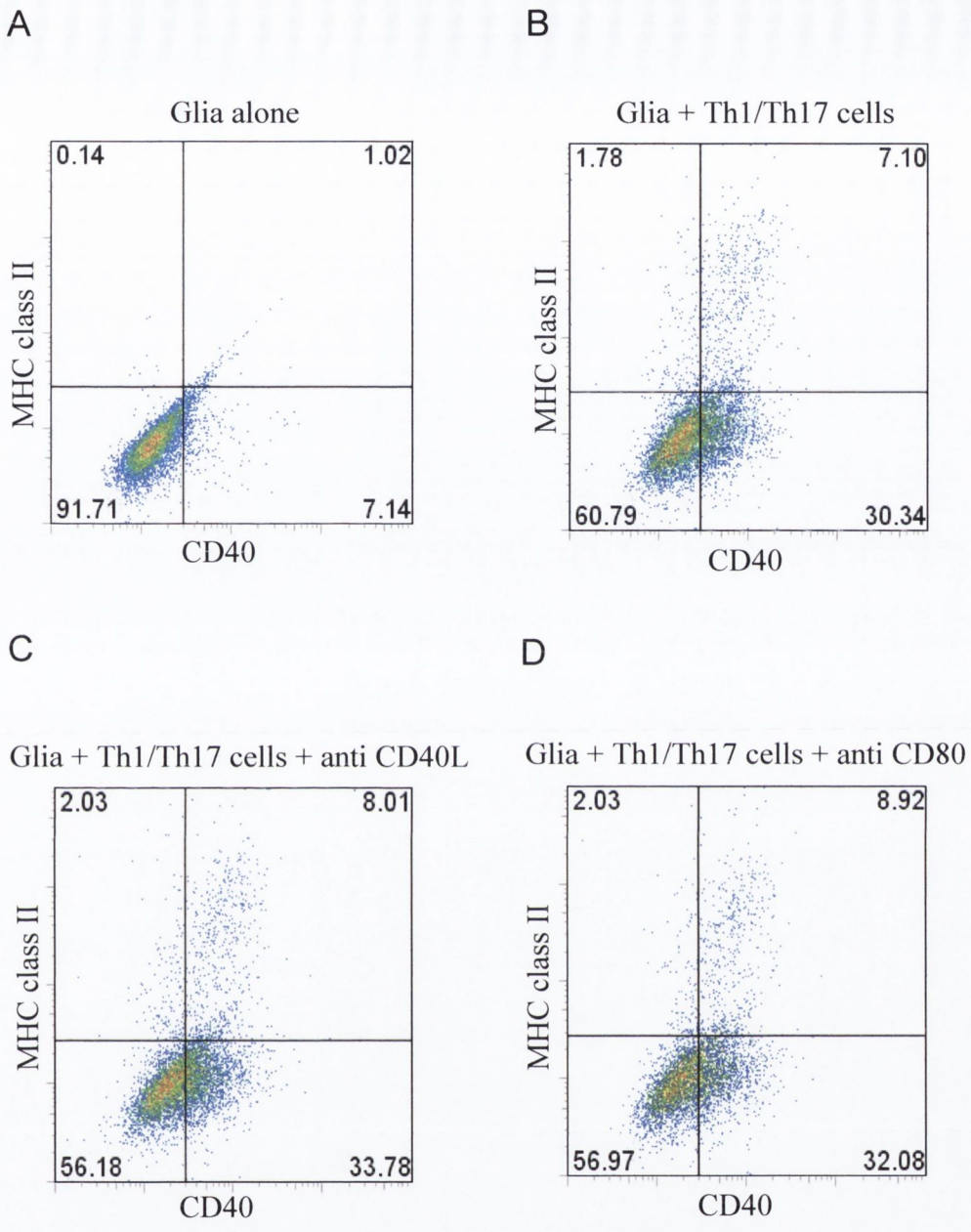

Figure 3.23. MOG-specific Th1/Th17 cell-induced upregulation of MHC class II and CD40 expression on microglia is unaltered by neutralising CD40L and CD80. Glia cells were co-cultured for 24 h with MOG-specific Th1/Th17 cells. Expression of MHC class II and CD40 was assessed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th1/Th17 cells, (C) microglia treated with Th1/Th17 cells and an anti-CD40L antibody (10 μg/ml), (D) microglia with Th1/Th17 cells and an anti-CD80 antibody (10 μg/ml). Numbers represent the percentage of cells in the gate. (n=4).

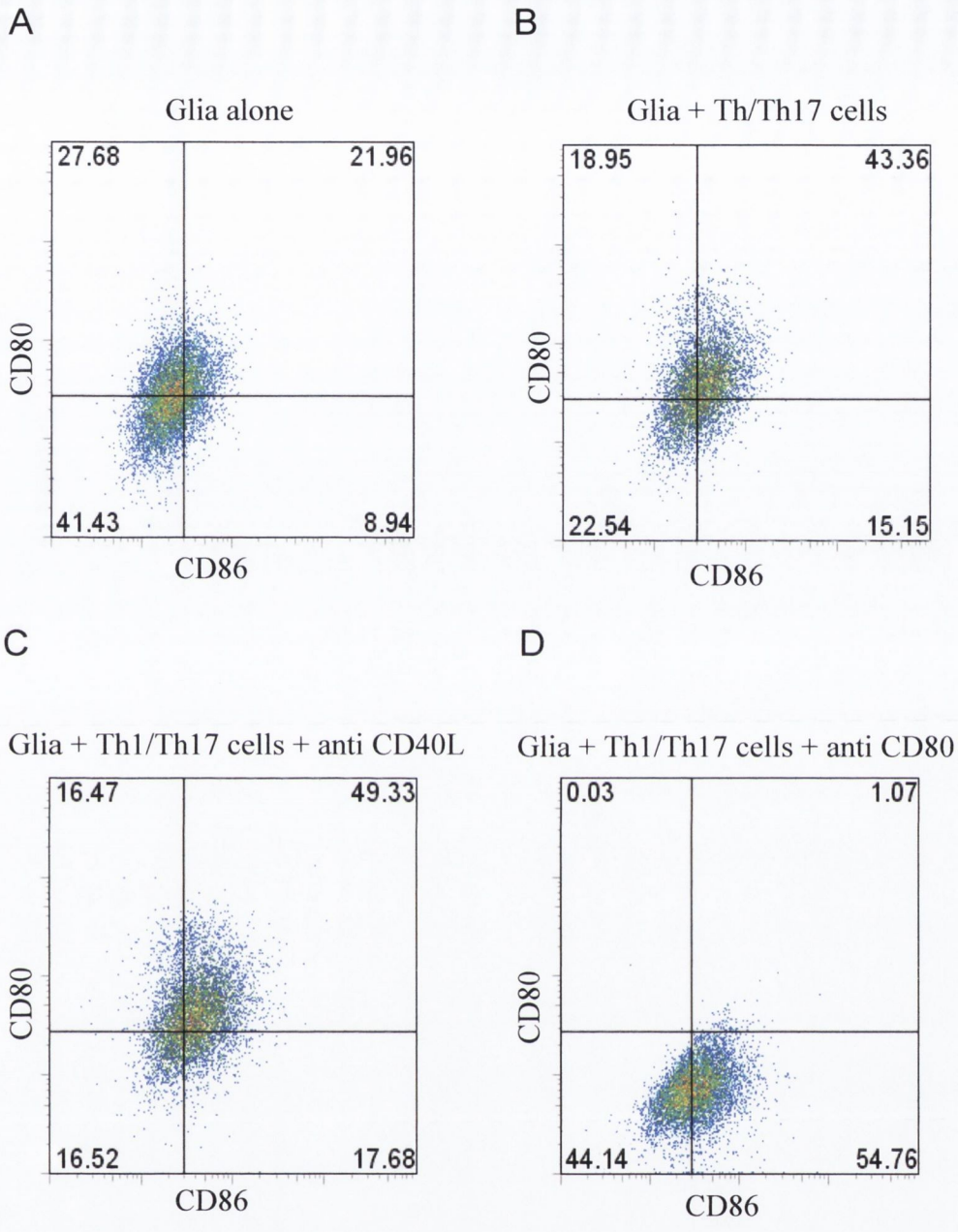

Figure 3.24. MOG-specific Th1/Th17 cell-induced upregulation of CD80 and CD86 expression on microglia is unaltered by neutralising CD40L and CD80. Glial cells were co-cultured for 24 h with MOG-specific Th1/Th17 cells. CD80 and CD86 expression was assessed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia in direct contact with Th1/Th17 cells, (C) microglia treated with Th1/Th17 cells and an anti-CD40L antibody (10 μg/ml), (D) microglia treated with Th1/Th17 cells and an anti-CD80 antibody (10 μg/ml). Numbers represent the percentage of cells in the gate. (n=4).

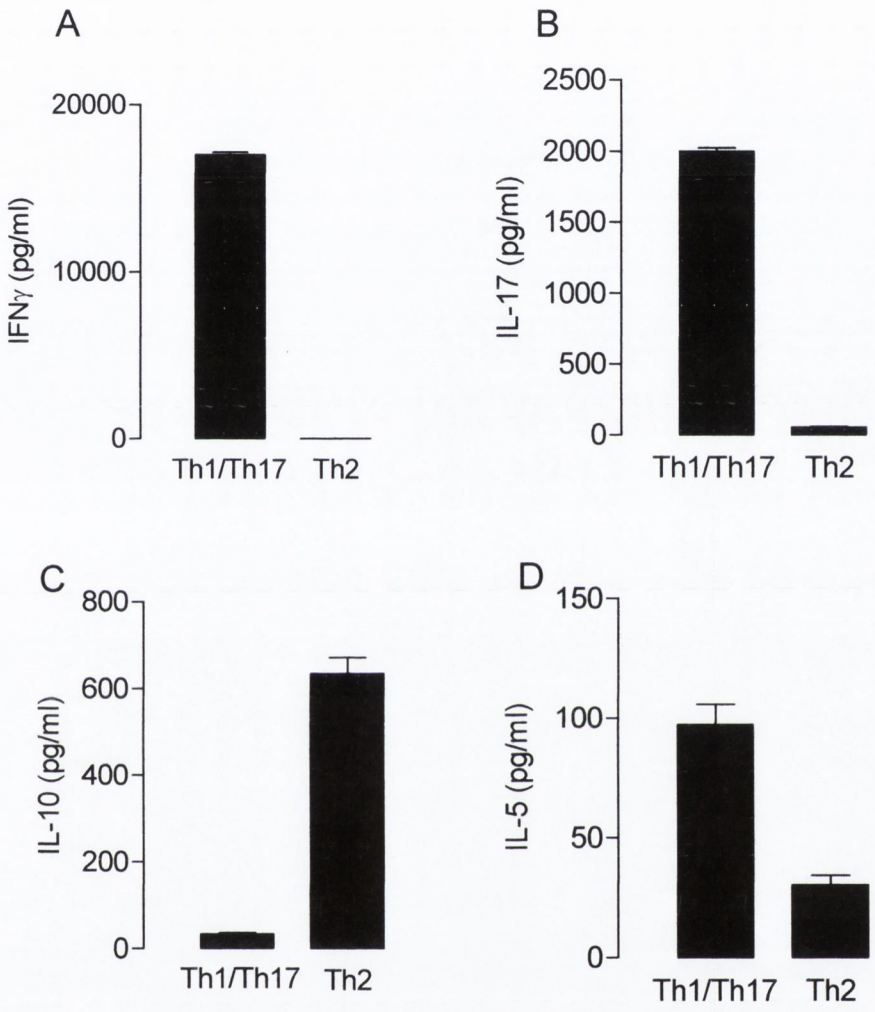

Figure 3.25. Cytokine profile of MOG-specific Th1/Th17 and Th2 cells. EAE was induced in C57BL/6 mice as described in method 2.2.11. After 7 days, the spleens and inguinal lymph nodes were removed and the cells stimulated with MOG (25 μg/ml) and Th1/Th17 polarising cytokines IL-23 (10 ng/ml), IL-1β (10 ng/ml) or Th2 polarising proteins dexamethasone (1 x 10^{-8} M) and IL-4 (20 ng/ml) . Day 7 of culturing, supernatants were removed and IFN-γ (A), IL-17 (B), IL-10 (C) and IL-4 (D) concentrations were quantified by ELISA.

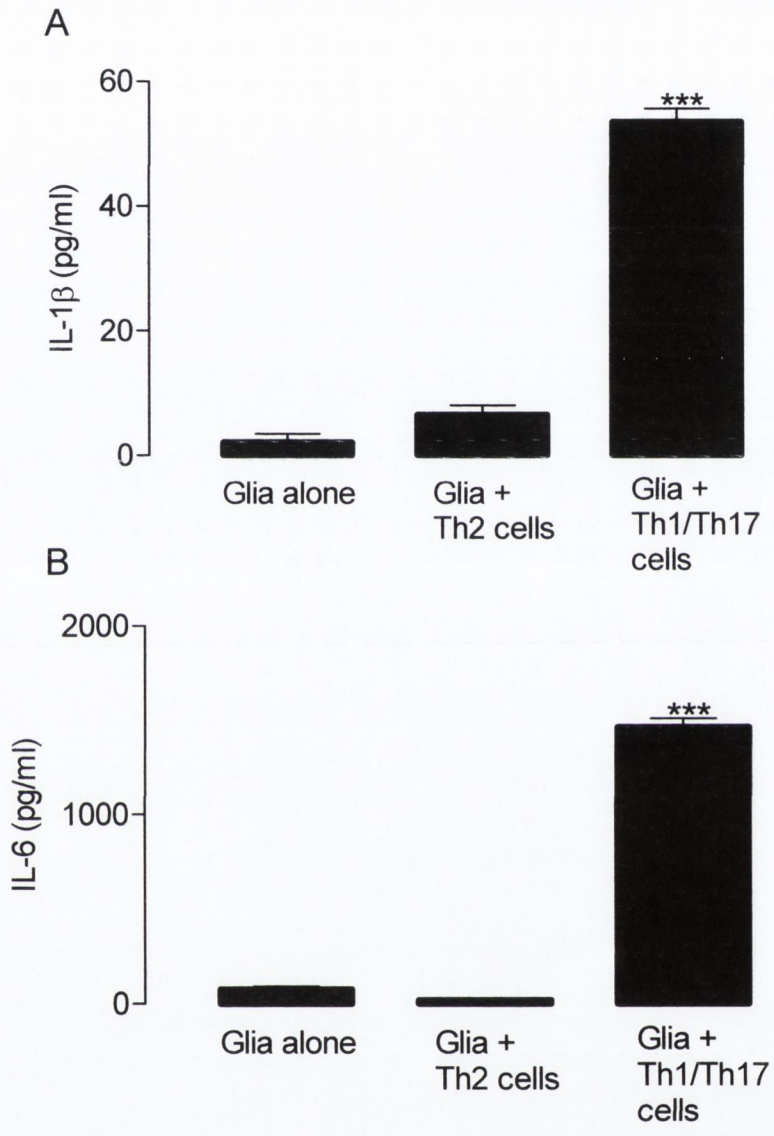

Figure 3.26. Co-cultures of glia and MOG-specific Th1/Th17 cells induce significantly greater IL-1β and IL-6 production compared with glia treated with MOG-specific Th2 cells. Glia were treated for 24 h with MOG-specific Th2 cells and Th1/Th17 cells. Supernatants were removed and IL-1β (A) and IL-6 (B) production was assessed by ELISA. ***$p<0.001$; versus medium-treated glia, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments.

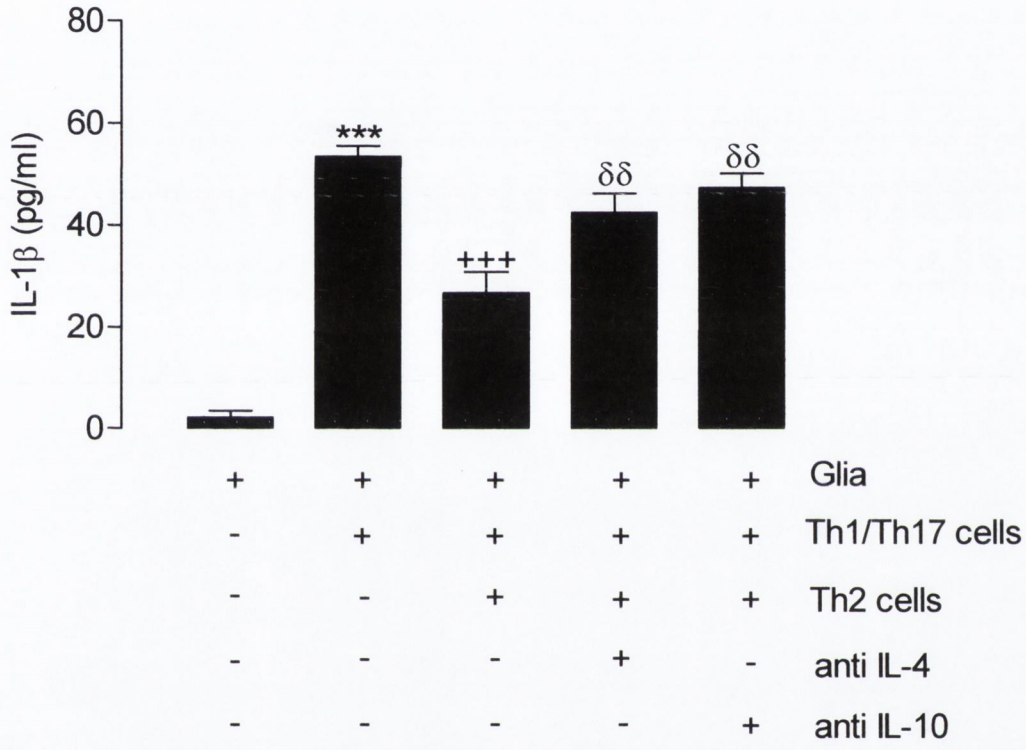

Figure 3.27. The inhibitory effect of Th2 cells is partially mediated by IL-4 and IL-10. MOG-specific Th1/Th17 cells and Th2 cells were co-cultured with glial cells at the ratio of 0.5:1 for 24 h, in the presence or absence of neutralising antibodies to IL-4 (10 μg/ml) and IL-10 (10 μg/ml). Supernatant was removed and IL-1β concentration was quantified by ELISA. ***$p<0.001$; versus medium-treated glia; $^{+++}p<0.001$; versus Th1/Th17 cell treated glia; $^{\delta\delta}p<0.01$; versus Th1/Th17 cell and Th2 cell treated glia, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments.

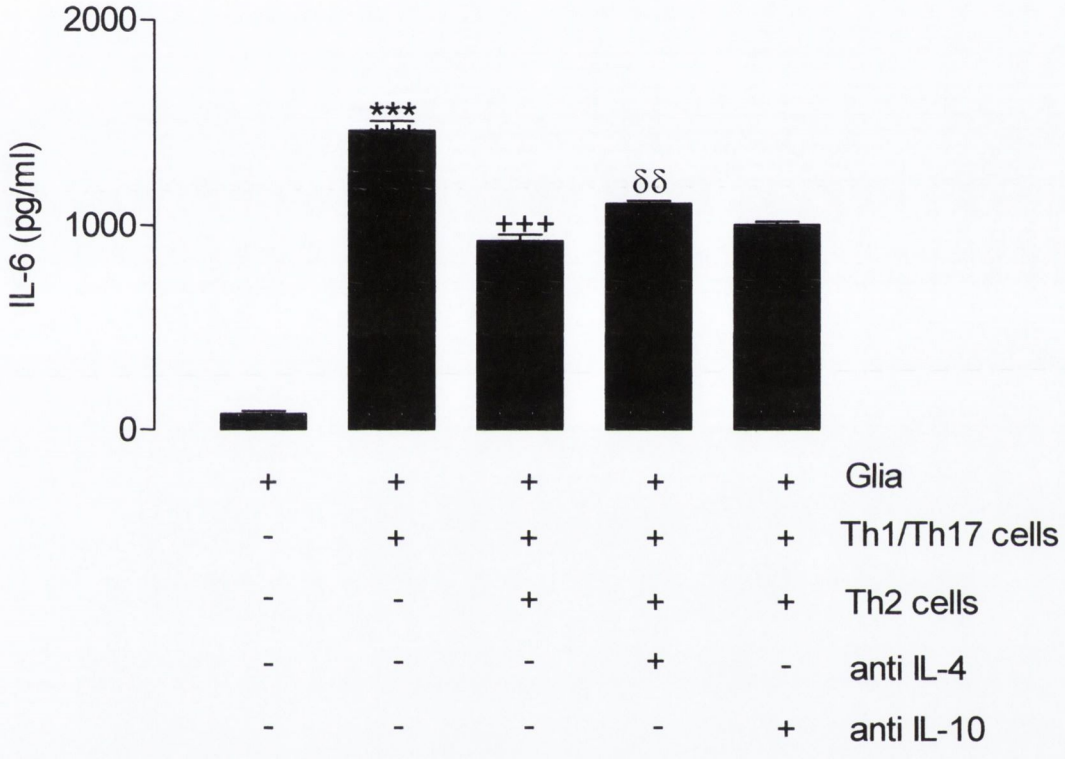

Figure 3.28. IL-4 partially mediates the inhibitory effect of Th2 cells on IL-6 production from co-cultures of glia and Th1/Th17 cells. MOG-specific Th1/Th17 cells and Th2 cells were co-cultured with mixed glial cells at a ratio of 0.5:1 in the presence or absence of neutralising antibodies to IL-4 (10 μg/ml) and IL-10 (10 μg/ml). After 24 h, IL-6 production was assessed by ELISA. ***$p<0.001$; versus medium-treated glia, $^{+++}p<0.001$; versus glia treated with Th1/Th17 cells, $^{\delta\delta}p<0.01$; versus glia treated with Th1/Th17 cells and Th2 cells, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments.

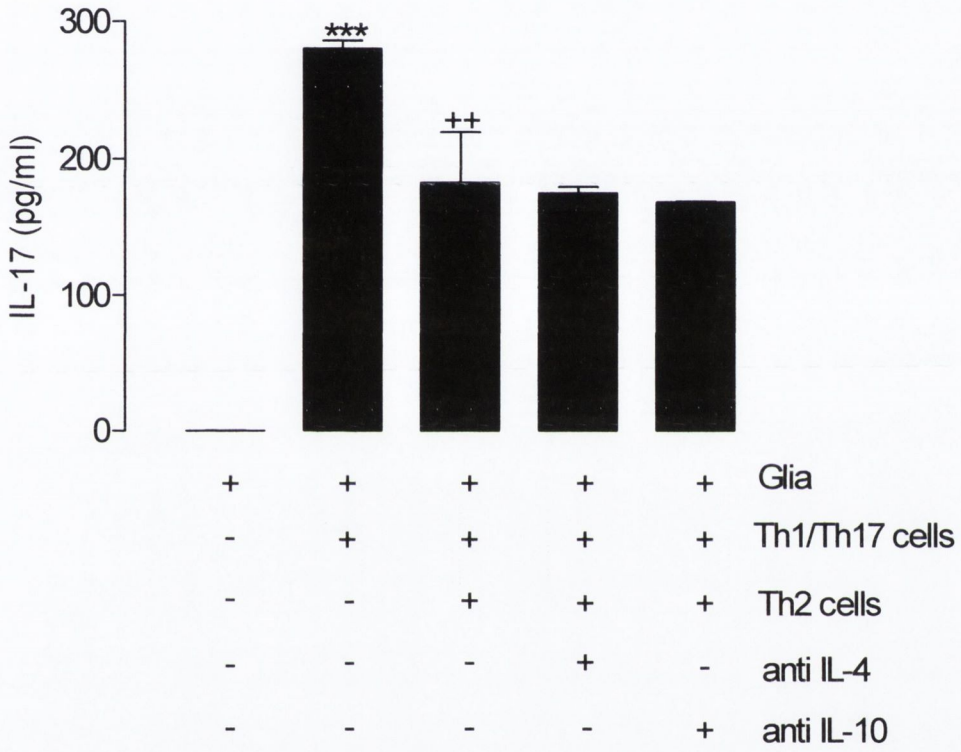

Figure 3.29. The inhibitory effect of Th2 cells on IL-17 production from Th1/Th17 cells is not mediated by IL-4 or IL-10. MOG-specific Th1/Th17 cells and Th2 cells were co-cultured with mixed glial cells at the ratio of 0.5:1 for 24 h in the presence or absence of neutralising antibodies to IL-4 (10 μg/ml) and IL-10 (10 μg/ml). ***$p<0.001$; versus medium-treated glia; $^{++}p<0.01$; versus glia treated with Th1/Th17 cells, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments.

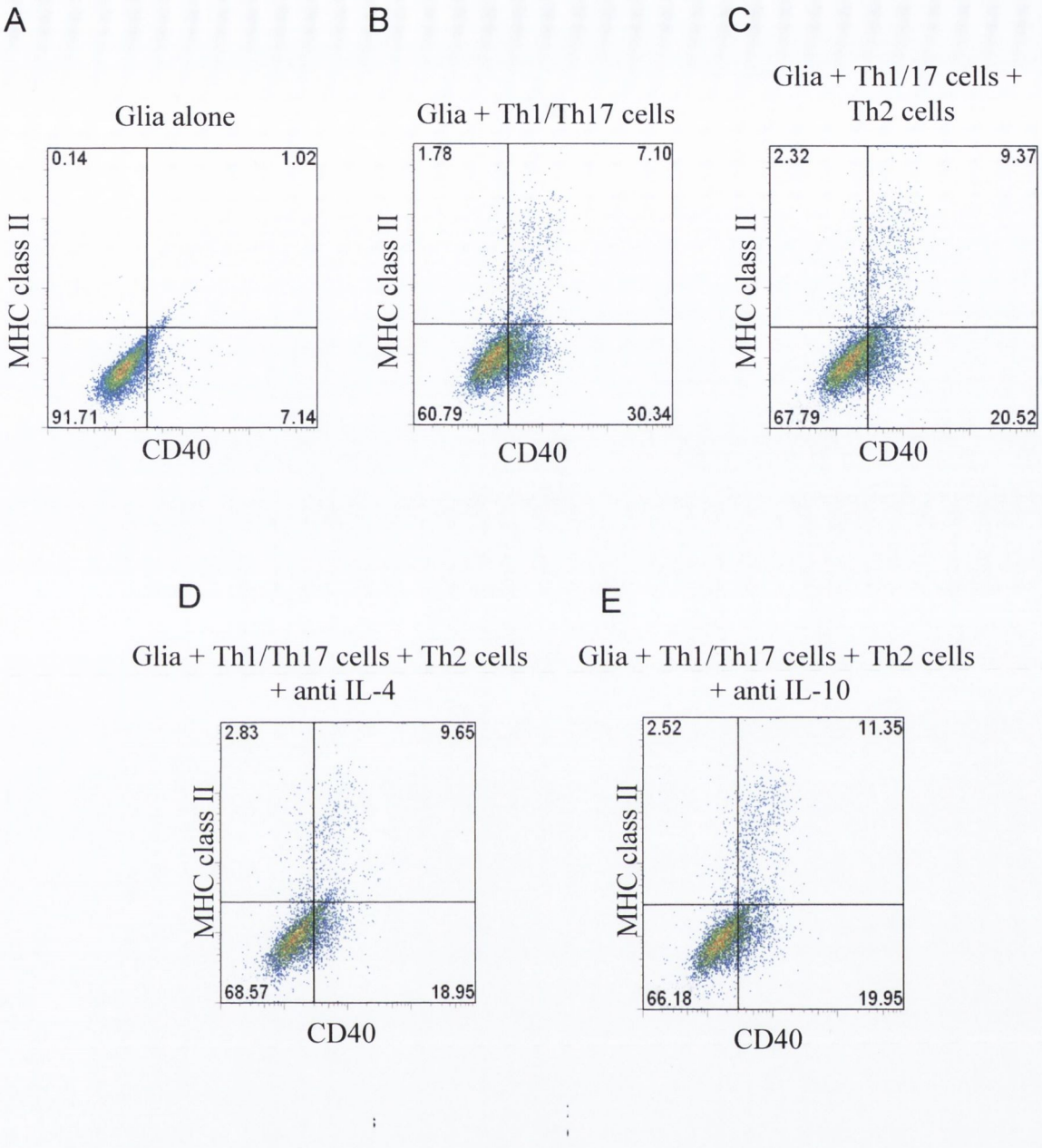

Figure 3.30. The attenuating effect of Th2 cells on Th1/Th17 cell-induced upregulation of MHC class II and CD40 on microglia is not reversed by neutralising IL-4 or IL-10. MOG-specific Th1/Th17 cells and Th2 cells were co-cultured with glial cells in the presence or absence of neutralising antibodies to IL-4 and IL-10. After 24 h, expression of MHC class II and CD40 on $CD11b^+$ cells was assessed by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th1/Th17 cells, (C) microglia treated with Th1/Th17 cells and Th2 cells, (D) microglia treated with Th1/Th17 cells, Th2 cells and anti-IL-4 antibody, (E) microglia treated with Th1/Th17 cells, Th2 cells and anti-IL-10 antibody. Numbers represent the percentage of cells in the gate.

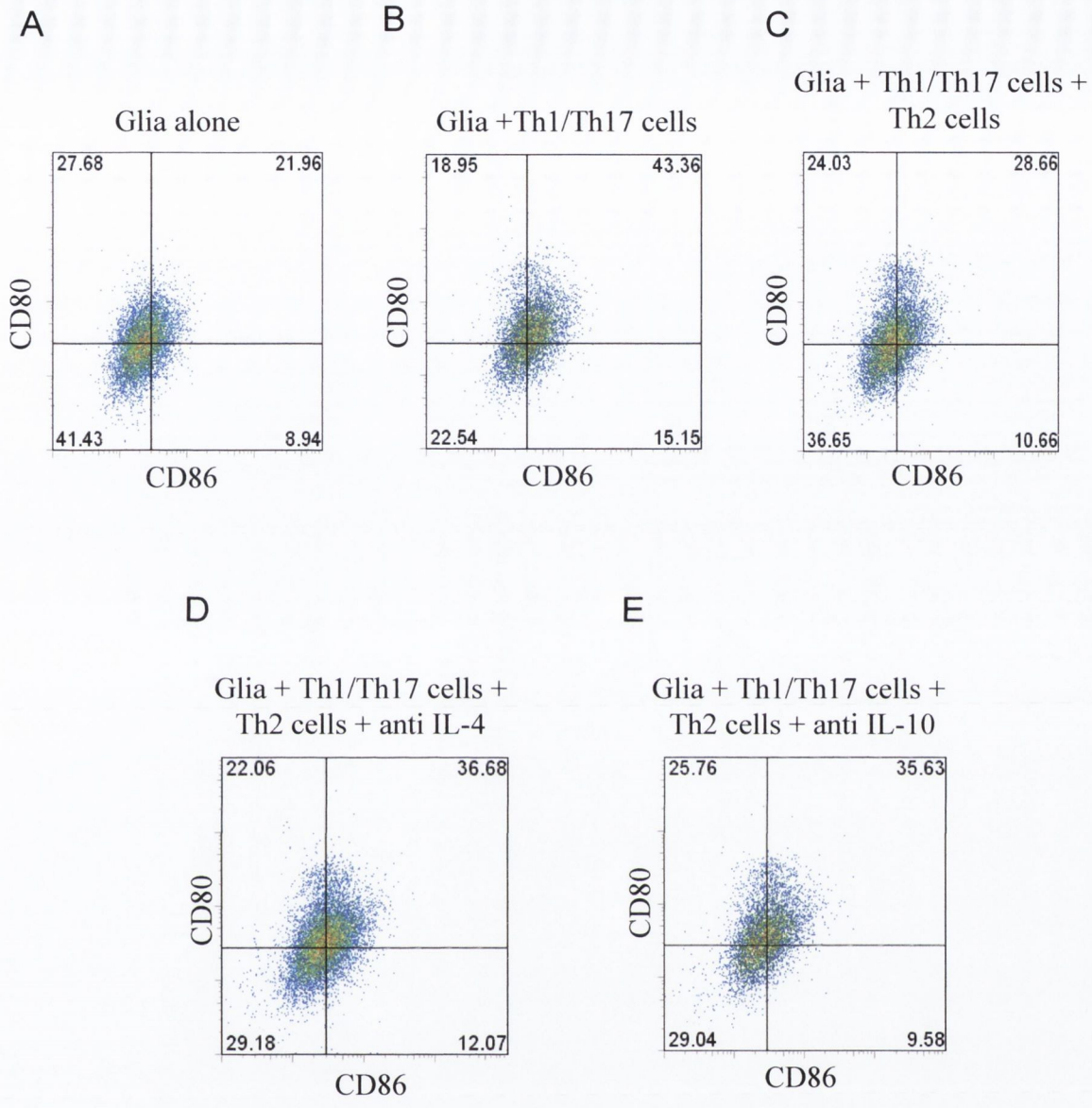

Figure 3.31. The attenuating effect of Th2 cells on Th1/Th17 cell-induced upregulation of CD80 and CD86 on microglia is partially reversed by neutralising IL-4 or IL-10. Glia were co-cultured with Th1/Th17 cells and Th2 cells in the presence or absence of neutralising antibodies to IL-4 (10 μg/ml) and IL-10 (10 μg/ml). After 24 h, CD80 and CD86 expression was assessed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th1/Th17 cells, (C) microglia treated with Th1/Th17 cells and Th2 cells, (D) microglia treated with Th1/Th17 cells, Th2 cells and anti-IL-4 antibody, (E) microglia treated with Th1/Th17 cells, Th2 cells and anti-IL-10 antibody. Numbers represent the percentage of cells in the gate.

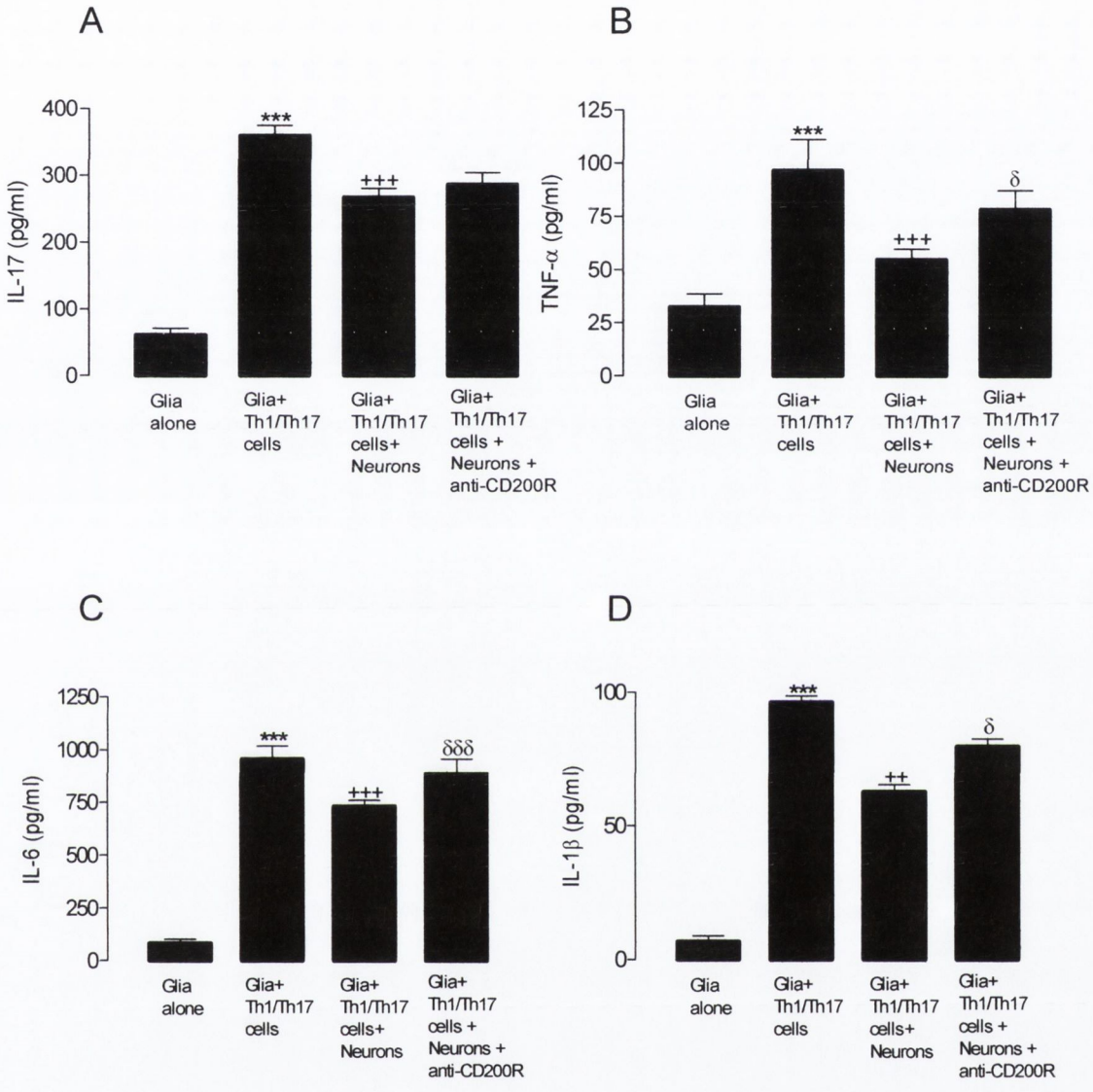

Figure 3.32. Blocking CD200R1 reverses the inhibitory effect of neurons on proinflammatory cytokine production in co-cultures of glia with glial : Th1/Th17 cells. MOG-specific Th1/Th17 cells were co-cultured with mixed glia and neurons for 24 h in the presence or absence of an anti-CD200R1 neutralising antibody. IL-17 (A), TNF-α (B), IL-6 (C) and IL-1β (D) production was assessed by ELISA. ***$p<0.001$; versus medium-treated glial cells, $^{+}p<0.05$; $^{++}p<0.01$;$^{+++}p<0.001$; versus glia treated with Th1/Th17 cells, $^{\delta}p,0.05$; $^{\delta\delta\delta}p<0.01$; versus co-incubation of Th17 cells and neurons with glia, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 2 independent experiments. (n=4)

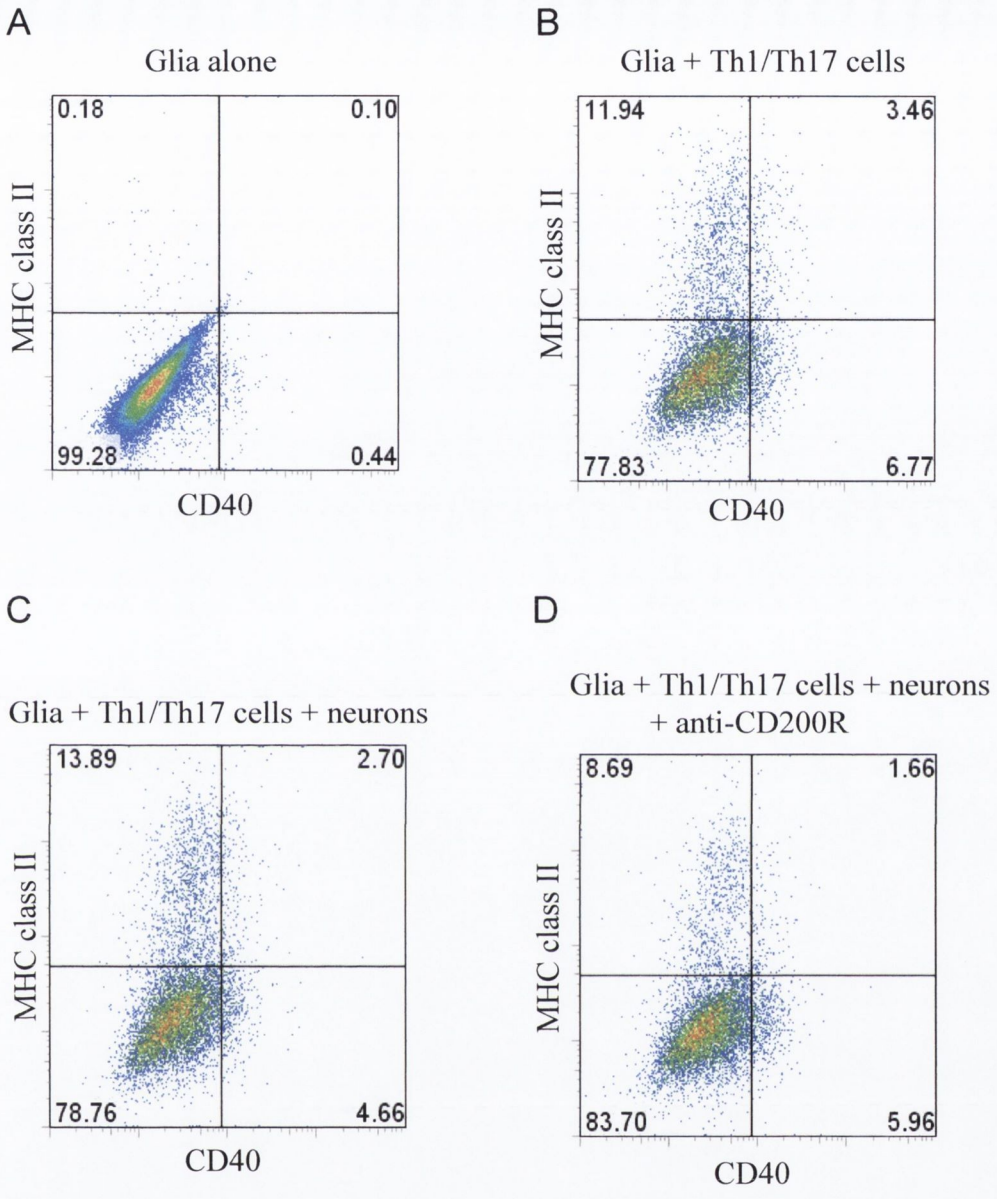

Figure 3.33. Neurons do not attenuate Th1/Th17 cell-induced upregulation of MHC class II and CD40 on microglia. Glial cells were co-cultured with medium, Th1/Th17 cells, Th1/Th17 cells plus neurons or Th1/Th17 cells plus neurons and anti-CD200R at the ratio of 8:4:1 (glia:Th1/Th17 cells:neurons) for 24 h. Expression of MHC class II and CD40 was analysed on $CD11b^+$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th1/Th17 cells, (C) microglia treated with Th1/Th17 cells and neurons (D) microglia treated with Th1/Th17 cells plus neurons plus anti-CD200R. Numbers represent the percentage of cells in the gate.

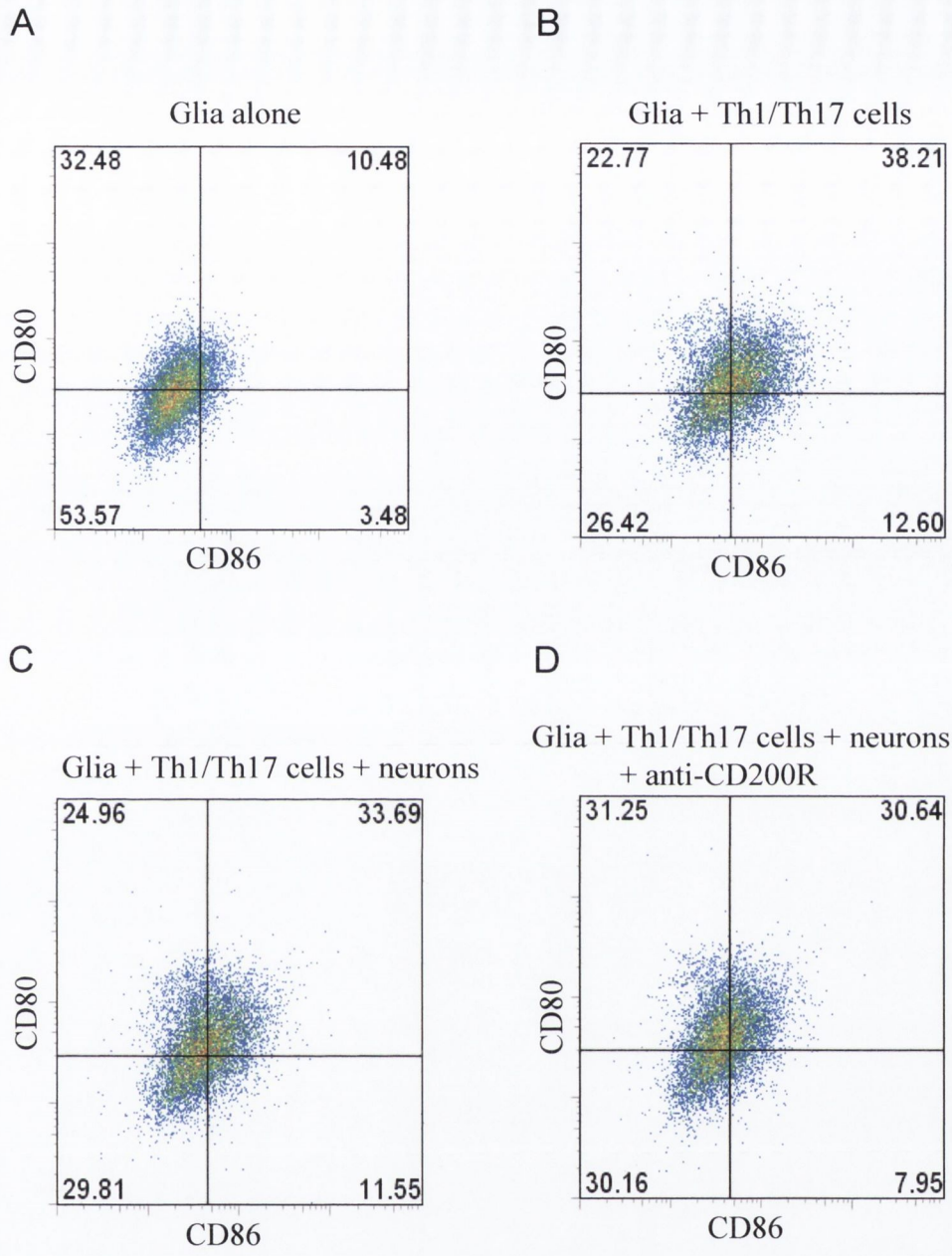

Figure 3.34. Neurons do not attenuate Th1/Th17 cell-induced upregulation of CD80 and CD86 on microglia. Glial cells were co-cultured with medium,Th1/Th17 cells,Th1/Th17 cells plus neurons or Th1/Th17 cells and neurons and anti-CD200R at the ratio of 8:4:1 (glia:Th1/Th17 cells:neurons) for 24 h. CD80 and CD86 expression was analysed on $CD11b^{+}$ cells by flow cytometry. (A) medium-treated microglia, (B) microglia treated with Th1/Th17 cells, (C) microglia treated with Th1/Th17 cells and neurons, (D) microglia treated with Th1/Th17 cells plus neurons plus anti-CD200R. Numbers represent the percentage of cells in the gate.

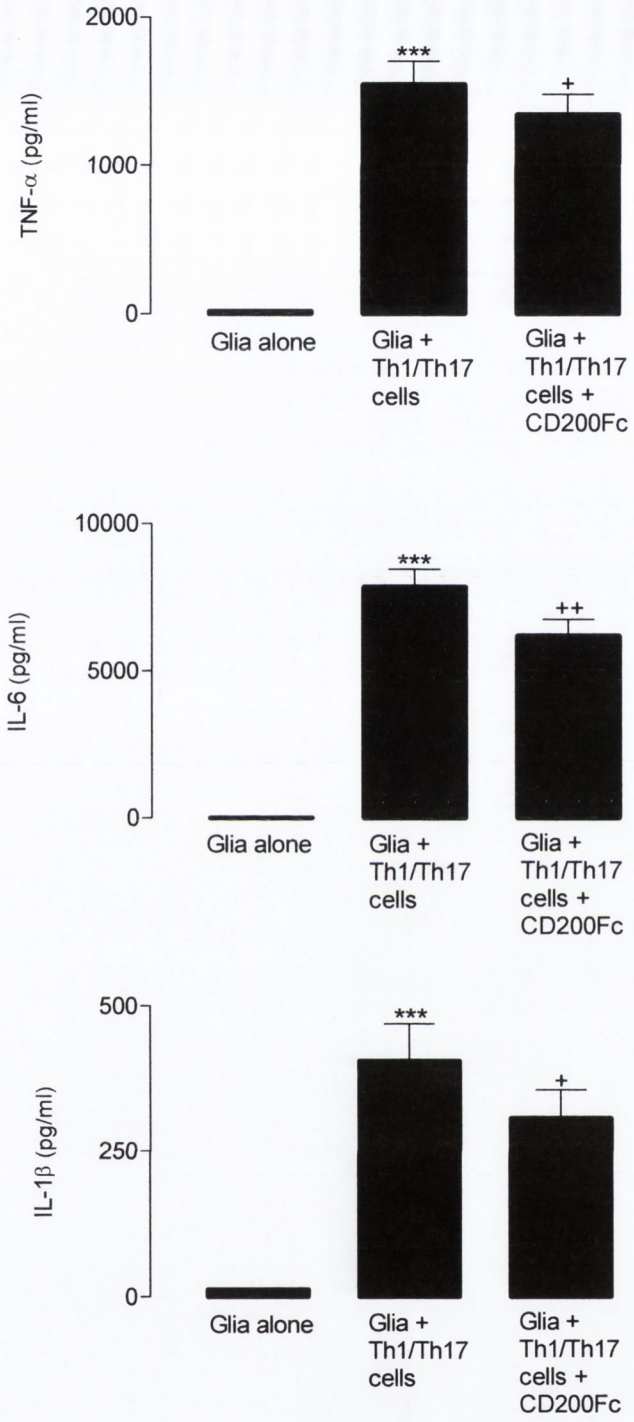

Figure 3.35. CD200 fusion protein attenuates TNF-α, IL-6 and IL-1β production in Th1/Th17 cell: glia co-cultures. Glia were cultured with medium, Th1/Th17 cells or Th1/Th17 cells and CD200Fc (4 μg/ml). After 24 h, supernatants were removed and TNF-α, IL-6 and IL-1β concentration was quantified by ELISA. **$p<0.01$; versus medium treated glia, $^{+}p<0.05$; $^{++}p<0.01$; versus glia treated with Th1/Th17 cells, by paired Student's *t* test. Data presented as mean ± SEM. (n=4).

Chapter 4
Antigen presenting cells in the CNS during EAE

4.1 Introduction

MOG_{35-55} induced EAE in C57BL/6 mice is a clinically chronic inflammatory demyelinating disease. Encephalitogenic T cells invading the CNS during disease interact with APC, which results in reactivation of the T cells and APC activation (Shrikant & Benveniste, 1996). Several potential APC are present in the CNS during disease, including resident astrocytes and microglia and infiltrating macrophages and DC.

Parenchymal microglia can become activated in response to immunological stimuli; this activation is accompanied by a morphological change to an amoeboid structure. Activated microglia exist in two states, activated non-phagocytic microglia found in the inflamed CNS and reactive phagocytic microglia, observed in areas of brain trauma (Minagar *et al.*, 2002). Microglia constitutively express CD45 at low levels and CD86, CD40 and ICAM-1, molecules necessary for microglial activation of CNS infiltrating T cells (Carson *et al.*, 1998). In response to immunological stimuli, the expression of CD45, MHC class II and CD80 is upregulated on microglia enhancing their ability to function as APC (Shrikant & Benveniste, 1996). Astrocytes have been shown to be relatively inefficient APC compared to microglia (Aloisi *et al.*, 2000b), however, are capable of presenting antigen and activating IL-17 and IFN-γ gene expression and protein synthesis in T cells (Miljkovic *et al.*, 2007).

DC and macrophages permeate the CNS and locate in the perivascular regions, meninges, choroid plexus and the subarachnoid spaces (Carson *et al.*, 2006). Hickey and Kimura (1988) found that induction of EAE required the expression of MHC class II on invading peripheral APC and suggested that MHC

class II expression on parenchymal microglia was not necessary for the initiation of EAE. Moreover, microglia are less effective than infiltrating DC and macrophages in driving T cell proliferation and this has been linked to microglial production of prostaglandins and NO (Carson & Sutcliffe, 1999). Prostaglandins and NO are inhibitors of T cell proliferation and also decrease the expression of MHC class II and costimulatory molecules on infiltrating APC entering the CNS. Therefore, microglia are thought to be less efficient than macrophages and DC at presenting antigen, this has been attributed to low expression of CD45 on microglia, since CD45 expression has been correlated with the ability to present antigen to naïve T cells (Carson *et al.*, 1998).

There are conflicting reports in the literature as to whether microglia are neuroprotective or neurotoxic when activated. It is well known that both microglia and macrophages produce TNF-α, IL-6 and IL-1β when activated and these proinflammatory cytokines have a pathogenic role in the CNS during diseases such as EAE. TNF-α potently induces inflammation by increasing antigen presentation, promoting astrocytic proliferation and altering chemokine and adhesion molecule expression, thus regulating cell trafficking into the CNS (Sedgwick *et al.*, 2000; Selmaj, 2000). IL-6 has also been implicated in T cell migration into the CNS. VCAM-1 and ICAM-1 are not enhanced on CNS endothelial cells in IL-$6^{-/-}$ mice during EAE; this finding suggests that IL-6 has a role in promoting T cell migration into the CNS (Eugster *et al.*, 1998). The proposed role of TNF-α and IL-6 in the development and promotion of EAE is supported by findings that decreased expression of TNF-α and IL-6 in the CNS is associated with attenuated EAE (Penkowa & Hidalgo, 2001). IL-1β expression in the brain of rats with EAE is correlated with the onset of clinical symptoms; IL-1β

production is increased as the disease progresses suggesting a crucial role for IL-1β in the pathogenesis of EAE (Bauer *et al.*, 1993). Furthermore, IL-1R1$^{-/-}$ mice are significantly resistant to the development of EAE (Sutton *et al.*, 2006).

Phagocytosis of apoptotic T cells by activated microglia may be an important mechanism for the termination of inflammation in the CNS (Magnus *et al.*, 2002). IFN-γ, secreted by encephalitogenic Th1 cells, activates phagocytic activity in microglia (Chan *et al.*, 2001), it has been proposed that augmentation of phagocytic activity by IFN-γ leads to accelerated clearance of inflammatory infiltrates in the CNS. Therefore activation of microglia and macrophages may have opposing roles in CNS inflammation that initiate neurotoxic and neuroprotective mechanisms.

Therefore the objectives of this chapter were to:

- investigate CD11b^{+}CD45^{hi} cell infiltration into the CNS during the progression of EAE.
- assess the expression of MHC class II, CD40, CD80 and CD86 on resident microglia and infiltrating macrophages in the brain and spinal cord over the course of the disease.
- determine the phagocytic activity of resident microglia and infiltrating macrophages in the CNS throughout disease.
- analyse proinflammatory cytokine production in the brain during disease.
- look for evidence of neuronal dysfunction during the development of EAE.

4.2. Results

4.2.1. Clinical course of EAE.

EAE was induced in C57BL/6 mice by s.c. injection of MOG_{35-55} (150 μg) emulsified in CFA, supplemented with 4 mg/ml H37 Ra *M. tuberculosis*. Mice were injected i.p. with PT (500ng) on days 0 and 2. Control animals were injected with PBS (200 μl) i.p. Animals were monitored daily for signs of clinical disease (Figure 4.1). 97% of mice had developed EAE by day 13 post immunisation. Clinical symptoms began with loss of tail tone (stage 1), and progressed through varying degrees of hind limb weakness and led ultimately to hind limb paralysis (stage 4). Mice were sacrificed 7, 10, 14 and 21 days post-immunisation and the brains and spinal cords were removed to assess microglial and infiltrating macrophage activation.

4.2.2. Increased frequency of $CD11b^{+}CD45^{hi}$ cells in the brain and spinal cord of mice with EAE.

Microglia can be distinguished from macrophages on the basis of CD45 expression. In normal and pathological CNS tissue, CD45 expression has been correlated with the ability to present antigen to naïve T cells (Carson *et al.*, 1998), activated microglia are known to upregulate their expression of CD45 to intermediate or high levels, thus improving their antigen presenting capabilities (Carson *et al.*, 1998). Mononuclear cells isolated from the brain and spinal cord of control mice and mice with EAE were stained with anti-CD11b and anti-CD45 to identify a resident microglial population ($CD11b^{+}CD45^{lo}$) and CNS infiltrating macrophage population ($CD11b^{+}CD45^{hi}$). The number of resident microglia in the

brain was not significantly altered throughout the disease. However, the percentage of infiltrating macrophages into the brain was markedly elevated at the onset of clinical symptoms (day 10) (***$p<0.001$, Figure 4.2B).

In the spinal cord, the number of resident microglia was not altered by disease (Figure 4.3A). In contrast, the number of infiltrating macrophages was significantly enhanced in the spinal cord at disease onset (day 10) and 14 days post immunisation (*$p<0.05$; **$p<0.01$ Figure 4.3B).

4.2.3. Increased co-stimulatory molecule mRNA in the spinal cord, striatum and cerebellum in C57BL/6 mice with EAE.

Traditionally the spinal cord in rodents has been considered the region most susceptible to EAE, with disease in the brain occurring later and with reduced severity. However, other areas of the brain involved in motor function are affected during EAE such as the striatum (Balkowiec-Iskra *et al.*, 2007) and the cerebellum (Tonra *et al.*, 2001). CD80, CD86 and CD40 are co-stimulatory molecules, expressed on APC, and their interaction with their ligands on T cells leads to T cell reactivation within the CNS and the production of a wide array of cytokines, chemokines and neurotoxins within the CNS (Chen *et al.*, 2006). Therefore, CD80, CD86 and CD40 mRNA levels were assessed in the spinal cord, cerebellum and striatum of control mice and mice with EAE. After prolonged disease, CD86 expression was enhanced in the spinal cord (***$p<0.001$, Figure 4.4), striatum (*$p<0.05$; Figure 4.4) and cerebellum (***$p<0.001$; Figure 4.4) of mice suffering from EAE compared to control mice. Concomitant with CD86 mRNA expression, CD80 mRNA expression was significantly enhanced in the spinal cord (*$p<0.05$; Figure 4.4) and cerebellum (**$p<0.01$; Figure 4.4) of mice

suffering from EAE. Disease did not significantly increase CD80 mRNA expression in the striatum of mice with EAE (Figure 4.4). Similarly, CD40 mRNA expression was significantly elevated in the spinal cord (*$p<0.05$; Figure 4.4) and cerebellum (**$p<0.01$; Figure 4.4) of mice suffering from EAE. CD40 mRNA was not detected in the striatum of control mice or mice with EAE. The data suggest that enhanced antigen presentation to encephalitogenic T cells can occur in brain areas known to be affected during EAE due to upregulation of CD80, CD86 and CD40 expression on the surface of APC.

4.2.4. Enhanced MHC class II and co-stimulatory molecule expression on resident microglia in the brain and spinal cord during EAE.

Having demonstrated the enhanced expression of co-stimulatory molecules at the mRNA level, this study examined the expression of MHC class II and co-stimulatory molecules on both resident microglia and infiltrating macrophages in the brain and spinal cord. In active lesions during MS, microglia exhibit an more activated morphology with increased expression of the cell surface markers MHC class II and the adhesion/co-stimulatory molecules CD54, CD40, CD80 and CD86 (Cannella *et al.*, 1995; Cannella & Raine, 1995). In the present study, MHC class II, CD40, CD80 and CD86 expression were assessed as an indicator of microglial activation over the course of EAE. The number of resident microglia ($CD11b^{+}$ $CD45^{lo}$) that express MHC class II increased in the brain over the course of EAE (Figure 4.5A). There was a significant increase in the number of CD40 expressing resident microglia in the brain at the onset of clinical symptoms, i.e. 10 days post immunisation (*$p<0.05$, Figure 4.5B). The data from the brain is consistent with data from the spinal cord during EAE,

which also showed a significant increase in the number of MHC class II expressing and CD40 expressing resident microglia at the onset of clinical symptoms (**$p<0.01$, ***$p<0.001$, Figure 4.6A and B). The number of CD80 expressing resident microglia in the brain was significantly increased 10 days post immunisation (*$p<0.05$, Figure 4.7A) while the number of CD86 expressing resident microglia in the brain was significantly elevated before clinical disease and at clinical disease onset (*$p<0.05$, Figure 4.7B). Consistent with this data, the number of CD80 expressing resident microglia in the spinal cord was significantly increased 10 days post immunisation (*$p<0.05$, Figure 4.8A), however the number of CD86 expressing microglia was increased in the spinal cord before disease onset and at clinical symptom onset but this increase was not significant.

4.2.5. Enhanced MHC class II and co-stimulatory molecule expression on CNS-infiltrating macrophages in the brain and spinal cord during EAE.

It had previously been shown that CNS-resident $CD11b^{+}CD45^{lo}$ microglia purified from the inflamed CNS were found to be largely incapable of activating either naïve or effector T cells whereas $CD11b^{+}CD45^{hi}$ macrophages efficiently presented endogenous myelin antigens to activate both pre-activated effector myelin-specific T cells and naïve T cells (Bailey *et al.*, 2007). Thus MHC class II, CD40, CD80 and CD86 expression was assessed on CNS-infiltrating macrophages over the course of EAE.The number of MHC class II expressing $CD11b^{+}CD45^{hi}$ cells was significantly enhanced 7, 10 and 14 days post immunisation in the brains of mice with EAE (*$p<0.05$; **$p<0.01$, Figure 4.9A). In addition, the number of CD40 expressing macrophages in the brain was significantly elevated at clinical disease onset (*$p<0.05$, Figure 4.9B). In contrast,

in the spinal cord of mice with EAE the greatest number of MHC class II expressing macrophages was observed 14 days after immunisation (**$p<0.01$, Figure 4.10A). The number of CD40 expressing $CD11b^{+}CD45^{hi}$ cells was significantly increased in the spinal cord 10 days post immunisation (*$p<0.05$, Figure 4.10B)

The number of CD80 and CD86 expressing infiltrating macrophages was significantly increased in the brain at disease onset (*$p<0.05$; Figure 4.11A and B). Data from the spinal cord show that the number of CD80 expressing $CD11b^{+}CD45^{hi}$ cells peaked 10 days post immunisation (*$p<0.05$, Figure 4.12A) while the number of CD86 expressing $CD11b^{+}CD45^{hi}$ cells was significantly increased at onset of clinical disease and remained elevated 14 days post immunisation (**$p<0.01$, Figure 4.12B).

4.2.6. Phagocytic activity of resident microglia and infiltrating macrophages in the brain and spinal cord over the course of EAE.

Apoptosis of T cells is a common pathway to terminate autoimmune inflammation in the brain during EAE (Pender *et al.*, 1991). Microglia cells are efficient phagocytes capable of ingesting apoptotic cells. Microglia cells significantly increased their phagocytic activity after stimulation with IFN-γ (Magnus et al., 2002), therefore, it was hypothesised that microglia and macrophages would become more phagocytic over the course of EAE.

The phagocytic activity of $CD11b^{+}CD45^{lo}$ cells and $CD11b^{+}CD45^{hi}$ cells was assessed by testing their ability to phagocytose fluorescent nano-particles called quantum dots, which were detected by flow cytometry.

The number of phagocytic resident microglia was significantly increased in the brain 10 and 14 days post immunisation (*$p<0.05$; **$p<0.01$, Figure 4.13A) and in the spinal cord later in disease (*$p<0.05$; ***$p<0.001$, Figure 4.14A). The number of phagocytic macrophages was greatest at the onset of clinical symptoms both in the brain (*$p<0.05$, Figure 4.15A) and in the spinal cord (*$p<0.05$, Figure 4.16A) of mice with EAE.

4.2.7. IL-1β, IL-6 and TNF-α production is significantly increased in the spinal cord, striatum and cerebellum during EAE.

It has been previously shown that induction of antigen-specific Th17 cells is an IL-1-dependent process with Th17 cell induction being abrogated in IL-1R1$^{-/-}$ mice (Sutton *et al.*, 2006). In addition to its role in promoting pathogenic Th17 cells in EAE, IL-1β also has proinflammatory effects within the CNS. BBB breakdown, followed by microglial and astrocyte activation and demyelination, is evident after increased expression of IL-1 in the striatum (Ferrari *et al.*, 2004). Other cytokines of importance in CNS inflammation include IL-6 and TNF-α. IL-6 is a crucial regulator of T cell infiltration into the CNS (Eugster *et al.*, 1998) with upregulation of IL-6 in the CNS correlating well with disease severity (Kennedy *et al.*, 1992). IL-6, together with TGF-β, supports Th17 cell differentiation, a process which is then amplified by IL-1β and TNF-α (Veldhoen *et al.*, 2006). TNF-α also has a role in controlling cell trafficking into the CNS due to its ability to alter chemokine and adhesion molecule expression and increase the antigen presenting capabilities of APC within the CNS (Sedgwick *et al.*, 2000; Selmaj, 2000). Thus IL-1β, IL-6 and TNF-α concentration were assessed in the brain during EAE.

IL-1β production was significantly increased in the spinal cord, striatum and cerebellum of mice with EAE (*p<0.05; **p<0.01, Figure 4.17). During disease, IL-6 concentration was significantly enhanced in the spinal cord and striatum (**p<0.01, Figure 4.17), however such an increase was not observed in the cerebellum (Figure 4.17). Similarly TNF-α production was not significantly increased in the cerebellum as a result of EAE (Figure 4.17), conversely TNF-α concentration was significantly increased in the spinal cord and striatum of mice with EAE (*p<0.05; **p<0.01, Figure 4.17).

IL-1β, IL-6 and TNF-α mRNA expression and protein production was assessed in the brain at various time points of EAE. IL-1β mRNA expression was significantly elevated before clinical disease onset (day 7) and was further increased at the onset of clinical symptoms (**p<0.01; ***p<0.001, Figure 4.18A). IL-1β protein production was significantly increased in the brain throughout the course of disease with the greatest increase observed 10 days post immunisation in mice with EAE (*p<0.05; **p<0.01; ***p<0.001, Figure 4.18B). TNF-α mRNA expression and protein production was significantly augmented in the brain at the onset of clinical symptoms (**p<0.01; ***p<0.001, Figure 4.19A and B). IL-6 mRNA expression was enhanced 7 and 10 days post immunisation (*p<0.05, Figure 4.20A) while IL-6 protein production was significantly increased in the brain at the onset of clinical disease but the highest production is observed before the onset of clinical symptoms (**p<0.01; ***p<0.001, Figure 4.20B).

4.2.8. The expression of CD200L mRNA in the brain and spinal cord during EAE

CD200 ligand is expressed on neurons and has an essential role in regulating microglial activation (Hoek *et al.*, 2000). Therefore, expression of CD200 ligand mRNA was assessed in isolated brain regions thought to be important in EAE i.e. spinal cord, striatum and cerebellum. CD200 ligand mRNA was significantly decreased in the spinal cord of mice with EAE (***$p<0.001$, Figure 4.21). In contrast, CD200 ligand mRNA expression was marginally increased in the striatum of mice with EAE (*$p<0.05$, Figure 4.21). CD200L mRNA expression was not significantly different in the cerebellum of mice with EAE compared to control mice (Figure 4.21). Later in the course of the disease, a significant decrease in CD200L was observed in the brain, indicative of neuronal dysfunction and/or degeneration (*$p<0.05$, Figure 4.22).

4.3. Discussion

The significant findings of this study are that infiltration of macrophages into the brain and spinal cord of mice increased at the onset of the clinical symptoms. This is coupled with an increase in the expression of MHC class II, the costimulatory molecules, CD80 and CD86, and the adhesion molecule, CD40, on resident microglia and infiltrating macrophages. The phagocytic activity of resident microglia and infiltrating macrophages is increased in the brain and spinal cord during later stages of disease, while the proinflammatory cytokines, IL-1β, TNF-α and IL-6, are differentially expressed in the brain throughout disease. Furthermore, neuronal dysfunction and degeneration was observed in later stages of disease.

Activated microglia express many of the common markers expressed by macrophages, including CD11b, F4-80 and CD45 (Sedgwick *et al.*, 1991; Carson *et al.*, 1998), and therefore it is hard to distinguish activated microglia from infiltrating macrophages. However, microglia and macrophages express significantly different levels of key surface molecules, indicating that these two cell types do exist in different stable physiological states. Most importantly, microglia express much lower levels of CD45, compared with acutely infiltrating macrophages (Sedgwick *et al.*, 1991). In response to CNS insult and inflammation, the expression of CD45 is increased on both macrophages and microglia. Despite this, the expression profile of CD45 on microglia remains at an intermediate level and does not reach that present on mature macrophages (Sedgwick *et al.*, 1991; Carson *et al.*, 1998). In the present study, $CD11b^{+}CD45^{+}$ cells were examined in the brain and spinal cord during EAE and microglia were

distinguished from macrophages based on their expression of CD45. The data showed that the numbers of resident microglia ($CD11b^{+}CD45^{lo}$) cells was not significantly altered in the brain and spinal cord during development of disease. This data are at variance with a study by Ponomarev and colleagues (2005), who reported that during EAE, the absolute number of resting $CD45^{lo}$ microglial cells progressively decreased until the peak of disease. It was postulated that this was due to activation of microglia and increased expression of CD45 on the surface of microglia to high levels comparable to that of infiltrating macrophages. Consistent with findings by Ponomarev and colleagues (2005), the present study showed infiltration of macrophages ($CD11b^{+}CD45^{hi}$ cells) was significantly increased in the brain and spinal cord at the onset of clinical symptoms.

There is conflicting data in the literature as to the relative contribution of resident microglia and infiltrating macrophages to antigen presentation in the CNS during EAE. Juedes and Ruddle (2001) demonstrated that both infiltrating macrophages and resident microglia are efficient APC during EAE since expression of MHC class II, CD80 and CD86 was upregulated on both populations. In contrast, Ford and colleagues (1995) reported that infiltrating macrophages isolated from the CNS, but not microglia, were effective APC for MBP-specific T cells. The data presented here show that expression of MHC class II on resident microglia in the brain increased over the course of the disease, but this increase was not significant, consistent with findings by Sedgwick and colleagues (1991), who reported that only a minority of $CD11b^{+}CD45^{lo}$ cells have enhanced expression of MHC class II molecules in response to inflammation. In contrast, expression of MHC class II on infiltrating macrophages in the brain and

spinal cord was significantly increased before clinical disease onset and in later stages of disease.

Expression of CD40 was increased on resident microglia and infiltrating macrophages at the onset of disease. Becher and colleagues (2001) demonstrated that expression of CD40 on CNS resident microglia is critical to the development and progression of EAE. Moreover, CD40 has been shown to be essential for full activation of microglia (Ponomarev *et al.*, 2006). Ponomarev and colleagues (2006) reported that initial activation of microglia during EAE consisted of glial proliferation and enhanced expression of MHC class II, CD40 and CD86 on microglia, while complete activation of microglia at the peak of disease was CD40-dependent. CD40-deficient microglia failed to further enhance their expression of activation markers and reduce proliferation. Failure of microglia to become fully activated during EAE correlated with the decline of clinical symptoms (Ponomarev *et al.*, 2006). Taken together, the data demonstrate a critical role for the expression of CD40 on microglia during pathogenesis of EAE.

The present study revealed that expression of CD80 was significantly increased on resident microglia and infiltrating macrophages in the brain and spinal cord at the onset of the clinical symptoms of EAE. A critical role for the expression of CD80 on microglia has been suggested during EAE pathology, since partial recovery from EAE is known to be correlated with decreased expression of CD80 as well as MHC class II on microglia (Juedes & Ruddle, 2001). Expression of CD86 was enhanced on microglia in the brain before clinical disease and 10 days post immunisation. This is consistent with the finding that expression of CD86 was up-regulated during the initial activation of microglia in EAE (Ponomarev *et al.*, 2006). In contrast to the findings presented here,

Ponomarev and colleagues (2005) reported that expression of MHC class II on $CD11b^{+}CD45^{lo}$ cells was increased at the onset and peak of EAE, but expression of CD86 or CD40 was not, suggesting that these $CD11b^{+}CD45^{lo}$ cells were alternatively activated microglia, which are inefficient APC.

Ponomarev and colleagues (2005) reported that microglial cells become activated before the infiltration of macrophages into the CNS and the onset of EAE. This is not consistent with the findings of this study, which indicated that microglia were activated at the onset of clinical symptoms. Furthermore, activation of microglia coincided with significant infiltration of $CD11b^{+}CD45^{hi}$ cells into the brain and spinal cord. The data presented in this study suggest that both resident microglia and infiltrating macrophages are capable of presenting antigen to encephalitogenic T cells in the CNS during EAE.

Expression of antigen presenting molecules was assessed in areas of the CNS known to be highly susceptible to inflammation during EAE. The spinal cord is traditionally considered as the area of the CNS most susceptible to inflammation. Both the cerebellum and the striatum are involved in the coordination of movement and are likely to be affected during EAE. The cerebellum is equally susceptible as the spinal cord to BBB breakdown and immune cell infiltration (Tonra *et al.*, 2001) while inflammation is also known to occur in the striatum during EAE (Balkowiec-Iskra *et al.*, 2007). The data presented in this study show that expression of CD80, CD86 and CD40 mRNA was significantly increased in the spinal cord and cerebellum of mice with acute EAE, however, expression of CD86, but not CD80 or CD40 was enhanced in the striatum. The data suggest that activation of microglia and infiltrating macrophages is more pronounced in the spinal cord and cerebellum than in the

striatum, suggesting that different regions of the CNS that are involved in movement are more susceptible to inflammation during EAE.

Apoptosis is an established mechanism to terminate an autoimmune T cell response in the brain, with up to 50% of infiltrating T cells undergoing apoptosis during EAE (Gold *et al.*, 1997). It is vital that apoptotic cells are removed quickly before they undergo secondary necrosis (Magnus *et al.*, 2002). Phagocytosis by activated microglia and macrophages is essential for the removal of apoptotic cells and limiting inflammation in the brain. The present study found that phagocytosis by activated microglia and macrophages was increased at the onset of symptoms and in later stages of disease. Magnus and colleagues (2002) demonstrated that production of proinflammatory cytokines from microglia was significantly decreased after microglia become phagocytically active. Similarly, macrophages produced less TNF-α, IL-1β, IL-10 and GM-CSF after phagocytosis of apoptotic cells (Fadok *et al.*, 1998). Therefore, the phagocytic activity of both microglia and macrophages, coupled with decreased production of proinflammatory cytokines, is indicative of phagocytosis of apoptotic T cells. This may lead to termination of an autoimmune T cell response in the CNS. Enhanced production of IL-1β was observed in the spinal cord, striatum and cerebellum of mice with acute EAE. In addition, expression of IL-1β mRNA and protein was significantly increased in the brain during development of EAE, with the greatest increase observed at the onset of clinical symptoms. Bauer and colleagues (1993) reported that the production of IL-1 from activated microglia and infiltrating macrophages in the brain of rats with EAE correlated with the onset of clinical symptoms, and that production of IL-1 was further increased in later stages of disease. The data show that TNF-α production was enhanced in the spinal cord

and striatum of mice with EAE but not in the cerebellum, expression of TNF-α mRNA and protein production in the brain were significantly augmented at the onset of clinical disease, however, the concentration of TNF-α protein detected were very low. IL-6 protein was significantly enhanced in the spinal cord and striatum of mice with EAE but not in the cerebellum. Expression of IL-6 mRNA in the brain during EAE was greater before clinical disease and at the onset of clinical symptoms. In contrast to the production of IL-1β and TNFα in the brain during EAE, production of IL-6 was greatest before the onset of clinical symptoms and remained elevated at the onset of clinical disease. This is consistent with findings by Okuda and colleagues (1999), who found that IL-6 had a role in the initiation of the disease. The importance of TNF-α and IL-6 production to the development of EAE was demonstrated in a study by Penkowa and Hidalgo (2001), who found that decreased expression of IL-6 and TNF-α in the CNS significantly reduced apoptotic cell death of neurons and oligodendrocytes and lead to the attenuation of EAE. Perhaps the phagocytic activity of resident microglia and infiltrating macrophages resulted in decreased production of IL-1β, TNF-α and IL-6 in the brain after the initial burst of cytokine production detected at the onset of clinical symptoms.

Although the expression of antigen presenting molecules and production of IL-1β, TNF-α and IL-6 by resident microglia and infiltrating macrophages had decreased in later stages of EAE, there was no remission from the disease. The data indicate that irreversible damage had already occurred within the brain, since decreased expression of CD200L mRNA in later stages of disease is indicative of neuronal dysfunction and degeneration. Expression of CD200L mRNA was also decreased in the spinal cord of mice with acute EAE, but not in the striatum or

cerebellum. This is consistent with the traditional view that the spinal cord is more susceptible to inflammation than the brain, furthermore, activation of microglia, as demonstrated by increased expression of CD11b and CD45, was increased in the spinal cord of $CD200^{-/-}$ mice (Hoek *et al.*, 2000).

The data demonstrate that at the onset of clinical symptoms, there was increased infiltration of macrophages into the CNS (Figure 2). In addition, expression of antigen presenting molecules on and production of proinflammatory cytokines by resident microglia and infiltrating macrophages was enhanced within the CNS. This suggests a role for both resident microglia and infiltrating macrophages in antigen presentation within the CNS during EAE. Neuroprotective mechanisms were also induced at the onset of clinical symptoms. Phagocytosis by microglia and macrophages increased at the onset of disease and remained elevated in later stages of disease (Figure 2). Despite this, irreversible neuronal dysfunction and degeneration were evident in later stages of disease. Therefore, strategies that decrease the expression of antigen presenting molecules and inhibit secretion of proinflammatory cytokines by resident microglia and infiltrating macrophages may potentially be neuroprotective.

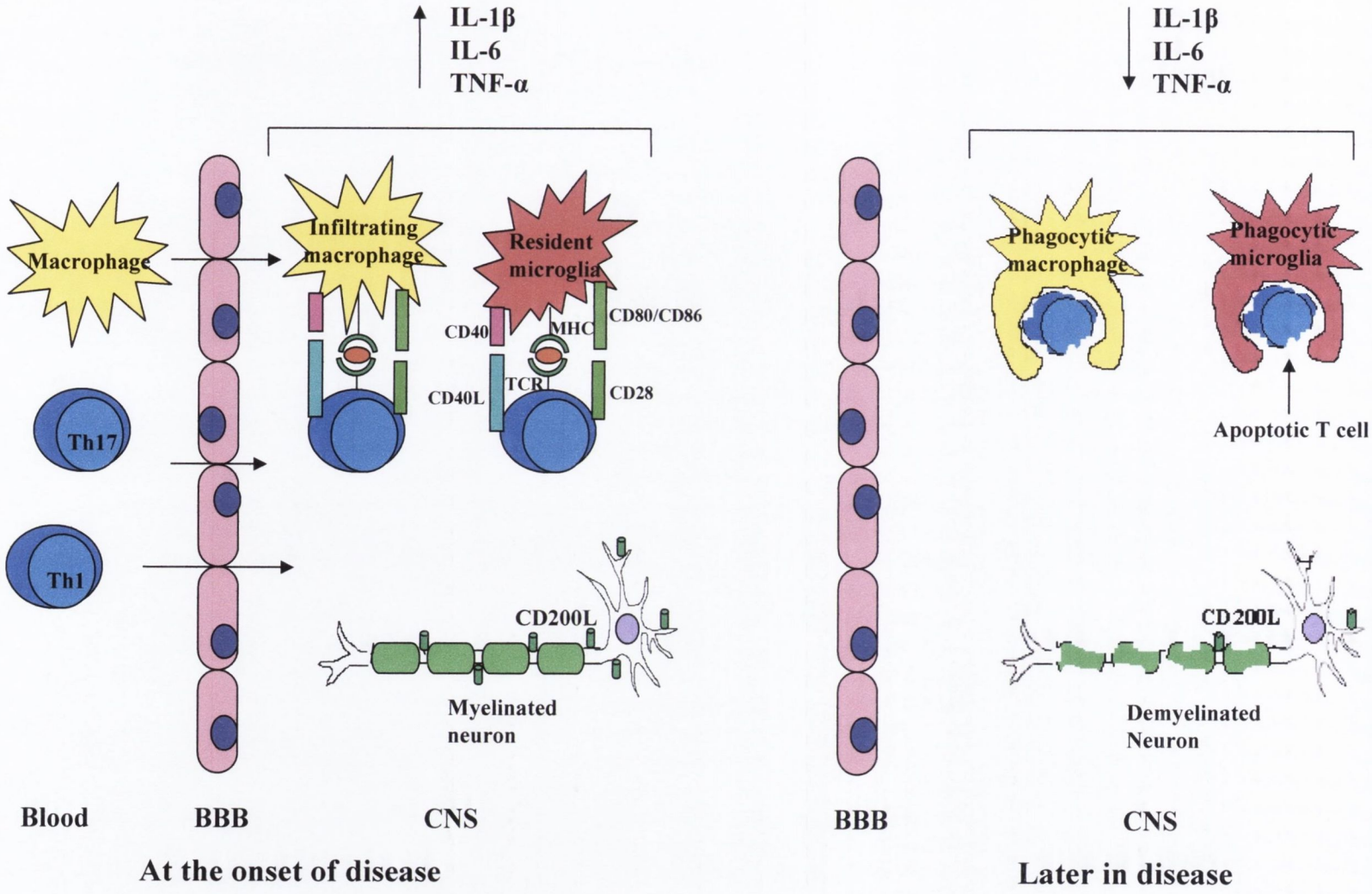

Figure 2. APC in the CNS during EAE. At the onset of disease, macrophages ($CD11b^{+}CD45^{hi}$) infiltrate the CNS, along with pathogenic Th1 and Th17 cells. In the CNS parenchyma, expression of MHC class II, CD40, CD80 and CD86 are increased on resident microglia and infiltrating macrophages, thus they are competent APC and can re-stimulate pathogenic T cells in the CNS. These activated APC secrete proinflammatory cytokines, such as IL-1β, TNF-α and IL-6. Later in disease, the phagocytic acitvity of resident microglia and infiltrating macrophages is increased. This may lead to phagocytosis of apopotic T cells and may contribute to the terminaton of the immune repsonse in the CNS. Production of proinflammatory cytokines by these cells is also decreased. However, CD200L expression on neurons is decreased, this is indicative of neuronal dysfunction and degeneration which leads to irreversible disability.

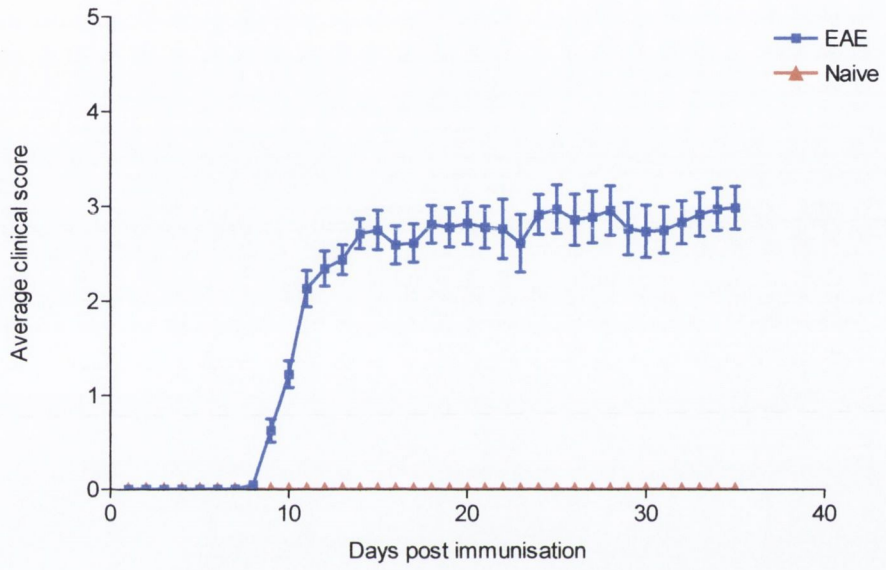

Figure 4.1. Clinical scores of C57BL/6 mice with EAE. EAE was induced in C57BL/6 mice by s.c. injection of MOG_{35-55} (150μg) emulsified in CFA supplemented with 4 mg/ml H37 Ra *Mycobacterium tuberculosis* on day 0 and i.p. injection of PT on day 0 and day 2. Control animals were injected i.p.with PBS (200μl) . Clinical scores were assessed daily and disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death.

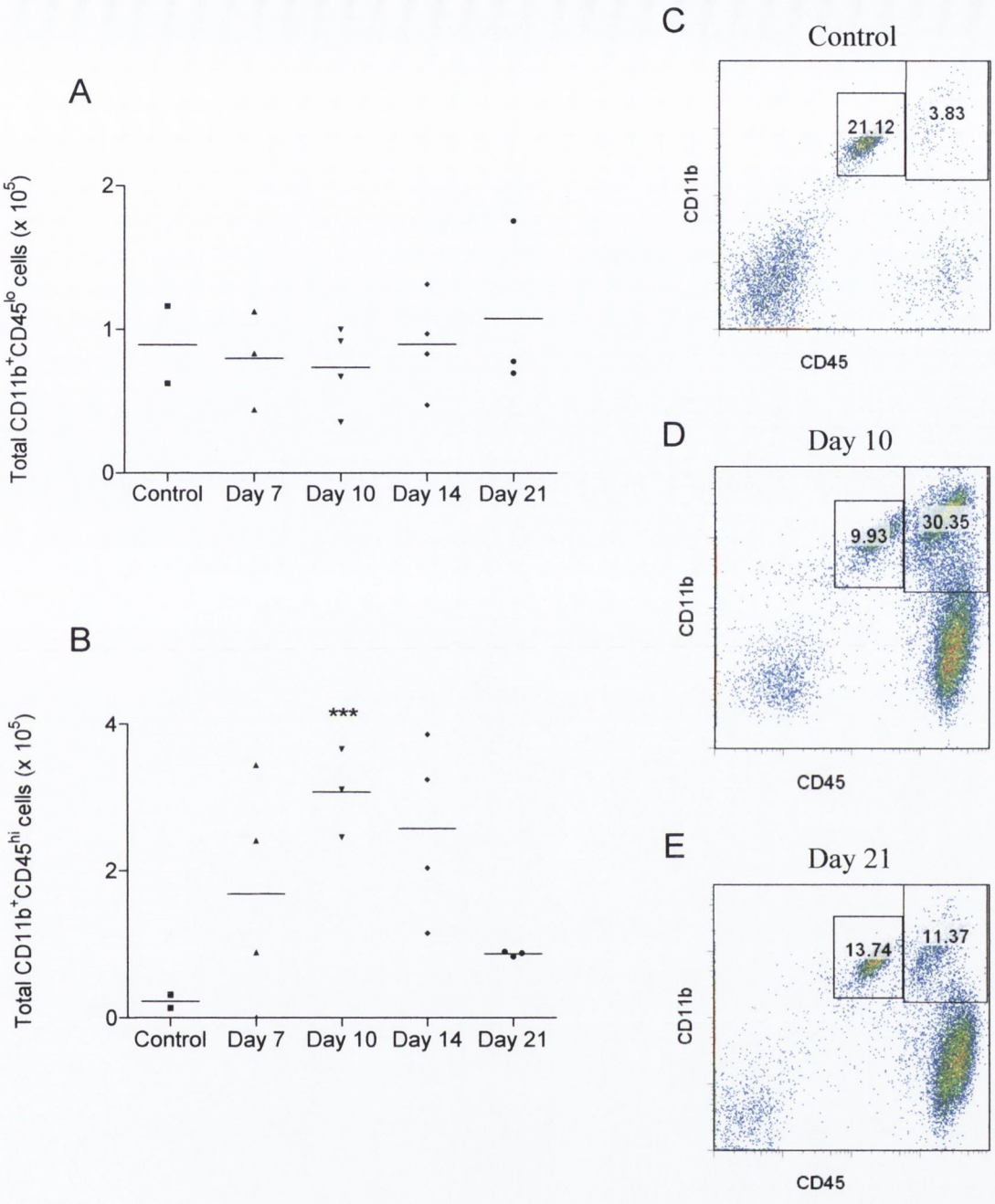

Figure 4.2. Increased frequency of $CD11b^{+}CD45^{hi}$ cells in the brain of mice with EAE. Mononuclear cells were isolated from the brain of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. Cells were stained with anti-CD11b and anti-CD45 to identify the resident microglial population (A; $CD11b^{+}CD45^{lo}$) and infiltrating macrophage population (B; $CD11b^{+}CD45^{hi}$). Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are percentage of total mononuclear cells. *$p<0.05$; ***$p<0.001$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

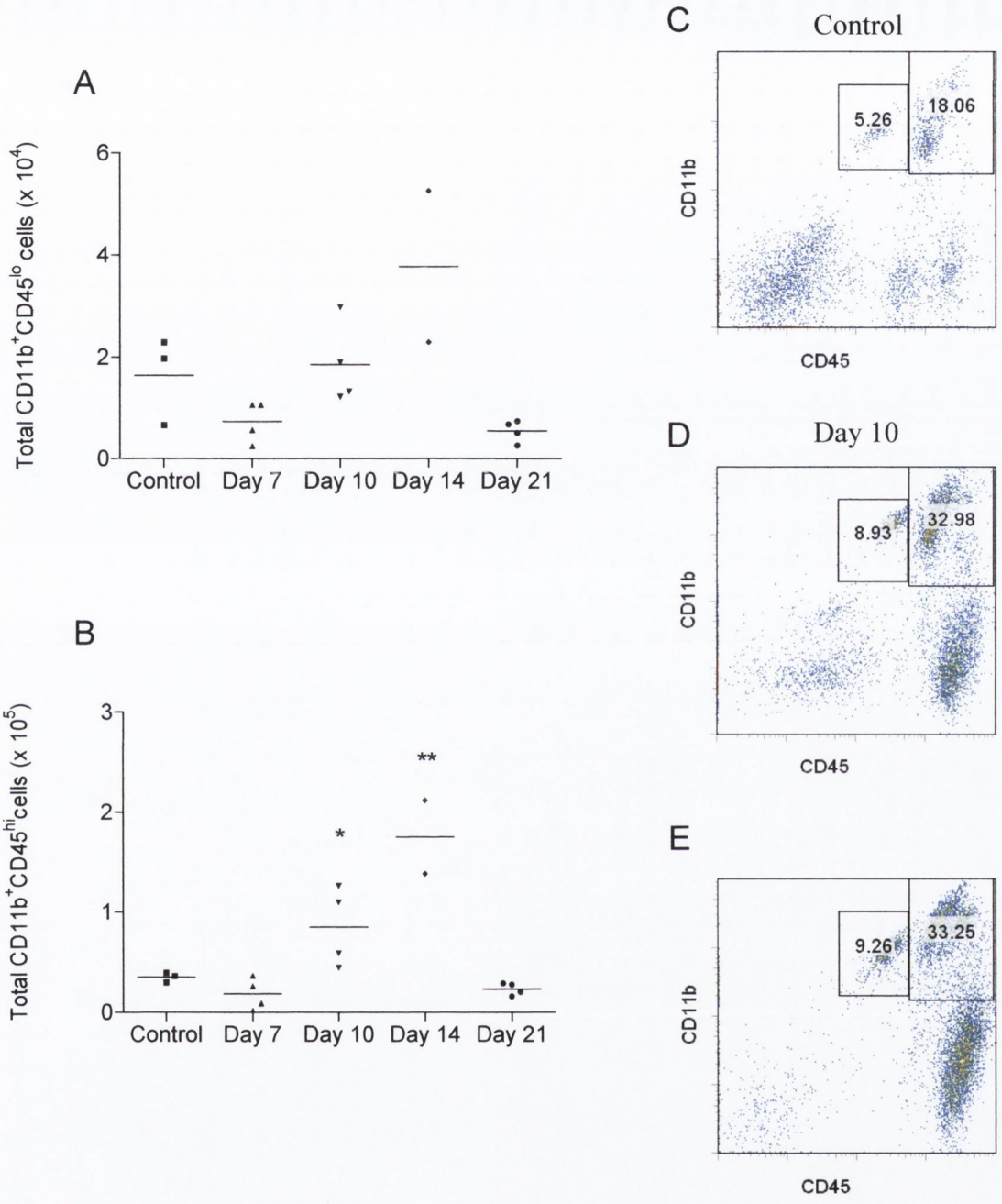

Figure 4.3. Increased frequency of CD11b^{+}CD45^{hi} cells in the spinal cord of mice with EAE. Mononuclear cells were isolated from the spinal cord of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. Cells were stained with anti-CD11b and anti-CD45 to identify the resident microglial population (A; CD11b^{+}CD45^{lo}) and infiltrating macrophage population (B; CD11b^{+}CD45^{hi}). Representative plots of CD45 versus CD11b in spinal cord from a control mouse (C) and a mouse with EAE at day10 (D) and day 21 (E). Numbers in gates are percentage of total number of mononuclear cells. *$p<0.05$; **$p<0.01$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

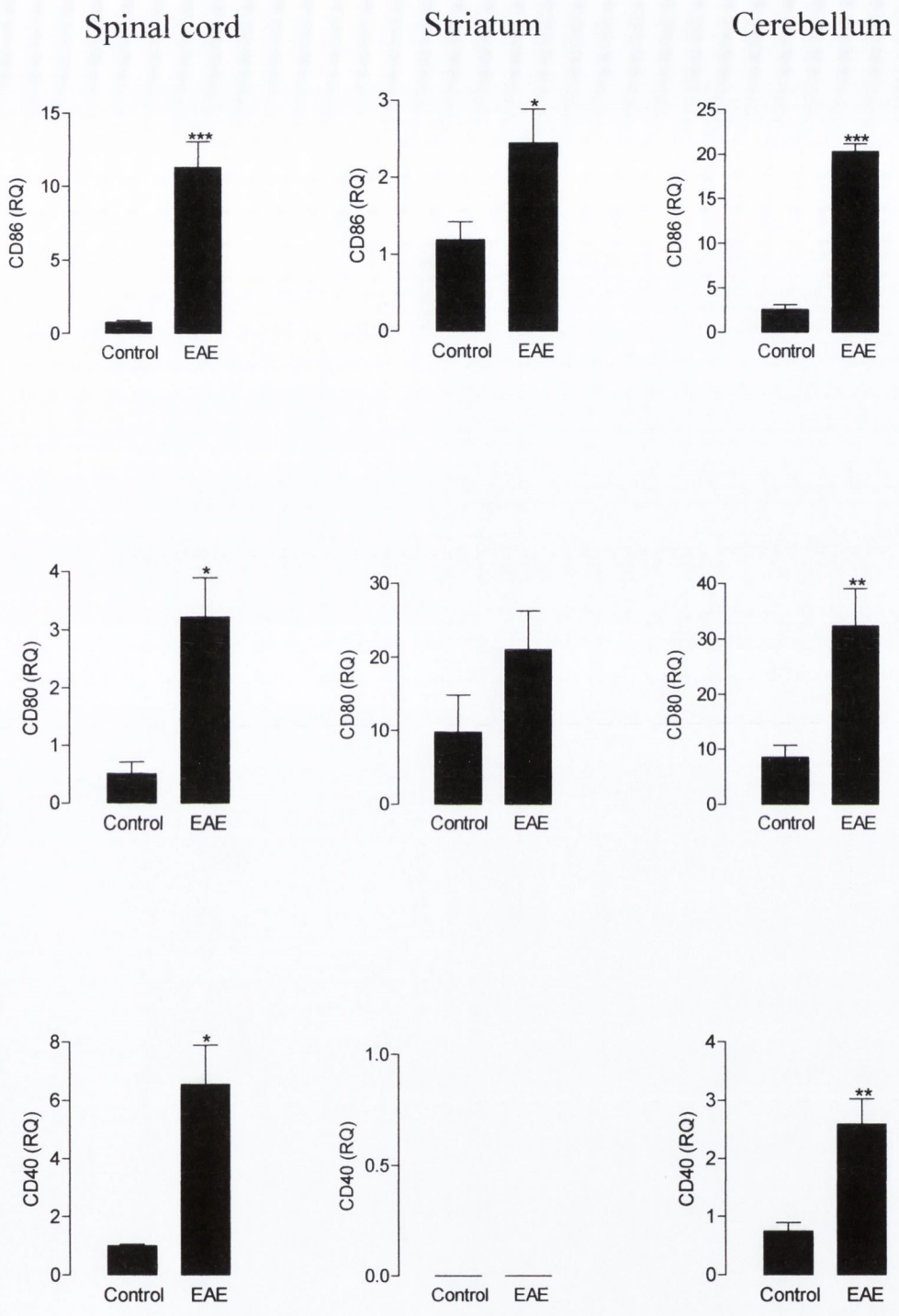

Figure 4.4. Increased mRNA expression of activation markers in the spinal cord, striatum and cerebellum in C57BL/6 mice with EAE. Mice with severe EAE were sacrificed, spinal cord, striatum and cerebellum were removed, expression of CD86, CD80 and CD40 mRNA were quantified by QPCR. Values are expressed as relative quantities (RQ) obtained from calculating the ratio of target gene mRNA to the endogenous control gene (18S). *p<0.05; **p<0.01; ***p<0.001; versus control mice, by Student's *t* test. (n=6-8).

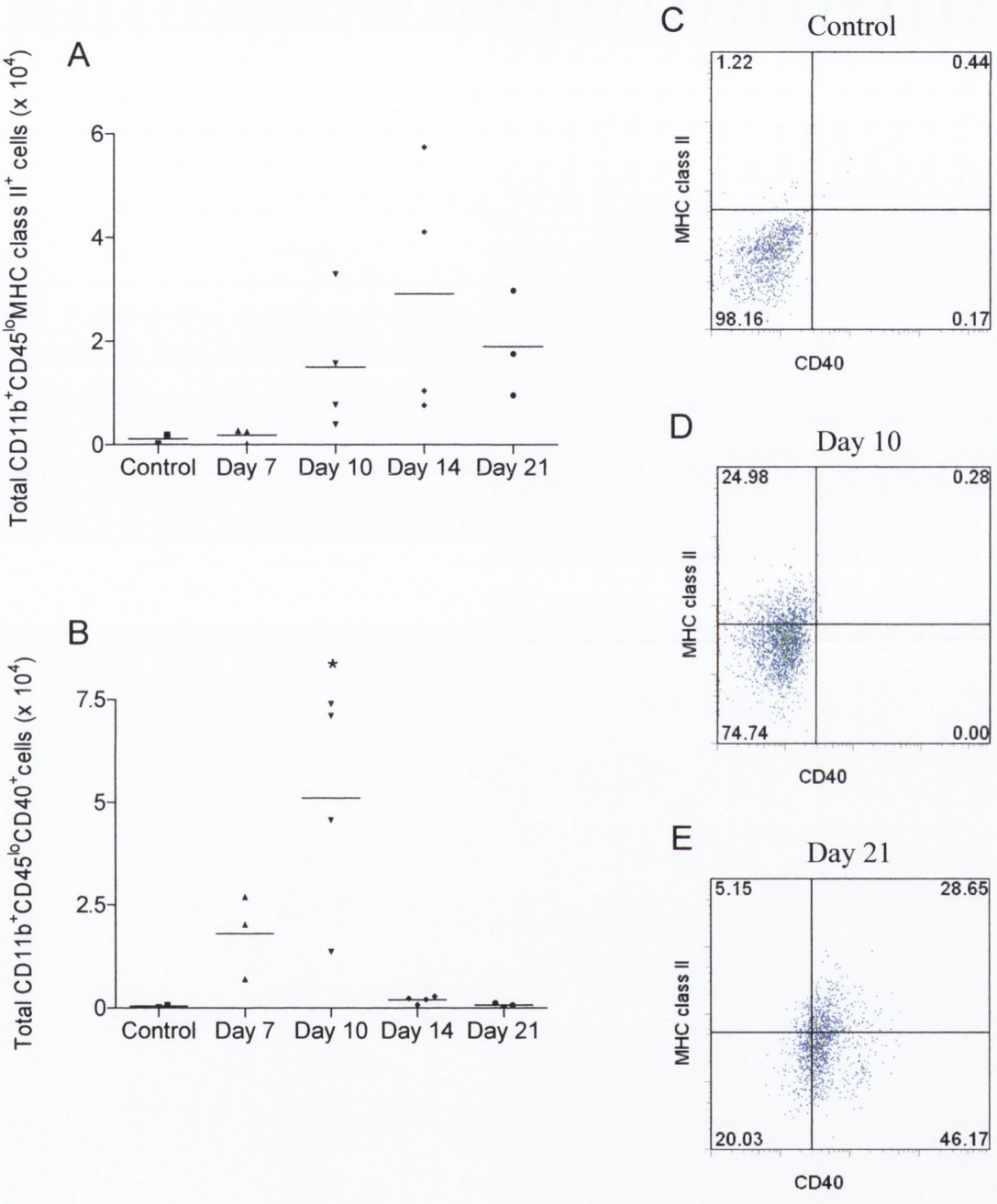

Figure 4.5. Increased expression of MHC class II and CD40 on resident microglia in the brain of mice with EAE. Mononuclear cells were isolated from the brain of control mice and immunised mice 7, 10, 14 and 21 days after induction of EAE. The cells were gated on CD11b and $CD45^{lo}$ expression, the total number of MHC class II expressing microglia (A) and CD40 expressing microglia (B) are shown in the scatter plots. Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are percentage of $CD11b^{+}CD45^{lo}$ cells. *$p<0.05$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

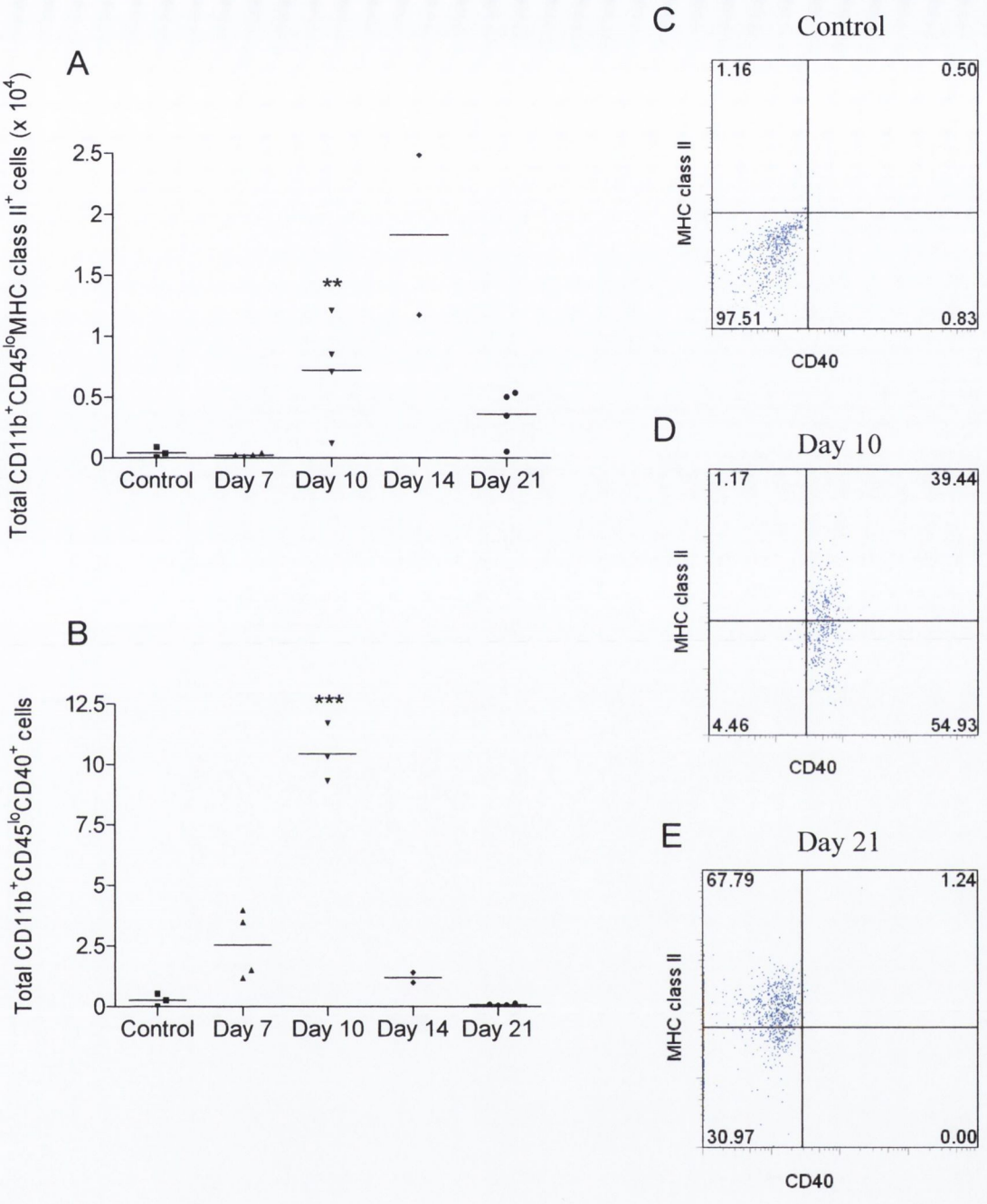

Figure 4.6. Increased MHC class II and CD40 expression on resident microglia in the spinal cord of mice with EAE. Mononuclear cells were isolated from the spinal cord of control mice and immunised mice 7, 10, 14 and 21 days after induction of EAE. The cells were gated on CD11b and $CD45^{lo}$ expression and the total number of $CD11b^{+}CD45^{lo}$ cells that express MHC class II (A) and CD40 (B) was assessed by flow cytometry. Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are percentage of $CD11b^{+}CD45^{lo}$ cells. $^{**}p<0.01$; $^{***}p<0.001$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point)

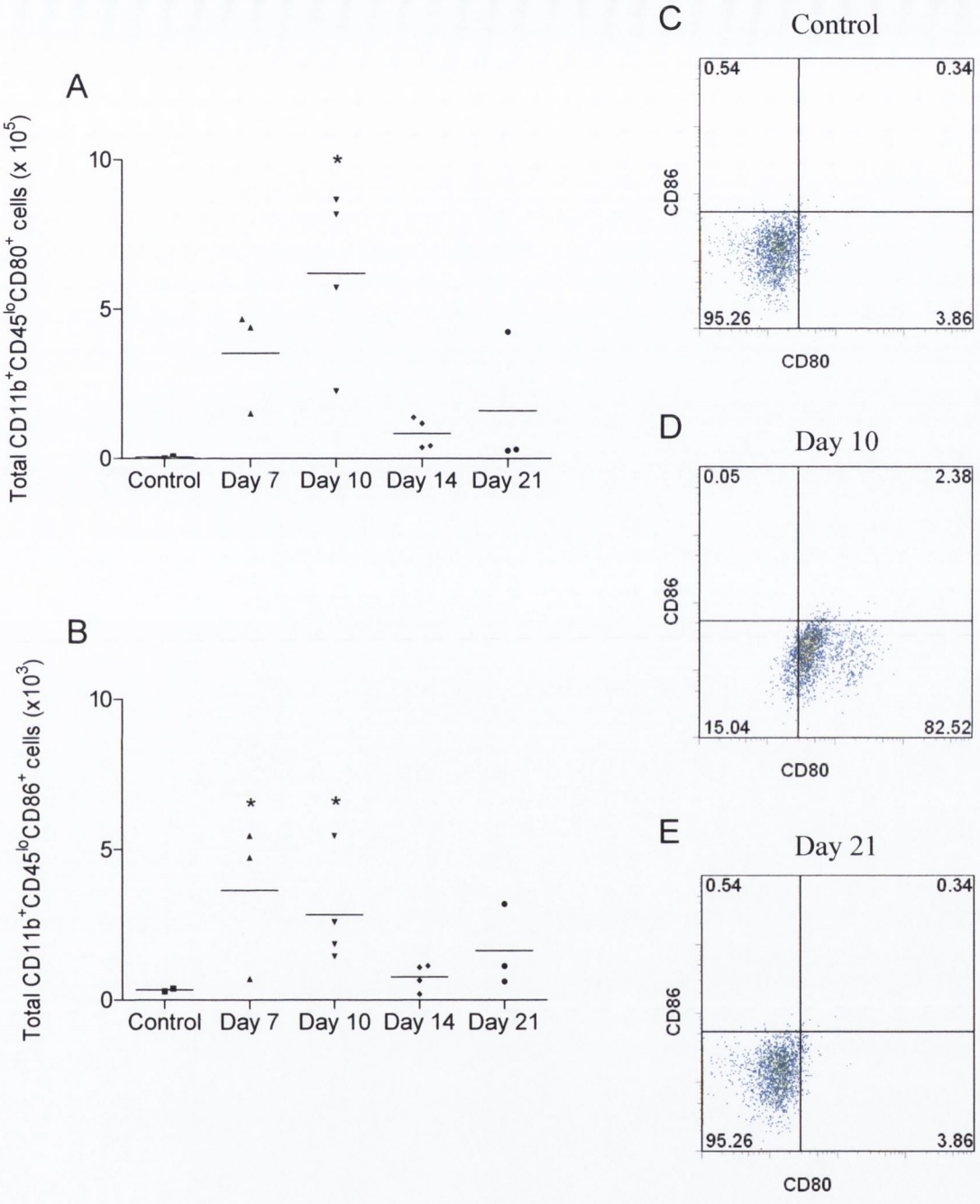

Figure 4.7. Increased CD80 and CD86 expression on resident microglia in the brain of mice with EAE. Mononuclear cells were isolated from the brain of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. The cells were gated on CD11b and $CD45^{lo}$ expression and the total number of microglia expressing CD80 (A) and CD86 (B) are shown in the scatter plots. Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are percentage of $CD11b^{+}CD45^{lo}$ cells. *$p<0.05$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per timepoint)

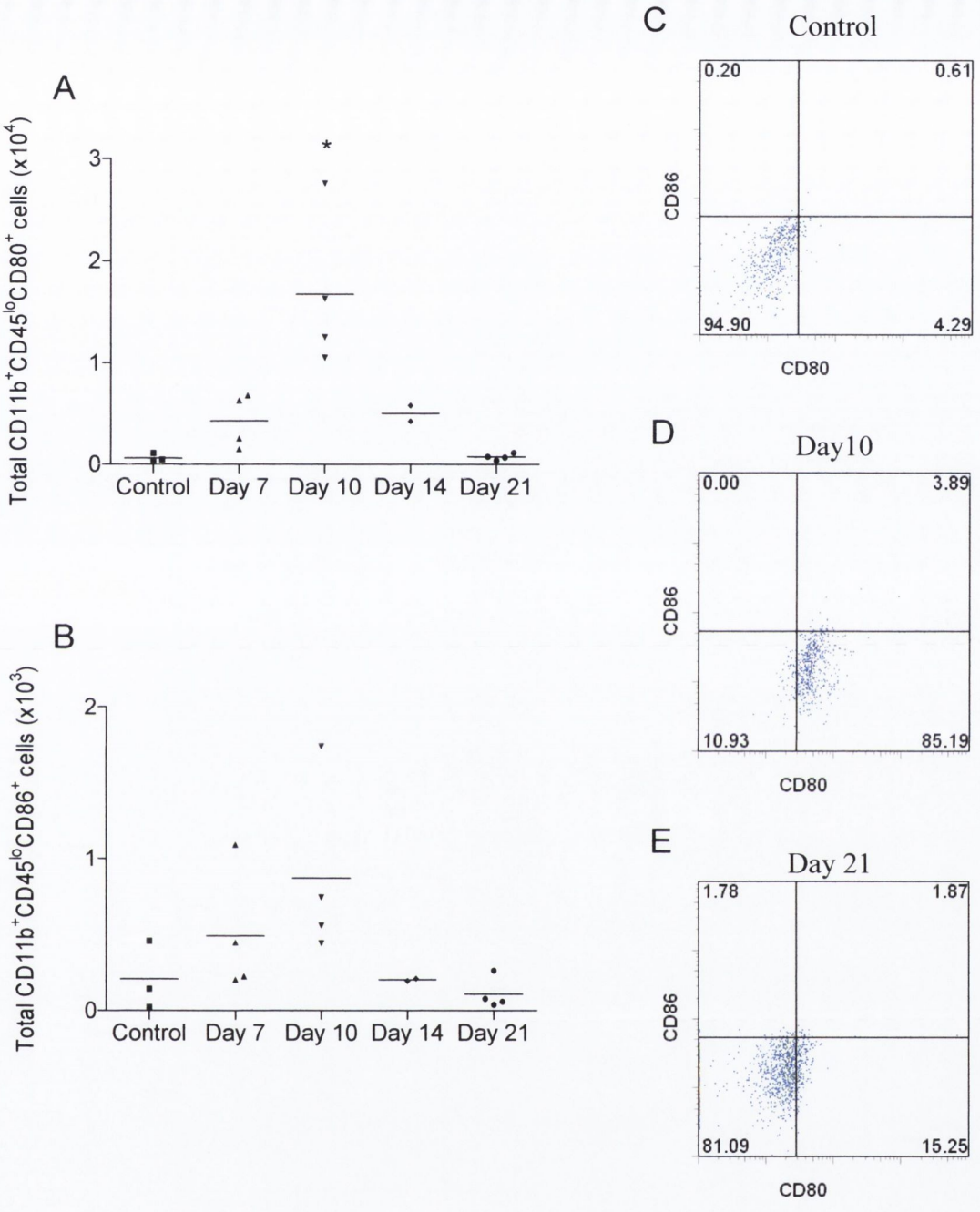

Figure 4.8. Increased frequency of CD80 and CD86 expressing resident microglia in the spinal cord of mice with EAE. Mononuclear cells were isolated from the spinal cord of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. The cells were gated on CD11b and $CD45^{lo}$ expression and the total number of microglia expressing CD80 (A) and CD86 (B) are shown in the scatter plots. Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are percentage of $CD11b^{+}CD45^{lo}$ cells. *$p<0.05$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

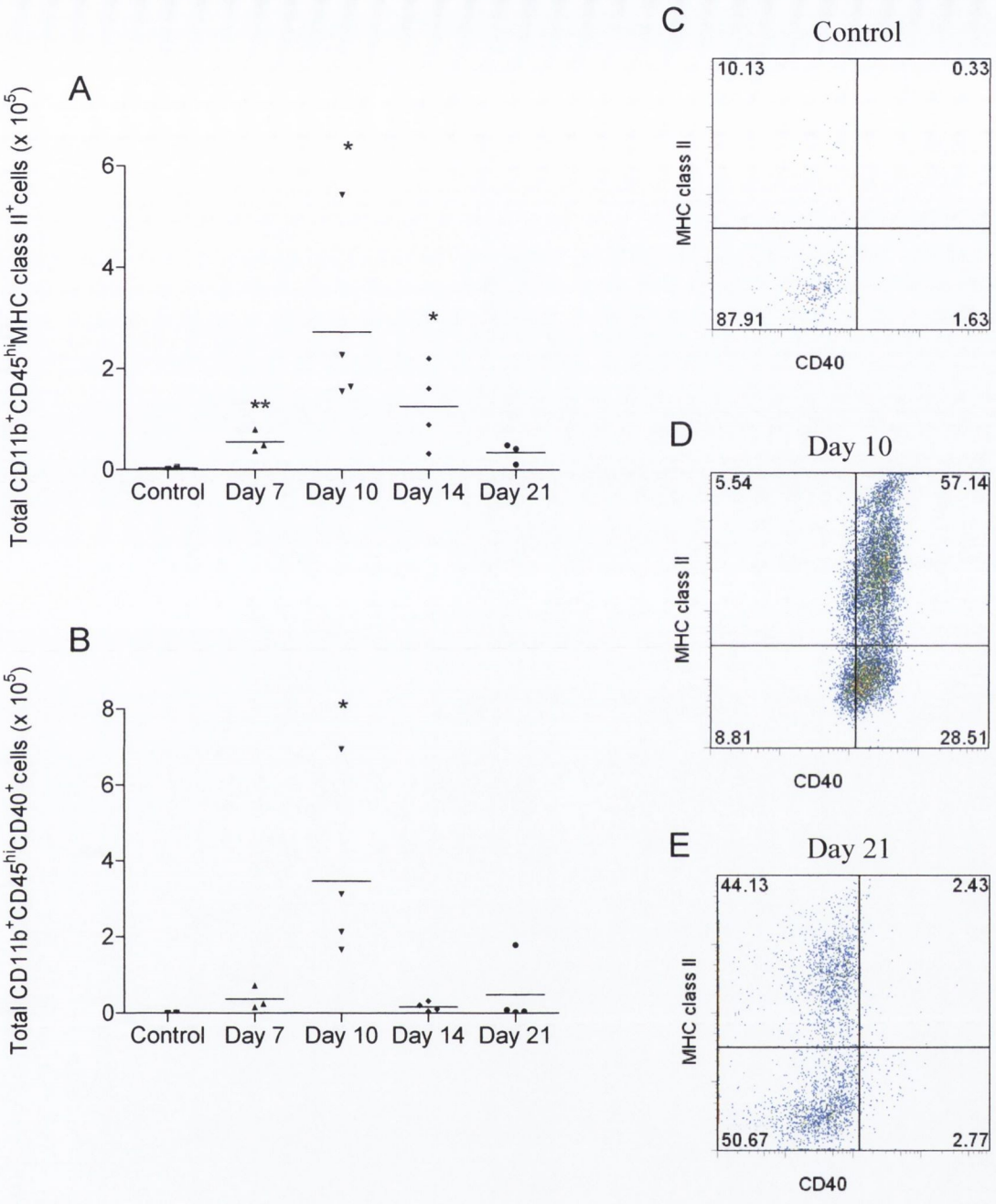

Figure 4.9. Increased MHC class II and CD40 expression on CNS-infiltrating macrophages in the brain of mice with EAE. Mononuclear cells were isolated from the brain of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. The cells were gated on CD11b and $CD45^{hi}$ expression and the total number of macrophages expressing MHC class II (A) and CD40 (B) are shown in the scatter plots. Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are percentage of $CD11b^{+}CD45^{hi}$ cells. *$p<0.05$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

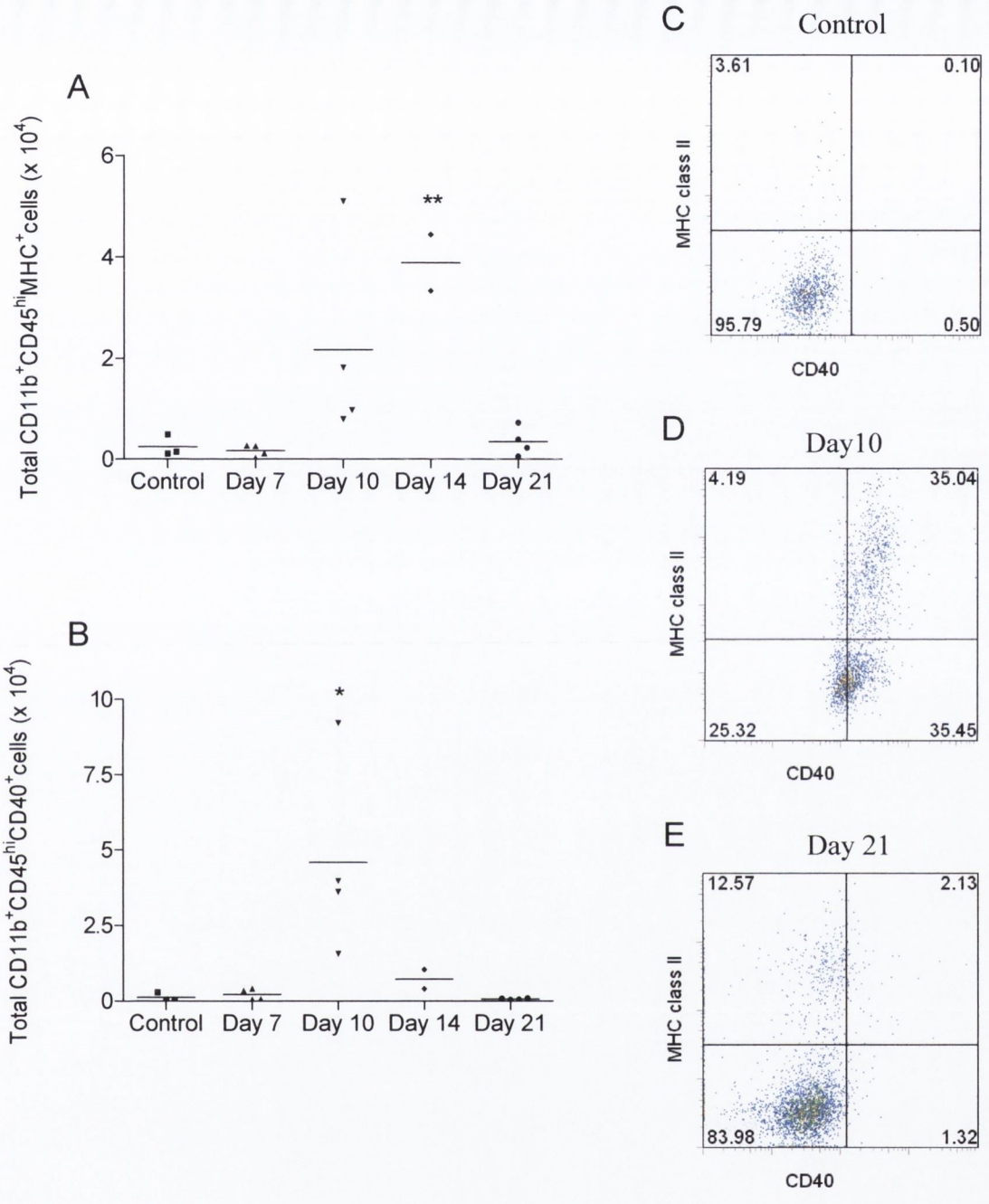

Figure 4.10. Increased frequency of MHC class II and CD40 expressing CNS-infiltrating macrophages in the spinal cord of mice with EAE. Mononuclear cells were isolated from the spinal cord of control mice and immunised mice 7, 10, 14 and 21 days after induction of EAE. The cells were gated on CD11b and $CD45^{hi}$ expression and the total number of macrophages expressing MHC class II (A) and CD40 (B) are shown in the scatter graphs. Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are percentage of CNS-infiltrating macrophages. *$p<0.05$; **$p<0.01$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

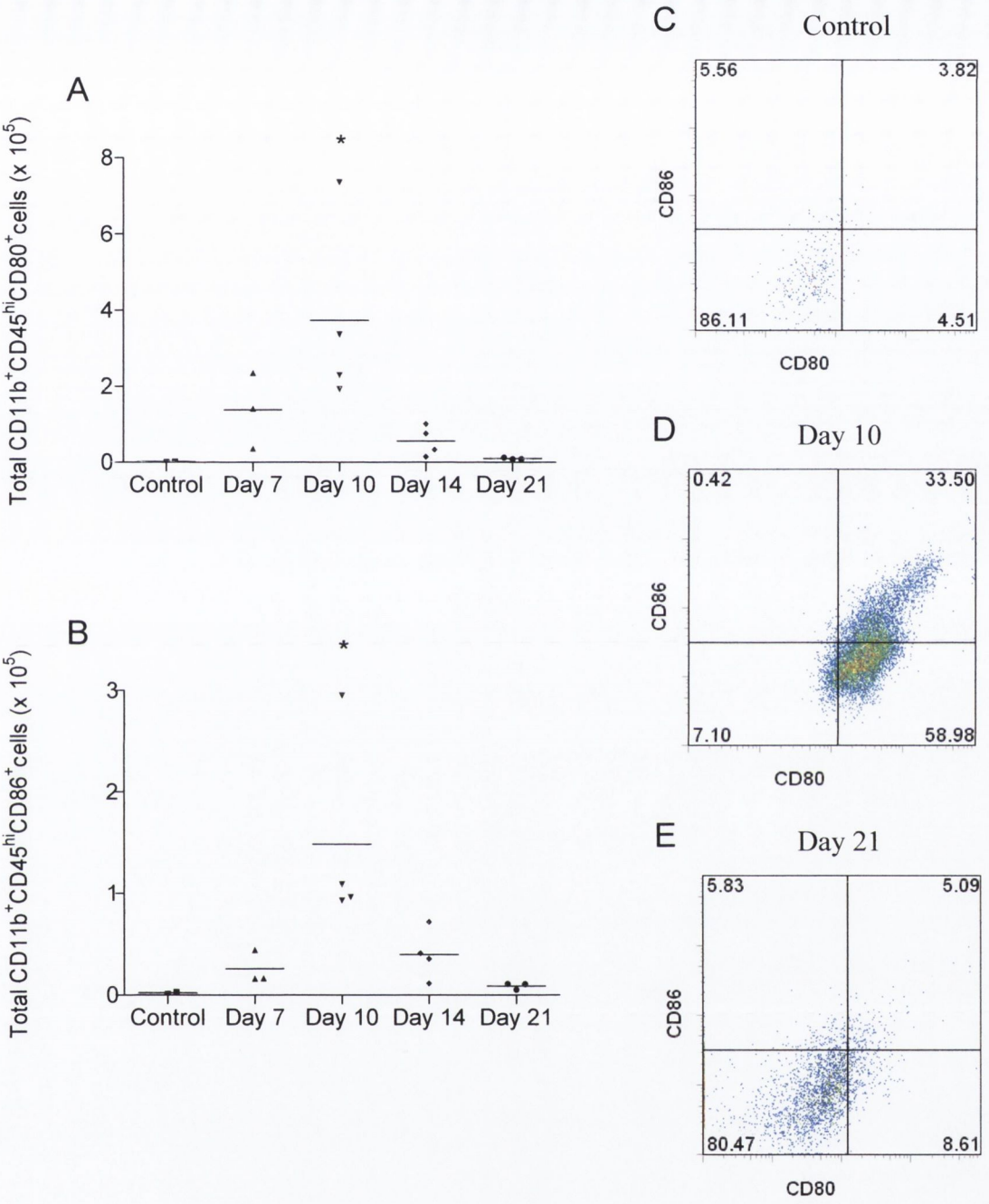

Figure 4.11. Increased number of CD80 and CD86 expressing CNS-infiltrating macrophages in the brain during EAE. Mononuclear cells were isolated from the brain of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. The cells were gated on CD11b and $CD45^{hi}$ expression and the total number of macrophages expressing CD80 (A) and CD86 (B) are shown in the scatter graphs. Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are the percentage of CNS-infiltrating macrophages cells. *$p<0.05$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

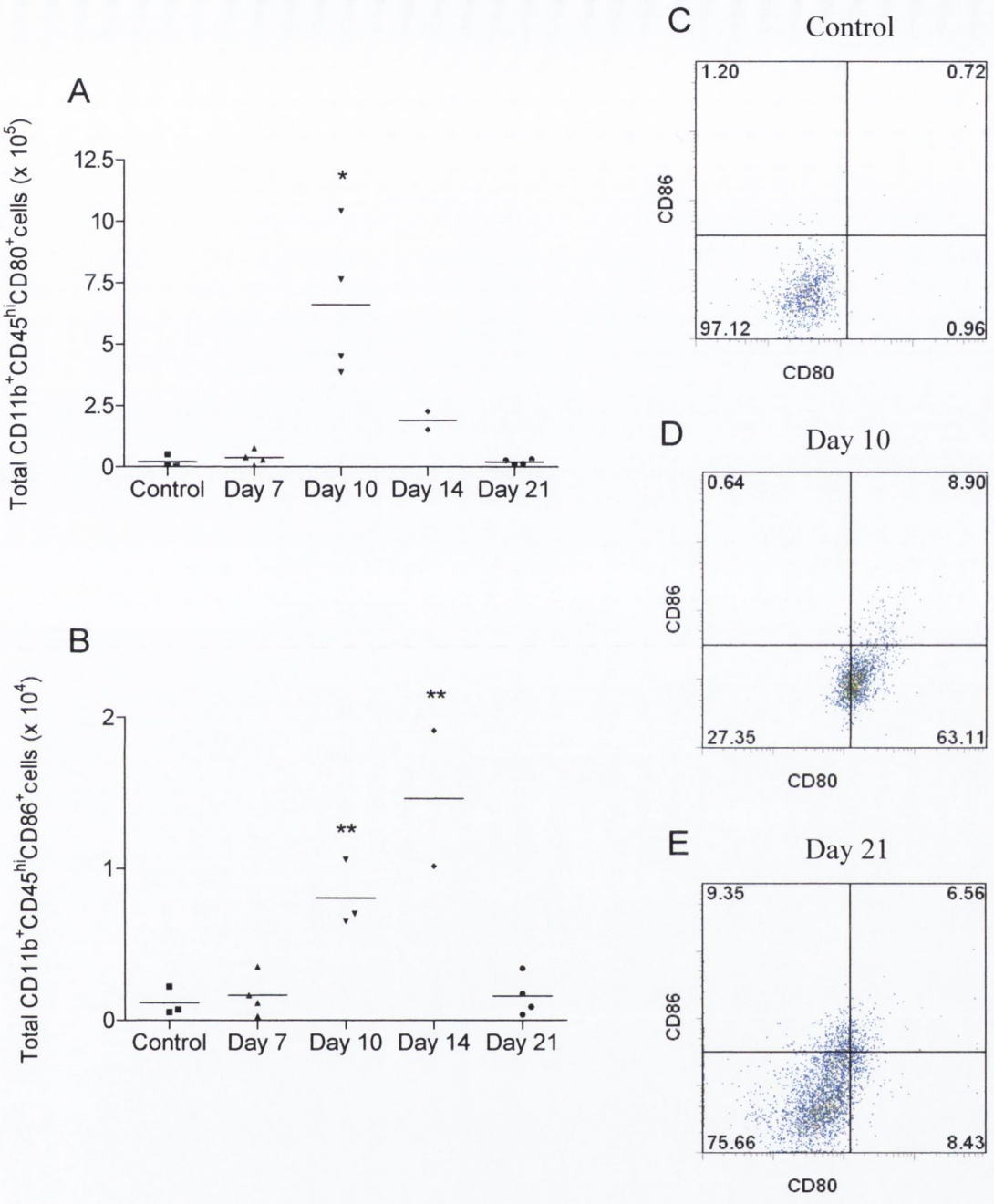

Figure 4.12. Increased frequency of CD80 and CD86 expressing CNS-infiltrating macrophages in the spinal cord of mice with EAE. Mononuclear cells were isolated from the spinal cord of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. The cells were gated on CD11b and $CD45^{hi}$ expression and the total number of macrophages expressing CD80 (A) and CD86 (B) are shown in the scatter graphs. Representative dotplots for control mice (C) and mice with EAE on day 10 (D) and 21 (E). Numbers in gates are the percentage of CNS-infiltrating macrophages. *$p<0.05$; **$p<0.01$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

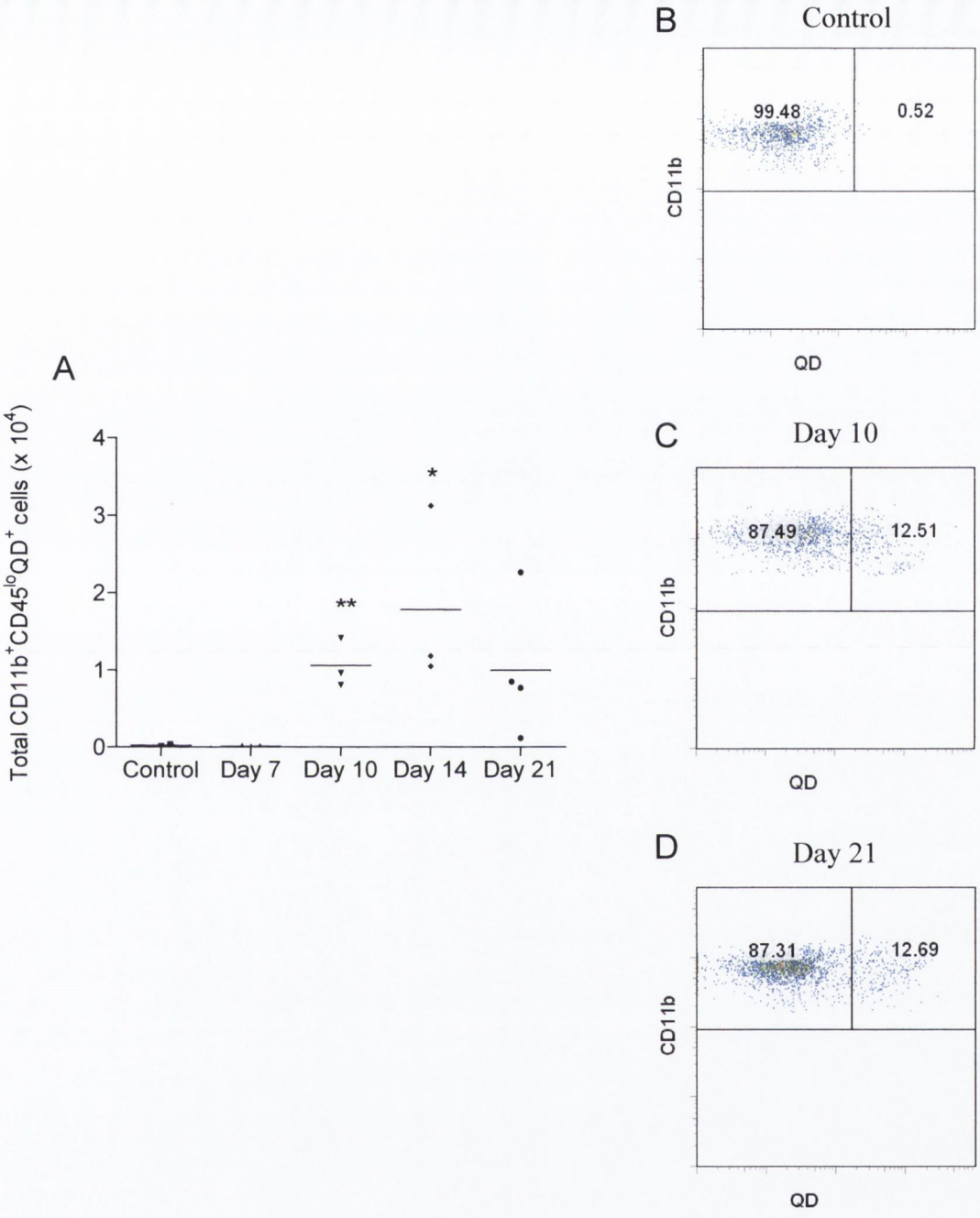

Figure 4.13. Phagocytic activity of resident microglia in the brain during development of EAE. Mononuclear cells were isolated from the brain of control mice and immunised mice 7, 10, 14 and 21 days after the induction of EAE. Cells were gated on their CD11b and $CD45^{lo}$ expression. The phagocytic activity of resident microglia was measured by testing their ability to phagocytose nano-particles. The total number of microglia that phagocytosed quantum dots are shown in the scatter plot (A). Representative dotplots for control mice (B) and mice with EAE on day 10 (C) and day 21 (D). Numbers in gates are percentage of resident microglia. *$p<0.05$; **$p<0.01$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

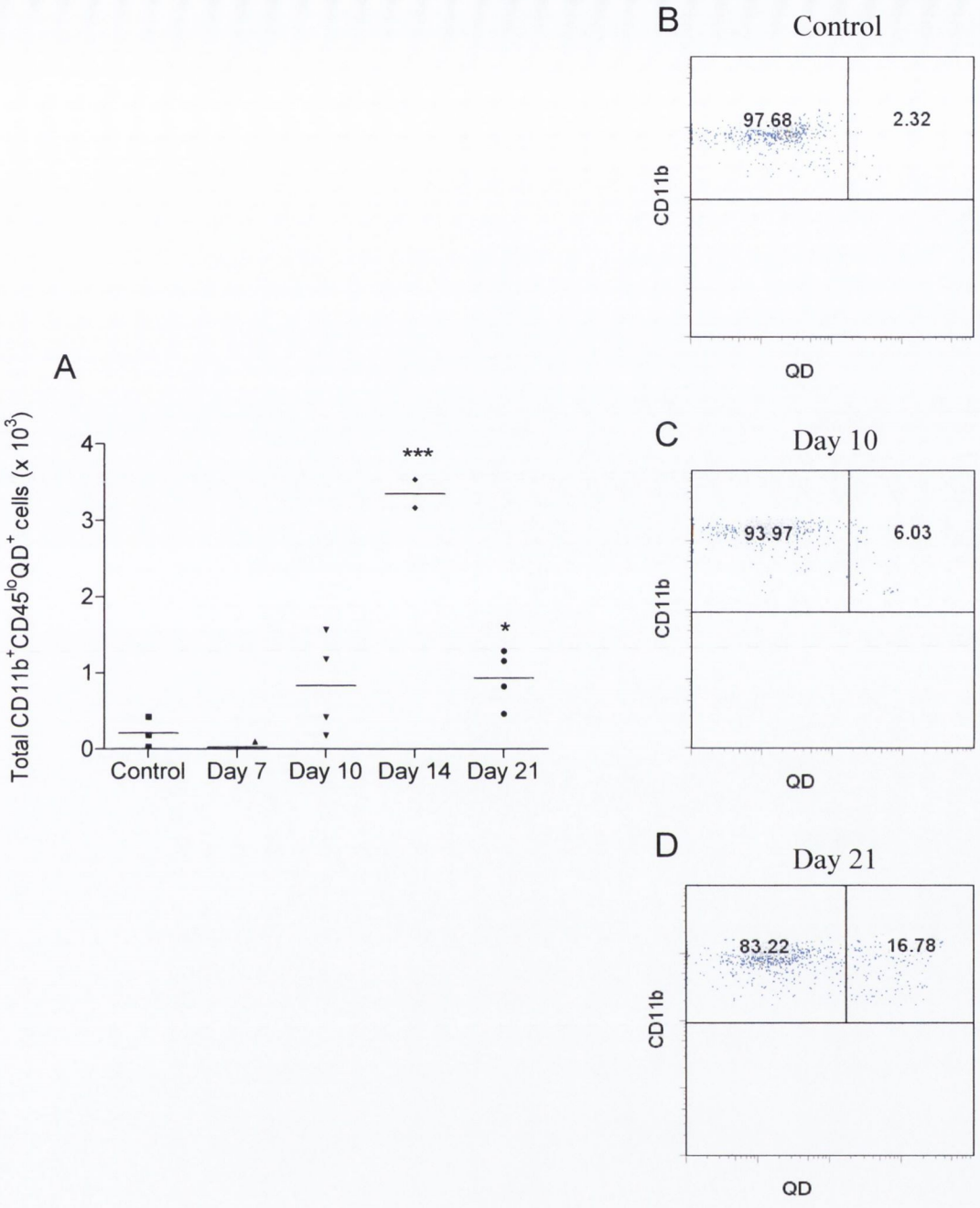

Figure 4.14. Phagocytic activity of resident microglia in the spinal cord during EAE. Mononuclear cells were isolated from the spinal cord of control mice and immunised mice 7, 10, 14 and 21 days after the induction of EAE. Cells were gated on their CD11b and $CD45^{lo}$ expression. The phagocytic activity of resident microglia ($CD11b^{+}CD45^{lo}$) was measured by testing their ability to phagocytose nano-particles. The total number of microglia that phagocytosed quantum dots are shown in the scatter plot (A). Representative dotplots for control mice (B) and mice with EAE on day 10 (C) and day 21 (D). Numbers in gates are percentage of resident microglia. *$p<0.05$; ***$p<0.001$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

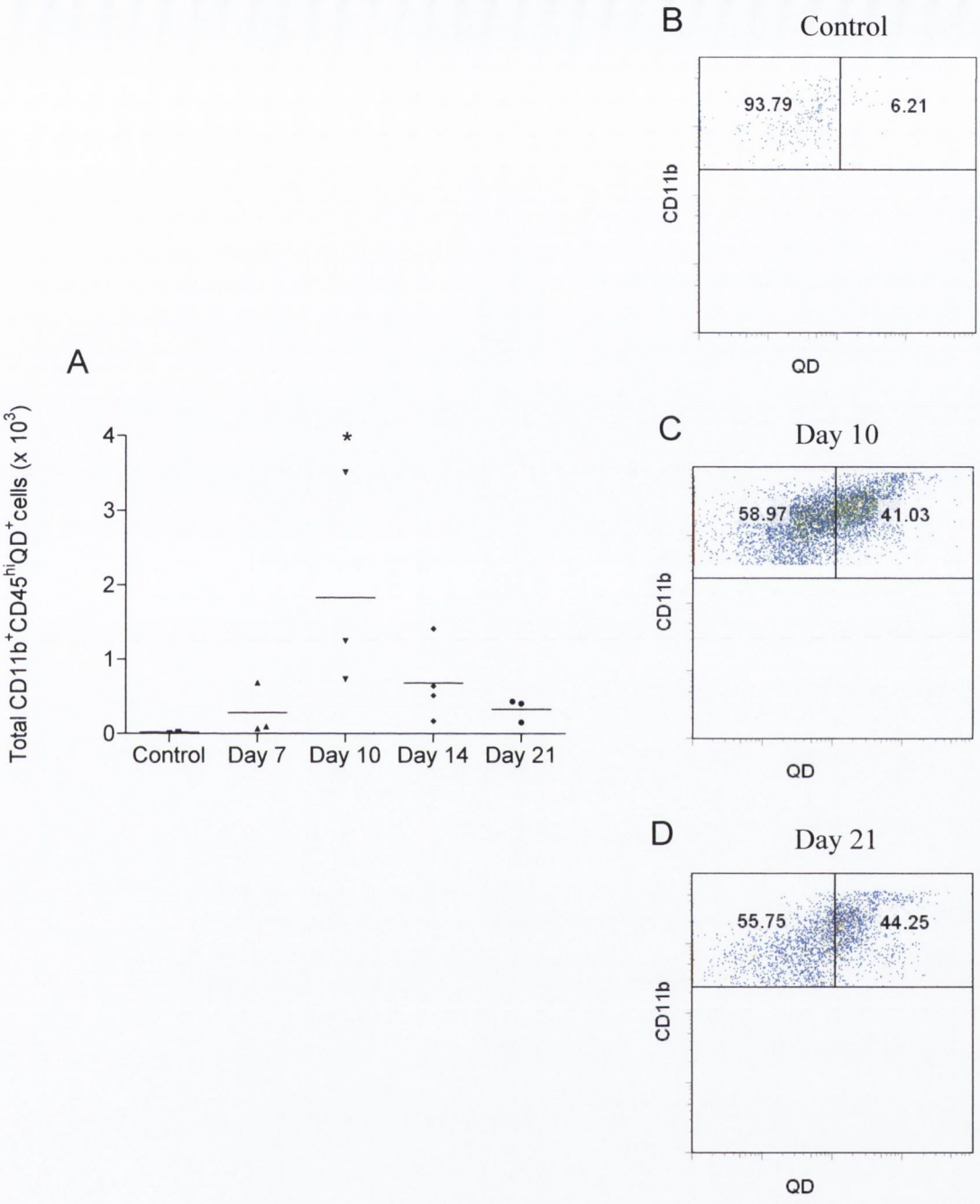

Figure 4.15. The phagocytic activity of CNS-infiltrating macrophages in the brain is significantly increase by EAE. Mononuclear cells were isolated from the brain of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. Cells were gated on their CD11b and $CD45^{hi}$ expression, the total number of phagocytically active macrophages are shown in the scatter plot (A). Representative dotplots for control mice (B) and mice with EAE on day 10 (C) and 21 (D). Numbers in gates are percentage of CNS-infiltrating macrophages. *$p<0.05$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

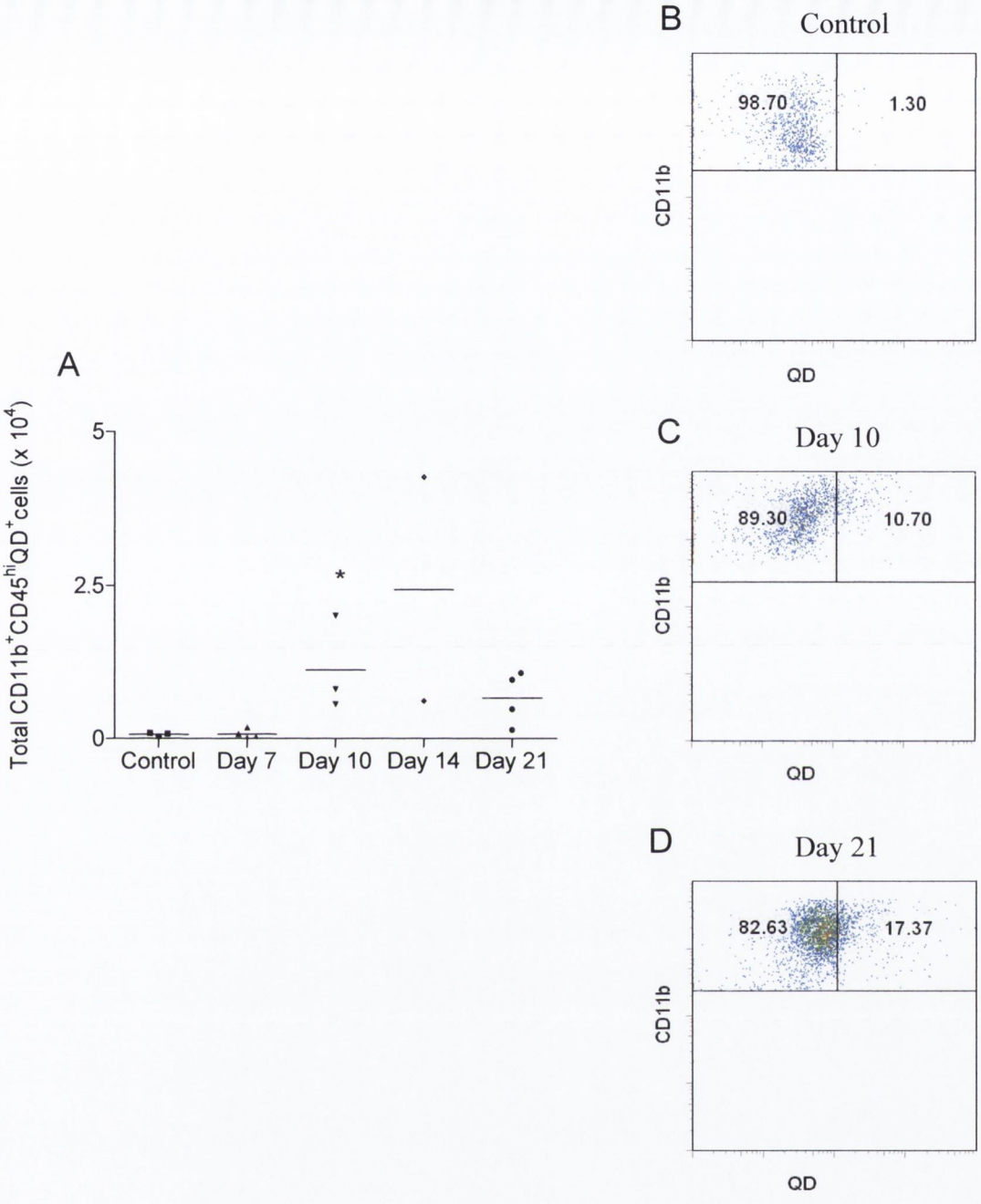

Figure 4.16. Phagocytic activity of CNS-infiltrating macrophages in the spinal cord is increased by EAE. Mononuclear cells were isolated from the spinal cord of control mice and immunised mice 7, 10, 14 and 21 days post immunisation. Cells were gated on their CD11b and $CD45^{hi}$ expression, the total number of phagocytically active macrophages are shown in the scatter plot (A). Representative dotplots for control mice (B) and mice with EAE on day 10 (C) and 21 (D). Numbers in gates are percentage of CNS-infiltrating macrophages. *$p<0.05$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as a scatterplot with the mean highlighted. (n=4 per time point).

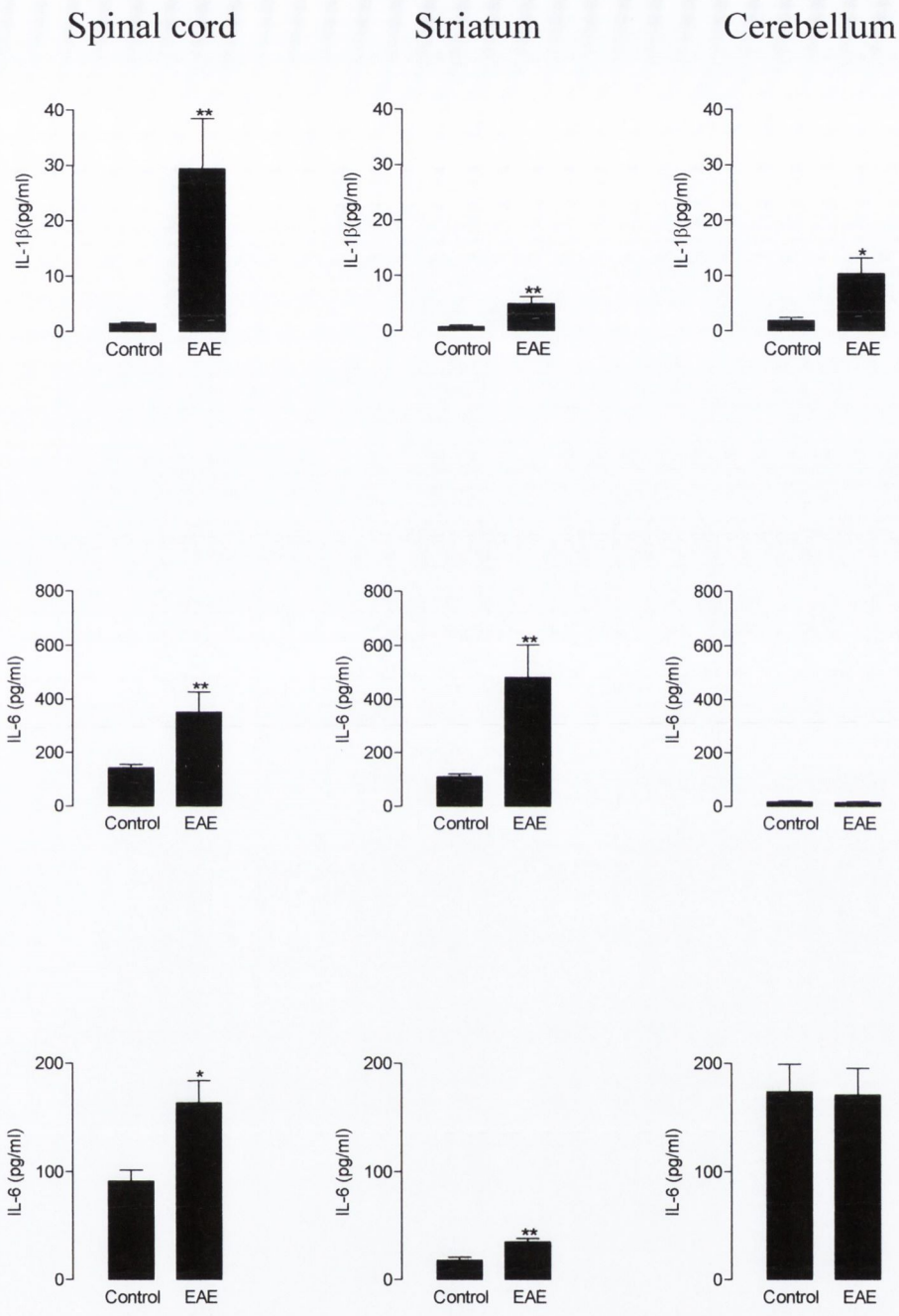

Figure 4.17. IL-1β, IL-6 and TNF-α production in the spinal cord, striatum and cerebellum of C57BL/6 mice during EAE. After the clinical symptoms of severe EAE were evident, mice were sacrificed, spinal cord, striatum and cerebellum were removed, IL-1β, IL-6 and TNF-α concentration was quantified by ELISA. *$p<0.05$; **$p<0.01$; versus control mice, by Student's *t* test. (n=6-8)

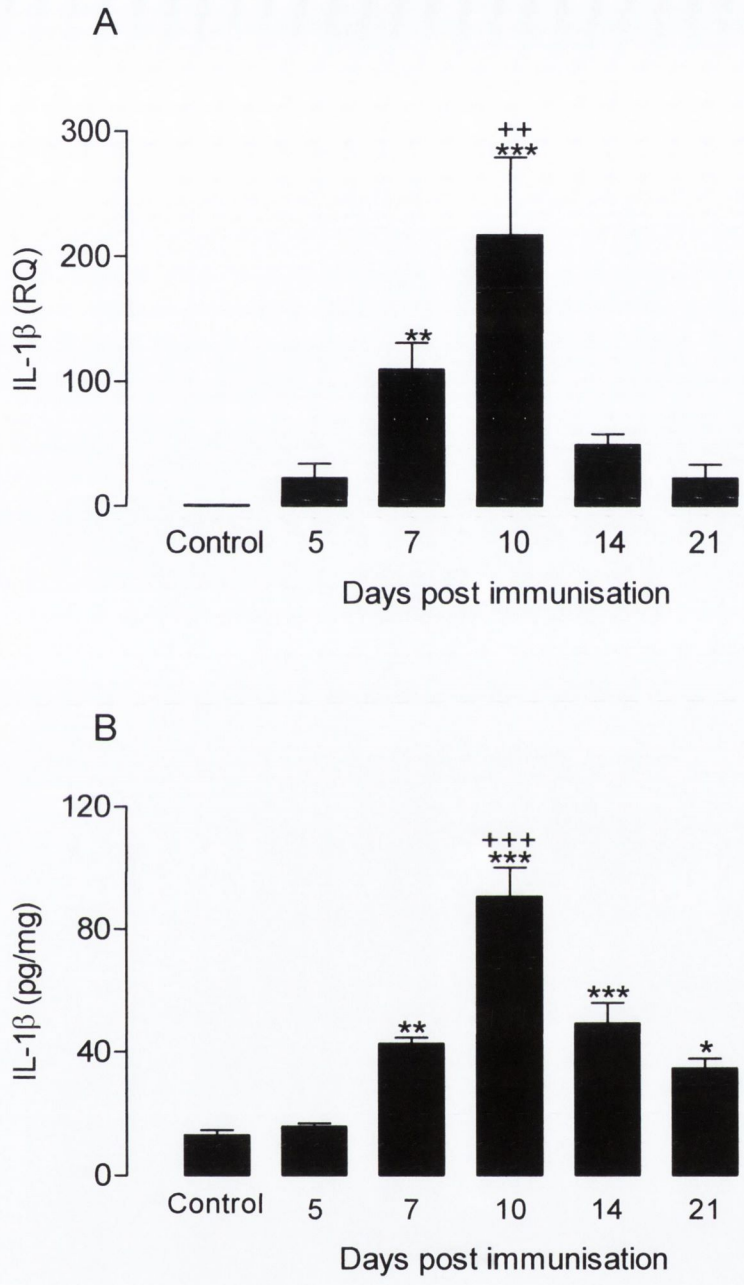

Figure 4.18. IL-1β mRNA expression and IL-1β protein production is significantly increased in the brain during EAE. Brains from control mice or mice with EAE were dissected at 5, 7, 10, 14 and 21 days post immunisation. RNA was extracted to assess IL-1β mRNA levels (A). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of IL-1β gene mRNA to the endogenous control gene, 18S. Tissue was homogenised and equalised in Krebs solution containing Ca^{2+} for IL-1β protein analysis (B). *$p<0.05$; **$p<0.01$, ***$p<0.001$; versus control mice, $^{++}p<0.01$; $^{+++}p<0.001$; versus mice with EAE 7 days post immunisation, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=4 per time point).

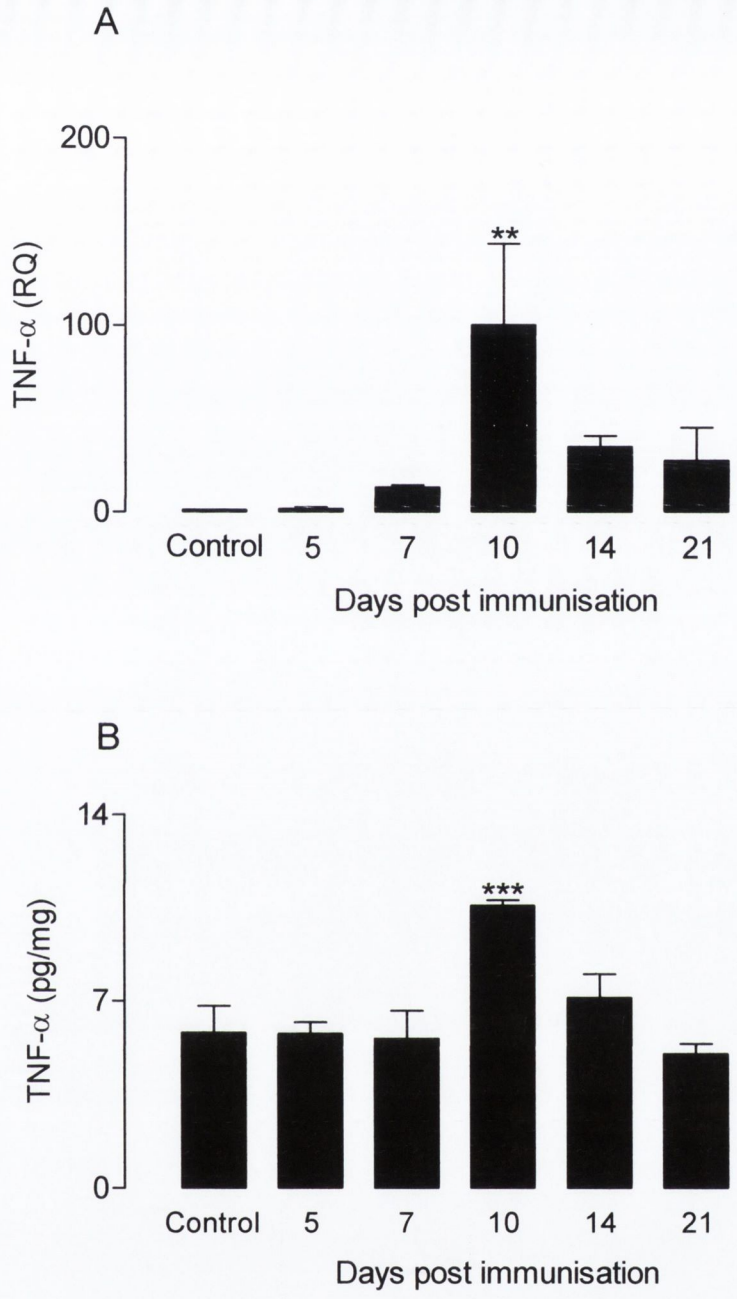

Figure 4.19. TNF-α mRNA expression and TNF-α protein production is significantly increased during EAE. Brains from control mice or mice with EAE were dissected at 5, 7, 10, 14 and 21 days post immunisation. RNA was extracted to assess TNF-α mRNA levels (A). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of TNF-α gene mRNA to the endogenous control gene (18S). Tissue was homogenised and equalised in Krebs solution containing Ca^{2+} for TNF-α protein analysis (B). **p<0.01; ***p<0.001; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=4 per time point).

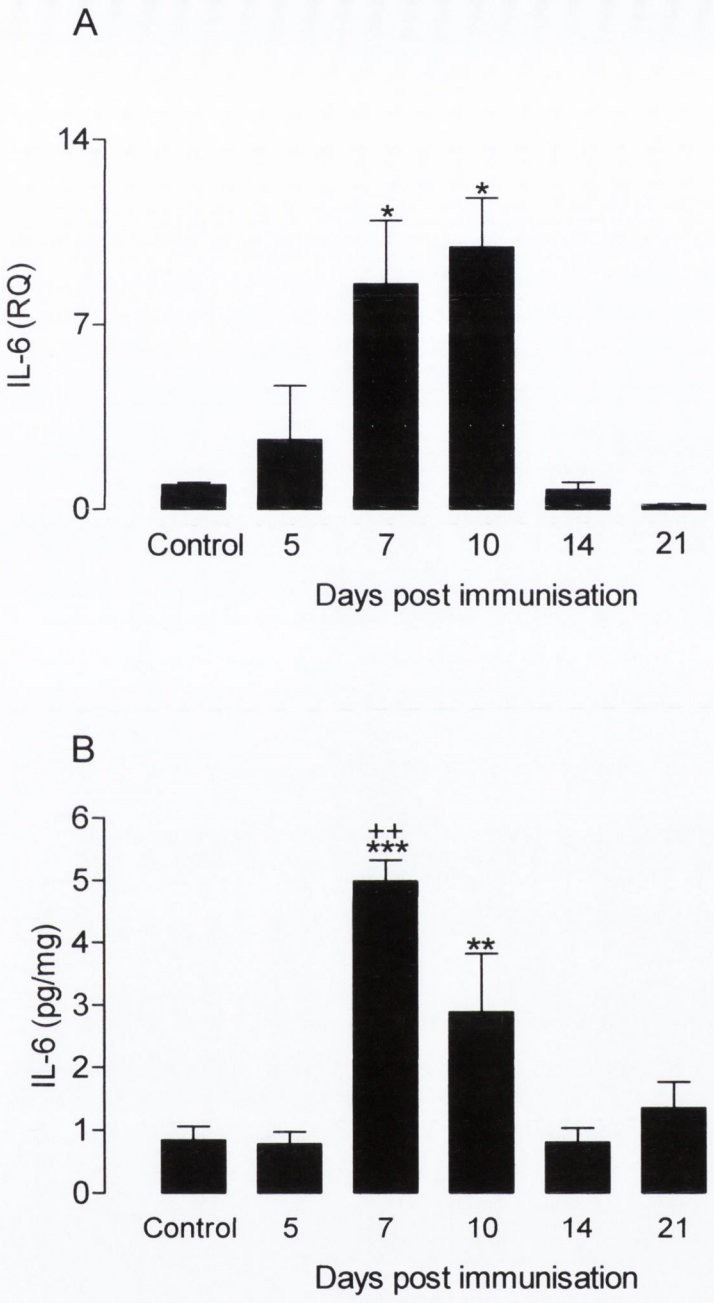

Figure 4.20. IL-6 mRNA expression and IL-6 protein production is significantly increased during EAE. Brains from control mice or mice with EAE were dissected at 5, 7, 10, 14 and 21 days post immunisation. RNA was extracted to assess IL-6 mRNA levels (A). Values are expressed as relative quantities (RQ) obtained from calculating the ratio of IL-6 gene mRNA to the endogenous control gene (18S). Tissue was homogenised and equalised in Krebs solution containing Ca^{2+} for IL-6 protein analysis (B). *$p<0.05$; **$p<0.01$; ***$p<0.001$; versus control mice, $^{++}p<0.01$; versus mice with EAE 10 days post immunisation, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=4 per time point).

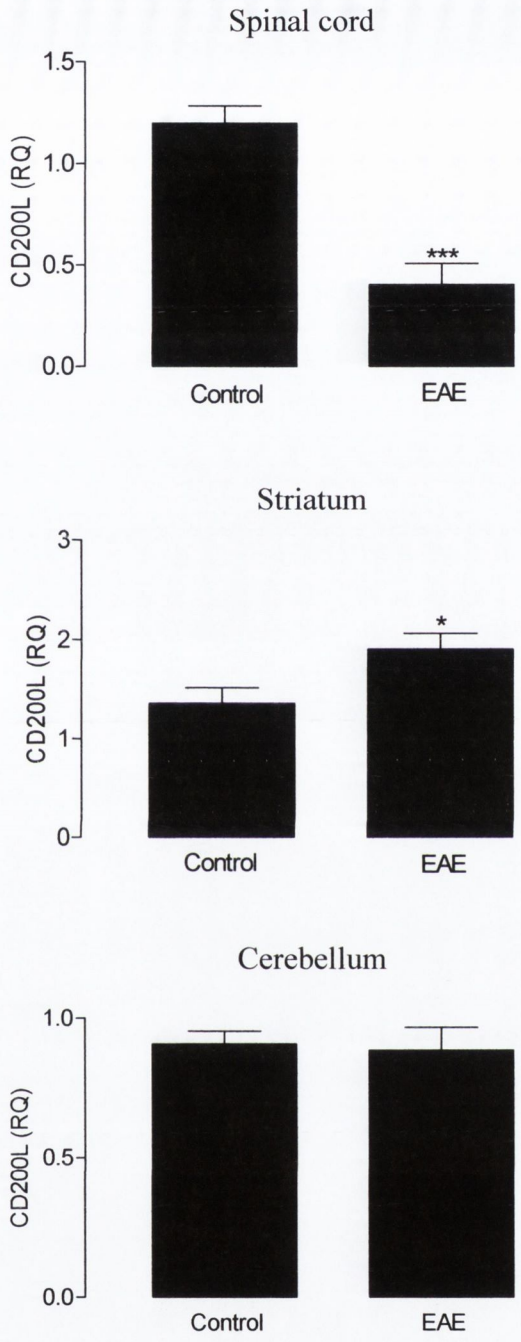

Figure 4.21. CD200 ligand mRNA is significantly decreased in the spinal cord but increased in the striatum of C57BL/6mice with EAE. Control mice and mice with EAE were sacrificed, spinal cord, striatum and cerebellum were removed, mRNA levels of CD200 ligand were quantified by QPCR. Values are expressed as relative quantities (RQ) obtained from calculating the ratio of CD200L gene mRNA to the endogenous control gene (β-actin). *$p<0.05$; ***$p<0.001$; versus control mice, by Student's *t* test. (n=6-8).

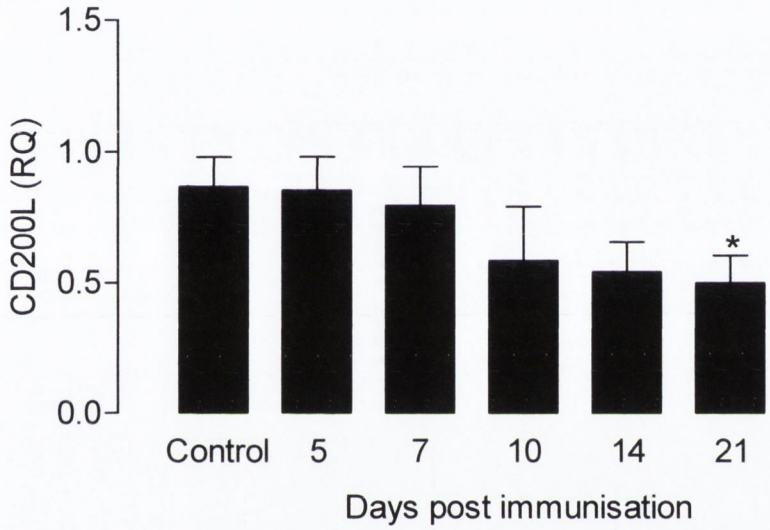

Figure 4.22. CD200 ligand mRNA significantly decreased in the brain during EAE. Brains were dissected from C57BL/6 control mice and mice with EAE 5, 7, 10, 14 and 21 days after induction of EAE. RNA was extracted and CD200L mRNA expression was analysed by QPCR. Values are expressed as relative quantities (RQ) obtained from calculating the ratio of CD200L gene mRNA to the endogenous control gene (18S). *$p<0.05$; versus control mice, by Student's *t* test. (n=4 per timepoint).

Chapter 5
The role of immunomodulatory molecules in EAE

5.1 Introduction

During EAE, macrophages and DC invade the CNS and act as APC, together with endogenous glia. The relative contribution of glia, macrophages and DC to antigen presentation within the CNS has been widely researched. Microglia are thought to be less efficient APC compared with DC and macrophages due to lower expression of CD45, a molecule that determines the efficiency of APC function (Carrieri *et al.*, 1998; Carson *et al.*, 1998). Expression of CD45 is enhanced to intermediate levels on activated microglia (Carson *et al.*, 1998) and to high levels on activated macrophages (Ponomarev *et al.*, 2005). In addition, antigen presentation by invading DC and macrophages within the CNS has been shown to be necessary for induction of EAE (Huitinga *et al.*, 1993). Blockade of macrophage infiltration significantly suppressed the severity of EAE (Huitinga *et al.*, 1993). Furthermore, Greter and colleagues (2005) demonstrate that antigen presentation by $CD11c^+$ DC in the CNS was necessary for the development and progression of EAE. These findings demonstrate an essential role for antigen presentation by macrophages and DC in induction of EAE. Another study compared the ability of CNS $CD11c^+CD11b^+$ DC and $CD11c^-CD11b^+$ microglia and macrophages to present antigen and found that macrophages and microglia express MHC class II and co-stimulatory molecules but to a lesser extent than DC. In addition, DC were more effective than microglia and macrophages in driving proliferation of antigen-specific T cells in the CNS (Deshpande *et al.*, 2007). In contrast, $CD45^{lo}$ microglia isolated from the CNS at the onset of clinical symptoms were found to be as efficient as CNS infiltrating $CD45^{hi}$ macrophages in stimulating proliferation and IFN-γ production from myelin-specific Th1 cells

(Mack *et al.*, 2003). Therefore, the role of glia, macrophages and DC as APC in the CNS during EAE is not fully elucidated.

Recently six new members of the IL-1 family of ligands have been identified; these proteins have been termed IL-1F5-IL-1F10 (Dunn *et al.*, 2001) and like the IL-1α, IL-1β and IL-1ra genes, all of the genes map to human chromosome 2 (Dunn *et al.*, 2001). Little is known about the signaling of these proteins. Our laboratory is the first to show that IL-1F5 binds to a member of the IL-1 receptor family termed single immunoglobulin IL-1 receptor-related molecule (SIGIRR) (Costelloe *et al.*, 2008). Binding of IL-1F5 to SIGIRR induced IL-4 production in CNS resident APC; however, the role of IL-1F5 in mediating anti-inflammatory effects in peripheral APC has not been established. The IL-1R family is characterized by the presence of three extracellular immunoglobulin domains and an intracellular Toll-IL-1R (TIR) domain. The IL-1R family alters gene expression via activation of NFκB and activating protein 1 (AP-1). However, SIGIRR is structurally and functionally different from the IL-1R and Toll superfamily with only one Ig domain in its extracellular portion (Thomassen *et al.*, 1998). SIGIRR does not activate NFκB and AP-1 and instead negatively modulates immune responses (Wald *et al.*, 2003). Overexpression of SIGIRR substantially reduces IL-1 and IL-18 mediated activation of NFκB (Wald *et al.*, 2003), thus SIGIRR has an inhibitory role in IL-1 signalling. SIGIRR$^{-/-}$ mice display a more potent inflammatory response (Wald *et al.*, 2003). A role for SIGIRR in EAE has not been elucidated.

There are conflicting reports in the literature on the role of IL-4 in EAE. IL-4 release from Th2 cells promotes the induction of Th2 cells from naïve T cells (Ohshima *et al.*, 1998) and is reported to mediate the protective effects of Th2

cells observed in studies on EAE (Falcone & Bloom, 1997). In the CNS, IL-4 can induce microglial activation and proliferation and decrease in IFN-γ-stimulated MHC class II expression on microglia (Suzumura *et al.*, 1994). There are conflicting results from studies investigating an endogenous role for IL-4 in EAE due to the use of different mice strains. Falcone and colleagues (1998) found that IL-4 deficiency in C57BL/6 mice resulted in more severe clinical symptoms but recovery occurred similiarly to wild type controls. However, on a Balb/c background, IL-$4^{-/-}$ mice had only a slightly more severe clinical episode and went into the recovery phase at the same timepoint as wild type controls (Falcone *et al.*, 1998). Analysis of cytokine levels suggested that IL-4 deficiency has a much greater effect on a C57BL/6 background compared with a Balb/c background. Coversely, Bettelli and colleagues (1998) demonstrated that deletion or overexpression of the IL-4 gene in C57BL/6 mice did not alter susceptibility to disease. In addition, no significant differences in the frequency, duration and severity of EAE were detected in IL-$4^{+/+}$, IL-$4^{+/-}$, IL-$4^{-/-}$ mice on the PL/J background (Liblau *et al.*, 1997). The effect of IL-4 deficiency on disease susceptibility in mice appears to be dependent on the strain of mice making it a difficult task to define an endogenous role for IL-4 in EAE.

It has been reported that *in vivo* administration of exogenous IL-4 can ameliorate EAE. Treatment of mice with atorvastatin prevented chronic and relapsing EAE by promoting Th2 cells and IL-4 production (Youssef *et al.*, 2002). Furthermore, intrasplenic electrotransfer of IL-4 encoding plasmid DNA was effective in preventing EAE (Ho *et al.*, 2006a). Peroxisome proliferator-activated receptors-γ (PPARs), are key regulators of lipid and glucose metabolism (Kliewer *et al.*, 1999). Agonists of the isoform PPAR-γ can modulate brain inflammation

and are protective in EAE (Niino *et al.*, 2001; Diab *et al.*, 2002; Feinstein *et al.*, 2002; Natarajan & Bright, 2002). Rosiglitazone, a synthetic agonist of PPAR-γ, induced the production of IL-4 in the brain and attenuated production of IL-1β and NO by microglia (Loane *et al.*, 2007). Therefore, it is of interest to investigate if rosiglitazone is neuroprotective during EAE.

In the brains of healthy aged animals, constitutive levels of IL-4 are decreased, proinflammatory cytokine production is increased (Nolan *et al.*, 2005) and microglia exhibit a more activated phenotype and increased expression of MHC class II (Godbout *et al.*, 2005). Therefore, induction of EAE in aged animals may result in more severe clinical disease compared to young animals.

Thus the aims of this chapter were to:

- compare the ability of DC, macrophages and glia to stimulate IFN-γ and IL-17 production from $CD4^+$ T cells.
- compare the ability of DC, macrophages and glia to induce T cell proliferation.
- assess the differential effects of IL-1F5 on DC, macrophages and glia.
- investigate if there is an endogenous role for SIGIRR in EAE.
- determine if there is an endogenous role for IL-4 in EAE.
- assess the effect of ageing on susceptibility to EAE.
- determine if rosiglitazone treatment is protective in EAE.

5.2. Results

5.2.1. Comparison of the ability of DC, macrophages and glia to induce IFN-γ and IL-17 production and T cell proliferation by $CD4^+$ T cells.

Microglia are the principal APC in the brain, however they differ from peripheral APC in their ability to present antigen in a number of important ways. In contrast to macrophages and DC, microglia do not express detectable MHC class I and II in their quiescent state (Perry *et al.*, 1993; Kreutzberg, 1996). It has been proposed that the relatively low expression of CD45 on microglial cells, compared with macrophages and DC, may account for their comparative deficits in antigen presenting cell capacity, since in normal and pathological CNS tissue CD45 expression has been correlated with the ability to present antigen to naïve T cells (Carson *et al.*, 1998). Thus, APC activity of microglia may depend not only on expression of MHC class II and costimulatory molecules, but also on sufficient CD45 expression (Carson & Sutcliffe, 1999). CNS-infiltrating macrophages and DC are known to be important APC during EAE. Hickey and Kimura (1988) showed that MHC class II expression on invading peripheral immune cells was sufficient for the initiation of EAE; MHC class II expression on parenchymal microglial cells was not required. The present study compared the antigen presenting capabilities of bone marrow-derived DC, peritoneal macrophages and glial cells through their ability to stimulate T cell cytokine production and to induce T cell proliferation.

$CD4^+$ T cells were purified from the spleen and inguinal lymph nodes of mice that were immunised with MOG_{35-55} in CFA and PT. $CD4^+$ T cells ($2x10^5$ cells/ml) were cultured with ascending ratios of DC, macrophages or glia in the

presence of MOG_{35-55} ($2x10^5$ $CD4^+$ T cells/ml: $3.2x10^3$, $1.6x10^4$, $8x10^4$, $4x10^5$ and $2x10^6$ APC/ml). After 5 days, MOG-specific IFN-γ production was quantified in cell supernatants by ELISA. DC and macrophages induced higher levels of IFN-γ production from MOG-specific $CD4^+$ T cells than glia, however, greater numbers of DC and Mφ were required for maximal IFN-γ induction. DC-induced IFN-γ production was greatest at the ratios $2x10^5$ T cell: $8x10^4$ DC and $4x10^5$ DC (**$p<0.01$; Figure 5.1). Macrophage-induced IFN-γ production was highest at the ratio $2x10^5$ T cell: $2x10^6$ macrophages (*$p<0.05$; Figure 5.1) while glial induced IFN-γ production was greatest at the lowest ratio of T cells: glia ($2x10^5$ T cell: $3.2x10^3$ glia) and declined as the ratios increased (***$p<0.001$; Figure 5.1).

The ability of glia to induce MOG-specific IL-17 release from $CD4^+$ T cells was less than that of DC and macrophages, with glia stimulating 2.5 times less IL-17 production from $CD4^+$ T cells. DC induced MOG-specific IL-17 production from $CD4^+$ T cells was significantly increased at T cell: DC ratios of $2x10^5$ T cell: $8x10^4$ DC, $4x10^5$ DC and $2x10^6$ DC (***$p<0.001$; Figure 5.2). IL-17 production from $CD4^+$ T cells increased with increasing ratios of macrophages with the greatest IL-17 production observed at the T cell: macrophages of $2x10^5$ T cell: $2x10^6$ macrophages (***$p<0.001$; Figure 5.2). Glial-induced IL-17 production from $CD4^+$ T cells was highest at the lowest T cell: glia ratios, $2x10^5$ T cell: $3.2x10^3$ glia and $1.6x10^4$ glia (***$p<0.001$; Figure 5.2).

After 5 days in culture, supernatants were removed, cells were pulsed with [^{3}H]-thymidine for 18 h and counts per minute were assessed using a liquid scintillation counter. DC induced the highest levels of $CD4^+$ T cell proliferation, with glial stimulated proliferation being half that of DC. Macrophages induced the

least T cell proliferation; the highest macrophage-induced T cell proliferation was 3 times less than that induced by glia. DC induced T cell proliferation was greatest at the highest ratios of T cells: DC (***$p<0.001$; Figure 5.3). Macrophages induced the greatest T cell proliferation when at a ratio of $2x10^5$ T cell: $2x10^6$ macrophages (*$p<0.05$; Figure 5.3). Interestingly, glial-induced IFN-γ and IL-17 production was highest at the lowest ratio of T cells: glia, however glia induced the greatest T cell proliferation at the higher ratios of $2x10^5$ T cell: $8x10^4$ glia and $4x10^5$ glia (***$p<0.001$; Figure 5.3).

5.2.2. IL-1F5 attenuates LPS-induced IL-1β production in mixed glial cultures.

IL-1F5, a novel member of the IL-1 family of ligands, has a high degree of sequence similarity to IL-1ra so it is thought that this protein may antagonise the function of IL-1β (Barton *et al.*, 2000). Therefore, it was hypothesised that IL-1F5 could abrogate the inflammatory effects of LPS. To test this possibility, mixed glial cultures prepared from 1 day old C57BL/6 neonatal mice were pre-treated with IL-1F5 (1 μg/ml) for 2 h followed by 24 h incubation with LPS (100 ng/ml), supernatant was removed and IL-1β concentration was determined by ELISA. LPS significantly increased the concentration in IL-1β in supernatant from mixed glial cells (***$p<0.001$; Figure 5.4). IL-1F5 significantly attenuated the LPS-induced IL-1β production by mixed glial ($^{+++}p<0.001$; Figure 5.4).

5.2.3. IL-1F5 fails to modulate LPS-induced IL-1β in mixed glial culture prepared from IL-4$^{-/-}$ mice.

IL-1F5 has been shown to induce IL-4 production from mixed glia (Costello et al., 2008) and this is a possible mechanism through which IL-1F5 may exert its anti-inflammatory effects. LPS (100 ng/ml) induced significant IL-1β production in cells prepared from C57BL/6 and IL-4$^{-/-}$ neonatal mice (**$p<0.01$, ***$p<0.001$; Figure 5.5A and B). However, the LPS-induced increase in IL-1β production was more profound in cells prepared from C57BL/6 mice compared with IL-4$^{-/-}$ mice. IL-1F5 (1 μg/ml) significantly attenuated the LPS-induced IL-1β production from glia from wild type mice ($^{++}p<0.01$; Figure 5.5A), but failed to suppress LPS-induced IL-1β in glia prepared from IL-4$^{-/-}$ mice. The data suggest that the anti-inflammatory actions of IL-1F5 are mediated by its ability to increase IL-4 expression.

5.2.4. IL-1F5 does not modulate the LPS-induced IL-1β production by DC or macrophages.

The data in Figures 5.1, 5.2 and 5.3 show that although DC, macrophages and glia act as efficient APC, they differ in their ability to induce IFN-γ and IL-17 production from CD4^{+} T cells and to stimulate T cell proliferation. IL-1F5 can modulate LPS-induced IL-1β production from CNS-resident glia; therefore the anti-inflammatory properties of IL-1F5 were assessed in DC and macrophages, which function as peripheral APC. Treatment with LPS induced significant IL-1β production from DC (***$p<0.001$; Figure 5.6) and macrophages (***$p<0.001$; Figure 5.7). In contrast, to the immunomodulatory effects of IL-1F5 on brain-

derived glial cells, IL-1F5 failed to suppress LPS-induced IL-1β production in DC or macrophages.

5.2.5. IL-1F5 failed to induce the production of IL-4 in DC and macrophages.

The finding that IL-1F5 failed to exert anti-inflammatory effects in cells prepared from IL-4$^{-/-}$ suggests that the immunomodulatory effects of IL-1F5 in the brain may be mediated by induction of IL-4. For this reason, the ability of IL-1F5 to induce IL-4 production from peripheral cells was assessed. Pretreatment of DC and macrophages with IL-1F5 for 2 h, followed by LPS treatment, failed to induce IL-4 production. However both DC and macrophages are capable of producing IL-4 as is shown by increased IL-4 release upon stimulation with the phorbolester, PMA (10 ng/ml), and the ionophore, ionomycin (1 μg/ml) (***$p<0.001$; Figure 5.8A and B).

5.2.6. SIGIRR$^{-/-}$ mice have reduced IL-4 production but similar disease severity to wild type mice following induction of EAE.

SIGIRR is an orphan receptor of the IL-1 family that negatively modulates the immune response (Wald et al., 2003) and it has previously been shown in the laboratory that SIGIRR may be a receptor for the anti-inflammatory cytokine IL-1F5 (Costelloe et al., 2008). SIGIRR$^{-/-}$ mice exhibit enhanced inflammation as a result of IL-1β challenge (Wald et al., 2003), therefore, it might be predicted that SIGIRR$^{-/-}$ mice may display an exaggerated response to immunisation with MOG_{35-55}. EAE was induced in C57BL/6 mice and SIGIRR$^{-/-}$ mice as described in method 2.2.11. It is evident from the data that there is no difference in the time of onset of clinical symptoms between SIGIRR$^{-/-}$ mice and C57BL/6 mice and

disease progression was unaltered in the SIGIRR$^{-/-}$ mice suggesting no endogenous role for SIGIRR in EAE (Figure 5.9).

Spleens were removed from C57BL/6 mice and SIGIRR$^{-/-}$ mice on day 26 of disease and cultured for 5 days with MOG_{35-55} (10 μg/ml or 100 μg/ml) or with PMA (10 ng/ml) and ionomycin (1 μg/ml). Supernatant was removed and IL-17, IFN-γ, IL-10 and IL-4 production was assessed by ELISA. IL-17 production, indicative of a Th17 response, was significantly higher from MOG_{35-55} treated cells from C57BL/6 and SIGIRR$^{-/-}$ mice with EAE compared to C57BL/6 and SIGIRR$^{-/-}$ control mice respectively (*$p<0.05$; **$p<0.01$; Figure 5.10).

In contrast, IFN-γ release, indicative of a Th1 response, was not significantly different from C57BL/6 controls or mice with EAE or from SIGIRR$^{-/-}$ controls or mice with EAE (Figure 5.10).

There was no difference in MOG-specific IL-10 production (Figure 5.10) or IL-4 production (Figure 5.10), from cells isolated from C57BL/6 or SIGIRR$^{-/-}$ control mice or C57BL/6 or SIGIRR$^{-/-}$ mice with EAE (Figure 5.10). Interestingly, cells from control SIGIRR$^{-/-}$ mice had higher constitutive levels of IL-10 compared with cells from C57BL/6 control mice ($p<0.05$; Figure 5.10), while cells from SIGIRR$^{-/-}$ mice with EAE produced significantly lower levels of IL-4 compared with cells from C57BL/6 mice with EAE (*$p<0.05$; Figure 5.10).

5.2.7. The role of IL-4 in development of pathogenic Th17 cells in EAE in young and aged mice.

Previous studies have shown that IL-4 is an important anti-inflammatory cytokine that may be involved in the amelioration of EAE (Furlan *et al.*, 1998; Ramirez & Mason, 2000; Youssef *et al.*, 2002; Ho *et al.*, 2006b). Treatment of mice with IL-4 delayed onset of EAE, significantly decreased clinical scores, significantly decreased perivascular and brain parenchyma inflammatory infiltrates and reduced demyelinated areas and axonal loss. These studies suggest a protective role for IL-4 in EAE, therefore, it was hypothesised that IL-$4^{-/-}$ mice would be more susceptible to EAE than wild type mice.

To test this hypothesis, EAE was induced in young C57BL/6 (12 week old) and young IL-$4^{-/-}$ (6-12 week old) mice. IL-$4^{-/-}$ mice displayed similar clinical scores as C57BL/6 mice suggesting that endogenous IL-4 does not have a role in controlling EAE (Figure 5.11A). Nevertheless, the levels of MOG-specific IL-17 produced were significantly higher from cells for young IL-$4^{-/-}$ mice compared with cells from young C57BL/6 mice (**$p<0.01$; Figure 5.12). In contrast, MOG-specific IFN-γ release was significantly lower from cells from young IL-$4^{-/-}$ mice compared with cells from young C57BL/6 mice (**$p<0.01$; ***$p<0.001$; ANOVA; Figure 5.12). The data suggest that endogenous IL-4 regulates IL-17 production but not IFN-γ production. MOG-specific IL-10 production was not significantly different in young C57BL/6 mice compared with young IL-$4^{-/-}$ mice suffering from EAE.Increased expression of proinflammatory cytokines, like IL-1β, is a feature of the aged brain along with a decrease in IL-4 concentration (Nolan et al., 2005). Thus, it was hypothesised that aged C57BL/6 mice would be more susceptible to EAE. To test the hypothesis, EAE was induced in young (12

week old) and aged C57BL/6 (10-12 month old) mice. Aged C57BL/6 mice with EAE were equally susceptible to EAE induction as young C57BL/6 mice (Figure 5.11B). MOG-specific IL-17 production was significantly greater from cells obtained from aged C57BL/6 mice compared with cells from young C57BL/6 mice (***$p<0.001$; Figure 5.13). Conversely, MOG-specific IFN-γ release was significantly lower from cells from aged C57BL/6 mice compared with cells from young C57BL/6 mice (**$p<0.01$; ***$p<0.001$; Figure 5.13). There was no significant difference in MOG-specific IL-4 or IL-10 production from cells isolated from young C57BL/6 mice or aged C57BL/6 mice with EAE (Figure 5.13). EAE was induced in aged C57BL/6 (10-12 month old) and aged IL-$4^{-/-}$ (10-12 month old) mice to examine the role of IL-4 in aged mice. It was hypothesised that aged IL-$4^{-/-}$ mice would display a more proinflammatory phenotype in the CNS compared to aged C57BL/6 mice and as a result would be highly susceptible to EAE. At the peak of the disease, aged IL-$4^{-/-}$ mice displayed more severe clinical symptoms than aged C57BL/6 mice, (*$p<0.05$; **$p<0.01$; Figure 5.11C). There was no significant difference in MOG-specific IL-17, IFN-γ or IL-10 production from cells isolated from aged C57BL/6 mice or aged IL-$4^{-/-}$ mice with EAE (Figure 5.14).

EAE was induced in young (6-12 week old) and aged IL-$4^{-/-}$ (10-12 month old) mice. Taking into to account the additional stress of ageing on the brain, accompanied by knock out of an important anti-inflammatory cytokine, it was thought that EAE induction would lead to greater disease severity in aged IL-$4^{-/-}$ mice compared to young IL-$4^{-/-}$ mice, however, young and aged IL-$4^{-/-}$ mice were equally susceptible to EAE. MOG-specific IL-17 and IL-10 production was not significantly different from cells from young IL-$4^{-/-}$ mice compared with cells

from aged IL-4$^{-/-}$ mice (Figure 5.15). In contrast, MOG-specific IFN-γ production was significantly lower by cells from aged IL-4$^{-/-}$ mice compared with cells from young IL-4$^{-/-}$ mice (***$p<0.001$; Figure 5.15).

5.2.8. Rosiglitazone does not significantly alter the disease severity of EAE in C57BL/6 mice.

Rosiglitazone, a PPAR-γ agonist, increases IL-4 mRNA and can reverse the age-related decrease in hippocampal IL-4 concentration (Loane *et al.*, 2007). Additionally, rosiglitazone failed to exert an effect in glia prepared from IL-4$^{-/-}$ mice, thereby suggesting that the anti-inflammatory actions of rosiglitazone are mediated by its ability to increase IL-4 expression (Loane *et al.*, 2007). Therefore it was hypothesised that rosiglitazone may be protective in EAE.

C57BL/6 mice were treated prophylactically and therapeutically with either a control diet or a diet containing rosiglitazone (6mg/kg/day) for 3 weeks prior to EAE induction and throughout disease. Contrary to expectations, rosiglitazone did not significantly alter disease incidence or the severity of symptoms over the course of the disease (Figure 5.16). MOG-specific IL-17 production was significantly enhanced in mice with EAE treated with rosiglitazone, similiarly MOG-specific IFN-γ production was highest from cells from mice with EAE treated with rosiglitazone (*$p<0.05$; Figure 5.17).

.5.3. Discussion

This study showed that DC, macrophages and glia are efficient APC and can induce T cell proliferation but at different T cell: APC ratios. The data show that IL-1F5 has differential effects in brain-derived and peripherally-derived APC. It is known that inflammation occurs in the CNS during EAE, therefore this study used different approaches to address the roles of anti-inflammatory proteins during EAE. SIGIRR, a negative regulator of immune responses and potential receptor for IL-1F5, was found to have no endogenous role in EAE. In addition, no endogenous role for the anti-inflammatory cytokine, IL-4, was found in EAE and rosiglitazone, a PPAR-γ agonist and promoter of IL-4 production in the brain, did not attenuate EAE. However, deletion of the IL-4 gene coupled with the inflammatory stress of ageing alters the disease course of EAE.

There are conflicting reports in the literature regarding the efficiency of APC to present antigen, stimulate cytokine production and induce proliferation of $CD4^+$ T cells. The relative contribution of DC, macrophages and glia to antigen presentation in the CNS during EAE is widely debated. The findings of the present study show that DC, macrophages and glia are competent APC, however, different numbers of APC are required for maximal production of IFN-γ and IL-17 from $CD4^+$ T cells. It has previously been reported that antigen presentation by myeloid DC to a $PLP_{139-151}$-specific $CD4^+$ T cells line induced greater IFN-γ and IL-17 production when compared to stimulation by macrophages (Bailey *et al.*, 2007). Conversely, in this study, both DC and macrophages induced equal IFN-γ and IL-17 production from $CD4^+$ T cells. Interestingly, higher numbers of glia are required for maximal stimulation of T cell proliferation than are necessary for

induction of T cell cytokine production. Glia induce greater T cell proliferation than macrophages, but less than DC. Consistent with this data, DC, isolated from the CNS during relapsing EAE (R-EAE), were shown to be the most efficient at stimulating T cell proliferation compared to macrophages and microglia (McMahon *et al.*, 2005). Moreover, a study by Deshpande and colleagues (2007) demonstrated that microglia, macrophages and DC could drive the proliferation of CNS antigen specific T cells but that DC were the most effective. The data presented in this study show that DC, macrophages and microglia are efficient APC and can induce T cell proliferation, albeit at different T cell: APC ratios.

APC also differ in their response to cytokine. IL-1F5, a member of the IL-1 family, has been shown to bind to the orphan IL-1 receptor, SIGIRR, which is expressed on glia. This leads to the production of IL-4 by glia (Costelloe *et al.*, 2008). Furthermore, IL-1F5 suppressed IL-1β and LPS-induced inflammatory responses in the brain via its ability to induce IL-4 production (Costelloe *et al.*, 2008). The data presented here show that the anti-inflammatory effects of IL-1F5 are brain specific. IL-1F5 did not induce IL-4 production in DC and macrophages and failed to attenuate LPS-induced IL-1β production from DC and macrophages. The data suggest that the anti-inflammatory actions of IL-1F5 are confined to cells derived from the brain with no anti-inflammatory effects observed in peripherally derived immune cells. This data indicates that glia, DC and macrophages, despite having similar immune functions, differ in their response to immunological stimuli.

SIGIRR is known to negatively modulate the immune response (Wald *et al.*, 2003), with SIGIRR$^{-/-}$ mice displaying a more potent inflammatory response. SIGIRR$^{-/-}$ mice have a reduced threshold to lethal endotoxin challenge, increased

cytokine production in response to TLR agonists and increased susceptibility to intestinal inflammation (Garlanda *et al.*, 2004), therefore it was hypothesised that SIGIRR$^{-/-}$ mice would be more susceptible to EAE than C57BL/6 mice. Surprisingly, deletion of the SIGIRR gene did not alter disease onset and severity of EAE despite previous reports of an inhibitory role for SIGIRR in IL-1 signalling (Costelloe *et al.*, 2008). Considering the pivotal role of IL-1 in the induction of Th17 cells (Sutton *et al.*, 2006) and in inflammation in the CNS during EAE (Loddick *et al.*, 1997), it was expected that SIGIRR$^{-/-}$ mice would have increased susceptibility to EAE. Consistent with the findings presented in this study, SIGIRR$^{-/-}$ mice have been shown to have normal susceptibility to systemic LPS toxicity (Garlanda *et al.*, 2004) implying a normal inflammatory phenotype. MOG-specific IL-4 production was reduced in SIGIRR$^{-/-}$ mice compared to C57BL/6 mice, this is consistent with the established link between the activation of SIGIRR and production of IL-4 (Costelloe *et al.*, 2008). Interestingly, basal concentrations of IL-10 were higher in SIGIRR$^{-/-}$ and C57BL/6 mice. IL-10 is a critical regulator of the immune response during EAE with IL-10$^{-/-}$ mice displaying an increased susceptibility to induction of EAE and disease severity (Bettelli *et al.*, 1998). Perhaps the elevated constitutive concentration of IL-10 in SIGIRR$^{-/-}$ mice is indicative of a compensatory immune mechanism that resulted in equal susceptibility to EAE in SIGIRR$^{-/-}$ mice and wild type mice. While there is no endogenous role for SIGIRR during EAE, this does not exclude the possibility that activation of SIGIRR and subsequent production of IL-4 may be of therapeutic value in EAE. Previous studies have shown that IL-4 is an important anti-inflammatory cytokine in the amelioration of EAE (Ramirez & Mason, 2000; Furlan *et al.*, 2001b; Youssef *et al.*, 2002; Ho *et al.*, 2006a).

Therefore, it was hypothesised that IL-4$^{-/-}$ mice would be more susceptible to EAE; however, there was no difference in the onset or severity of disease between young C57BL/6 mice and young IL-4$^{-/-}$ mice, despite increased production of MOG-specific IL-17 in IL-4$^{-/-}$ mice. Production of MOG-specific IFN-γ was decreased in IL-4$^{-/-}$ mice, this may account for the increase in IL-17 production since IFN-γ inhibits Th17 cell development (Harrington *et al.*, 2005). The data suggest that endogenous IL-4 regulates IL-17 production *in vivo,* but not IFN-γ, which contradicts the reported suppressive effects of IL-4 on Th1 differentiation (Zheng & Flavell, 1997). The finding that there is no endogenous role for IL-4 in EAE is consistent with previous studies where IL-4$^{-/-}$ mice were found to have equal susceptibility and severity of clinical symptoms to EAE as wild type mice. Furthermore, mice overexpressing IL-4 develop similar disease compared to their non transgenic littermates (Bettelli *et al.*, 1998). Furthermore, Liblau and colleagues (1997) reported that there was no significant difference in the frequency, severity and duration of actively induced EAE in IL-4$^{+/+}$, IL-4$^{+/-}$ and IL-4$^{-/-}$ mice. In contrast, the present study demonstrates that deletion of the IL-4 gene increased the severity of EAE in aged animals. The healthy aged brain displays a more inflamed phenotype, with increased activation of microglia (Griffin *et al.*, 2006) coupled with increased production of proinflammatory cytokines and a decline in anti-inflammatory cytokine production (Nolan *et al.*, 2005) (Godbout & Johnson, 2004).The absence of IL-4 production along with the added stress of ageing on the brain may contribute to the increased susceptibility of aged IL-4$^{-/-}$ mice to EAE.

There is no endogenous role for IL-4 during EAE in young animals, however, previous reports suggested that induction of IL-4 may have an

ameliorating effect on the development of EAE (Youssef *et al.*, 2002; Ho *et al.*, 2006a). Rosiglitazone, a PPAR-γ agonist, is reported to have anti-inflammatory effects in the brain mediated by induction of IL-4 (Loane *et al.*, 2007). However, rosiglitazone did not attenuate EAE. In contrast, Feinstein and colleagues (2002) demonstrated that rosiglitazone reduced disease incidence and symptoms late in disease. However, other PPAR-γ agonists have been shown to be more effective than rosiglitazone at ameliorating EAE (Feinstein *et al.*, 2002) (Niino *et al.*, 2001; Natarajan & Bright, 2002).

The present findings suggest that DC, macrophages and glia differ in their ability to respond to cytokine, induce T cell proliferation and stimulate production of T cell cytokines. The data demonstrate that there is no endogenous role for the immunoregulatory receptor, SIGIRR, in EAE. While there is no endogenous role for IL-4 in EAE in young animals and rosiglitazone, a promoter of IL-4 production in the brain, was not effective at ameliorating EAE, IL-4 may have a protective effect during EAE in aged animals.

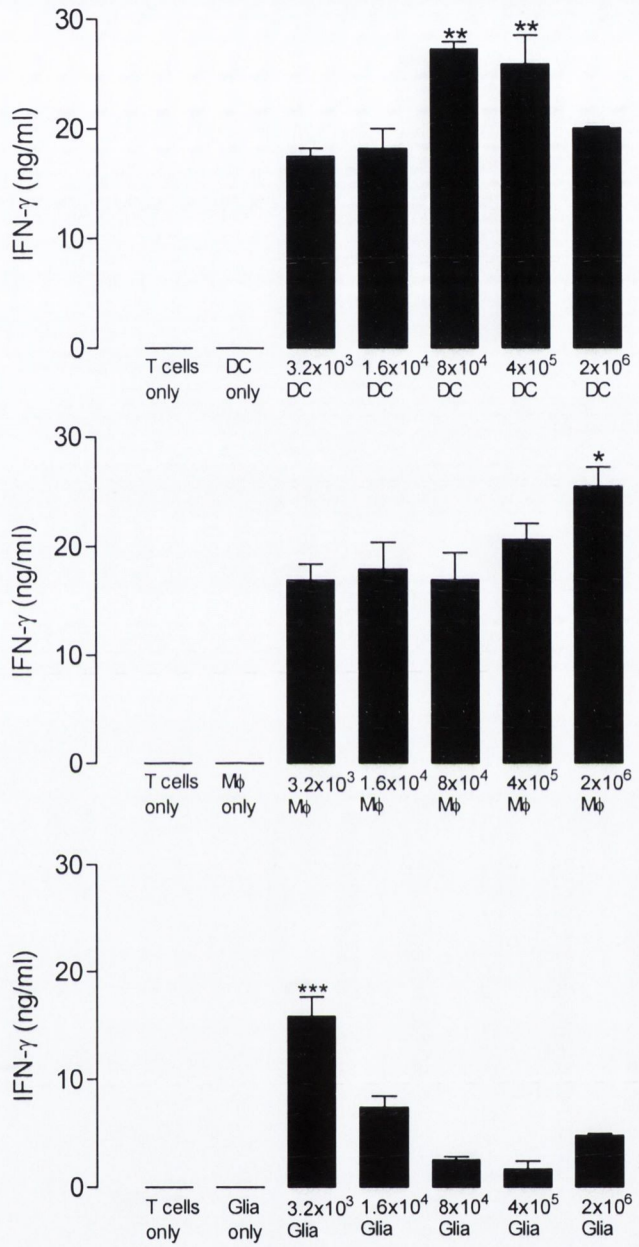

Figure 5.1. Comparison of the ability of DC, Mφ and glia to induce IFN-γ production from MOG-specific $CD4^+$ T cells. $CD4^+$ T cells were isolated from the spleen and inguinal lymph nodes of mice with EAE. $CD4^+$ T cells ($2x10^5$ cells/ml) were cultured for 5 days with ascending ratios of DC, macrophages or glia in the presence of MOG_{35-55} ($2x10^5$ T cells/ml: $3.2x10^3$, $1.6x10^4$, $8x10^4$, $4x10^5$ and $2x10^6$ APC/ml). MOG-specific IFN-γ production was assessed by ELISA. **$p<0.01$; versus $2x10^5$ T cell: $2x10^6$ DC, *$p<0.05$; versus all Mφ ratios, ***$p<0.001$; versus all glia ratios, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM.

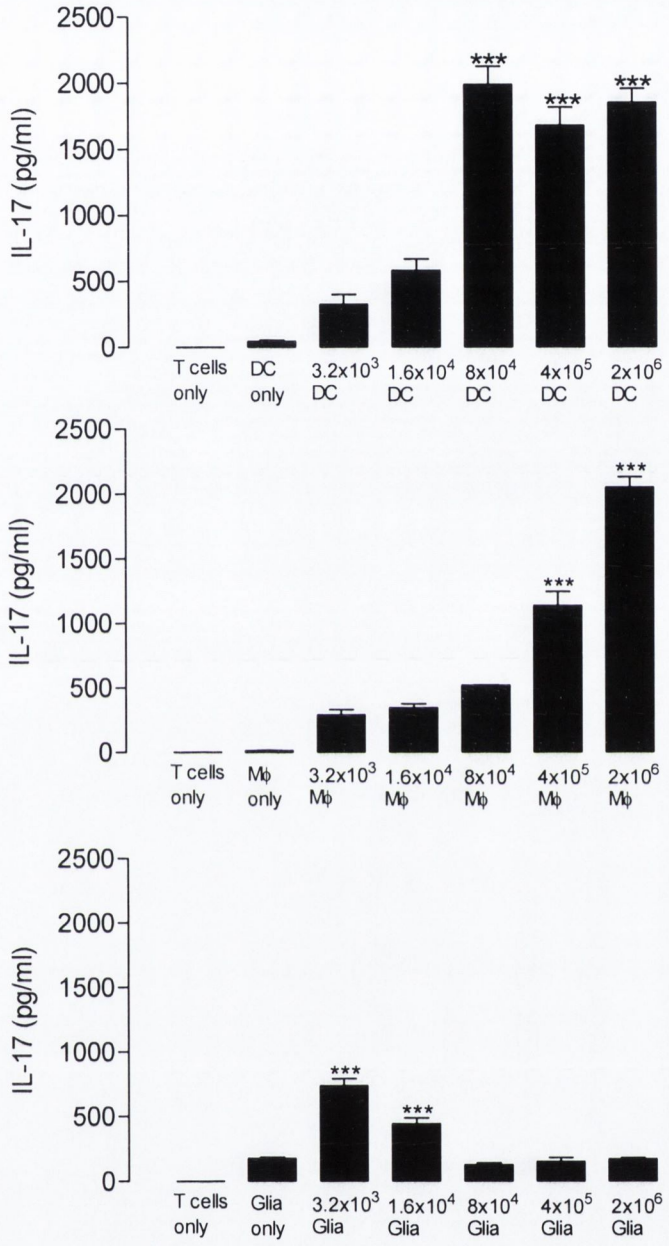

Figure 5.2. Comparison of the ability of DC, Mφ and glia to induce IL-17 production from MOG-specific CD4$^+$ T cells. CD4$^+$ T cells were isolated from the spleen and inguinal lymph nodes from mice suffering from EAE. CD4$^+$ T cells ($2x10^5$ cells/ml) were cultured for 5 days with ascending ratios of DC, macrophages or glia in the presence of MOG_{35-55} ($2x10^5$ T cells/ml: $3.2x10^3$, $1.6x10^4$, $8x10^4$, $4x10^5$ and $2x10^6$ APC/ml). MOG-specific IL-17 production was assessed by ELISA. ***$p<0.001$; versus ratios of $2x10^5$ T cell: $3.2x10^3$ and $1.6x10^4$ DC, ***$p<0.001$; versus lower Mφ ratios, ***$p<0.001$; ANOVA; versus higher glial ratios, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM.

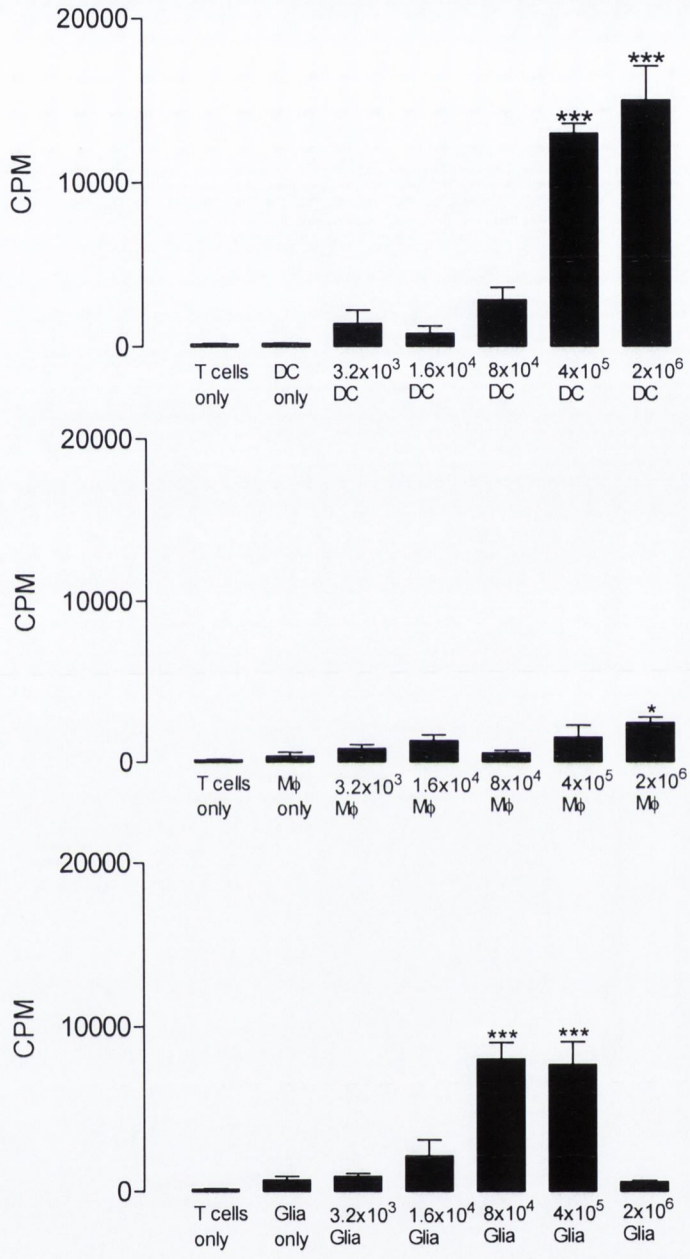

Figure 5.3. Comparison of the ability of DC, Mφand glia to induce proliferation of MOG-specific CD4$^+$ T cells. CD4$^+$ T cells were isolated from the spleen and inguinal lymph nodes from mice suffering from EAE. CD4$^+$ T cells were cultured with ascending ratios of DC, macrophages or glia in the presence of MOG_{35-55} ($2x10^5$ T cells/ml: $3.2x10^3$, $1.6x10^4$, $8x10^4$, $4x10^5$ and $2x10^6$ APC/ml). After 5 days, cells were pulsed with [^{3}H]-thymidine for 18 h and counts per minute were assessed using a liquid scintillation and luminescence counter. ***$p<0.001$; versus lower DC ratios, *$p<0.05$; versus $2x10^5$ T cell : $3.2x10^3$ Mφ, ***$p<0.001$; versus all other glial ratios, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM.

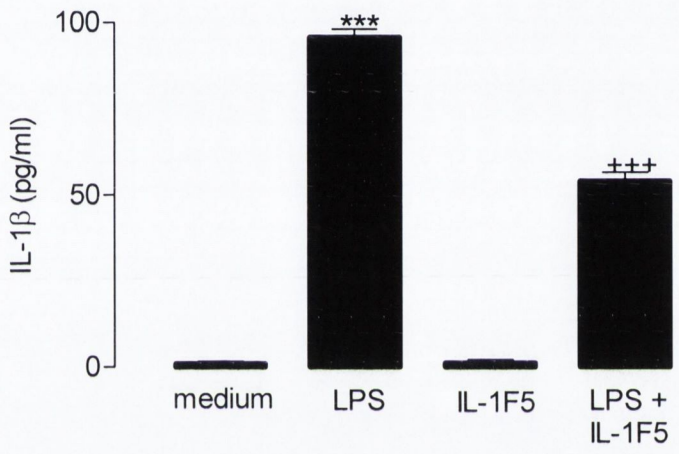

Figure 5.4. IL-1F5 attenuates LPS-induced IL-1β production in mixed glial cultures. Mixed glia were prepared from the cortices of C57BL/6 neonatal mice. After 10 days, the glial cells were pretreated with medium or IL-1F5 (1 μg/ml) for 2 h followed by treatment with medium or LPS (100 ng/ml). After 24 h, supernatant was removed IL-1β production was assessed by ELISA. ***$p<0.001$; versus medium-treated glia, $^{+++}p<0.01$; versus LPS treated glia, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=6)

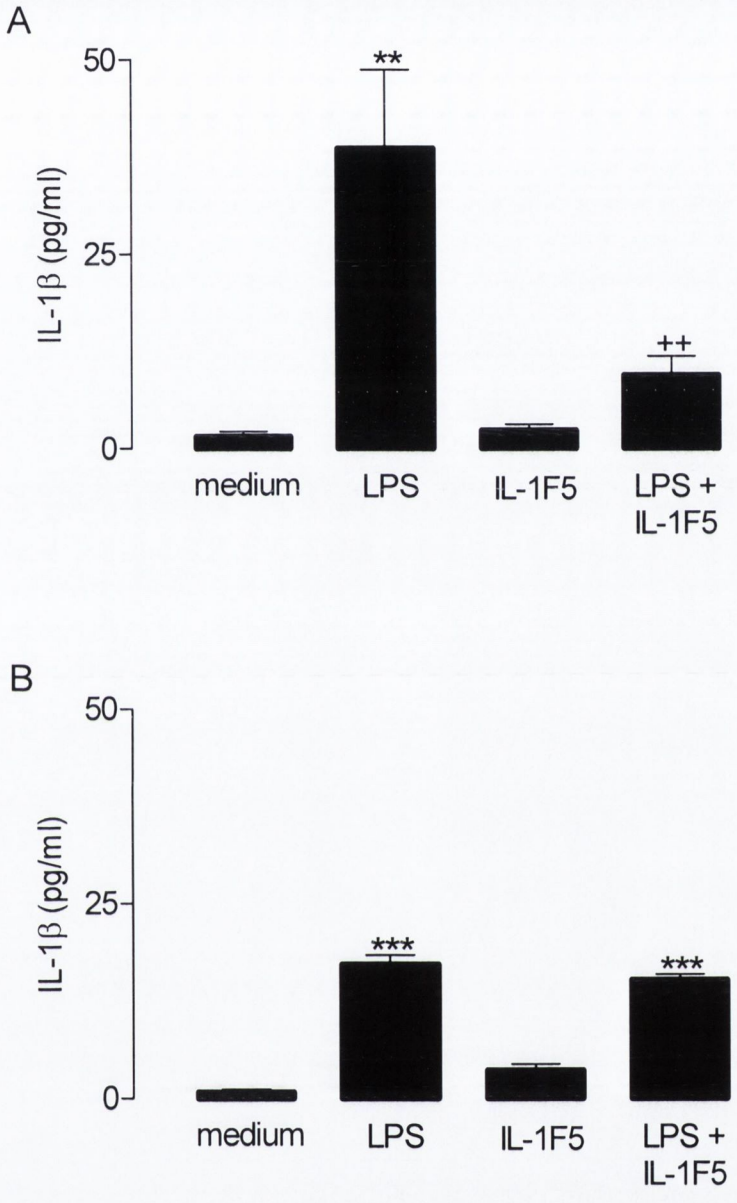

Figure 5.5. IL-1F5 fails to modulate LPS-induced IL-1β in mixed glial cultures prepared from IL-4$^{-/-}$ mice. Mixed glia were prepared from the cortices of C57BL/6 and IL-4$^{-/-}$ neonatal mice. On day 10, the glial cells were pretreated with medium or IL-1F5 (1 μg/ml) for 2 h followed by treatment with medium or LPS (100 ng/ml). After 24 h, supernatant was removed for IL-1β quantification by ELISA. (A) glia from C57BL/6 mice, (B) glia from IL-4$^{-/-}$ mice. ***$p<0.001$; **$p<0.01$; versus medium-treated glia, $^{++}p<0.01$; versus LPS treated glia, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=6)

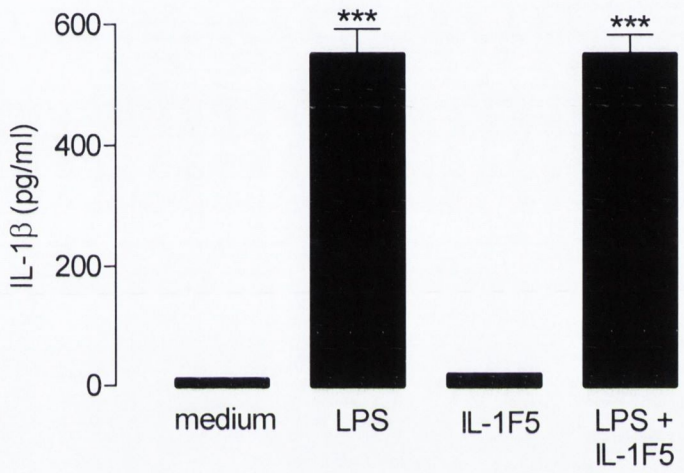

Figure 5.6. IL-1F5 does not reverse the LPS-induced increase in IL-1β production in bone marrow-derived DC. Bone marrow-derived DC were prepared from C57BL/6 mice and pretreated with medium or IL-1F5 (1 μg/ml) for 2 h followed by treatment with medium or LPS (100 ng/ml). Supernatant was removed after 24 h and IL-1β concentration was assessed by ELISA. ***$p<0.001$; versus medium-treated DC, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 4 independent experiments.

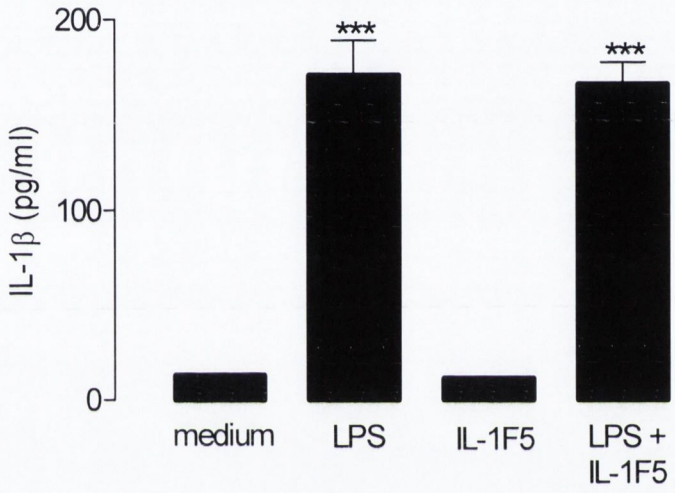

Figure 5.7. IL-1F5 does not attenuate the LPS-induced IL-1β production by peritoneal macrophages. Macrophages were isolated from the peritoneal cavity of C57BL/6 mice and pretreated with medium or IL-1F5 (1 μg/ml) for 2 h followed by treatment with medium or LPS (100 ng/ml). After 24 h, supernatant was removed and IL-1β production was assessed by ELISA. ***$p<0.001$; versus medium-treated macrophages, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 4 independent experiments.

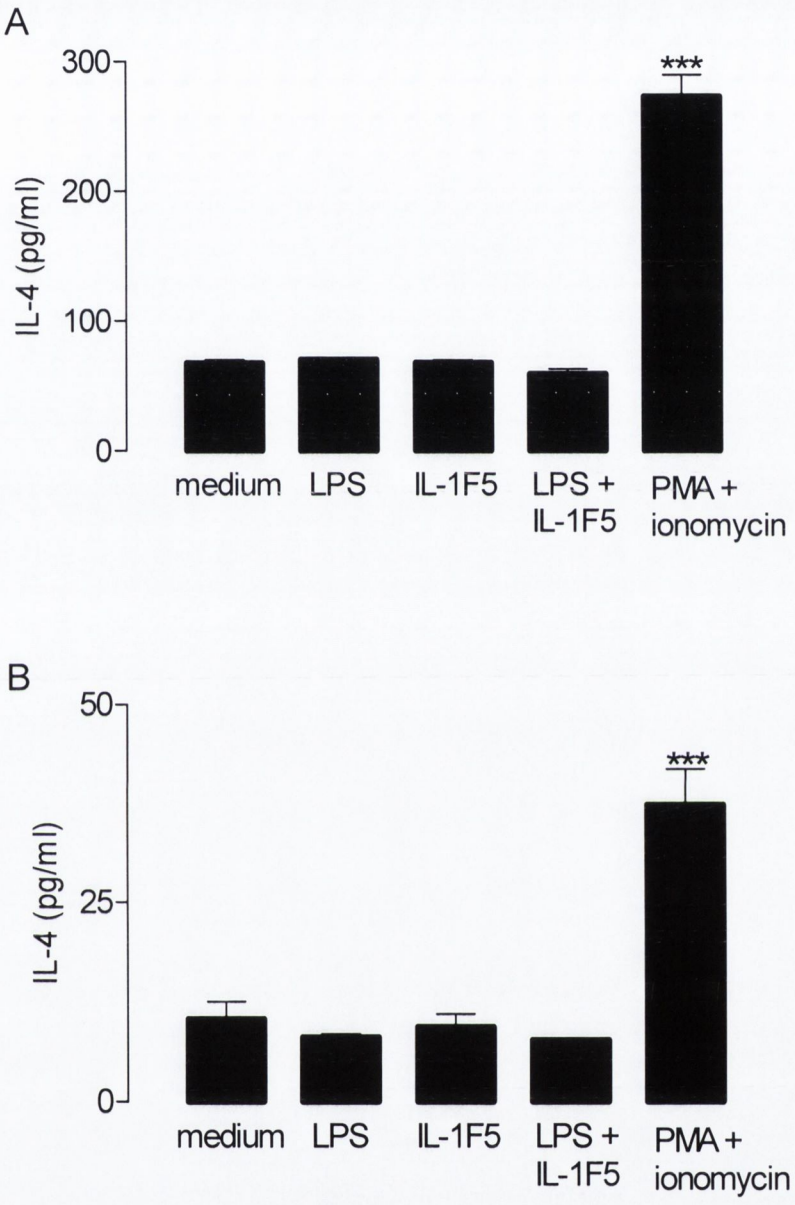

Figure 5.8. IL-1F5 failed to induce IL-4 production in bone marrow-derived DC and peritoneal macrophages. Bone marrow-derived DC and peritoneal macrophages were pretreated with medium or IL-1F5 (1 μg/ml) for 2 h followed by treatment with medium or LPS (100 ng/ml). Cells were also treated with PMA and ionomycin as a positive control. After 24 h, supernatant was removed and IL-4 production was assessed by ELISA. (A) DC, (B) macrophages. ***$p<0.001$; versus IL-1F5 treated cells, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. Representative of 4 independent experiments.

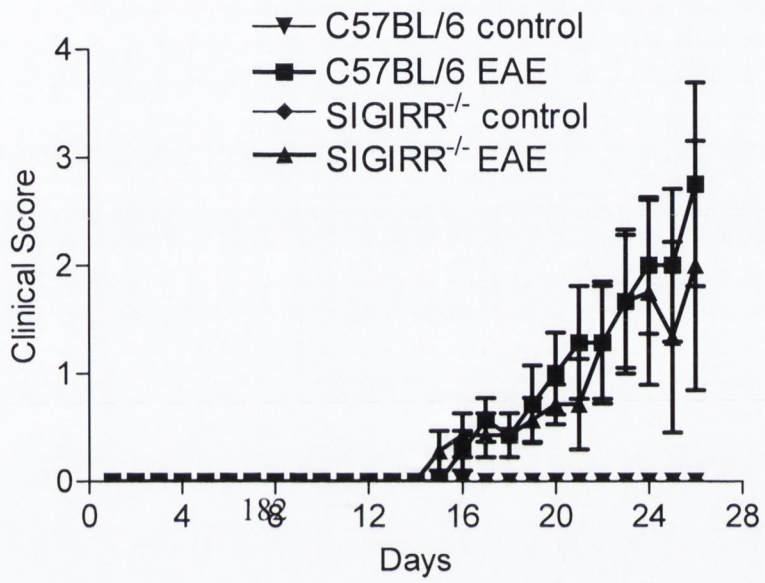

Figure 5.9. Onset of EAE and disease severity is not significantly altered in SIGIRR$^{-/-}$ mice compared with C57BL/6 mice. EAE was induced in C57BL/6 mice and SIGIRR$^{-/-}$ mice. Clinical scores were assessed daily and disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death. Mice were sacrificed on day 26 and spleens were removed for analysis of T cell phenotype. (n=7)

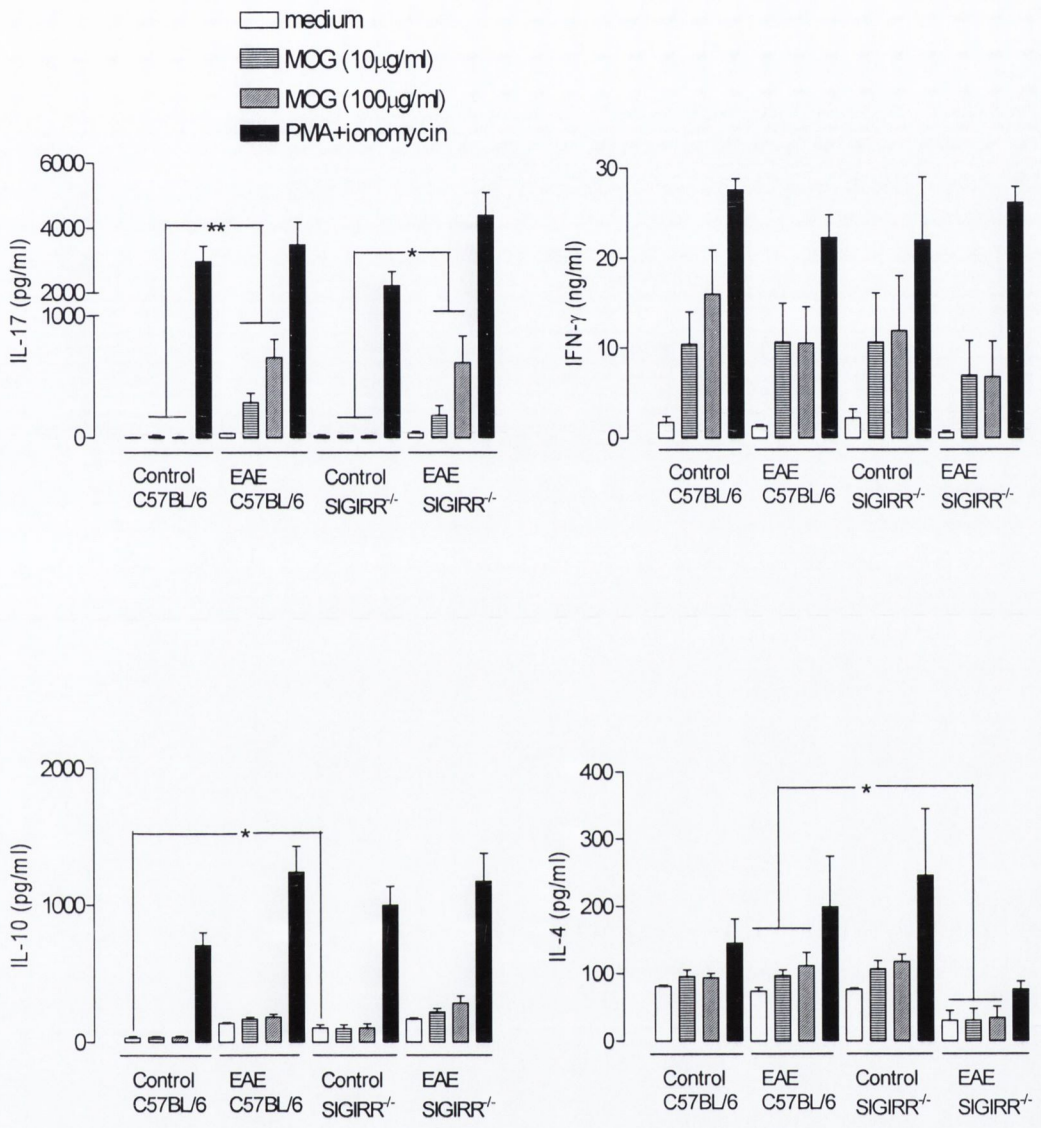

Figure 5.10. MOG-specific IL-17, IFN-γ, IL-10 and IL-4 production from spleen cells from C57BL/6 and SIGIRR$^{-/-}$ control mice and mice with EAE. Spleens were removed from C57BL/6 and SIGIRR$^{-/-}$ control mice and mice with EAE on day 26 and cultured for 5 days with MOG_{35-55} (10 μg/ml or 100 μg/ml) or with PMA (10 ng/ml) and ionomycin (1μg/ml). IL-17, IFN-γ, IL-10 and IL-4 concentration was assessed by ELISA. *$p<0.05$; **$p<0.01$, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=7).

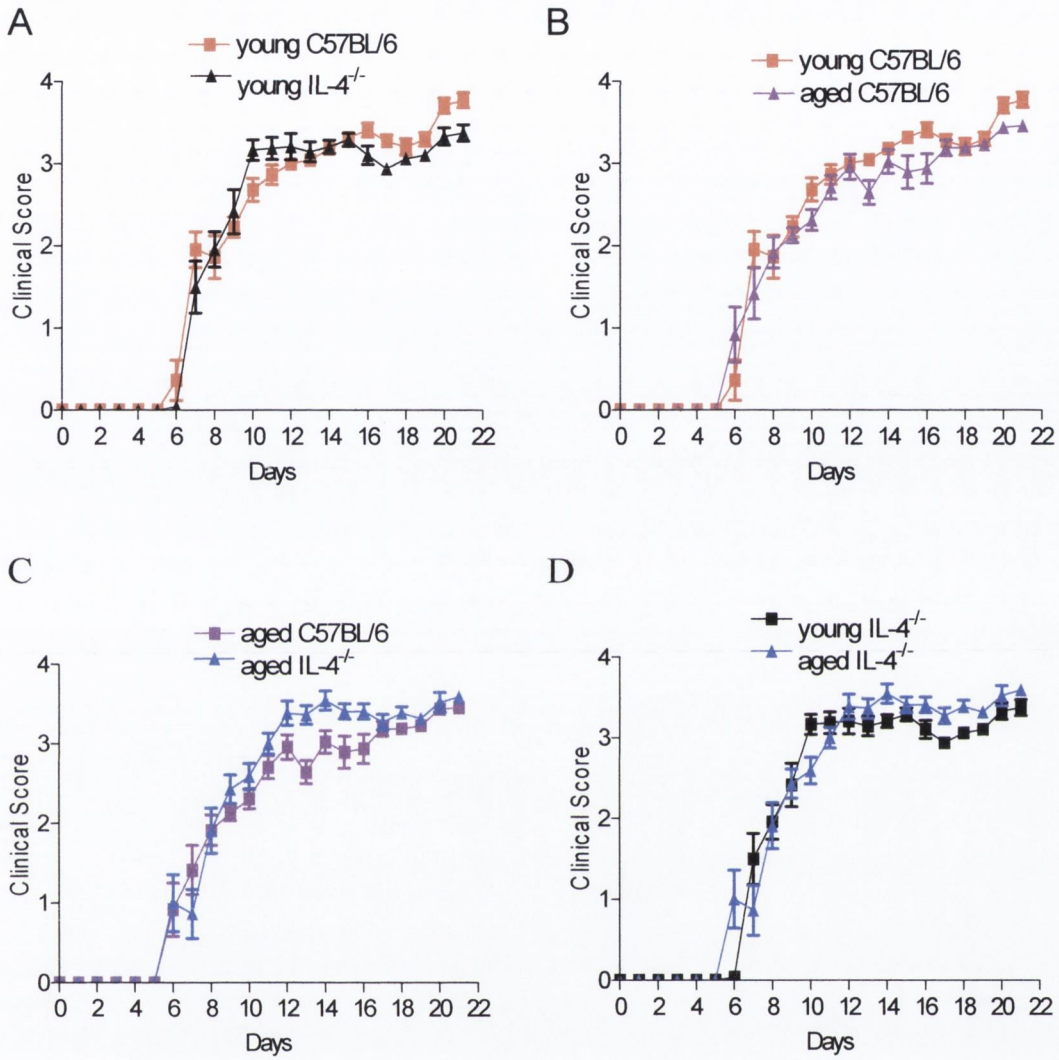

Figure 5.11. The role of IL-4 and ageing on MOG-induced EAE. EAE was induced in young or aged C57BL/6 and young or aged IL-4$^{-/-}$ mice by s.c. injection of MOG_{35-55} (150μg) emulsified in Complete Freund's Adjuvant supplemented with H37 Ra *Mycobacterium tuberculosis* (4mg/ml) on day 0 and i.p. injection of pertussis toxin on day 0 and day 2. Animals were monitored daily for signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death. (A) young C57BL/6 mice versus young IL-4$^{-/-}$ mice (B) young C57BL/6 mice versus aged C57BL/6 mice, (C) aged C57BL/6 mice versus aged IL-4$^{-/-}$ mice, *$p<0.05$, by two-way ANOVA, (D) young IL-4$^{-/-}$ mice versus aged IL-4$^{-/-}$ mice. (n=12 per group)

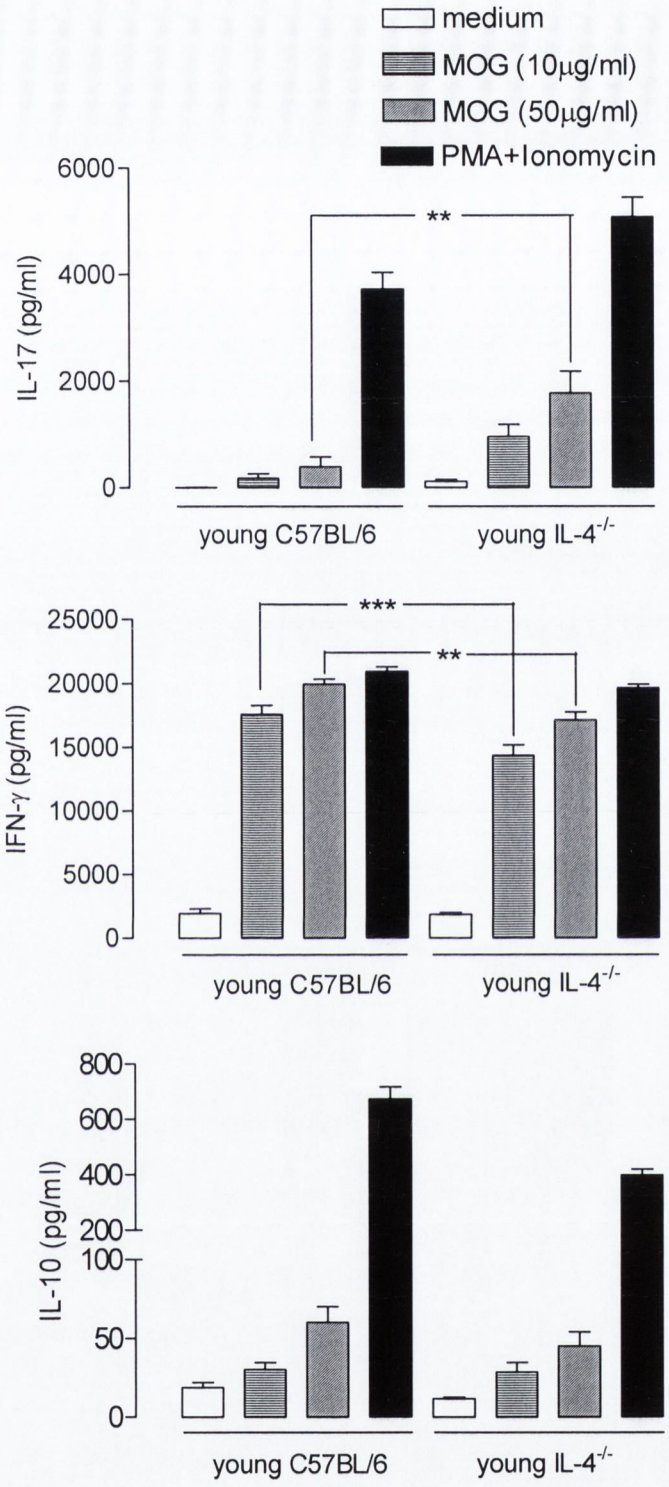

Figure 5.12. MOG-specific IL-17, IFN-γ and IL-10 production from spleen cells from young C57BL/6 mice and young IL-4$^{-/-}$ mice with EAE. Spleens were removed on day 22 from young C57BL/6 mice and IL-4$^{-/-}$ mice with EAE and cultured for 5 days with MOG_{35-55} (10 μg/ml or 50 μg/ml) or with PMA (10 ng/ml) and ionomycin (1 μg/ml). IL-17, IFN-γ and IL-10 release was assessed by ELISA. **p<0.01; ***p<0.001, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=12).

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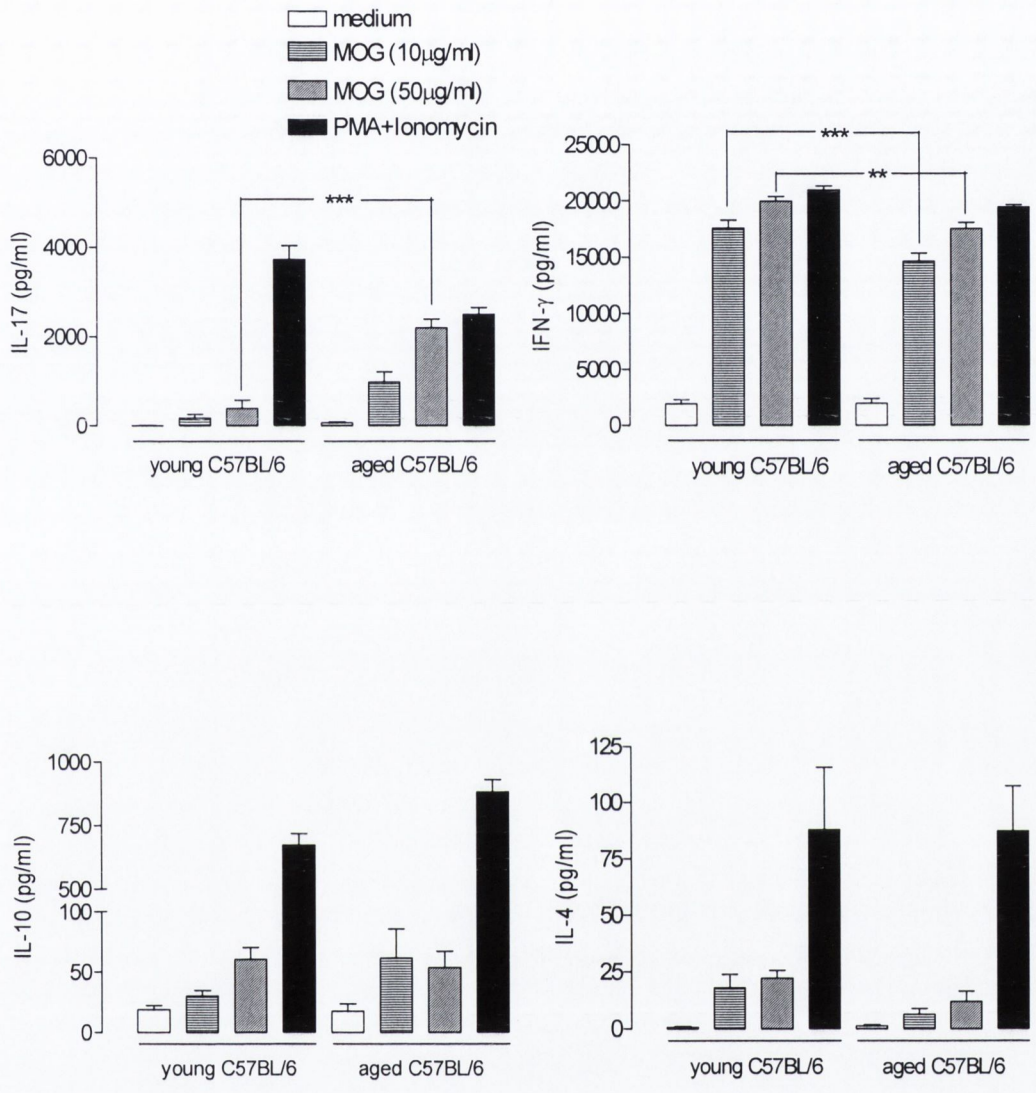

Figure 5.13. MOG-specific IL-17, IFN-γ, IL-10 and IL-4 production from spleen cells from young C57BL/6 mice and aged C57BL/6 mice with EAE. Spleens were removed on day 22 from young and aged C57BL/6 mice with EAE and cultured for 5 days with MOG_{35-55} (10 μg/ml or 50 μg/ml) or with PMA (10 ng/ml) and ionomycin (1μg/ml). IL-17, IFN-γ, IL-10 and IL-4 release was assessed by ELISA. **p<0.01; ***p<0.001, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=12).

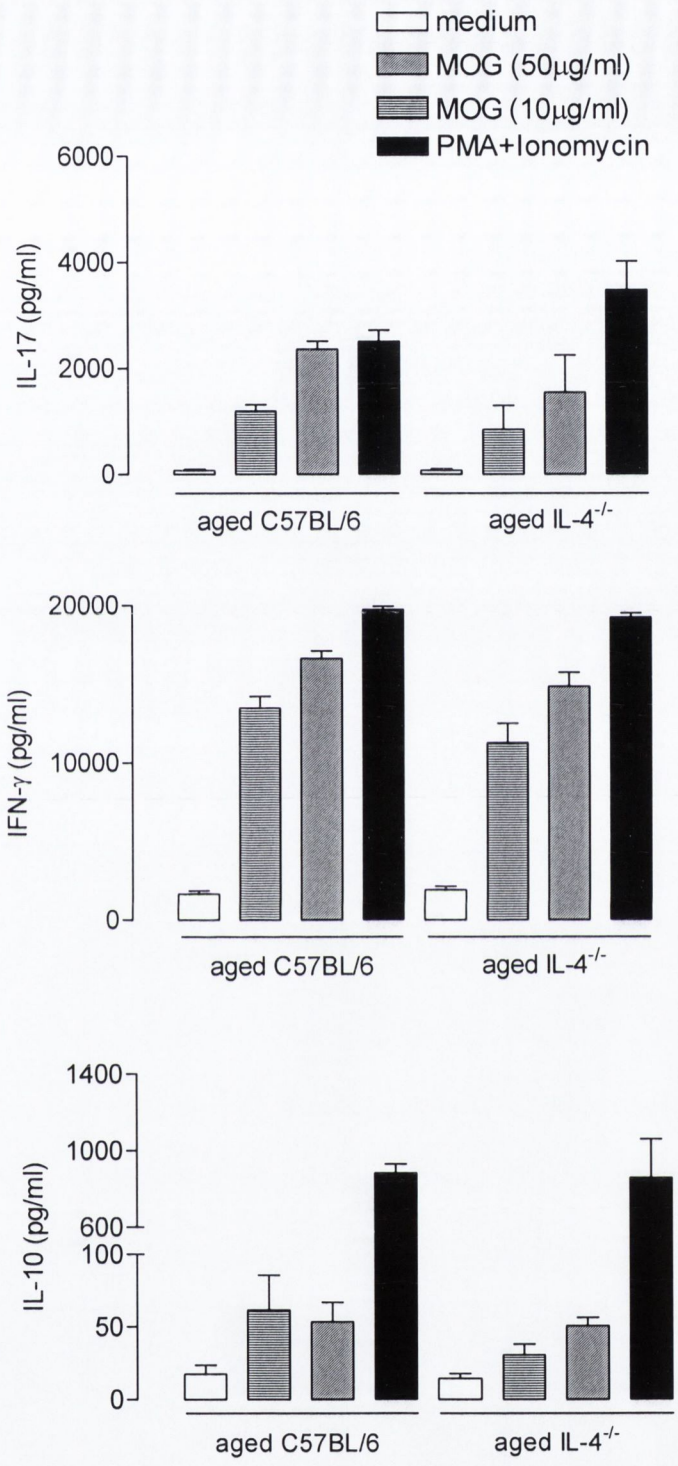

Figure 5.14. MOG-specific IL-17, IFN-γ and IL-10 production from spleen cells from aged C57BL/6 mice and aged IL-4$^{-/-}$ mice with EAE. Spleens were removed on day 22 from aged C57BL/6 and aged IL-4$^{-/-}$ mice with EAE and cultured for 5 days with MOG_{35-55} (10 μg/ml or 50 μg/ml) or with PMA (10 ng/ml) and ionomycin (1μg/ml). IL-17, IFN-γ and IL-10 production was assessed by ELISA.

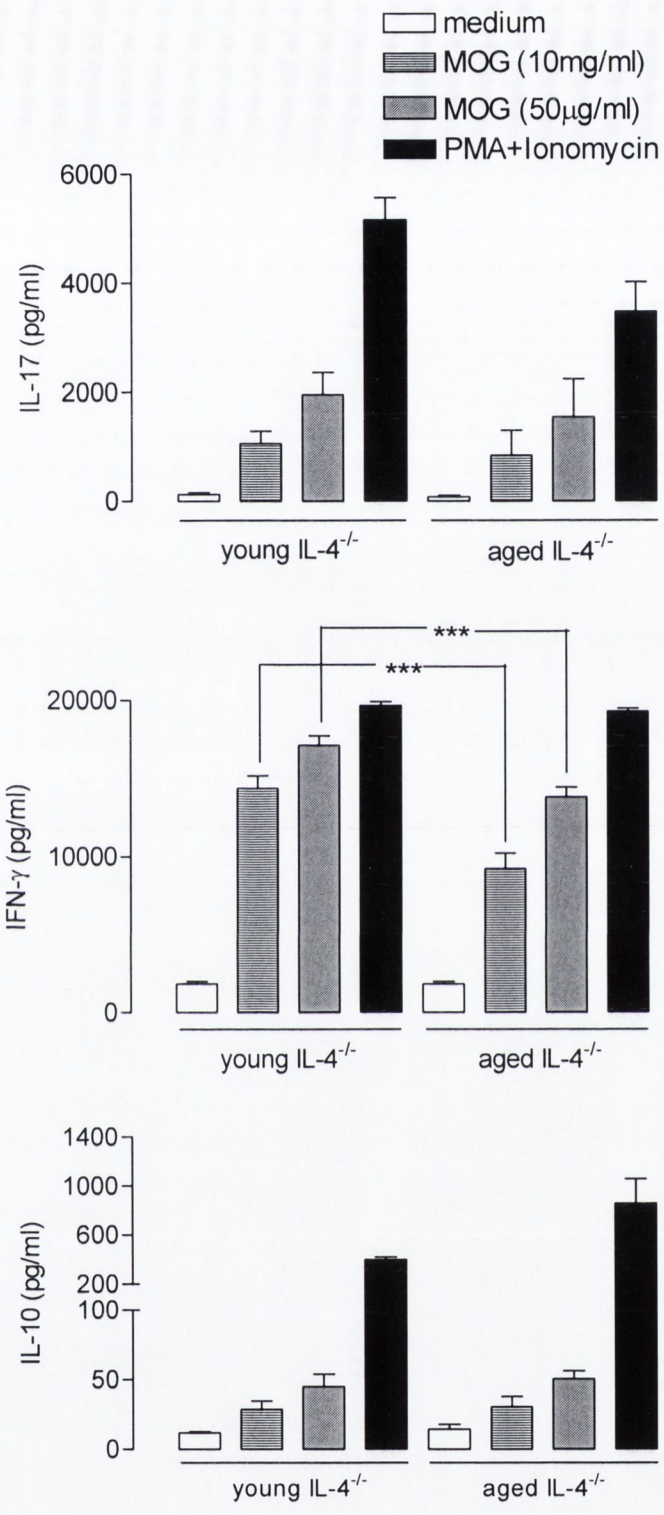

Figure 5.15. MOG-specific IL-17, IFN-γ and IL-10 production from spleen cells from young IL-4$^{-/-}$ mice and aged IL-4$^{-/-}$ mice with EAE. Spleens were removed on day 22 from young IL-4$^{-/-}$ and aged IL-4$^{-/-}$ mice suffering from EAE and cultured for 5 days with MOG_{35-55} (10 µg/ml or 50µg/ml) or with PMA (10 ng/ml) and ionomycin (1µg/ml). IL-17, IFN-γ and IL-10 release was assessed by ELISA. ***$p<0.001$, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=12).

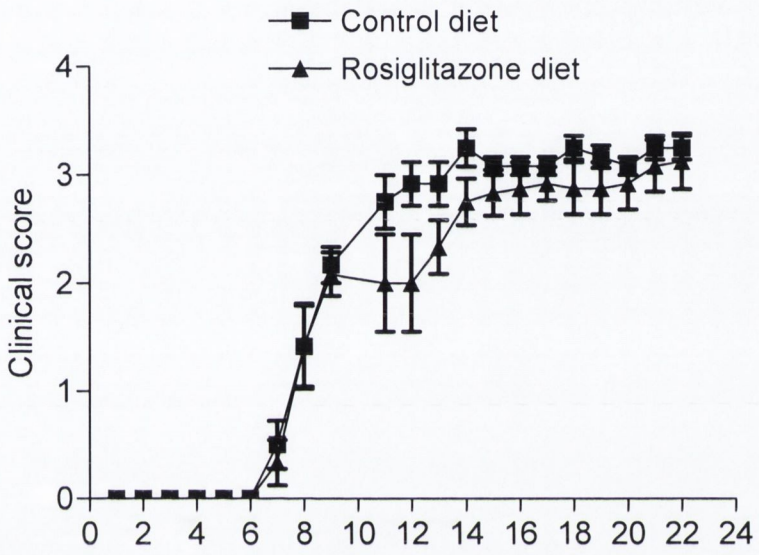

Figure 5.16. Effect of rosiglitazone on progression of EAE in C57BL/6 mice. C57BL/6 mice were treated prophylactically and therapeutically with a control diet or with Rosiglitazone (6mg/kg/day) for 3 weeks prior to induction of EAE and throughout disease. EAE was induced as described in method 2.2.11. Mice were monitored daily for signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death. (n=8)

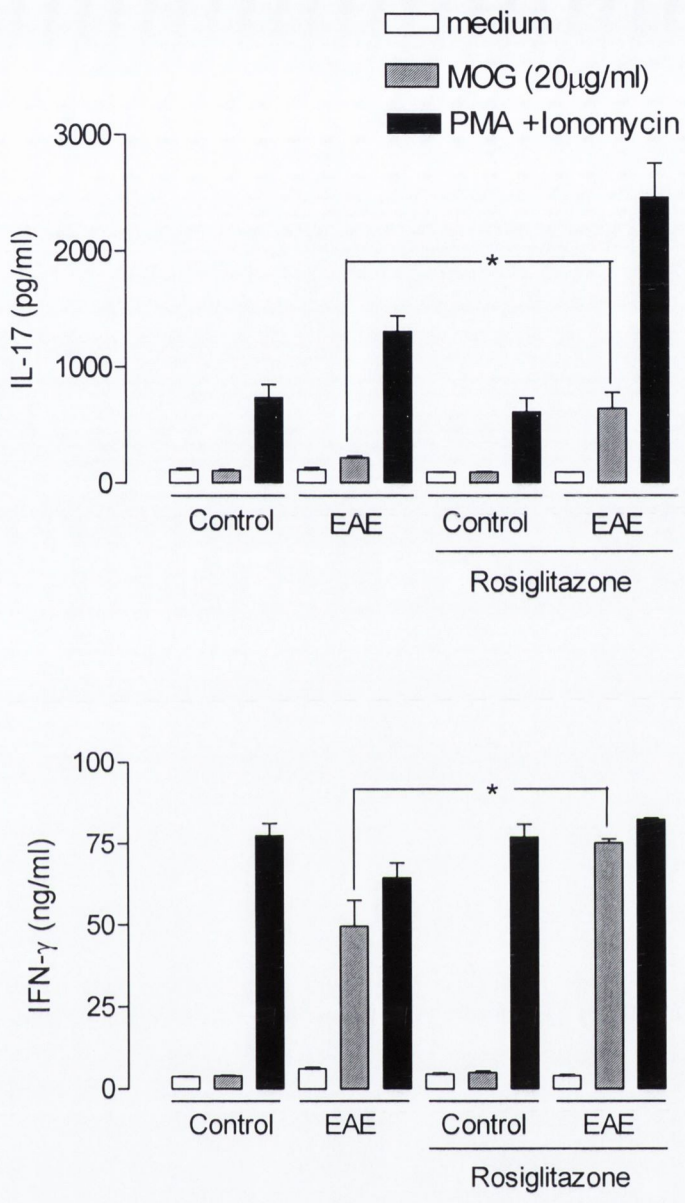

Figure 5.17. MOG-specific IL-17 and IFN-γ production from spleen cells from control treated and rosiglitazone treated C57BL/6 mice with EAE. Mice were treated prophylatically and therapeutically with control diet or rosiglitazone (6mg/kg/day). Spleens were removed from C57BL/6 control mice or mice with EAE treated with or without Rosiglitazone on day 22 and cultured for 5 days with MOG_{35-55} (20 μg/ml) or with PMA (10 ng/ml) and ionomycin (1 μg/m). IL-17 and IFN-γ release was assessed by ELISA. *$p<0.05$, by one-way ANOVA, Student Newman-Keuls. Data presented as mean ± SEM. (n=8).

Chapter 6
General Discussion

6.1. Discussion

Dissecting the pathogenesis of a complex disease such as MS in man has many limitations, not the least of which is the clinical and genetic heterogeneity of the disease. Much of our understanding of the disease comes from the animal model of MS, EAE. A major focus of research in this area has been to understand the sequence of events during development of disease, the interaction of infiltrating immune cells and CNS resident cells and the consequences of these interactions. To this end, the EAE model and *in vitro* culture systems have been invaluable. This thesis has described the timing of events during MOG-induced EAE, from the infiltration of $CD11b^{+}CD45^{hi}$ cells into the CNS to the expression of antigen presenting molecules, the production of cytokine and the phagocytic activity of resident and infiltrating APC. This study has addressed the endogenous role of the anti-inflammatory cytokine IL-4, the immunomodulatory receptor SIGIRR and ageing in EAE. In addition, this study has examined the interaction of pathogenic T cells with CNS resident glia *in vitro* and demonstrated a role for both peripheral and brain derived cells in modulating these interactions.

Infiltration of macrophages into the brain and spinal cord during EAE coincided with the onset of clinical disease. The expression of MHC class II, CD80, CD86 and CD40 were enhanced on the surface of both resident microglia and infiltrating macrophages in the brain and spinal cord. The data suggest that infiltrating T cells can be reactivated by both microglia and macrophages in the CNS, since both cell types express comparable levels of antigen presenting molecules. A comparison of the APC function of glia, macrophages and DC *in vitro* demonstrated that each APC could induce IFN-γ and IL-17 production from

$CD4^+$ T cells, but glia were the least efficient. In addition, glia, macrophages and DC can stimulate T cell proliferation, however, DC were the most effective. The reduced ability of glia to induce production of cytokines from T cells compared with DC and macrophages, has been attributed to lower expression of CD45 on their surface (Carson *et al.*, 1998).

At the acute stage of disease, expression of MHC class II remained elevated on microglia and macrophages, however, expression of CD40 was completely downregulated to levels observed on microglia and macrophages from control mice. Expression of CD80 and CD86 was also decreased as disease progressed but not to basal levels. Therefore, this pattern of MHC class II and co-stimulatory molecule expression during the later stages of EAE may initiate a regulatory mechanism within the CNS. T cells may become anergic due to presentation of antigen by MHC class II expressing microglia and macrophages that lack the co-stimulatory signals required for complete T cell activation. Phagocytic activity by microglia and macrophages can provide a suppressive effect on inflammation within the CNS. Phagocytic cells may limit inflammation by removal of apoptotic cells and by reduced release of proinflammatory cytokines (Magnus *et al.*, 2002). Proinflammatory cytokine production in the brain was greatest at the onset of clinical symptoms. This promotes the infiltration of peripheral immune cells into the CNS, and can cause direct damage to neurons and their myelinating cells, the oligodendrocytes. Proinflammatory cytokine production by microglia and macrophages was inversely correlated to phagocytic activity; as phagocytic activity increased, proinflammatory cytokine production decreased. The elucidation of these events during EAE highlights the timing of pathogenic mechanisms that require attenuation. The balance of these pathogenic

and immunoregulatory mechanisms resulted in a steady state of clinical symptoms, however, irreversible neuronal damage occurred later in disease.

Previously, inflammation was reported to localise predominantly in the spinal cord in the "classic" form of EAE (Sakuma *et al.*, 2004; Stromnes & Goverman, 2006). Conversely, the present study showed that inflammation manifests in both the brain and spinal cord during EAE. The extent of macrophage infiltration and expression of MHC class II and co-stimulatory molecules on microglia and macrophages was similar in both areas of the CNS. Stromnes and colleagues (2006) claim that the localisation of inflammation to the spinal cord is a limitation of this "classic" EAE model, since inflammation during MS involves both the brain and spinal cord. This study demonstrated that inflammation occurs in the brain and spinal cord during "classic" EAE, thus "classic" EAE may better represent inflammation in the CNS during MS than previously thought.

Several anti-inflammatory proteins could potentially have important protective roles in EAE. Since there are many conflicting reports in the literature regarding the role of IL-4 in EAE (Liblau *et al.*, 1997; Bettelli *et al.*, 1998; Falcone *et al.*, 1998), this study investigated the endogenous role of IL-4 in EAE. The data show that there was no endogenous role for IL-4 in EAE in young mice, with IL-$4^{-/-}$ mice showing equal susceptibility to EAE and suffering from similar disease severity as wild type mice. However, there is evidence in the literature to suggest that treatments that promote the production of IL-4 are therapeutically beneficial in EAE (Youssef *et al.*, 2002; Ho *et al.*, 2006a). Previously in our laboratory, rosiglitazone was shown to increase production of IL-4 in the brain, therefore, it was hypothesised that treatment with rosiglitazone may be able to reduce the symptoms of EAE. Contrary to expectations, rosiglitazone did not

attenuate the clinical symptoms of EAE. The data suggest that neither endogenous IL-4 nor administration of agents that induce IL-4 production in the CNS are beneficial in EAE. However, the lack of IL-4 production during EAE in aged mice exacerbated disease symptoms, this data suggests that IL-4 may play a protective role during EAE in aged mice.

SIGIRR is known to negatively regulate immune responses (Wald *et al.*, 2003). Although data in this thesis showed SIGIRR had no endogenous role in EAE, activation of SIGIRR by its ligand, IL-1F5, may be therapeutically beneficial in EAE. IL-4 is produced after binding of IL-1F5 to SIGIRR, a phenomenon that is confined to the brain (Costelloe *et al.*, 2008). Therefore, administration of IL-1F5 could potentially be protective in EAE with brain-specific anti-inflammatory effects and limited peripheral effects. Although, it must be considered that increased IL-4 production in the brain may not be sufficient for the amelioration of EAE, since the protective effect of Th2 cells during EAE has been attributed to increased IL-4 production in the lymphoid system and not the CNS (Falcone & Bloom, 1997).

The presence of IL-4 in the brain is decreased with age (Nolan *et al.*, 2005), this is coupled with an increase in the production of proinflammatory cytokines (Godbout & Johnson, 2004; Nolan *et al.*, 2005) and a heightened activation state of microglia (Godbout *et al.*, 2005). The present study showed that IL-4 deficiency in addition to a more inflamed CNS phenotype due to ageing was associated with increased severity of EAE in aged mice. Aged IL-4 defective mice showed increased production of MOG-specific IL-17 but decreased IFN-γ production by T cells from the spleen and lymph nodes after induction of EAE,

demonstrating that administration of exogenous IL-4 to the lymphoid microenvironment may be therapeutic in EAE in older mice.

This study provided further indirect evidence that IL-4 may have an anti-inflammatory role in the CNS during EAE. The attenuating effects of Th2 cells on cytokine production in co-cultures of Th1/Th17 cells and glia are mediated by IL-4, as well as IL-10. Furthermore, neutralisation of IL-4 and IL-10 reversed the inhibitory effect of the Th2 cells on the expression of CD80 and CD86 on microglia, demonstrating that IL-4 and IL-10 may play a role in regulating the expression of CD80 and CD86 on activated microglia. Th2 cells mediated their suppressive effects via interaction with glial cells directly, since addition of Th2 cells to co-cultures of Th1/Th17 cells and glia decreased the expression of CD40, CD80 and CD86. Th2 cells did not attenuate the expression of MHC class II on microglia. Th2 cells may have a protective effect during EAE by inducing T cell anergy in the CNS via downregulation of the expression of co-stimulatory and adhesion molecules on microglia.

Neurons may also have an immunoregulatory role during EAE, since neurons were found to attenuate proinflammatory cytokine production from cultures of Th1/Th17 cells and glia *in vitro*. The suppressive effect of neurons was mediated by CD200 ligand-receptor interactions. Neurons failed to attenuate IL-17 production from Th1/Th17 cells in the absence of glia; this data suggests that neuronal-glial interactions are crucial for the inhibitory effect of neurons on proinflammatory cytokine production. Interestingly, neurons did not affect the expression of MHC class II, CD40, CD80 and CD86 on microglia. However, the role of astrocytes in the cultures of glia with Th1/Th17 cells must be considered. Astrocytes are classified as non-professional APC and expression of MHC class I

and II and cosimulatory molecules is enhanced on activated astrocytes (Fontana *et al.*, 1984; Nikcevich *et al.*, 1997), thus, they are capable of presenting antigen to T cells. In addition, astrocytes release IL-1, IL-6 and TNF-α in response to a variety of immunological stimuli (Dong & Benveniste, 2001). Furthermore, astrocytes are reported to express CD200R (Chitnis *et al.*, 2007). Therefore, it is possible that neuronal interaction with astrocytes may contribute to the suppressive effect of neurons on proinflammatory cytokine production in cultures of glia with Th1/Th17 cells.

The role of Th1 cells and Th17 cells in the pathogenesis of EAE remains unresolved. Both T cell subsets have been shown to have pathogenic roles during EAE (Kroenke *et al.*, 2008; O'Connor *et al.*, 2008; Stromnes *et al.*, 2008). The present study showed that interaction of Th1 and Th17 cells with glia resulted in differential production of IL-1β, TNF-α and IL-6, which is predominantly mediated by cell contact between the T cells and the glia. In the case of MOG-specific T cells, a mixed population of Th1/Th17 cells consistently induced greater proinflammatory cytokine production when cultured with glia, compared to Th1 cells. This data suggests that the interaction of Th1 cells and Th17 cells with glia could result in a differential immune response in the CNS, which may contribute to the complexity of their distinct pathogenic pathways during EAE. Cell contact between glia and T cells was important for the production of proinflammatory cytokines and enhanced expression of MHC class II, CD40, CD80 and CD86 on microglia. However, this study showed that CD40L-CD40 and CD80/CD86-CD28 interactions did not mediate this cell contact. It is possible that other unidentified activation markers may have overcome the effect of neutralising CD40 and CD80 interactions. There may be a specific interaction

between Th1 or Th17 cells and glia that has not yet been identified and blockade of this interaction may attenuate the inflammatory response induced after T cell: glial contact. Thus, blocking the interactions of Th1 or Th17 cells and glia may be therapeutic during EAE.

A major focus of future research of immune cell interactions during inflammation in the CNS would be to describe the differential interactions of Th1 and Th17 cells with CNS resident cells *in vivo*. However, studies of this nature are complicated by the fact that inflammatory responses within the CNS differ from those in the periphery. The microenvironment of the CNS is crucial to the suppression of immune responses; therefore, the role of CNS resident cells during diseases such as EAE and MS cannot be underestimated. Thus, it is of the utmost importance to define the interactions between pathogenic T cell subsets and CNS resident cells and their consequences during CNS inflammation and autoimmunity.

Chapter 7
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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.

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-

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-

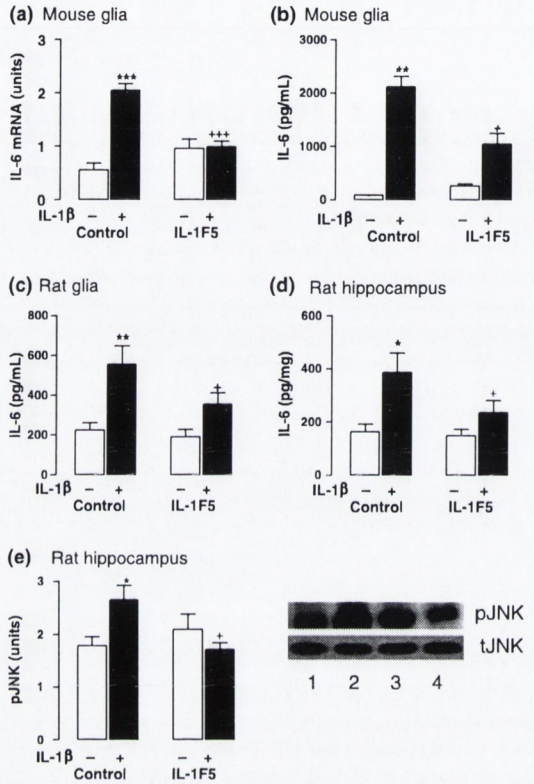

Fig. 1 IL-1F5 antagonizes IL-1β-induced IL-6 in brain tissue *in vitro* and *in vivo*. Mouse glia (a and b) or rat glia (c) cells were cultured with IL-1β (3.5 ng/mL), following 2 h pre-treatment with IL-1F5 (3 μg/mL). After 24 h, IL-6 protein concentrations in supernatants were quantified by ELISA or 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.

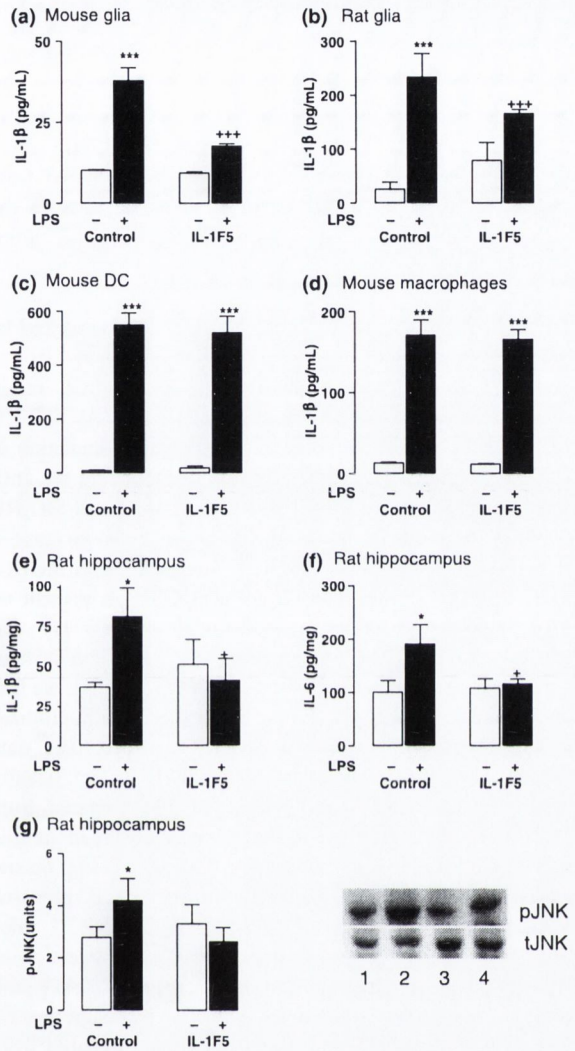

Fig. 2 IL-1F5 antagonizes LPS-induced IL-1β in brain tissue *in vitro* and *in vivo*. Mouse glia (a), rat glia (b), mouse DC (c) or mouse macrophages (d) were cultured in the presence or absence of LPS (100 ng/mL), following 2 h pre-treatment with IL-1F5 (1 and 3 μg/mL in the case of mouse and rat cells 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.

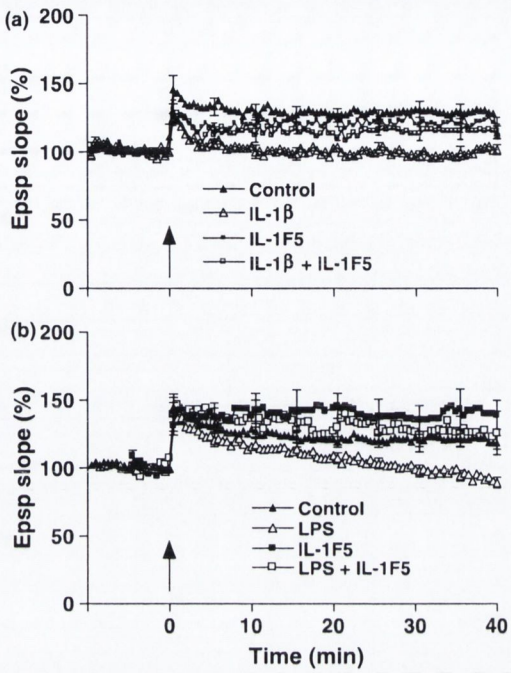

Fig. 3 IL-1F5 reverses the inhibitory effects of IL-1β or LPS on tetanus-induced LTP in dentate gyrus. Rats were injected i.c.v. with 5 μL saline or 30 ng/mL of IL-1F5 and 5 min later were treated i.c.v. with (3.5 ng/mL; 5 μL) IL-1β (a) or i.p. with 100 μg/kg LPS (b). Rats were assessed for their ability 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

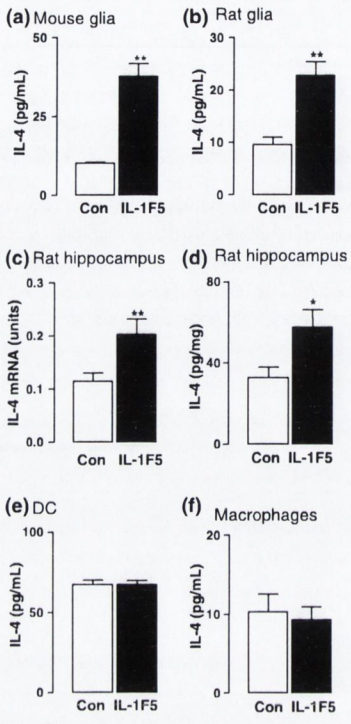

Fig. 4 IL-1F5 increases in IL-4 in brain tissue. Mouse glia (a), rat glia (b), mouse DC (e) or mouse macrophages (f) were cultured with IL-1F5 (1 and 3 μg/mL in the case of mouse and rat cells respectively) or medium only (control). After 24 h, IL-4 protein concentrations were quantified in supernatants 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.

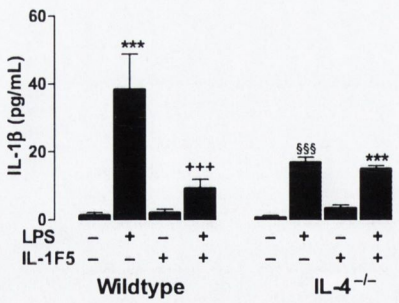

Fig. 5 IL-1F5 abrogates the LPS-induced increase in IL-1β in glia from WT but not IL-4$^{-/-}$ mice. Glial cells from WT and IL-4$^{-/-}$mice stimulated with medium only, LPS (100 ng/mL) or IL-1F5 (3 μg/mL) or both and IL-1β concentration were determined in the supernatants 24 h later. Values are mean ± 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.

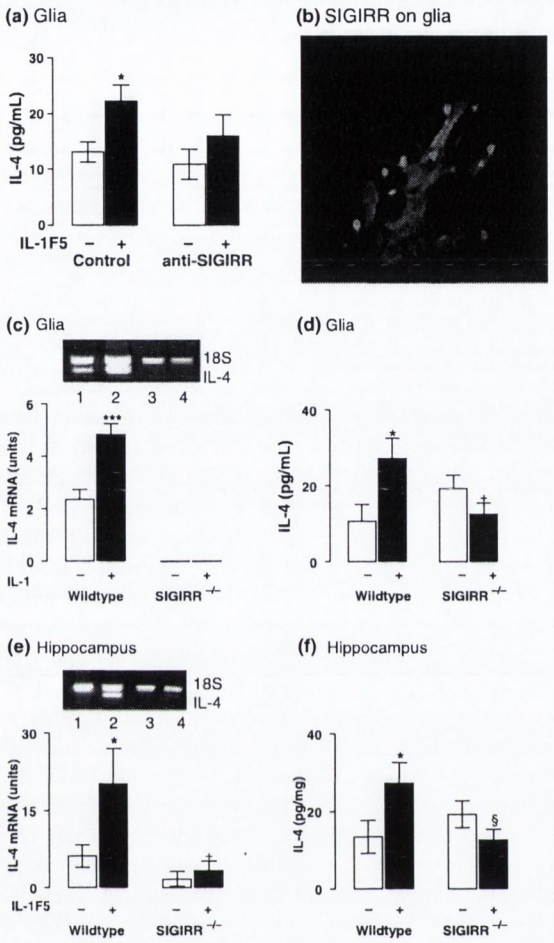

Fig. 6 IL-1F5 fails to induce IL-4 in SIGIRR$^{-/-}$ mice. (a) Mouse glial cells were cultured with IL-1F5 (3 μg/mL) or medium only in the presence or absence of anti-SIGIRR antibody (20 μg/mL). (b) SIGIRR is expressed on glia. (c and d) Mouse glial cells from WT or SIGIRR$^{-/-}$ mice were cultured 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.

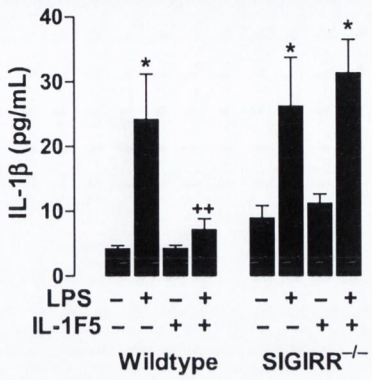

Fig. 7 IL-1F5 attenuates LPS-induced IL-1β in WT but not SIGIRR$^{-/-}$ mice. Glia cells from WT and SIGIRR$^{-/-}$ mice were stimulated with medium only, LPS (100 ng/mL), IL-1F5 (3 μg/mL) or LPS and IL-1F5. After 24 h, IL-1β concentration were determined in supernatants by ELISA. Values are mean ± SEM;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

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.

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

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$^{-/-}$

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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